Mutant p53-reactivating compound APR-246 synergizes with asparaginase in inducing growth suppression in acute lymphoblastic leukemia cells

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INTRODUCTION

TP53 is the most frequently mutated gene in cancer [1]. The most common type of TP53 mutations is missense mutation that results in expression of functionally deficient p53 protein that fails to bind DNA and transactivate p53 target genes. Mutant p53 can exert a dominant-negative effect on wild-type p53, as recently demonstrated in myeloid malignancies [2], and may in some cases display so called gain-of-function activities that promote tumor development in various ways [3–5]. The mutant p53-targeting compound APR-246 (Eprenetapopt/PRIMA-1Met) is currently being tested in phase II and III clinical trials in several hematological malignancies with mutant TP53. Here we present Cellular Thermal Shift Assay (CETSA) data indicating that ASNS is a direct or indirect target of APR-246 via the active product methylene quinuclidinone (MQ). Furthermore, combination treatment with asparaginase and APR-246 resulted in synergistic growth suppression in ALL cell lines. Our results thus suggest a potential novel treatment strategy for ALL.

Asparaginase depletes extracellular asparagine in the blood and is an important treatment for acute lymphoblastic leukemia (ALL) due to asparagine auxotrophy of ALL blasts. Unfortunately, resistance occurs and has been linked to expression of the enzyme asparagine synthetase (ASNS), which generates asparagine from intracellular sources. Although TP53 is the most frequently mutated gene in cancer overall, TP53 mutations are rare in ALL. However, TP53 mutation is associated with poor therapy response and occurs at higher frequency in relapsed ALL. The mutant p53-reactivating compound APR-246 (Eprenetapopt/PRIMA-1Met) is currently being tested in phase II and III clinical trials in several hematological malignancies with mutant TP53. Here we present Cellular Thermal Shift Assay (CETSA) data indicating that ASNS is a direct or indirect target of APR-246 via the active product methylene quinuclidinone (MQ). Furthermore, combination treatment with asparaginase and APR-246 resulted in synergistic growth suppression in ALL cell lines. Our results thus suggest a potential novel treatment strategy for ALL.

Thus, given the documented polypharmacology of APR-246/MQ, it is conceivable that this compound targets other proteins and pathways in cancer cells.

For the past four decades, asparaginase (ASNase) has been an important therapeutic agent for acute lymphoblastic leukemia (ALL) [12]. Despite its long clinical use, the exact molecular mechanism of action of ASNase in ALL is not fully understood [12, 13]. It is thought that ASNase depletes asparagine in the bloodstream, and while normal cells can synthesize asparagine intracellularly via the enzyme asparagine synthetase (ASNS), ALL cells have defective ASNS expression and thus rely on the uptake of extracellular asparagine from the blood [12–15]. It has also been proposed that the glutaminase activity of ASNase is important for its mechanism of action and contributes to its antitumor activities [12, 16, 17], since cancer cells are often dependent on glutamine [18]. Development of resistance to ASNase is an important clinical problem [12] and for a long time such resistance has been associated with ASNS expression [15, 19–22]. However, it remains unclear whether ASNS expression can predict ASNase response [23]. TP53 mutations are rare in ALL in general but occur more frequently in relapsed ALL and are strongly associated with poor therapy response and poor prognosis [4, 24, 25].

Cellular Thermal Shift Assay (CETSA) is a biophysical method that was initially developed to study in situ drug–target engagement from lysate, cells, or tissues [26–28]. Upon heat
of all thermally stabilized hits by MS-CETSA after MQ treatment in OVCAR-3 cells as shown in MS-CETSA identifications ASNS as a putative MQ target. A overview of experimental set-up for MQ treatment of OVCAR-3 cells and MS-CETSA 2 h after treatment. B Total glutathione (GSH + GSSG) measured by enzymatic recycling assay in OVCAR-3 cells after 2 or 6 h of 6 μM MQ treatment. Untreated control was harvested at the 6 h time point. \( n = 3 \). C Confluence of OVCAR-3 cells at the indicated MQ and APR-246 concentrations as assessed by Incucyte. Gradients indicate treatment concentrations of 1.5, 3, 6, and 12 μM MQ and 5, 7.5, 10, and 15 μM APR-246, respectively. \( n = 2–3 \) (except for 15 μM where \( n = 1 \)). D Dot plot of the dose–response trend and relative shift of all thermally stabilized hits by MS-CETSA after MQ treatment in OVCAR-3 cells as shown in A. ASNS is indicated in purple. E MS-CETSA thermal shift response of ASNS with increasing concentrations of MQ at the indicated temperature in OVCAR-3 after 2 h of treatment. Different colors of the same temperature indicate separate MS runs. Relative protein stability is compared to vehicle control. MQ concentrations used were 0.0025, 0.01, 0.05, 0.2, 0.8, 3.1, and 12.5 μM.

RESULTS

MS-CETSA identifies ASNS as a putative MQ target

Since APR-246 is converted to the active product MQ, a Michael acceptor that reacts reversibly with cellular thiols [6, 11], it is plausible that APR-246 targets a number of proteins other than what is known to date. Thus, we used MS-CETSA to identify novel proteins affected by MQ/APR-246. We selected the ovarian cancer cell line OVCAR-3, which carries the well-characterized TP53 hot spot missense mutation R248Q, as an appropriate cell model for these studies. We have previously reported that this cell line responds to APR-246 treatment [11], which we document in further detail here (see below). We treated OVCAR-3 (TP53 R248Q) with a range of concentrations of MQ for 2 h (discussed further below), after which cells were harvested and heated at 37, 46, 52, and 58 °C to denature and precipitate unfolded proteins (Fig. 1A). After cell lysis and removal of denatured/precipitated proteins by centrifugation, the supernatant was analyzed by MS/MS (Table S1). Proteins that were thermally stabilized or destabilized upon MQ treatment were considered potential hits that were either targeted by covalent MQ binding or indirectly affected via, for example, posttranslational modifications, redox modification, protein–protein interactions or binding of cellular metabolites, such as nucleotides or amino acids [29–31].

While treatment with up to 12.5 μM of MQ for 2 h did not affect cell viability (Fig. S1A), it drastically depleted total glutathione (GSH + GSSG; Fig. 1B). However, after 6 h the total glutathione levels increased, indicating that the cells had begun to replenish their antioxidant reservoir. Similarly, increased xCT expression was observed after 2 h of MQ treatment but was not sustained at the 24-h time point (Fig. S1B). Antipporter xCT is regulated by master antioxidant sensor nuclear factor erythroid 2-related factor 2 (NRF2) and provides glutathione building blocks by importing cystine [35, 36]. MQ only showed inhibition of recombiant glutathione reductase (GR) activity at 100-fold higher concentrations than the concentrations used (Fig. S1C) and is therefore unlikely to affect the measurement in the GR recycling assay. Although cell viability was not affected at the harvesting time point with treatment up to 12.5 μM MQ (Fig. S1A), cell death was
observed at later time points (Fig. S1D), as also shown by cell confluence (Fig. 1C) and caspase-3 cleavage (Fig. S1E). MQ was more potent than its prodrug APR-246 at the same concentrations (Fig. 1C) and induced caspase-3 cleavage at an earlier time point (Fig. S1E) as expected, since conversion of APR-246 to MQ takes certain time. Taken together, these data demonstrate that the concentrations of MQ chosen for the MS-CETSA study were sufficient to elicit anticancer phenotypes associated with APR-246 treatment and should therefore allow identification of biologically relevant targets.

ASNS, an enzyme that synthesizes asparagine from aspartate (Fig. S1F), was among the top five thermostabilized hits (Fig. 1D) and showed strong thermal shift at 58 °C (Fig. 1E). Expression of ASNS has been implicated in resistance to the important anti-leukemic therapy ASNS [15, 19–22], which has been in clinical use for decades to treat ALL, and thus we selected the hit ASNS for follow-up studies given the potential clinical relevance in using APR-246 as a novel strategy to overcome ASNase resistance in this disease. In summary, our MS-CETSA study identified ASNS as a thermally stabilized protein upon treatment with APR-246 active product MQ, suggesting that MQ may directly or indirectly alter ASNS activity.

**MQ thermostabilizes ASNS in ALL cells**

Analysis of pharmacogenomic datasets available through the DepMap portal [37] with >300 cancer cell lines of various origins did not show any significant difference in PRIMA-1 (APR-246 analog) sensitivity between cells with high and low ASNS expression (Fig. S2A). Thus, the effect of APR-246/MQ on ASNS activity may not be biologically relevant in a pan-cancer setting. However, as resistance to ASNS in ALL is associated with increased ASNS expression [15, 19–22], targeting ASNS has therapeutic relevance in ALL [13]. Therefore, we first sought to validate MQ-induced thermal stabilization of ASNS in the ALL cell line CCRF-CEM. Western blot-based CETSA (WB-CETSA) was performed as previously described (Fig. 1A) but with 10 and 15 µM MQ and additional temperatures around 58 °C to achieve a full melting curve (Fig. 2A) where the ASNS shift was observed in the MS-CETSA (Fig. 1E). WB-CETSA did not show any change in ASNS protein level at 37 °C but both concentrations of MQ induced a temperature-dependent stabilization of ASNS at 57 and 59 °C (Fig. 2B and S2B). SOD1 was used as thermally stable loading control as described [38] and was unaffected by treatment and temperature. Ponceau staining indicated temperature-dependent protein degradation while protein loading within the same temperature was similar (Fig. S2B). Quantification of the WB-CETSA showed a thermal shift of ASNS from 56.6 to 57.5 °C upon incubation with MQ at the aggregation temperature \( T_{agg} \) when half of the protein has aggregated and been removed from the soluble fraction (Fig. 2C and S2C). Thus, WB-CETSA in a tumor model relevant to ALL biology indicates that MQ, the active product of APR-246, directly or indirectly modulates ASNS.

**Markers for ASNase and APR-246 sensitivity in ALL cells**

Demir et al. demonstrated increased APR-246 efficacy in mutant TP53 ALL cell lines and patient-derived ALL xenografts in mice [32], in line with many of our previous studies showing that cancer cells with mutant TP53 are more sensitive to APR-246 than wild-type TP53 cells [6, 7, 11, 39]. However, other studies have demonstrated p53-independent sensitivity to APR-246 [40, 41]. Furthermore, we have recently shown that neither TP53 status, GSH content, nor drug accumulation alone can fully determine APR-246 sensitivity [11]. We did not detect any correlation between p53 protein expression levels and APR-246 sensitivity in the ten ALL cell lines included in this study (Figs. 3A, B and S3A). For example, KARPAS-45 and CCRF-CEM with hot spot TP53 mutations (Table S2) and very high levels of mutant p53 expression were only moderately sensitive to APR-246 and their half-maximal inhibitory concentration (IC50) values (Table S3) did not differ from those of other ALL lines with lower levels of p53 expression. Interestingly, CCRF-SB that carries wild-type TP53 was highly resistant to APR-246 (Table S3). Database analysis of 22 ALL cell lines in the DepMap portal did not show any difference in PRIMA-1 sensitivity depending on TP53 status (Fig. 3C) as has also been observed in a Pan-Cancer setting and overall in tumors of hematological lineages [42].
Markers for ASNase and APR-246 sensitivity in ALL cells. A. p53 protein levels in untreated cells plotted against cell viability after 72 h treatment with APR-246. B. Western blot of untreated ALL cell lines and a heat map indicating growth suppression as assessed by resazurin after 72 h of APR-246 treatment. C. Analysis of data from the DepMap portal of the Broad Institute of 22 ALL cancer cell lines showing PRIMA-1 activity area under the curve (AUC) grouped into TP53 status. WT = wild type, Miss. Mut. = missense mutation, F.s. = frameshift, Nons. = nonsense, Del. = deletion. D. Analysis of data from the DepMap portal of the Broad Institute of 22 ALL cancer cell lines showing PRIMA-1 activity area under the curve (AUC) grouped into xCT/SLC7A11 mRNA expression below or above median. Unpaired t-test, p = 0.07, r = 0.12. E. xCT protein levels in untreated cells plotted against cell viability after 72 h treatment with APR-246. F. xCT levels were obtained by quantification of the western blot in F. Cell viability was assessed by resazurin (area under the curve [AUC]). See Table S5. Spearman correlation r = 0.07, p = 0.9. G. Western blot of untreated ALL cell lines and a heat map indicating growth suppression as assessed by resazurin after 72 h of APR-246 treatment (same as in B but in different order). H. Total glutathione (GSH + GSSG) measured by enzymatic recycling assay in untreated ALL cell lines after 72 h in culture plotted against cell viability after 72 h of APR-246 treatment. Cell viability was assessed resazurin (area under curve [AUC]). Cell lines are specified in the figure legend box. I. ASNS protein levels in untreated cells plotted against growth suppression after 72 h treatment with 0.003 U/ml ASNase. ASNS levels were obtained by quantification of the western blot in J. Cell viability was assessed by resazurin (area under the curve [AUC]). See Table S5. Spearman correlation r = 0.5, p = 0.1. J. Western blot of untreated ALL cell lines and a heat map indicating growth suppression as assessed by resazurin after 72 h of asparaginase (ASNase) treatment. Detailed information and n regarding growth suppression are given in Table S4. The same GAPDH control blot is shown in panels F and J since both xCT (F) and ASNS (J) were examined on the same blot. The blot was divided into an upper part which was probed with ASNS antibody and a lower part which was probed with xCT antibody and then re-probed with GAPDH antibody. Thus, the same GAPDH blot serves as control for both xCT and ASNS.
MQ can trigger p53-independent cell death by induction of oxidative stress [6, 40, 43]. This effect can be inhibited by various antioxidative compounds and cellular mechanisms. For example, expression levels of the cystine/glutamate antiporter xCT (SLC7A11), which imports glutathione building blocks, shows one of the highest correlations to PRIMA-1 resistance in a DepMap analysis of >700 cancer cell lines [11] and has been suggested as a predictive biomarker for APR-246 sensitivity [42, 44]. Surprisingly, when stratifying for only ALL cell lines in the DepMap database, we found no difference in PRIMA-1 sensitivity between ALL cell lines with high and low xCT/SLC7A11 mRNA levels (Fig. 3D). Similarly, there was no correlation between APR-246 sensitivity and xCT protein levels among the ten ALL cell lines included in the study (Fig. 3E, F) nor with antioxidant TrxR1 protein levels (Fig. S3B). Antioxidant capacity reflected by total glutathione (GSH + GSSG) did show a weak correlation with APR-246 sensitivity (r = 0.6, p = 0.1) but only when the APR-246-resistant cell line CCRF-SB (IC50 > 9 μM) was excluded (Figs. 3G and S3C, D). The DepMap analysis also did not show any significant correlation between reduced glutathione (GSH) and PRIMA-1 activity in 22 cell lines of ALL origin (Fig. S3E). Asparagine and PRIMA-1 activity (area under the curve (AUC)) did not correlate (Fig. S3F), but interestingly, aspartate was inversely correlated to PRIMA-1 activity in 22 ALL cell lines in the DepMap analysis (Fig. S3G) (r = −0.5, p = 0.03). Thus, ALL cells with high aspartate are more sensitive to PRIMA-1, while this correlation was not apparent in a pan-cancer setting (>700 cell lines) (Fig. S3H, I). Western blot quantification of ASNS indicated a weak correlation (r = 0.5, p = 0.1) between high ASNS expression and APR-246 resistance (Fig. 3H). This would be expected if MQ binding inhibits the enzyme, since more MQ will be needed to inhibit ASNS function if more enzyme is present.

In agreement with other studies [19–22], we observed a clear correlation (r = −0.7, p = 0.03) between low ASNS expression and high ASNS activity (Fig. 3I). RS4;11 and SUP-B15 were the most sensitive lines to ASNS treatment with no visible ASNS expression according to Western blot analysis, while CCRF-SB, Jurkat A3 cells, and Reh had low ASNS sensitivity with a high expression of ASNS. All other cell lines that had visible ASNS bands showed intermediate ASNS sensitivity. We conclude that ALL has a distinct metabolic landscape and that APR-246 sensitivity in ALL, unlike in solid tumors, is not correlated to mutant p53 or xCT expression levels.

APR-246 synergizes with ASNase in ALL cells

Given that ASNase resistance is associated with ASNS expression, and that our data suggest that APR-246/MQ may target ASNS, we hypothesized that APR-246 may sensitize ALL cells to ASNase treatment. After determining the effect of APR-246 or ASNase monotherapy, we measured cell viability in a panel of ten ALL cell lines following a 72-h treatment with a concentration response matrix consisting of APR-246 and three different ASNase concentrations ranges (low, mid, and high) (Fig. 4A). In agreement with another study [42] showing that hematological lineage tumor cells are more sensitive to APR-246 treatment than solid cancer cell lines, we observed that the APR-246 IC50 values (Table S3) in most of the tested ALL cell lines were more than tenfold lower compared to the IC50 values of multiple previously tested solid cancer cell lines [11]. Furthermore, incubation with APR-246 reduced cell viability after ASNase treatment in several of the tested ALL cell lines, for example, in MOLT-16 and Jurkat A3 with low p53 levels and CCRF-CEM cells with high p53 levels (Figs. 4B and S4A). We observed an increased growth suppression at several tested concentrations (Fig. S4B). Synergistic growth suppression was found at several concentration combinations as determined using the web application Synergy-Finder over the concentration response matrices (Fig. 4C).

In general, APR-246 sensitivity was increased (as demonstrated by decreased AUC) in the combination with ASNase (Fig. 4D).

According to the three tested synergy models, the combination of APR-246 and ASNase resulted in synergistic growth suppression (scores > 0; Fig. 4E). Eight out of ten tested ALL cell lines exhibited synergy, although to varying extent (Fig. 4F). We did not observe any correlation with p53, xCT, or ASNS expression and synergy scores (Fig. S4C–E). For example, Jurkat A3, Reh, and CCRF-SB, which are among the most ASNS-resistant cell lines with the highest ASNS expression (Fig. 3J), exhibited variable degrees of synergy scores (Fig. 4F), similar to the most ASNS-sensitive cell lines with no detectable ASNS expression, RS4;11 and SupB15.

In summary, combination treatment with APR-246 and ASNase results in synergistic growth suppression in several ALL cell lines independently of p53, xCT, and ASNS.

DISCUSSION

The mutant p53-targeting compound APR-246 is currently being tested in phase III clinical trials in patients with TP53 mutant MDS and several phase I and II studies in other hematological malignancies with mutant TP53 or solid cancers independent of TP53 status. The produg APR-246 is converted to the Michael acceptor MQ that reactivates mutant p53 and also induces oxidative stress by targeting redox regulators such as TrxR1, thioredoxin, and glutathione, leading to tumor cell death by apoptosis [45]. Given the thiol-binding properties of MQ, we reasoned that additional MQ targets may play variable roles in APR-246-induced tumor cell death, depending on the cellular context.

Our CETSA screen demonstrated that ASNS is thermally stabilized in MQ-treated cells (Figs. 1 and 2). This could result from direct binding of MQ or indirect effects including, for instance, binding to another protein that is stabilized by MQ or from stabilizing posttranslational modification induced by MQ. Our data suggest that stabilization of ASNS is associated with inhibition of the enzyme, as indicated by the observed correlation between APR-246 efficacy and ASNS protein levels (Fig. 3H). Furthermore, MQ is a very reactive molecule with a preference for “soft” nucleophiles like the thiol of a cysteine [11], such as the cysteine in the N-terminal active site that was reported to be essential for the glutamine-dependent activity of ASNS [46]. Taken together, these data suggest that the thermal stabilization may be derived from MQ binding to this active site cysteine in ASNS, and if so, it is conceivable that MQ inhibits enzyme activity as demonstrated for TrxR1 [9] and other redox enzymes [10].

During the past decades, ASNS has been successfully used as standard-of-care treatment for ALL [12, 47]. For instance, in childhood ALL, recombinant Escherichia coli ASNase treatment alone can induce complete remission in up to 40–60% of the patients. This effect is largely attributed to ASNase-mediated depletion of circulating asparagine in the blood, by converting it to aspartate. However, it should be noted that ASNase also has glutaminase activity that contributes to antitumor effect [12]. Normal cells can import aspartate and synthesize asparagine intracellularly through ASNase (Fig. S1F). Asparagine is important for tumor growth as a substrate for protein synthesis and due to its role in cellular amino acid homeostasis [48] (Fig. 5), but it may also have other regulatory functions [49]. ASNS is part of the amino acid response (AAR) pathway with AAR elements and nutrient-sensing response elements in its promoter. Consequently, upon asparagine depletion (but also depletion of other amino acids), ASNS will be upregulated via activating transcription factor 4 (ATF4) [50, 51]. ALL cells are asparagine auxotrophs, relying on circulating asparagine, and are therefore highly sensitive to ASNase treatment (Fig. 5B). Although ASNS mRNA is upregulated upon asparagine deprivation [21], the protein is not expressed [49], possibly as a result of promoter methylation [52] or epigenetic changes [53] in ALL cells. ASNS upregulation is one mechanism of ASNase resistance, enabling cells to synthesize aspartate and thereby survive and grow even when circulating asparagine is depleted [12, 19] (Fig. 5C).
Fig. 4 APR-246 synergizes with ASNase in ALL cells. A Single treatment ($n = 2–7$) and experimental overview of combination treatments. Cell viability as assessed by resazurin after 72 h single treatment with either asparaginase (ASNase) or APR-246. Three different ASNase treatment groups (low, mid, high) for the combination treatments were used; APR-246 concentrations were the same for all cell lines. B Cell viability as assessed by resazurin after 72 h of increasing concentrations of ASNase in combination with APR-246. Each dot indicates an individual experiment. C Synergy landscape determined by ZIP model over APR-246 and ASNase concentrations of one representative experiment. Concentrations of APR-246 and ASNase are as in Fig. S4A. D Cell viability (area under the curve [AUC]) as assessed by resazurin after 72 h of APR-246 alone or with two different concentrations of ASNase (0.04–0.05 and 0.4–0.5 U/ml for all cell lines except for RS4;11 and SupB15 that were treated with 0.009 and 0.003 U/ml). Each dot indicates one ALL cell line. See Table S5 for more details and $n$ for each cell line. E Box-and-whisker plot of synergy scores of most synergistic area determined by three different synergy models. Central band indicates median, boxes indicated 25th and 75th percentile, and whiskers show min. and max. values. Each dot indicates one ALL cell line. See Table S6 for more details, including $n$ for each cell line. F Synergy score of most synergistic area determined by the ZIP model in 10 different ALL cell lines after ASNase treatment +/− APR-246 for 72 h. Each dot indicates one experiment.
Fig. 5  Proposed mechanisms of synergy between APR-246 and ASNase. A Asparaginase (ASNase) depletes extracellular asparagine (ASN) by converting it to aspartic acid (ASP). Normal cells can synthesize ASN by asparagine synthetase (ASNS). B Acute lymphoblastic leukemia (ALL) cells have defective ASNS expression and are therefore sensitive to ASNase treatment. C ASNase resistance is associated with ASNS expression and the resistant cells can therefore synthesize ASN from ASP. D Combination treatment with mutant p53 reactivating compound APR-246 directly or indirectly inhibits ASN, resulting in enhanced sensitivity in ASNS-resistant ALL cells.

Although TP53 mutations are infrequent in ALL overall, TP53 is mutated at a higher frequency in relapsed ALL and this is associated with poor therapy response [4, 24, 25]. Wild-type p53 can downregulate ASNS transcription [54] and mutant p53 can upregulate ASNS expression by binding to its promoter according to another study [55]. This suggests that mutant p53 could play a role in ASNase resistance (Fig. 5C). In support of this notion, our TCGA-PanCancer analysis demonstrated that patients with wild-type TP53 show lower ASNS expression compared to patients with missense or truncating TP53 mutations (Fig. S4F).

Apart from ALL, ASNase has been used for treatment of other hematological malignancies including AML [17], for which APR-246 has received FDA fast track designation. This raises interesting future perspectives for combination treatment. A screen of >900 cancer cell lines identified aberrant ASNS promoter hypermethylation resulting in lack of ASNS protein expression in gastric and hepatic cancer cell lines. Mouse xenograft models with these cell lines showed high sensitivity to ASNase treatment [56]. As TP53 mutation is common in gastric cancer [57] and a driver mutation and frequent event (> 30%) in hepatic cancer [58], the combined targeting of mutant p53 with APR-246 and depletion of asparagine with ASNase may also be fruitful approach in some solid cancers with low ASNS expression.

Another interesting tumor type for the combination treatment with APR-246 and ASNase is KRAS-driven non-small-cell lung cancer (NSCLC). Around 20% of these tumors carry mutation in Kelch-like ECH-associated protein 1 (KEAP1) or NRF2 [59]. Tumors with mutation in master antioxidant response regulator NRF2 or its negative regulator KEAP1 have high antioxidant capacity, for example, via induction of antioxidant xCT (SLC7A11) expression, resulting in efflux of glutamate and influx of cystine, which may be used for antioxidant glutathione synthesis. LeBoeuf et al. demonstrated that xCT-driven glutamate efflux makes KEAP1-mutated tumors ASNase sensitive as they rely on the uptake of non-essential amino acids, including asparagine [59]. Additionally, high xCT activity renders tumors glutamine dependent as glutamine is utilized for generating glutamate that is exported with the imported cystine [60–62]. This may increase their sensitivity to the glutaminase activities of ASNase [16]. Both xCT [44] and KEAP1 [42] are correlated to APR-246 resistance, and so it is conceivable that ASNase may sensitize APR-246-resistant tumors. Furthermore, wild-type KEAP1 tumor cells may be sensitized to ASNase treatment by induction of oxidative stress [59]. Thus, as APR-246 (via MQ) depletes glutathione (Fig. 1B), APR-246 may also sensitize NSCLC without KEAP1 mutations to ASNase treatment.

Our data indicate that the mutant p53 reactivator APR-246, currently in phase III clinical trials in MDS, targets ASNS and thereby possibly inhibits its activity. Mutant p53 reactivation by APR-246 has previously been demonstrated in patient-derived ALL xenografts in mice [32]. The mutant p53-reactivating and ASNS-targeting activities of APR-246 might exert a dual effect on ASNS-expressing cells (Fig. 5D), resulting in synergistic growth suppression upon combination treatment with ASNase. Since TP53 mutation is associated with poor treatment response and ASNS expression is associated with ASNase resistance, targeting both mutant p53 and ASNase with APR-246 in combination with ASNase, which depletes circulating asparagine, appears as an attractive strategy that warrants further investigation in ALL, especially in light of the benign safety profile of APR-246 [63].

MATERIALS AND METHODS
Cell lines and culture conditions
OVCAR-3, RS4;11, SupB15, and KARPAS-45 were cultured in RPMI-1640 media containing Hepes (HyClone). Insulin–Transferrin–Selenium (51300044, Thermo Fisher Scientific) was freshly added into the media for OVCAR-3. Molt-16 was cultured in Iscove’s Modified Dulbecco’s Medium (HyClone) media. Molt-4, Reh, Jurkat, Jurkat A3, CCRF-CEM, and CCRF-SB were cultured in RPMI-1640 media All media were supplemented with L-glutamine and 10% fetal bovine serum. Cells were incubated for 2 h in 37 °C at 5% CO2. After incubation, the media was poured out and cells were washed once with phosphate-buffered saline (PBS) and incubated with 2 ml trypsin (HyClone) at 37 °C. When cells detached, 5 ml Hank’s Balanced Salt Solution (HBSS)
with isolation window at 1.2 resolution of 70,000 and AGC target of 3e6: top 12 dd-MS2 70,000, and 3e6 scaling step was performed, followed by curve data analysis and visualization by our in-house developed mineCETSA package only master proteins in the protein group were used for further downstream relative fold changes as compared to vehicle control at different temperatures showing thermal stabilization in 46, 52, or 58 °C but not in 37 °C were compared to vehicle were included for hit selection. Additionally, only hits target proteins with at least three PSM and a minimum fold change of 30% as labeling peptides and labeling efficiency was checked. Isobaric Tandem Mass Tags (TMT)−10plex (90110, Thermo Scientific) was used for online chromatography, performed using Dionex UltiMate 3000 UPLC system coupled to a Q Exactive HF mass spectrometer (Thermo Scientific). Using a 50 cm × 75 μm (ID) EASY-Spray analytical column (Thermo Scientific) in 70 min gradient of programmed mixture of solvent A (0.1% FA in H2O) and solvent B (99.9% acetonitrile, 0.1% FA), each fraction was separated. MS data were acquired using a top 12 data-dependent acquisition method. Full-scan MS spectra were acquired in the range of 300–600 m/z at a resolution of 70,000 and AGC target of 3e6: top 12 dd-MS2 70,000, and 3e6 with isolation window at 1.2 m/z.

Cell viability measurement by resazurin
Drug combination effects were determined by resazurin cell viability measurements were performed as described previously [64, 65]. All cells were diluted to 2 × 104 live cells/ml in fresh media. APR-246 (100 μM in DMSO) and AS3-250 (30 μM, 0.3% Tween-20) were dispensed into clear bottomed 384-well plates (Corning) using the D300e digital dispenser (Tecan), and solvents were normalized across the plates. Subsequently, cells were seeded in 50 μl/well using a MultiDrop (Thermo Fisher) to give a final seeding concentration of 1000 cells/well. Plates were incubated at 37 °C and 5% CO2 for 72 h in a humidity chamber, until Resazurin (R17017, Sigma Aldrich) dissolved in PBS was added to a final concentration of 0.01 mg/ml. After 4-6 h of Resazurin incubation, fluorescence at 530/ 590 nm (ex/em) was measured by Hitachi Fluorescence Microplate Reader. Wells containing only media and solvent were used to subtract background, and data were normalized to vehicle-treated wells.

Total glutathione (GSH + GSSG) measurements by enzymatic re-cycling assay
ALL cells were seeded into standing 25-cm² cell culturing flask (Sarstedt) in 10 ml media at a cell density of 0.2 × 10⁶ cells/ml except RS4;11 and SupB15, which were seeded at double density due to lower proliferation rate. After 24 or 72 h of incubation at 37 °C and 5% CO₂, cells were harvested by 5 min 500 x g centrifugation, washed in PBS, counted, centrifuged, and resuspended in 100 μl of 10 mM HCl. Samples were centrifuged at 20,800 x g for 20 min at 4 °C, 60 μl of the supernatant was transferred to new tubes, and frozen at −20 °C. Samples were thawed to measure total GSH + GSSG as described [11].

Glutathione Reductase (GR) activity assay
Recombinant GR activity assays were performed as described [11].

Confluence assessment by Incucyte
The day before treatment, 10000 OVCA-3 cells were seeded per well in a 96-well plate with 100 μl of media. The next day, indicated treatments were added from a diluted stock into the media and incubated in the IncuCyte® S3 real-time video imaging system (Essen BioScience, USA) stored in a cell incubator at 37 °C and 5% CO₂. Caspase-3 dye was incubated at the same time as drug treatments. Each well was imaged every third hour up to 72 h in duplicate wells and 4 images per well. Confluen was based on bright-field images and caspase 3 activity was assessed by measuring green
fluorescence and normalized to confluence in each image. Confluence analysis was performed by the Incucyte software and normalized to starting time point for each condition.

Data analysis and statistics
Results presented in figures are mean and standard error of the mean unless otherwise specified. Adobe Illustrator was used to put together figure panels and draw illustrative figures. GraphPad Prism 9 was used to prepare heat maps, histograms, scatter plots, and perform statistical analysis. Normal distribution of dataset was tested by Shapiro–Wilk’s test, and depending on the outcome, parametric or nonparametric statistical tests were performed. Data for correlation analysis of PRIMA-1, gene expression, and metabolites were downloaded from Cancer Dependency Map (https://www.depmap.org) [56, 66, 67] and analyzed in GraphPad Prism 9. Synergy was determined as previously described [11] using SynergyFinder web application 2.0 by FIMM (https://synergyfinder.fimm.f.u/u/) using ZIP, Bliss, or HSA synergy [68].

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COMPETING INTERESTS

This study was funded in part by Aprea Therapeutics, a company that develops p53-based cancer therapy, including APR-246. K.G.W. and V.J.N.B. are co-founders and shareholders of Aprea Therapeutics; K.G.W. is also a member of its Clinical Advisory Board. K.G.W. and V.J.N.B. have received a salary from Aprea Therapeutics. L.A. is Chief Scientific Officer of Aprea Therapeutics. P.N. is the inventor of the C4TA method, which is patented by Pelago Biosciences AB. P.N. is a member of the Board of Directors and shareholder of Pelago Bioscience AB.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study did not require ethical approvals.

ADDITIONAL INFORMATION

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