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APR-246 induces early cell death by ferroptosis in acute myeloid leukemia.

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Abstract:

APR-246 is a promising new therapeutic agent that targets p53 mutated proteins in myelodysplastic syndromes and in acute myeloid leukemia. APR-246 reactivates the transcriptional activity of p53 mutants by facilitating their binding to DNA target sites. Recent studies in solid cancers have found that APR-246 can also induce p53-independent cell death. In this study, we demonstrate that AML cell death occurring early after APR-246 exposure is suppressed by iron chelators, lipophilic antioxidants and inhibitors of lipid peroxidation, and correlates with the accumulation of markers of lipid peroxidation, thus fulfilling the definition of ferroptosis, a recently described cell death process. The capacity of AML cells to detoxify lipid peroxides by increasing their cystine uptake to maintain major antioxidant molecule glutathione biosynthesis after exposure to APR-246 may be a key determinant of sensitivity to this compound. The association of APR-246 with induction of ferroptosis (either by pharmacological compounds, or genetic inactivation of SLC7A11 or GPX4) had a synergistic effect on the promotion of cell death, both in vivo and ex vivo.
**Introduction**

Acute myeloid leukemias (AML) are highly heterogeneous diseases with a constant activation of oncogenic signaling\(^1\). Recent years have witnessed major breakthroughs in their treatment with the approval of midostaurin, venetoclax and IDH mutant inhibitors\(^2\)–\(^5\). However, AML has a poor prognosis and there is still an urgent need for new treatments. APR-246, also known as PRIMA-1\(^{MET}\), is a promising new therapeutic agent that targets TP53 mutated cancers\(^6\)–\(^8\). This compound is being evaluated in AML and myelodysplastic syndromes (MDS) with TP53 mutations and appears to be highly effective against this poor prognosis disease\(^8\)–\(^11\). Mechanistically, APR-246 is converted to a reactive product (methylene quinuclidinone, MQ) that reacts with nucleophiles and thus alkylates thiol groups in proteins\(^12\). APR-246 reactivates the transcriptional activity of p53 mutants by facilitating their binding to DNA target sites. Specific cysteines in the core domain of mutant p53 proteins are critical targets for their reactivation by APR-246/MQ\(^13\). APR-246 also triggers p53-independent cell death mechanisms\(^14\),\(^15\). Accordingly, using esophageal cancer as a model, it has been shown that APR-246 causes a decrease in glutathione (GSH) content resulting in an increased amount of reactive oxygen species (ROS) and of lipid peroxides in particular\(^16\). In this report, we investigated the mechanisms of cell death induced by APR-246 in AML and we demonstrated that early cell death in AML is due to ferroptosis induction.

**Methods**

**Cell lines and reagents:** HL60, MOLM14, SET2, MV4-11, OCI-AML2, OCI-AML3, K562, THP1, UT7-EPO, SKM1, NB4 and KASUMI AML cell lines were used. Patients provided written informed consent in accordance with the Declaration of Helsinki. Bone marrow (BM) samples were obtained from 5 patients with newly diagnosed AML (characteristics provided in Supplemental Table 1). Cells were cultured in RPMI with glutamine (Gibco61870, Life Technologies® Saint Aubin, France) supplemented with 10% fetal bovine serum (FBS) and 4 mM glutamine. All AML cell lines were certified using their microsatellite identity (characteristics provided in Supplemental Table 2). Ferrostatin-1, necrostatin-1, necrostatin-1S, necrosulfonamide, QVD-OPH, APR-246 for the in vitro study, erastin and RSL3 were sourced from Selleckchem (Houston, TX). Chloroquine and doxycycline were obtained from Sigma-Aldrich (Saint-Louis, MO). FINO\(_2\) was purchased from Cayman Chemicals (Ann Arbor, MI). The APR-246 reagent used in the in vivo study was provided by APREA therapeutics (Solna, Sweden).

**Constructs:** Inducible shRNAs targeting SLC7A11 or GPX4 were constructed as previously described\(^17\) using the following sequences: SLC7A11#2, GCTGAATTGGGAACAGCTATA; SLC7A11#3, GCAGTTGCAGCCGTGATTAT; GPX4#1, GTGAGGCAAGACCGAAAGTAAA; GPX4#2, CTACACGTCATAATTCGATAT.
**Lentivirus production and AML cell line infection:** 293-T packaging cells were used to produce lentiviral constructs through co-transfection with plasmids encoding lentiviral proteins. Supernatants were collected and ultracentrifuged for 48 h after transfection over two consecutive days and subsequently stored at -80°C. AML cell lines were plated at 2x10^6/ml and 2-10μl aliquots of lentiviral supernatants were added for 3 h. Cells were then grown in 10% FCS medium and further selected with puromycin. For shRNA induction, 200μg/ml doxycycline was added to the culture medium.

**Flow cytometry-based assay:** Data acquisition and data analysis were conducted at the Cochin Cytometry and Immunobiology Facility. For glutathione measurements using monochlorobimane (MCB; Thermofisher; Waltham, MA), 2x10^5 cells were labeled with 40 μM MCB in 1 mL of warm complete medium for 20 min in a tissue culture incubator (37°C, 5% CO2) in the dark. The reaction was terminated using 1 mL of cold complete medium, followed by centrifugation (200 x g, 1 min). The pelleted cells were then re-suspended in 0.5 mL of cold complete medium and placed on ice in the dark until analysis by flow cytometry (FCM). The MCB-GSH signal was detected using a 355 nm laser through a 450/50-nm filter. FCM data were collected using a BD Fortessa flow cytometer with DIVA software. 10,000 events were recorded for analysis. Data analysis was then carried out with KALUZA software. For lipid peroxide production measurements using C11-BODIPY (581/591) (2 μM) (Thermofischer, Waltham, MA), 2x10^5 cells were labeled with C11-BODIPY in 1 mL of warm complete medium for 10 min in a tissue culture incubator (37°C, 5% CO2) in the dark. Cells were then washed twice and resuspended in 200 μl of fresh PBS. For cystine uptake measurements using BioTracker Cystine-FITC Live Cell Dye (5 μM) (Thermofischer, Waltham, MA), 2x10^5 cells were labeled with Biotracker cystine in 1 mL of warm complete medium for 120 minutes in a tissue culture incubator (37°C, 5% CO2) in the dark. FCM data were collected using a C6 Accuri flow cytometer (Becton Dickinson, Le Pont de Clai, France) with CFlow Plus software. 10,000 events were captured for subsequent analysis with CFlow Plus software (Becton Dickinson, Le Pont de Clai, France).

**Western blotting:** Whole-cell extraction and western blotting were performed as previously described. Anti-GPX4 antibody was purchased from Proteintech (Manchester, UK). Anti-PARP, caspase 8, caspase 3, cleaved caspase3, MLKL, pMLKL, p53 and SLC7A11 antibodies were sourced from Cell Signaling Technology (Danvers, MA).

**Viability assay:** AML cells were plated at 20x10^4/ml in 100μl of 10% FBS-supplemented RPMI prior to the addition of compounds. Cells were cultured in the presence of the test compounds for 24 to 48 h at 37°C. Viability was quantified using the fluorescence based Uptible blue assay (Interchim, Montluçon, France). Uptible blue was added to each well in 10 μl aliquots. Fluorescence was then measured with a Typhoon FLA9500 scanner (GE Healthcare; IL). Fluorescence values were normalized to DMSO-treated controls for each AML cell line. IC50 values were calculated using a four parameter non-linear
regression curve with Graph Pad Prism v8 (GraphPad, La Jolla, CA). For primary AML cells, viability was assayed by FCM analysis using FSC vs SSC.

Measurement of synergistic effects: Cell viability was calculated for every dose combination of APR-246 and ferroptosis inducer using the Synergy Finder webtool (https://synergyfinder.fimm.fi/) and compared to each agent alone. Calculations were based on the ZIP model.

Tumor xenografts in NOD/SCID IL-2 receptor γ-chain-null mice: Xenograft tumors were generated by randomly injecting 1×10^6 MOLM14 shCTRL or shSLC7A11 cells into the tail veins of NOD/SCID IL-2 receptor g-chain-null mice (NSG) aged 6–9 weeks. Fourteen days after injection, doxycycline (200μg/ml) and sucrose (1% weight:volume) were added to the drinking water of these animals. After 3 days, the mice were randomly treated with a daily intraperitoneal injection of APR-246 (100 mg/kg) or vehicle (PBS) for 4 days. All experiments were conducted in accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. Animals were used in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee of Région Midi-Pyrénées (France). Bone marrow (mixed from tibias and femurs) were dissected and flushed in Hanks balanced salt solution with 1% FBS. MNCs from bone marrow were labeled with PE-conjugated anti-hCD33, PerCP-Cy5.5-conjugated anti-mCD45.1 and APC-conjugated anti-hCD45 (all antibodies from Becton Dickinson, BD) to determine the fraction of human blasts (hCD45+mCD45.1−hCD33+ cells) using flow cytometry (FCM). Acquisition of data was performed on a CytoFLEX (Beckman Coulter) flow cytometer with CytExpert software. The number of AML cells in the bone marrow was determined using CountBright beads (Invitrogen, CA) in accordance with the manufacturer's protocol. Data analysis was performed with flowJo software.

Results

APR-246 induces ferroptosis in AML cells.

To determine the activity of APR-246 in AML, we assayed a set of 12 AML cell lines carrying diverse and representative molecular abnormalities, and 5 primary AML samples (Supplemental table 1). Most of the AML cell lines and primary AML cells were sensitive to cell death induction by APR-246 (Fig. 1-a, b). The IC_{50} of APR-246 at 24 hours for each cell line ranged from 11 to more than 200 μM, independently of their TP53 mutational status (Fig. 1-c). Previous in vivo human data have shown that the plasma concentrations of APR-246 in the 12 hours following its intravenous administration range from 50 to 500 μM, suggesting that concentrations above 50 μM are suitable for in vitro studies of the early effects of APR-246. In our subsequent experiments, we selected five AML cell lines sensitive to APR-246 in these concentration ranges, with or without TP53 mutations.
To investigate the mechanisms underlying APR-246 activity against AML cells, we exposed the cells treated with APR-246 to inhibitors of various cell death pathways (Fig. 2-a). The decrease in cell viability was almost completely rescued by either iron chelation via deferoxamine (DFO) or by the lipophilic antioxidant ferrostatin-1 (Fer1), demonstrating that cell death is both iron and ROS dependent. Necrostatin-1 (Nec1) also consistently prevented cell death induced by APR-246 after short-term incubation in all AML cell lines. Nec1 has been used to define necroptotic cell death, but can also protect against ferroptosis through a target which is as yet unknown. Induction of necroptosis after treatment with APR-246 in our cells was excluded due to the absence of protection by more specific necroptosis inhibitors (Necrostatin 1s and Necrosulfonamide\textsuperscript{21,22}, and due to the absence of MLKL phosphorylation, a key marker in necroptosis\textsuperscript{21} (Supplemental Fig 1-a, b). Inhibitors of autophagy (chloroquine) or apoptosis (QVD-OPH) failed to block APR-246-induced cell death. We confirmed that the mechanism of APR-246 induced cell death is distinct from apoptosis, as evidenced by the absence of caspases 3/8 or PARP1 cleavage, including in TP53 mutated AML cell lines (Fig. 2-b; Supplemental Fig 2-a, b, c). Notably, the protection against cell death observed with Fer1, DFO or Nec1 was partially lost at higher doses of APR-246 (Fig. 2-c) and at later time points (Fig. 2-d).

However, apoptosis did not appear to be the mechanism of this late death, since caspases 3/8 and PARP1 were not cleaved and QVD-OPH was still unable to prevent the cell death (Supplemental Fig 2-a, b, c, d, e). Examination of the ultrastructural changes induced by APR-246 treatment did not reveal any characteristic features of apoptosis (i.e. no plasma membrane blebbing, chromatin condensation or nuclear fragmentation) or autophagy (absence of autophagosomes). Necrotic cells were rare, and some mitochondria showed membrane rupture and reduced cristae (Fig.3-a, Supplemental Fig.3). We analyzed the levels of endogenous lipid peroxidation – a hallmark of ferroptosis induction – following APR-246 treatment by flow cytometry analysis with C11-BODIPY staining. We observed a high induction of lipid ROS in AML cell lines and primary AML cells from 2 patients treated with APR-246 ex vivo. This increase in lipid ROS was fully reversed by Fer1, indicating that lipid peroxides had been newly generated (Fig3-b, c). All these results allowed to conclude that APR-246 induces early cell death by ferroptosis, a recently described non-apoptotic form of regulated cell death that links together membrane lipid peroxidation, cysteine and iron metabolism, glutathione peroxidase activity and oxidative stress (as summarized in Fig.4-a\textsuperscript{23-25}). As previously reported\textsuperscript{14,16}, we observed that APR-246 treatment induced a dramatic decrease in GSH levels in AML cell lines (Fig.4-b). Cysteine is the main biosynthetic precursor of GSH. Cysteine can be transported into cells via membrane transporters for neutral amino acids. However, in the extracellular space, cysteine is rapidly reduced to cystine. Thus the main source of intracellular cysteine comes from the entry of cystine into the cell via system xc\textsuperscript{26}. \(\beta\)-mercaptoethanol (\(\beta\)-ME) can promote cystine uptake through an alternative pathway\textsuperscript{27}. \(\beta\)-ME was able to completely rescue the cell death and GSH
depletion induced by APR-246 (Fig.4-c, d). Cysteine treatment showed similar results as Fer1 treatment (Fig.4-c, d). Altogether, these results demonstrate that APR-246 induces GSH depletion which induces ferroptosis in AML cells irrespective of their TP53 mutational status, and that ferroptosis induction is the main mechanism of cell death after early exposure to APR-246.

**Cystine uptake determined the sensitivity of AML cells to APR-246.**

Since cysteine is a biosynthetic precursor of GSH, we asked whether the ability of cells to provide cysteine for GSH synthesis underlies the sensitivity to APR-246. Using FITC-labeled cystine, we showed that after exposure to APR-246, AML cells increased their uptake of cystine from the extracellular space (Fig.5-a). Western blot analysis of the protein levels of SLC7A11 showed an increased amount of SLC7A11 (Fig.5-b). This suggests that the cells enhanced cystine uptake by increasing SLC7A11 protein levels to maintain intracellular GSH levels after exposure to APR-246. We modulated the cystine uptake through SLC7A11 overexpression or inhibition. SLC7A11 overexpression in MOLM-14 and OCI-AML2 (supplemental Fig.4-a) decreased the cell death and prevented the depletion of GSH induced by APR-246 (Fig.5-c, d). Then we showed that targeting the SLC7A11 cystine transporter by RNA interference reduced the basal uptake of cystine (supplemental Fig.4-b, c) and had a very little effect on cell death (supplemental Fig.4-d), but strongly reduced cell proliferation in AML cells in vitro (supplemental Fig.4-e). Inhibition of SLC7A11 with RNA interference increased cell death and viability impairment, GSH depletion, and the accumulation of lipid peroxides induced by APR-246 (Fig.6-a, b, c; supplemental Fig.4-f). Interestingly, basal GSH levels were not consistently affected by β−ME addition, cysteine addition, or the overexpression or inhibition of SLC7A11, which suggests that the amount of GSH in basal conditions is not a reliable marker of cell cystine uptake ability (Fig.4-d; 5-d, 6-c). Finally, targeting SLC7A11 with erasin, a potent inhibitor of system x_c^−28 that showed variable sensitivity in our AML cell lines (Supplemental Fig.4-g), had synergistic activity with APR-246 both on cell death and on cell viability impairment (Fig.6-d, e, Supplemental Fig.5). The association of erasin and APR-246 also had a synergistic effect on cell viability in 5 primary AML samples (Fig. 6-f). There was no correlation between the basal levels of GPX4 and SLC7A11 proteins and the sensitivity to APR-246 (Supplemental Fig.6-a, b). Altogether, these data suggest that the ability of AML cells to prevent lipid peroxides accumulation by increasing their cystine uptake to support GSH after exposure to APR-246 is a key determinant of the sensitivity to this compound.

**The association of APR-246 and ferroptosis inducers has a synergistic anti-leukemic activity in vitro.**

We next determined whether targeting ferroptosis pathways in combination with APR-246 can increase the anti-leukemic activity of this compound, mimicking SLC7A11 inhibition. Downregulation
of GPX4 by RNA interference resulted in cell death (Supplemental Fig. 7-a, b). Our AML cell lines panel showed variable sensitivity to two ferroptosis-inducing drugs: RSL3, a direct GPX4 inhibitor; and FNO2, an indirect GPX4 inhibitor and direct iron oxidant (Supplemental Fig. 8). We observed that knockdown of GPX4 increased the impairment of cell viability induced by APR-246 (Fig. 7-a). RSL3 and FNO2 in association with APR-246 synergistically decreased cell viability in AML cell lines (Fig. 7-b, c; Supplemental fig. 9-10). Collectively, these results show that pharmacological or genetic activation of ferroptosis enhances the anti-leukemic activity of APR-246 in AML.

**Genetic invalidation of SLC7A11 has synergistic anti-leukemic activity with APR-246 in vivo.**

We then examined whether inhibition of GSH synthesis through SLC7A11 inhibition could interfere with AML persistence and could enhance APR-246 activity in vivo. We engrafted MOLM14 cells transduced with either control (shSCR) or anti-SLC7A11 (shSLC7A11) doxycycline-inducible small hairpin (sh) RNA (Fig. 8-a). After induction of shRNA expression in vivo, we treated the mice with a 4-day APR-246 regimen in order to mimic the therapeutic schedule used in clinical trials of APR-246. This treatment scheme varied from those previously published in mice in terms of treatment duration. Indeed, in these studies, APR-246 was administrated for 7 to 28 days, and reduction of tumor volume after 4 days of treatment was minimal at best. However, our aim was not to assess the efficacy of APR-246 alone but to demonstrate that its association with SLC7A11 inhibition enhanced its anti-leukemic activity. As expected, APR-246 alone did not reduce bone marrow tumor cell burden. SLC7A11 knockdown significantly reduced tumor cell burden in bone marrow (Fig. 8-b, c). Moreover, the decrease in bone marrow tumor cell burden was enhanced when APR-246 treatment was combined to SLC7A11 knockdown (Fig. 8-b, c). Overall, these results showed that inhibition of anti-ferroptosis mechanisms enhanced the anti-leukemic activity of APR-246 in vivo.

**Discussion**

APR-246 can restore the wild type conformation of mutant p53 protein, therefore inducing apoptosis and inhibition of tumor growth in mice. Thus, APR-246 is one of the most promising compounds in clinical development for TP53 mutated cancers. Controversies exist over the TP53 mutation status dependencies of APR-246. Some studies reported that APR-246 acts independently of its ability to reactivate mutant p53 protein. Tessoulin et al. demonstrated that myeloma cells are highly sensitive to APR-246, independently of their TP53 status. In this cancer, APR-246 induces cell death by impairing GSH/ROS balance and acts synergistically with L-buthionine sulfoximine to inhibit myeloma growth in vivo. In ARID1A-deficient cancers, GSH was the major target of APR-246 and was the basis of the high sensitivity of these cancer cells to this compound. In esophageal cancer,
Liu et al. showed that mutants p53 bind to the antioxidant transcription factor NRF2, leading to a decreased expression of SLC7A11 which sensitizes cells to GSH depletion by APR-246.16 Paradoxically, while APR-246 clinical development is the most advanced in AML with TP53 mutation, the effects of APR-246 have been little studied in this disease. Two studies showed that APR-246 induced in vitro cell death in a large number of leukemic cells from patients, alone or in association with chemotherapies.39,40 In both studies, the cytotoxicity of APR-246 was independent of the TP53 mutational status. The mechanisms of action of APR-246 was investigated in AML cell lines with TP53 mutations, and more specifically studied its association with 5-azacytidine which is currently used in clinical trials.8 It was shown that in TP53-mutated myelodysplastic syndromes (MDS) and AML, APR-246 can reactivate the p53 pathway and induce an apoptotic transcriptional program, with synergistic effects of APR-246 and azacytidine.

In this context, our study strongly showed that APR-246 induced cell death in AML cells irrespective of their TP53 mutational status. APR-246 depleted intracellular GSH and induced lipid peroxide production, which led to ferroptosis induction. The ability of AML cells to detoxify lipid peroxides primed their sensitivity to APR-246 treatment. Additionally, we uncovered that inhibition of anti-ferroptosis mechanisms enhanced the anti-leukemic activity of APR-246 both in vitro and in vivo. We confirm the TP53 independence and GSH depletion and we demonstrated that APR-246 induces ferroptosis.14,16 Ferroptosis is rapidly induced after GPX4 inactivation and cell death occurs in the first 24 hours post-treatment or administration.23,29 The observation that protection against cell death by ferroptosis inhibitors decreases after 24 hours of exposure suggests that other cell death mechanisms might be involved after this early phase and that they may have masked the earlier induction of ferroptosis. The effect of APR-246 might be also different in AML cells in comparison to solid cancers.

Our study might have several important implications for the management of MDS and AML patients. First, since APR-246 acts independently of TP53 mutational status, this treatment could be used in a broader panel of AML patients. Future study will need to identify predictive elements of the sensitivity of AML to APR-246 and the induction of ferroptosis. The mechanism of action of APR-246, which is based on GSH depletion and induction of ferroptosis, makes it the first ferroptosis-inducing agent currently used therapeutically in humans. Using ferroptosis induction to treat cancer is an emerging field in oncology research. Renal cancer cells have been reported as highly dependent on the GSH pathway for ROS detoxification, including lipid peroxides, and targeting components of this pathway is an effective strategy for the treatment of this disease.41 Other studies have elegantly highlighted the higher sensitivity to ferroptosis of cancer cells that are resistant to conventional therapy.42,43 In AML, data about ferroptosis are scarce. An in vitro study showed that the ferroptosis inducer erastin enhances sensitivity of AML cells to chemotherapeutic agents.44 Jones et al. recently
reported that cysteine depletion leads to GSH exhaustion and ROS-low leukemic stem cell eradication in AML. Thus, APR-246 could act on these cell pools that are poorly sensitive to conventional therapy, and which are at the origin of frequent therapeutic failures in AML.

From a clinical perspective, this mechanism of action might be relevant. Indeed, iron chelators are frequently used for the treatment of iron overload due to red blood cell transfusions and dyserythropoiesis in MDS/AML patients. Several studies have reported beneficial effects of iron chelation therapy on overall survival in MDS patients with iron overload. However, iron chelators are recognized as canonical ferroptosis inhibitors. Therefore, caution should be exercised regarding co-administration of iron chelators which may antagonize the anti-leukemic activity of APR-246, as we observed in vitro. Moreover, the phase 2 studies of APR-246 in MDS/AML reported the occurrence of neurological adverse events in over a third of patients treated with APR-246.

Recently, ferroptosis has been implicated in the pathogenesis of several neurological disorders, especially neurodegenerative disorders. One hypothesis could be that the neurological side effects observed after administration of APR-246 are linked to its ability to deplete GSH in neuronal cells. Consequently, anti-ferroptosis agents, such as iron chelators or vitamin E, could be valuable drugs to treat these side effects. Finally, our study highlights that ferroptosis induction may represent a new target in AML, opening new therapeutic strategies based on disease-specific vulnerabilities. The effect of ferroptosis induction-based treatments on normal hematopoietic cells and their value compared to standard-of-care AML therapies will be important to evaluate in the future.

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Authorship Contributions

JD, LCA, EG, TH performed experiments. CL, JES, JT, NG and MG performed in vivo experiments. NC, OK and MF provided AML samples. NC, PM, JES, NJ and JT analyzed the results and corrected the
manuscript. RB performed experiments, analyzed data, and wrote the manuscript. DB designed and supervised the research program, analyzed data, and wrote the manuscript. All authors approved the final version of the manuscript.

**Disclosure of conflicts of interest**

The authors declare no competing financial or other interests in relation to this study.
References


**Figure 1** - APR-246 induces cell death in AML cells irrespective of their TP53 mutational status.

(a) Viability curves for the indicated cells at 24 h post APR-246 treatment. Error bars, ± s.d.

(b) Viability curves for the indicated primary AML cells at 24 h post APR-246 treatment. Patient characteristics are provided in supplemental table 1.

(c) IC₅₀% of APR-246 treatment for 24 h across a panel of AML cell lines based on cell viability (n=3).

In our subsequent experiments, we selected five AML cell lines sensitive to APR-246 in these concentration ranges, with or without TP53 mutations (highlighted in bold font). wt: wild type; fs: frameshift; del: deletion.

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**Figure 2** - APR-246 induces ferroptosis in AML cells.

(a) Cell viability (%) for the indicated cells at 16 h post-APR-246 treatment (60 µM) with or without ferrostatin-1 (10 µM), DFO (100 µM), necrostatin-1 (20 µM), chloroquine (20 µM) or QVD-OPH (25 µM) (n=3). Error bars, ± s.e.m. All compounds were added 2 h prior to APR-246 in the medium. Statistics, 2-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.0001.

(b) Immunoblotting analysis of PARP, caspase 8 and caspase 3 in MOLM-14 cells treated for 16 h with DMSO, APR-246 (60 µM) or puromycin (1 µg/mL). β-actin was used as a loading control (n=2).

(c) Viability curves for the indicated cells at 16 h post APR-246 treatment with or without ferrostatin-1 (10 µM), DFO (100 µM), necrostatin-1 (20 µM), chloroquine (20 µM) or QVD-OPH (25 µM) (n=3). Error bars, ± s.e.m.

(d) Cell death (%) of the indicated cells at 16 h and 24 h post-APR-246 treatment (50 µM) with or without ferrostatin-1 (10 µM) (n=3). Error bars, ± s.d.

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**Figure 3** - APR-246 induces ferroptosis in AML cells.

(a) Electron microscopy analysis of MOLM-14 cells treated with or without APR-246 (60 µM, H16). The white arrowhead indicates a mitochondrion showing membrane rupture and reduced cristae.

(b, c) Detection of lipid peroxidation using C11-BODIPY and flow cytometry (FCM) at 14 h post APR-246 treatment in AML cell lines (b) and in primary AML cells (c). APR-246 was used at a 100 µM concentration for MOLM-14 and 50 µM for other AML cell lines. Left panels show representative FCM quantification (n=3). Error bars, ± s.d.

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**Figure 4** - APR-246 induces GSH depletion in AML cells.

(a) Summary of the cellular pathways involved in ferroptosis. Ferroptosis execution is triggered by an iron-catalyzed excessive peroxidation of polyunsaturated fatty acids (PUFA)-containing phospholipids (PL-PUFA). GSH and glutathione peroxidase 4 (GPX4) are the two key elements controlling the elimination of lipid peroxides. Solute carrier family 7 member 11 (SLC7A11) encodes the transporter
subunit of the heterodimeric cystine-glutamate antiporter named system \( x_c^- \). System \( x_c^- \) mediates cystine entry into the cell in exchange for glutamate\(^{39}\). Once inside the cell, cystine is rapidly reduced to cysteine which is the limiting amino acid for GSH synthesis. SLC7A11 inhibition results in cellular cysteine depletion, which leads to the exhaustion of intracellular pool of GSH. GPX4 is a pleiotropic selenoprotein that uses GSH to selectively reduce lipid hydroperoxides to lipid alcohols, in order to protect the cells against membrane lipid peroxidation\(^{29}\). GPX4 inhibition is either due to its direct inhibition or downregulation, or to GSH depletion via direct or indirect processes. The inhibition of GPX4 results in uncontrolled polyunsaturated fatty acid phospholipid (PL-PUFA) oxidation and fatty acid radical generation, leading to ferroptotic cell death. ACSL4: acetyl-CoA synthetase long chain family member 4; LPCAT 3: Lysophosphatidylcholine Acyltransferase 3; ALOX: Arachidonate lipooxygenase; PUFA: polyunsaturated fatty acid; PL: phospholipids; PE: phosphatidylethanolamine; GPX4: Glutathione peroxidase 4.

(b) GSH (mBCI) measurement in AML cell lines by FCM at 14 h post APR-246 and fer1 (10µM) treatment. APR-246 was used at 100 µM for MOLM-14 and 50 µM for other AML cell lines. Fer1 was associated to prevent cell death and allowed analysis of GSH depletion. Left panels show representative FCM quantification. n=3. Error bars: ± s.d. Statistics: t-test. *p < 0.05, **p < 0.01, ***p < 0.0001.

(c-d) Cell death (%) (c) and GSH (mBCI) measurement (d) for MOLM-14 at 24 h post-APR-246 treatment (60µM) with or without B-ME (50 µM), Cysteine (50µM) or Fer1 (10µM). Error bars, ± s.d. Statistics: t-test; *p < 0.05, **p < 0.01, ***p < 0.0001.

Figure 5- SLC7A11 overexpression prevents GSH depletion and cell death following APR-246 exposure.

(a) Cystine uptake in MOLM-14 and OCI-AML2 cells lines at 16 h post-APR-246 (100µM) and Fer1 (10µM) treatment. Fer1 was associated to prevent cell death and allowed analysis of cystine uptake.
(b) Immunoblotting analysis of SLC7A11 in MOLM-14 cells treated for 16 h with DMSO or APR-246 (n=2). β-actin was used as a loading control.
(c-d) Cell death (%) (c) and GSH (mBCI) measurement (d) of the indicated cells at 20 h post-APR-246 treatment (n=3). For GSH measurement, Fer1 was associated to prevent cell death and allowed analysis of GSH depletion. Error bars, ± s.d.; Statistics: t-test. *p < 0.05, **p < 0.01, ***p < 0.0001.

Figure 6- SLC7A11 inhibition sensitizes cells to APR-246.

(a) Viability curves for MOLM-14 and OCI-AML2 with shSCR or shSLC7A11 cells at 16 h post APR-246 treatment. Prior to adding APR-246, the cells were treated for 3 days with doxycycline (n=3). Error bars, ± s.d.
(b-c) Cell death (%) (b) and GSH (mBCI) measurement (c) of the indicated cells at 20 h post-APR-246 (MOLM-14 30 µM, OCI-AML2 10 µM) treatment (n=3). For GSH measurement, Fer1 was associated to prevent cell death and allowed analysis of GSH depletion. Prior to adding APR-246, the cells were treated for 3 days with doxycycline (n=3). Error bars, ± s.d.; Statistics: t-test. *p < 0.05, **p < 0.01, ***p < 0.001.
(d) Cell death (%) for the indicated cell types at 24 h post APR-246 (MOLM-14 30 µM, OCI-AML2 10 µM) and or Erastin (MOLM-14 100 nM, OCI-AML2 1 µM). Error bars, ± s.d. Statistics, t-test; *p < 0.05, **p < 0.01.
(e) Illustrative synergy map (left panel) of 24 h co-treatment of MOLM-14 cells with APR-246 and erastin. The mean cell viability of three independent experiments was used. Mean synergy scores of the most synergistic area of 24 h co-treatment of AML cell lines with APR-246 and erastin (n=3).
(f) Mean synergy score of the 48h co-treatment of primary AML cells with APR-246 and erastin (n=1).

Figure 7: The combination of APR-246 with ferroptosis inducers has synergistic anti-leukemic effects in AML in vitro.

(a) Viability curves for MOLM-14 and OCI-AML2 cells with or without GPX4 inducible shRNA at 16 h post APR-246 treatment. Prior to adding APR-246, the cells were treated for 2 days with doxycycline (n=3). Error bars, ± s.d.
(b) Illustrative synergy map (left panel) of 24 h co-treatment of MOLM-14 cells with APR-246 and RSL3. The mean cell viability of three independent experiments was used. Mean synergy scores of the most synergistic area of 24 h co-treatment of AML cell lines with APR-246 and RSL3 (n=3).
(c) Illustrative synergy map (left panel) of 24 h co-treatment of MOLM-14 cells with APR-246 and FINO2. The mean cell viability of three independent experiments was used. Mean synergy scores of the most synergistic area of 24 h co-treatment of AML cell lines with APR-246 and FINO2 (n=3).

Figure 8: The combination of APR-246 with SLC7A11 inhibition has synergistic anti-leukemic effects in AML in vivo.

(a) Design of the in vivo experiment.
(b) Representative FCM analysis of bone marrow cells marked with mCD45.1 and hCD45 from each treatment subgroup.
(c) Viable mCD45.1- hCD45+ hCD33+ cell counts in the bone marrow of the different treatment subgroups. Bars represent mean of all experiments and errors denote ± s.d. Statistics, t-test; *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 1-
Figure 2-
Figure 3-
Figure 4-
Figure 5-
Figure 6
**Figure 7-**

(a) Graphs showing % Relative cell viability versus APR-246 concentrations for OCI2 and MOLM14 cells.

(b) ZIP synergy score: 4.988

(c) ZIP synergy score: 9.517

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a

IV injection of MOLM14 shSCR or MOLM14 shSLC7A11 cells in NSG mice

Disease development

+ Doxycycline

IP injection of PBS 1x or APR-246 100 mg/kg/day

Cell engraftment in bone marrow cell sorting

D0

D14

D17

D20

D21

b

Group 1 (n=7): shSCR + Vehicle
Group 2 (n=8): shSLC7A11 + Vehicle
Group 3 (n=7): shSCR + APR-246
Group 4 (n=8): shSLC7A11 + APR-246

Bone marrow and spleen

Viable hCD45+ hCD33+

(millions)

Figure 8-
**Supplemental Figure 1**

(a) Cell death (%) in MOLM-14 cells at 16 h post-APR-246 treatment (60µM) with or without ferrostatin-1 (10µM), necrostatin-1 (20 µM), necrostatin-1s (20 µM) and Necrosulfonamide (2µM) (n=3). Error bars, ± s.e.m. All compounds were added 2 h prior to APR-246 in the medium. Statistics, 2-way ANOVA; *p < 0.05, **p < 0.01, ***p < 0.0001.

(b) Immunoblot showing MLKL and pMLKL protein levels in MOLM-14 and OCI-AML2 cells with or without APR-246 at 16 h. b-actin was used as a loading control (n=2).

**Supplemental Figure 2**

(a) Immunoblotting analysis of PARP, caspase 8 and caspase 3 in NB4 cells treated for 16 h with DMSO, APR-246 (60 µM) or puromycin (1 µg/mL). b-actin was used as a loading control (n=2).

(b) Cell death (%) and C11-BODIPY positive cells (%) of the indicated cells at 16 h and 14 h post-APR-246 treatment (60 µM) with or without ferrostatin-1 (10 µM) (n=3). Error bars, ± s.d.

(c-d) Immunoblotting analysis of PARP, caspase 8 and caspase 3 in OCI-AML2 (c) or MOLM-14 (d) cells treated for 16 h and 24 h with DMSO, APR-246 (30 µM), ferrostatin-1 (10µM) or puromycin (1 µg/mL). b-actin was used as a loading control (n=2).

(d) Immunoblotting analysis of PARP, caspase 8 and caspase 3 in OCI-AML2 cells treated for 16 h with DMSO, APR-246 (30 µM), ferrostatin-1 (10µM) or puromycin (1 µg/mL). b-actin was used as a loading control (n=2).

(e) Cell death (%) for the indicated cells at 16 h and 24 h post-APR-246 treatment (60µM) with or without ferrostatin-1 (10µM) and/or QVD-OPH (25µM) (n=3). Error bars, ± s.e.m. All compounds were added 2 h prior to APR-246 in the medium. Statistics, t-test; *p < 0.05, **p < 0.01, ***p < 0.0001.

**Supplemental Figure 3**

Electron microscopy analysis of MOLM14 cells treated with vehicle, chloroquine (autophagy inhibitor, 20µM), puromycin (apoptosis inducer, 1 mg/mL), erastin (ferroptosis inducer, 10 µM), RSL3 (ferroptosis inducer, 1 µM) or APR-246 (60 µM) at 16 h post treatment. The cells treated with chloroquine showed an accumulation of autophagolysosomes (*). Puromycin-treated cells showed a nuclear condensation typical of apoptosis induction (white arrow). However, cells treated with the ferroptosis inducers did not show these characteristics.

**Supplemental Figure 4**

(a) Immunoblotting analysis of SLC7A11 protein levels in MOLM14 and OCI-AML2 cells with or without SLC7A11 overexpression. b-actin was used as a loading control (n=2).

(b) Immunoblotting analysis of SLC7A11 protein levels in MOLM14 and OCI-AML2 cells with or without shRNA targeting SLC7A11 at 72 h post doxycycline induction. b-actin was used as a loading control (n=2).

(c) Cystine uptake in MOLM-14 cells with or without shRNA targeting SLC7A11 at 72 h post doxycycline induction.

(d) Cell death (%) for the indicated cells at 3- and 7-days post doxycycline induction. Error bars, ± s.e.m. All compounds were added 2 h prior to APR-246 in the medium. Statistics, t-test; *p < 0.05, **p < 0.01, ***p < 0.0001.

(e) Cell count for the indicated cells at 3- and 7-days post doxycycline induction. Error bars, ± s.e.m. All compounds were added 2 h prior to APR-246 in the medium. Statistics, t-test; *p < 0.05, **p < 0.01, ***p < 0.0001.

(f) Detection of lipid peroxidation (using C11-BODIPY) by FCM at 14 h post APR-246 (50 µM) treatment in the MOLM14 cell line. Statistics, t-test; *p < 0.05, **p < 0.01, ***p < 0.0001.

(g) Viability curves for the indicated cells at 24 h post-erastin treatment. Error bars, ± s.d.
**Supplemental Figure 5**

Synergy score for co-treatment with APR-246 and erastin in the indicated AML cell lines (24 hours). The mean AML cell line viability of three independent experiments was used. One experiment was used for primary AML cells.

**Supplemental Figure 6**

(a) Immunoblotting analysis of SLC7A11, GPX4, and TP53 protein level in AML cell lines. b-actin was used as a loading control (n=2).

(b) Correlation analysis between SLC7A11 or GXP4 protein level and APR-246 IC50. Statistics: Pearson index. *p < 0.05, **p < 0.01, ***p < 0.0001.

**Supplemental Figure 7**

(a) Immunoblotting analysis of GPX protein levels in MOLM14 and OCI-AML2 cells with or without shRNA targeting GPX4 (Day 3 post-doxycycline induction). b-actin was used as a loading control (n=2).

(b) Cell death (%) for the indicated cell types at day 2-3-4 h post-doxycycline treatment (n=3). Error bars, ± s.d.

**Supplemental Figure 8**

Viability curves for the indicated cells and compounds at 24 h post-treatment. Error bars, ± s.d.

**Supplemental Figure 9**

Synergy score for co-treatment with APR-246 and RSL3 in the indicated AML cell lines (H24). The mean cell viability of three independent experiments was used.

**Supplemental Figure 10**

Synergy score for co-treatment with APR-246 and FINO-2 for the indicated AML cell lines (H24). The mean cell viability of three independent experiments was used.

**Supplemental Table 1**

Primary AML sample characteristics.

**Supplemental Table 2**

AML cell lines characteristics.
**Supplemental Figure 1**

(a) Graph showing the percentage of cell death (PI) for different treatments:
- Vehicle
- Fer1 (10 µM)
- Nec1 (20 µM)
- Nec1S (20 µM)
- NSA (2 µM)

(b) Western blot analysis showing levels of pMLKL (S358), MLKL, and Actin in MOLM14 and OCI2 cells treated with Vehicle or APR-246 (60 µM).
Supplemental Figure 2

(a) Western blot analysis showing the effect of APR246 and puromycin on cell death markers in NB4 H16 and NB4 H14 cells.

(b) Graphs showing the percentage of cell death and % cells C11 Bodipy + in NB4 H16 and NB4 H14 cells treated with different conditions.

Markers:
- PARP: 116 kDa, 89 kDa
- Caspase 8: 57 kDa, 43 kDa, Cleaved Caspase 8
- Caspase 3: 35 kDa, Cleaved Caspase 3
- Actin: 19 kDa, 17 kDa

Conditions:
- Vehicle
- APR246 60 µM
- Puromycin

Cell lines:
- NB4 H16
- NB4 H14

Graphs display the percentage of cell death and % cells C11 Bodipy + for each condition.
Supplemental Figure 2-C

Vehicle
APR-246 60 µM H24
APR-246 60 µM + fer1 H24
APR 60 µM + Nec1 + fer1 H24
APR 60 µM + Nec1s + fer1 H24
APR 60 µM + NSA + fer1 H24
Puromycin

Full length PARP
PARP
Caspase 8
Cleaved Caspase 8
Caspase 3
Cleaved Caspase 3
Actin

% viable cells

[Bar chart showing % viable cells for different treatments]

Vehicle
APR-246 60 µM
APR-246 + Fer1
Puromycin 1 mg/mL
Supplemental Figure 2-
Supplemental Figure 5-
Supplemental Figure 6-
Supplemental Figure 7-
**Supplemental Figure 8**

The figure shows the concentration-response curves for RSL3 and FINO2 for different cell lines.

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Supplemental Figure 9
Supplemental Figure 10-
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