Targeting 6-phosphogluconate dehydrogenase in the oxidative PPP sensitizes leukemia cells to anti-malarial agent dihydroartemisinin

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Abstract

The oxidative pentose phosphate pathway (PPP) is crucial for cancer cell metabolism and tumor growth. We recently reported that targeting a key oxidative PPP enzyme, 6-phosphogluconate dehydrogenase (6PGD), using our novel small molecule 6PGD inhibitors Physcion and its derivative S3, shows anti-cancer effects. Notably, humans with genetic deficiency of either 6PGD or another oxidative PPP enzyme, glucose-6-phosphate dehydrogenase (G6PD), exhibit non-immune hemolytic anemia upon exposure to aspirin and various anti-malarial drugs. Inspired by these clinical observations, we examined the anti-cancer potential of combined treatment with 6PGD inhibitors and anti-malarial drugs. We found that stable knockdown of 6PGD sensitizes leukemia cells to anti-malarial agent dihydroartemisinin (DHA). Combined treatment with DHA and Physcion activates AMP-activated protein kinase, leading to synergistic inhibition of human leukemia cell viability. Moreover, our combined therapy synergistically attenuates tumor growth in xenograft nude mice injected with human K562 leukemia cells and cell viability of primary leukemia cells from human patients, but shows minimal toxicity to normal hematopoietic cells in mice as well as red blood cells and mononucleocytes from healthy human donors. Our findings reveal the potential for combined therapy using optimized doses of Physcion and DHA as a novel anti-leukemia treatment without inducing hemolysis.

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Conflict of interest

The authors declare no conflict of interest.

Keywords

Cancer metabolism; hemolytic anemia; 6-phosphogluconate dehydrogenase; anti-malarial agent; leukemia cell proliferation

Introduction

G6PD and 6PGD are the first and third enzymes of the oxidative PPP, which links glycolysis to anabolic biosynthesis and produces NADPH to regulate redox status in mammalian cells. Inhibition of G6PD results in attenuated cell growth as well as H_2O_2-mediated cell death, likely due to lack of reducing power in the form of NADPH [1]. We recently reported that 6PGD coordinates anabolic biosynthesis and redox homeostasis in cancer cells, providing a proliferative advantage to tumor cells [2]. Furthermore, we identified and developed novel, selective small molecule 6PGD inhibitors, Physcion and its derivative S3, which demonstrate promising efficacy and minimal non-specific toxicity in inhibition of cancer cell proliferation and tumor growth *in vitro* and *in vivo*, respectively, as well as in treatment of human primary leukemia cells from leukemia patients [2]. G6PD deficiency is the most common human enzyme defect, affecting more than 400 million people worldwide [3]. Individuals with G6PD deficiency exhibit non-immune hemolytic anemia with acute hemolysis, characterized by abnormal red blood cell (RBC) breakdown in response to infection, consumption of broad beans, or exposure to certain medications including aspirin and most anti-malarial drugs. In addition, 6PGD deficiency has recently been identified in a Spanish family and an Italian family, with afflicted individuals exhibiting chronic hemolytic anemia [4, 5]. However, little is understood regarding the molecular mechanisms underlying the chemical induction of hemolytic anemia in 6PGD-deficient patients. Inspired by these clinical observations, together with the notion that like RBCs, tumor/leukemia cells are highly glycolytic, we tested the hypothesis that there may exist an appropriate dosing window within which combined treatment with our novel, selective 6PGD inhibitors [2] and anti-malarial drugs may result in synergistic inhibition of leukemia cell proliferation without perturbing the viability of RBCs and inducing hemolytic anemia.

Results

Targeting 6PGD and G6PD sensitizes human leukemia cells to anti-malarial agent DHA

We began by testing combined treatment with Physcion + DHA in BCR-ABL positive K562 human leukemia cells. Physcion selectively inhibits 6PGD but not G6PD (Figure 1A) and attenuates K562 cell proliferation in a dose dependent manner (Figure 1B). We chose to focus on anti-malarial agent dihydroartemisinin (DHA) due to its high therapeutic efficacy and safety profile in humans, which also inhibits K562 cell proliferation in a dose dependent manner (Figure 1C). We first determined whether deficiency of 6PGD or G6PD in K562 cells would increase sensitivity to DHA treatment. Indeed, we found that stable knockdown of 6PGD and G6PD (Figure 1D) results in decreased 6PGD and G6PD enzyme activity, respectively (*left panels*; Figure 1E) and reduced oxidative PPP flux rate (*right panels*; Figure 1E), as well as increased sensitivity to treatment with DHA (Figure 1F and Supplemental Figure 3A, respectively), compared to vector control cells. In consonance with

these results, combined treatment with Physcion + DHA results in synergistic inhibition of K562 cell proliferation (Figure 2A) and induces synergistic apoptosis in K562 cells (Figure 2B). Moreover, Physcion + DHA treatment also results in synergistic inhibition of cell proliferation of diverse human leukemia cells including KG1a, Molm14 and HEL cells (Figure 2C). Together these results suggest that combined treatment with Physcion + DHA may represent a common anti-leukemia treatment strategy.

Combined treatment with 6PGD inhibitor Physcion + DHA synergistically inhibits human leukemia cells through AMPK activation

We next explored the molecular mechanisms by which the combined therapy synergistically inhibits leukemia cell proliferation. RBCs predominantly rely on glycolysis for carbohydrate metabolism, similar to cancer cells, and the oxidative PPP is the only pathway for RBCs to produce NADPH. The current explanation of the chemical induction of hemolytic anemia in G6PD or 6PGD-deficient patients suggest that defects in the oxidative PPP due to G6PD or 6PGD-deficiency results in decreased NADPH production and subsequently insufficient reductive power to maintain appropriate redox homeostasis; this in turn sensitizes RBCs to chemically induced oxidative damage by medications such as aspirin and anti-malarials, which ultimately has profound effects on erythrocyte viability [3]. However, we found that, although Physcion + DHA treatment causes a significant increase in ROS level in K562 cells compared to cells treated with single agents (Figure 3A), which is reversed by treatment with an antioxidant agent N-acetylcysteine (NAC) (Figure 3B), NAC does not rescue reduced K562 cell viability by Physcion + DHA (Figure 3C). In addition, we also tested another usual suspect, cyclooxygenase-2 (COX2), since both DHA and chloroquine have been reported to inhibit gene expression of COX2 in cancer cells [6, 7] and COX2 is expressed in most cancer cells with gradually increased levels during malignant development. However, Physcion + DHA treatment did not significantly alter the mRNA and protein expression levels of COX2 in K562 cells (Figures 3D), while combined treatment with Physcion and COX2 inhibitor Celecoxib did not result in synergistic inhibition of K562 cell proliferation (Figure 3E). These results suggest that combined treatment with Physcion + DHA does not inhibit leukemia cell viability by inducing oxidative damage or inhibiting COX2 expression or activity.

We next sought to test whether Physcion + DHA treatment signals through AMPK. AMPK is a master regulator of cellular energy homeostasis and is activated in response to stresses that deplete cellular ATP supplies such as low glucose and hypoxia. AMPK activation may lead to autophagy or apoptosis, depending on the environmental nutrient availability. Anti-cancer effects of AMPK activation have been well documented [8] and AMPK activators including A-769662 have been shown to be effective as anti-cancer agents in a number of cancer cell types, including human prostate cancer cells [9] and human breast cancer cells [10, 11]. Recently, both aspirin and chloroquine treatments were reported to promote phosphorylation and activation of AMPK [10, 11]. We also reported that 6PGD regulates the intracellular level of its product Ru-5-P to inhibit LKB-AMPK signaling, and treatment with 6PGD inhibitor Physcion results in activation of AMPK [2]. Indeed, we found that treatment with Physcion + DHA effectively activates AMPK as assessed by increased T172 phosphorylation level of AMPK. Furthermore, we also found that NAC treatment does not

abrogate the synergistic activation of AMPK seen in Physcion + DHA treated cells (Supplemental Figure 1). ACC1 plays a key role in fatty acid synthesis, where its major function is to synthesize malonyl-CoA as a substrate for *de novo* lipid synthesis, a critical metabolic process for proliferating cells. Phosphorylation of ACC1 at S79 by AMPK inhibits ACC1 enzyme activity [12], leading to decreased lipid biosynthesis and cell proliferation [13]. To further delineate the pathway through which Physcion + DHA signal to inhibit leukemia cell proliferation, we assessed phosphorylation of AMPK substrate acetyl-CoA carboxylase 1 (ACC1). As expected, we found that phosphorylation of ACC1 follows the activation pattern of AMPK (Figure 4A), leading to attenuated lipid synthesis rate after Physcion + DHA treatment (Figure 4B). Conversely, we found that treatment with AMPK inhibitor Compound C (Figure 4C) or inhibition of AMPK by shRNA-mediated knockdown (Figure 4D) effectively rescues decreased cell viability upon combined treatment with Physcion + DHA. In addition, combined treatment with Physcion and AMPK activator A769662 [14–16] results in synergistic inhibition of cell viability (Figure 5A) and induction of apoptosis in K562 cells (Figure 5B), as well as inhibition of cell viability of diverse leukemia cells including KG1a, HEL and Molm14 (Figure 5C). Furthermore, we previously demonstrated that 6PGD-mediated production of ribulose-5-phosphate (Ru-5-P) inhibits AMPK activation by disrupting the active LKB1 complex [2]. In consonance with this, we found decreased Ru-5-P level as well as increased LKB1 kinase activity in K562 cells with combined drug treatment (Supplemental Figure 2A). In addition, in a control experiment, DHA + Physcion treatment of cells with stable knockdown of CaMKK2, an alternative upstream kinase of AMPK, display increased phosphorylation of AMPK and ACC1, while increased phosphorylation was predominantly abolished in LKB1 knockout cells treated with Physcion + DHA (Supplemental Figure 2B–2C). Taken together, our data suggest that LKB1 is the relevant upstream activator of AMPK with combined drug treatment and the effect of Physcion + DHA is predominantly mediated through Ru-5-P dependent regulation of LKB1 in cells.

Combined treatment with 6PGD inhibitor and DHA inhibits tumor growth of human leukemia cells in xenograft nude mice

We previously showed that treatment with Physcion derivative S3 shows minimal toxicity to human cells *in vitro* and is well tolerated with minimal toxicity in nude mice (20mg/kg/day for 30 days), and that 20mg/kg/day was efficacious to inhibit tumor growth in xenograft nude mice injected with human cancer cells including K562 leukemia cells [2]. However, the principal concern in considering the feasibility of Physcion + DHA combination treatment as an anti-cancer therapy is its potential to induce hemolysis at the whole organism level. To address this concern, we performed chronic toxicity studies by injecting S3 + DHA into nude mice for 30 days. We found that 5 mg/kg/day S3 + 2.5 mg/kg/day DHA administered intraperitoneally is a well-tolerated dose, and did not significantly alter body weight (data not shown) of nude mice. Importantly, we observed that this dose of S3 + DHA did not significantly affect the hematopoietic properties of nude mice, and that both hemoglobin (Hb) and RBC levels fell within the normal range, suggesting no evidence of RBC damage (Table 1). These results demonstrate that S3 + DHA combination treatment at such doses has minimal toxicity *in vivo*. To determine whether such doses of S3 and DHA are sufficient to inhibit tumor growth *in vivo*, we next performed *in vivo* drug treatment using a xenograft

model in which nude mice were subcutaneously injected with K562 cells on the right flank. Three days post-injection, mice were randomly divided into four groups and treated with DMSO, S3 (5 mg/kg/day), DHA (2.5 mg/kg/day), or S3 + DHA (5 mg/kg/day + 2.5 mg/kg/day) for 15 days. We found that S3 + DHA treatment did not cause any noticeable damage to diverse organs and tissues, nor to the overall morphology or composition of the bone marrow (Figure 6A), but resulted in significantly decreased K562 leukemia cell-derived tumor growth (Figure 6B) and masses (Figure 6C–6D) compared to tumors derived from mice treated with DMSO, S3, or DHA alone. Moreover, tumor cell lysates from S3 + DHA treated mice demonstrated increased levels of phospho-AMPK compared to DMSO, S3 or DHA treated mice (Figure 6E), suggesting that S3 + DHA activates AMPK to inhibit tumor growth *in vivo*.

Combination therapy inhibits cell viability of primary human leukemia cells with minimal toxicity

Lastly, we found that Physcion + DHA treatment significantly inhibited viability of primary leukemia cells from representative patients with diverse types and subtypes of leukemia (Figure 7A), including the acute promyelocytic leukemia (APL) subtype of AML, AML, and CML. Moreover, Physcion + DHA treatment resulted in increased levels of phospho-AMPK compared to DMSO, Physcion or DHA treated primary leukemia cells from a representative AML patient (Figure 7B). In contrast, despite increased AMPK phosphorylation induced by Physcion + DHA treatment in mononucleocytes (WBC) isolated from the PB of healthy human donors (Supplemental Figure 4A–4B), neither drug alone nor the combination affected AMPK phosphorylation in RBC cells (Supplemental Figure 4C) or cell viability of WBC or RBCs isolated from the PB of healthy human donors (Figure 7C and Supplemental Figure 4D–4E), suggesting promising anti-leukemia potential with minimal toxicity to human blood cells.

Discussion

Taken together, inspired by the clinical observations in hemolytic anemia patients with G6PD or 6PGD-deficiency, we developed a novel therapeutic strategy and provide "proof of principle" to suggest that combining 6PGD inhibitors with anti-malarial agent DHA is a promising anti-leukemia therapy. Most importantly, although deficiency of oxidative PPP enzymes G6PD and 6PGD renders red blood cells in human patients sensitive to treatment with anti-malarial agents, we propose that because leukemia cells might be rewired by leukemogenic signals to become "addicted" to certain signaling and metabolic processes such as attenuated LKB1-AMPK pathway, we are able to determine a dosing window in which we can inhibit 6PGD at a concentration that renders leukemia cells, but not normal cells, sensitive to anti-malarial treatment, which synergistically activates LKB-AMPK signaling. This is supported by the results showing that, although Physcion + DHA treatment resulted in increased AMPK phosphorylation in both normal WBCs from healthy donors and primary leukemia cells from patients, only the primary leukemia cells responded to the combined treatment with reduced cell viability (Figure 7 and Supplemental Figure 4).

Our studies also suggest that G6PD and 6GPD, although both similarly implicated in hemolytic anemia, signal differentially in leukemia cells to promote cell viability. We found that, while G6PD and 6PGD deficient cells were similarly sensitive to DHA treatment (Supplementary Fig. 3A and Figure 1F), the decreased cell viability in G6PD deficient cells could not be rescued by AMPK inhibition (Supplementary Fig. 3B–C). Together, these findings demonstrate that, unlike 6PGD, sensitivity to DHA in G6PD knockdown cells may be regulated in an AMPK-independent manner. These data are also consistent with our previous work which shows that AMPK phosphorylation as well as cell proliferation rate remain unaltered in G6PD knock-down cells ([2], suppl. Figure 8c). Moreover, these results suggest differential molecular mechanisms underlying the sensitivity of G6PD-deficient hemalytic anemia patients to DHA or other anti-malarial agents compared to 6PGD-deficient patients.

Further studies are warranted to fully decipher the mechanisms underlying distinct enzymes in the oxidative PPP in cancer cell proliferation and survival, and to explore the potential involvement of AMPK activation in hemolysis induced by food (fava beans) and chemicals (aspirin and many anti-malarial agents) in individuals with genetic deficiency of 6PGD, which may inform improved treatment strategies for these patients.

Materials and methods

Cell lines

All cell lines were originally obtained from American Type Culture Collection (ATCC) and their identity was authenticated by Short Tandem Repeat (STR) profiling.

Reagents

Stable KD of endogenous h6PGD was achieved using lentiviral vector harboring shRNA construct (Open Biosystems; 5'-CCGGGTGGATGATTTCATCGAGAAACTCGAGTTTCTCGATGAAATCATCCACTTTT T -3'). The shRNA is designed to target the 3' non-coding region of h6PGD-A mRNA and shows no effect on the plasmid directed expression of 6PGD cDNA in cells. Stable KD of endogenous hG6PD was achieved using lentiviral vector harboring shRNA construct (Open Biosystems; 5'-CCGGCCCTGAAGTGACTGAGACAATCTCGAGATTGTCTCAGTCACTTCAGGGTTT TT-3'). Stable KD of endogenous hAMPK was achieved using lentiviral vector harboring shRNA construct (Open Biosystems; 5'-CCGGGCATAATAAGTCACAGCCAAACTCGAGTTTGGCTGTGACTTATTATGCTTTT T -3'). Antibodies against AMPK (cat# 5831), phospho-AMPK (T172) (cat# 2535), ACC1 (cat# 3662), and phospho-ACC1 (S79) (cat# 11818), G6PD (cat# 12263), and LKB1 (cat# 3050) were from Cell Signaling Technology (CST); antibody against 6PGD was from Novus (cat# NBP1-31589); antibody against β-actin was from Sigma (cat# A1978); prediluted Ki67 antibody was from Invitrogen (cat# 08-0156). Physcion was purchased from Santa Cruz Biotechnology. 1-hydroxy-8-methoxy-anthraquinone (S3) was purchased Sigma. DHEA was purchased from Calbiochem. DHA was purchased from TCI America. A769662 was purchased from LC Laboratories. Compound C was purchased from EMD Millipore.

Cell culture

K562, HEL, KG1a, and Molm14 cells were cultured in RPMI 1640 medium with 10% FBS and penicillin/streptomycin. K562 6PGD stable KD cells were cultured in the presence of 6μg/mL puromycin. K562 G6PD and AMPK stable KD cells were cultured in the presence of 2μg/mL puromycin.

G6PD and 6PGD assays

Purified 6PGD and G6PD proteins were treated with increasing concentrations of Physcion or vehicle (DMSO) for 4 hours, followed by enzyme activity assays. G6PD activity was determined by the NADPH production rate from G6PD and 6PGD, then subtracting that of 6PGD, since a product of G6PD, 6-phosphogluconolactone, is rapidly hydrolyzed to a substrate of 6PGD, 6-phosphogluconate, in cells. To obtain the combined dehydrogenase activity, substrates for both dehydrogenase enzymes were added to a cuvette. In another cuvette, substrates for the second enzyme, 6PGD, were added to obtain the activity of this enzyme. Substrate concentrations were as follows: 0.2 mM glucose 6-phosphate, 0.2 mM 6-phosphogluconate, and 0.1 mM NADP$^+$. 10 μg of cell lysates were added to a cuvette containing buffer (50 mM Tris/1 mM MgCl$_2$, pH 8.1) and the reaction was then initiated by NADP$^+$. The increase in 341 nm absorbance (OD$_{340}$) as a measure of NADPH production was measured every 20 seconds for 10 minutes on a DU800 Spectrophotometer (Beckman Coulter).

Oxidative PPP flux assay using $^{14}CO_2$ Release

Cells were seeded on 6-cm dishes that are placed in a 10-cm dish with 2 sealed pinholes on the top. $^{14}CO_2$ released from cells was collected by completely sealing the 10-cm dish, in which the cells on the 6-cm dish were incubated in 2 mL of medium containing [1–^{14}C]- or [6–^{14}C]-glucose, respectively, at a final specific activity of 10 μCi/mL glucose at 37 °C for 3 h. The oxidative PPP flux was stopped by injecting 0.3 mL of 50% TCA through one of the holes on the top, and at the same time $^{14}CO_2$ released was trapped by injecting 0.3 mL of Hyamine Hydroxide into a small cup placed on the 10-cm dish through the second hole. Krebs cycle measurements, obtained in parallel samples incubated with [6–^{14}C]-glucose, were used to correct the oxidative PPP flux measurements obtained from samples incubated with [1–^{14}C]-glucose. Each dish was completely re-sealed with parafilm and incubated overnight at room temperature. Hyamine Hydroxide in the small cup was dissolved into 60% methanol and directly subjected to scintillation counting.

Cell viability assays

0.5×10^6 cells were seeded in 6-well plates and incubated with indicated drug concentrations at 37°C for 36–48 hours. Relative cell viability was determined by trypan blue exclusion using a TC10 Automated Cell Counter (BioRad). Combination index (CI) values were calculated using ComboSyn program.

Apoptosis assay

1×10^5 cells were treated with indicated drug concentrations for 24 hours, then collected and stained using FITC-conjugated annexin V labeling reagent (BD Pharmingen) and PI (Sigma) followed by FACS analysis for apoptotic cell population.

Intracellular reactive oxygen species (ROS) production

The amount of intracellular ROS was measured by detecting dichlorodihydrofluorescein, which is the cleavage product of carboxy-H_2DCFDA (Invitrogen) by ROS. 1×10^5 cells were seeded in 6-well plate and treated with drug at indicated concentrations. Two hours after treatment, cells were washed with PBS and loaded with 5μM carboxy-H_2DCFDA for 30 min. The cells were harvested, resuspended in PBS and analyzed using a FACS (BD Biosciences; excitation and emission at 490 and 530 nm, respectively).

Lipid synthesis assay

Cells were pre-incubated in complete growth medium in the absence of D-(14C-U)-Glucose for 2 hours. Lipids were extracted by the addition of 500μl of hexane/isopropanol (3:2 v/v), air dried, and resuspended in 50 μl of chloroform and subjected to scintillation counting.

Complete blood counts

For complete blood counts, blood was collected retro-orbitally and immediately applied to a HemaVet 950FS (Drew Scientific) for generation of complete hematology profile.

Primary tissue samples from patients with leukemia and healthy donors

Approval of use of human specimens was given by the Institutional Review Board of Emory University School of Medicine. All clinical samples were obtained with informed consent with approval by the Emory University Institutional Review Board. Clinical information for the patients was obtained from the pathologic files at Emory University Hospital under the guidelines and with approval from the Institutional Review Board of Emory University School of Medicine and according to the Health Insurance Portability and Accountability Act. Only samples from patients that were not previously treated with chemotherapy or radiation therapy were used. Mononuclear cells and RBCs were isolated from PB and bone marrow samples from patients with leukemia or from healthy donors using lymphocyte separation medium (Cellgro). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin and incubated with indicated drug concentrations for 48 hours.

Xenograft studies

Approval of use of mice and designed experiments was given by the Institutional Animal Care and Use Committee of Emory University. Nude mice (nu/nu, female 4–6-week-old, Harlan Laboratories) were subcutaneously injected with 5×10^6 K562 cells on the *right* flank. 3 days after injection, mice were randomly divided into four treatment groups. Treatment was administered by daily intraperitoneal injection (S3: 5 mg/kg/day; DHA: 2.5 mg/kg/day; S3 + DHA: 5 + 2.5 mg/kg/day). Tumor growth was recorded by measurement of two perpendicular diameters using the formula $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$. The tumors

were harvested and weighed at the experimental endpoint, and the masses of tumors (g) treated with vehicle control (DMSO) and drug treated groups were compared by two-way ANOVA.

Statistics

In studies in which statistical analyses were performed, a 2-tailed Student's t test was used to generate P values. P values less than or equal to 0.05 were considered significant. A two-way ANOVA was used for tumor volume and tumor weight. Combination indexes were calculated using CalcuSyn software, where CI=1 indicates an additive effect, CI < 1 indicates synergism, and CI > 1 indicates antagonism in drug combinations.

Study Approval

Approval of use of human specimens was given by the Institutional Review Board (IRB) of Emory University School of Medicine, and participants were given informed consent in accordance with IRB policy.

Reproducibility of experiments

The results of one representative experiment from at least two independent experiments are shown except data obtained from primary patient samples and animal experiments shown in Figs 6 and 7. There is no estimate of variation in each group of data and the variance is similar between the groups. No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All data are expected to have normal distribution.

Supplemental materials and methods

CRISPR-mediated gene knock out, LKB1 *in vitro* kinase assay, intracellular Ru-5-P measurement are described in "Expanded material and methods" of supplementary information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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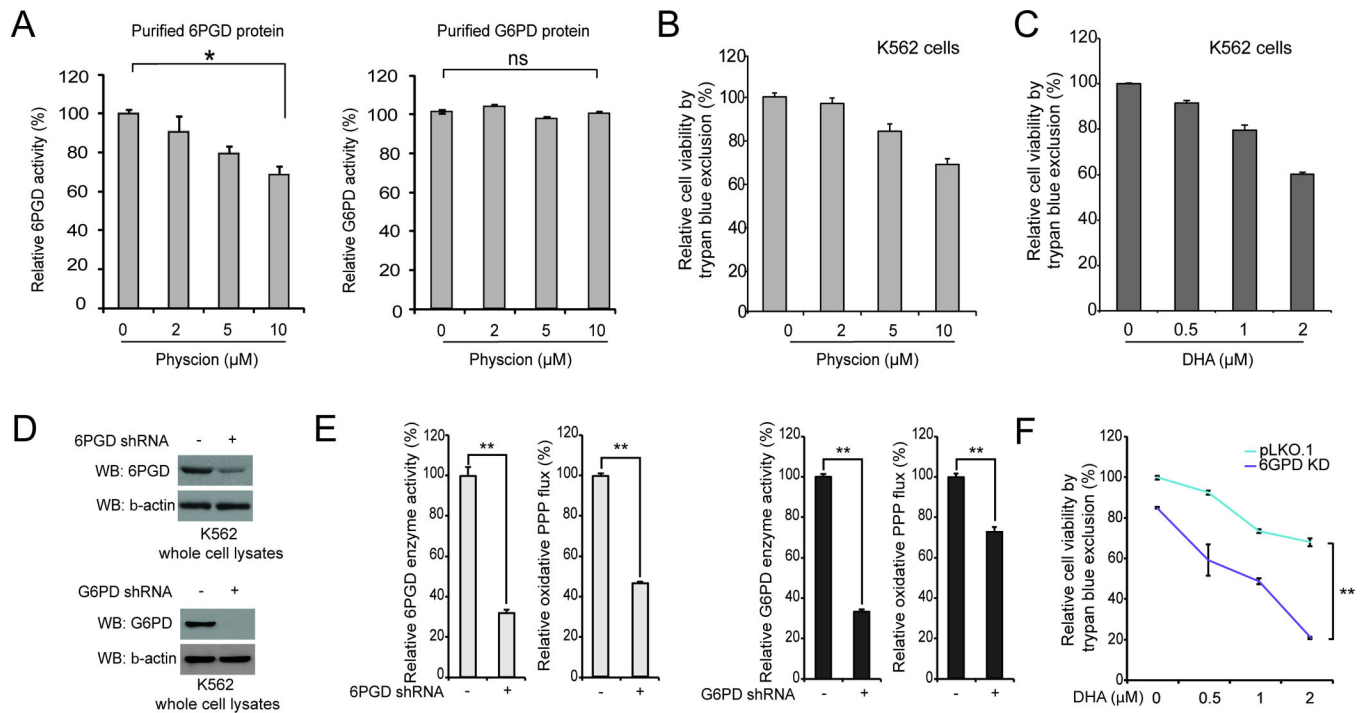
Figure 1. Knockdown of G6PD or 6PGD sensitizes human K562 leukemia cells to anti-malarial agent DHA

(**A**) Purified 6PGD (*left*) and G6PD (*right*) are assayed for 6PGD and G6PD enzyme activity, respectively, in the presence of increasing concentrations of Physcion. (**B**) K562 cells were treated with increasing concentrations of Physcion for 36 hours, followed by cell viability assay assessed by cell numbers. (**C**) K562 cells were treated with increasing concentrations of DHA for 36 hours, followed by cell viability assay assessed by cell numbers. (**D**) Results of Western blot experiments detecting 6PGD (*upper*) and G6PD (*lower*) protein levels in 6PGD (*upper*) and G6PD (*lower*) stable knockdown cells, respectively. (**E**) 6PGD (*left*) or G6PD (*right*) stable knockdown K562 cells and control cells harboring an empty vector were tested for enzyme activity and oxidative PPP flux rate. (**F**) 6PGD stable knockdown K562 cells and control vector cells were treated with increasing concentrations of DHA for 36 hours, followed by cell viability assay. The error bars represent mean values ±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (*$0.01 < p < 0.05$; (**$0.001 < p < 0.01$; ns, not significant). The results of one representative experiment from at least two independent experiments are shown.
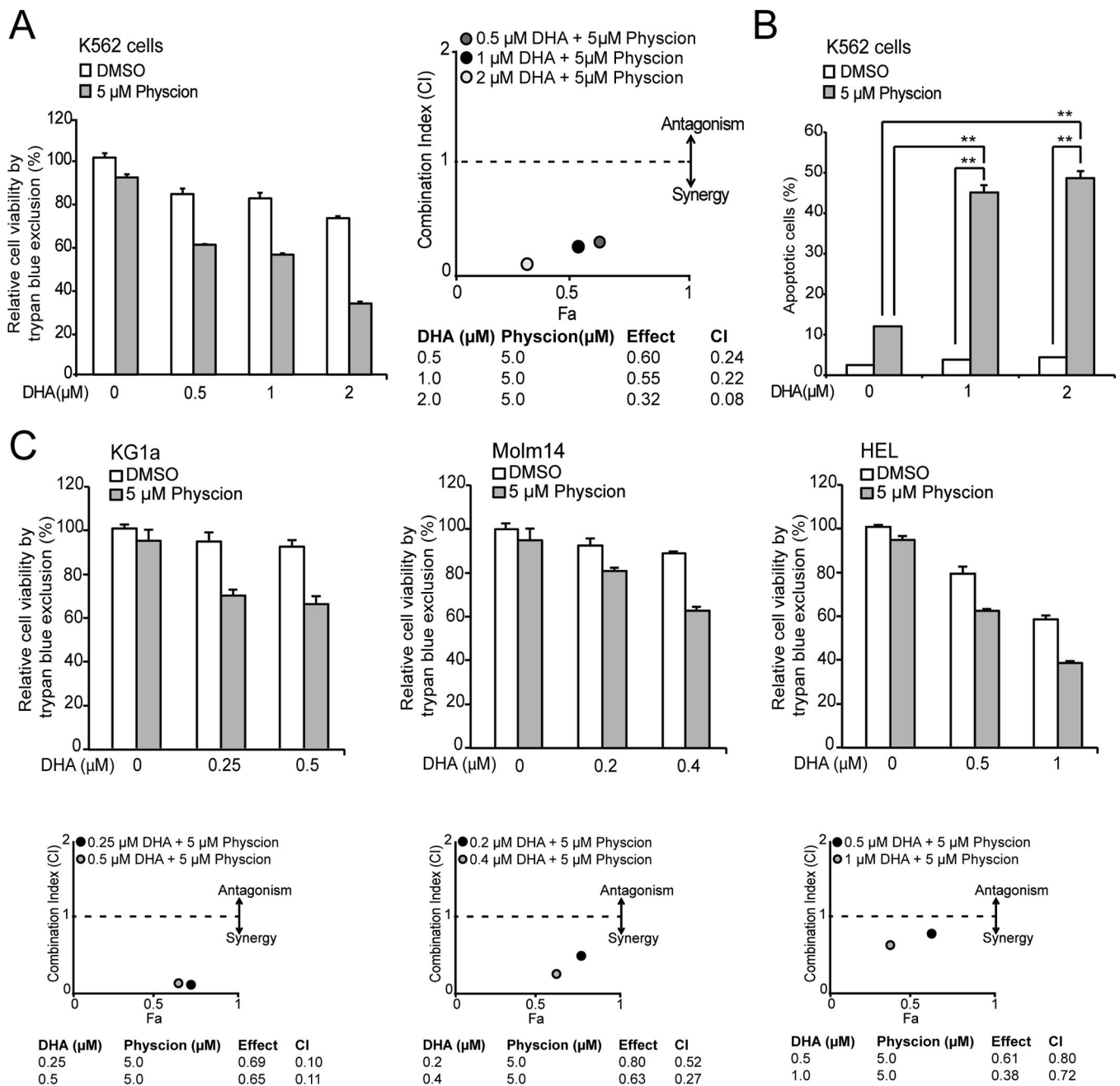
Figure 2. Treatment with 6PGD inhibitor Physcion with DHA results in synergistic inhibition of diverse human leukemia cells

(**A**) *Left*: K562 cells were treated with increasing concentrations of DHA in the presence or absence of Physcion for 36 hours, followed by cell viability assay. *Right*: The combination index (CI) plot of results shows the CI for combined treatment with Physcion + DHA in K562 cells. CI<1 denotes synergy. (**B**) K562 cells were treated with increasing concentrations of DHA in the presence or absence of Physcion for 24 hours, followed by apoptosis assay. The error bars represent mean values ±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (**0.001 < p < 0.01).
(**C**) *Upper panels*: KG1a (*left*), Molm14 (*middle*) and HEL (*right*) cells were treated with

increasing concentrations of DHA in the presence or absence of Physcion for 36 hours, followed by cell viability assay. *Lower panels*: The combination index plots show the CI for combined treatment with Physcion + DHA in KG1a (*left*), Molm14 (*middle*) and HEL (*right*) cells. CI<1 denotes synergy. The results of one representative experiment from at least two independent experiments are shown.
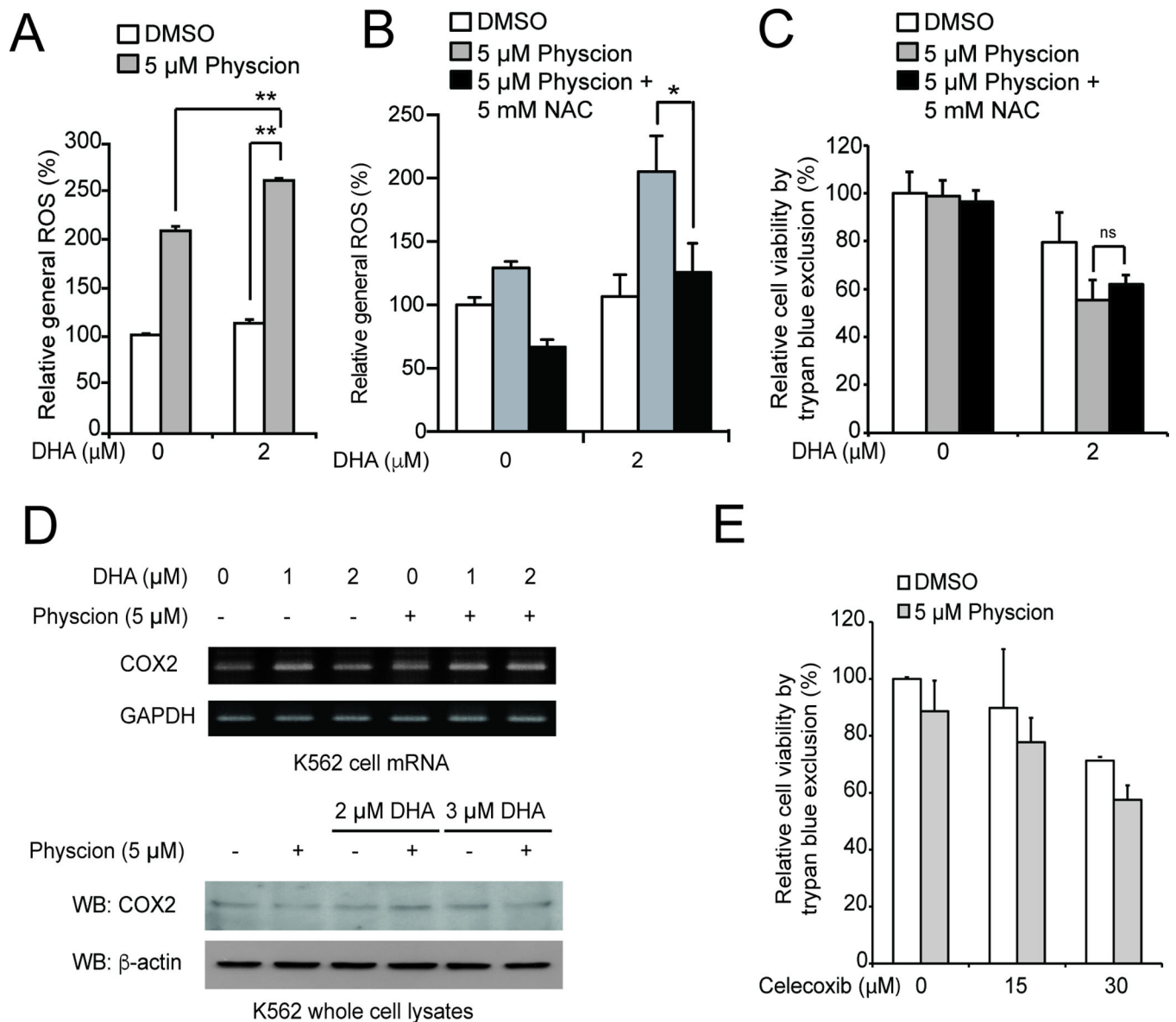
Figure 3. Combined treatment with Physcion + DHA does not signal through COX2

(**A**) K562 cells treated with or without DHA in the presence or absence of Physcion were tested for ROS levels. K562 cells treated with or without NAC for 36 hours, followed by ROS assay (**B**) or cell viability assay (**C**). (**D**) The mRNA (*upper*) and protein expression (*lower*) levels of COX2 in K562 cells treated with increasing concentrations of DHA in the presence or absence of Physcion were determined by RT-PCR and Western blot assays, respectively. (**E**) The effect of combined treatment with Physcion and COX2 inhibitor Celecoxib on K562 cell viability was examined. The error bars represent mean values ±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (*0.01 < p < 0.05; (**0.001 < p < 0.01; ns, not significant). The results of one representative experiment from at least two independent experiments are shown.
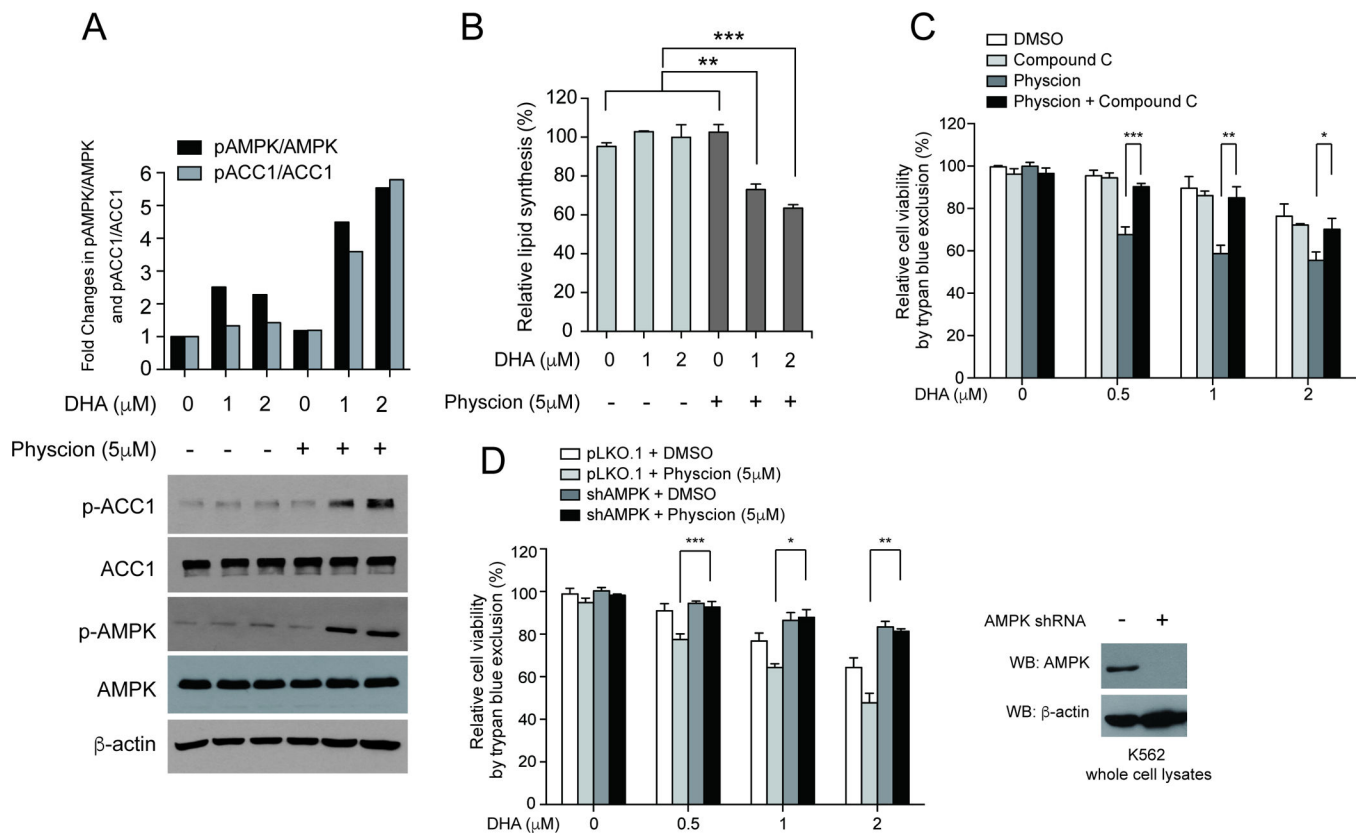
Figure 4. Combined treatment with Physcion + DHA activates AMPK
(**A–B**) K562 cells were treated with increasing concentrations of DHA in the presence or absence of Physcion for 4 hours, followed by (**A**) Western blot to detect protein expression and phosphorylation levels of both AMPK (T172) and downstream substrate ACC1 (S79); (**B**) Lipid biosynthesis assay to detect *de novo* lipogenesis. (**C**) K562 cells were treated with increasing concentrations of DHA in the presence or absence of Physcion alone or combined treatment with Physcion and AMPK inhibitor Compound C for 36 hours, followed by cell viability assay. (**D**) *Left*: AMPK stable knockdown K562 cells and control vector cells were treated with increasing concentrations of DHA in the presence or absence of Physcion for 36 hours, followed by cell viability assay. *Right*: Result of Western blot experiment detecting AMPK protein levels in AMPK stable knockdown cells compared to control vector cells. The error bars represent mean values ±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (*0.01 < p < 0.05; **0.001 < p < 0.01; ns, not significant). The results of one representative experiment from at least two independent experiments are shown.
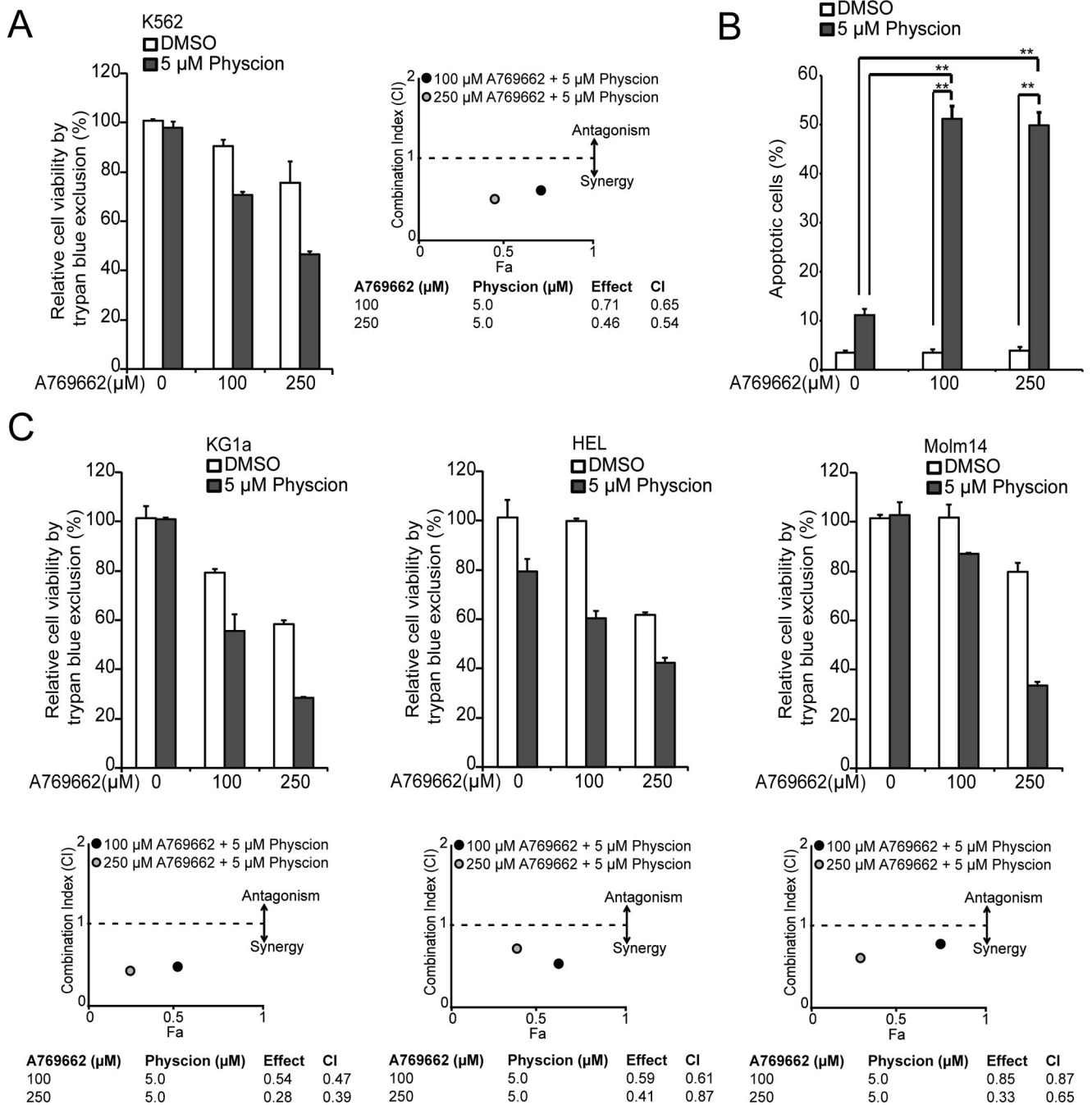
A

K562

☐DMSO
■ 5 μM Physcion

A769662 (μM)	Physcion (μM)	Effect	CI
100	5.0	0.71	0.65
250	5.0	0.46	0.54

B

☐DMSO
■ 5 μM Physcion

C

KG1a

☐DMSO
■ 5 μM Physcion

A769662 (μM)	Physcion (μM)	Effect	CI
100	5.0	0.54	0.47
250	5.0	0.28	0.39

HEL

☐DMSO
■ 5 μM Physcion

A769662 (μM)	Physcion (μM)	Effect	CI
100	5.0	0.59	0.61
250	5.0	0.41	0.87

Molm14

☐DMSO
■ 5 μM Physcion

A769662 (μM)	Physcion (μM)	Effect	CI
100	5.0	0.85	0.87
250	5.0	0.33	0.65

Figure 5. Combined treatment with Physcion and AMPK activator results in synergistic inhibition of diverse leukemia cells

(**A**) *Left*: K562 cells were treated with increasing concentrations of AMPK activator A769662 in the presence or absence of Physcion for 48 hours, followed by cell viability assay. The error bars represent mean values ±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (*0.01 < p < 0.05; (**0.001 < p < 0.01; ns, not significant). Right: The combination index plot shows the CI for combined treatment with Physcion and A769662 in K562 cells. CI<1 denotes synergy. (**B**)

K562 cells were treated with increasing concentrations of AMPK activator A769662 in the presence or absence of Physcion for 24 hours, followed by apoptosis assay. (**C**) *Upper panels*: KG1a (*left*), Molm14 (*middle*) and HEL (*right*) cells were treated with increasing concentrations of A769662 in the presence or absence of Physcion for 48 hours, followed by cell viability assay. *Lower panels*: The combination index plots show the CI for combined treatment with Physcion + DHA in KG1a (*left*), Molm14 (*middle*) and HEL (*right*) cells. CI<1 denotes synergy.
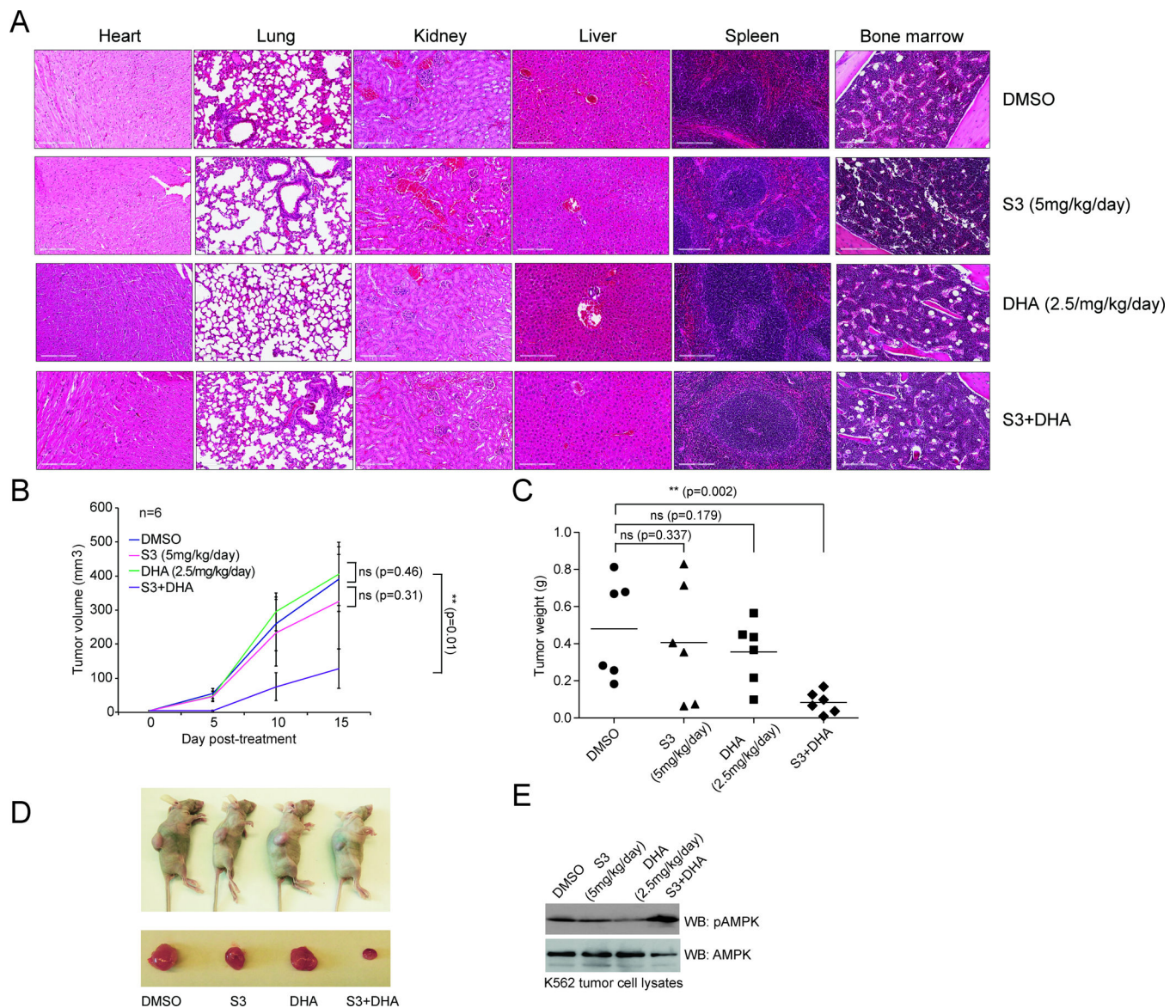
Figure 6. Combined treatment with S3 and DHA attenuates tumor growth in xenograft nude mice injected with K562 cells

(**A**) Histological morphology of hematoxylin-eosin stained tissue sections of representative K562 xenograft nude mice in control DMSO, single agents DHA or S3, and S3+DHA treated groups. Mice were treated with vehicle control, S3 alone (5 mg/kg/day), DHA alone (2.5 mg/kg/day), or S3+DHA for 15 days. The vital organs were collected for histopathological analysis. Histopathologic tissue sections (kidney, lung, liver, spleen, and bone marrow) from representative nude mice stained with hematoxylin-eosin did not reveal significant differences among control DMSO, single agents DHA or S3, and S3+DHA treated groups. Images were analyzed and captured using ImageScope software (Aperio Technologies Inc.) without any additional or subsequent image processing (high power images are 20×; low power images are 4×). Scale bars are indicated. (**B**) Tumor growth curve in xenograft nude mice injected with K562 cells were compared among the group of

mice treated with control DMSO, single agents DHA or S3, or S3+DHA. (**C**) Tumor size in xenograft nude mice injected with K562 cells were compared between groups of mice treated with control DMSO, single agent Physcion derivative S3 or DHA, and combined therapy using S3 and DHA (n=6/group). p values were determined by a two way ANOVA test (**0.001 < p < 0.01; ns, not significant). (**D**) Dissected tumors in representative nude mice treated with control DMSO, single agents DHA or S3, or S3+DHA. (**E**) Western blot results detecting protein expression and phosphorylation (T172) levels of AMPK in tumors harvested from K562 xenograft mice with distinct drug treatment is shown.
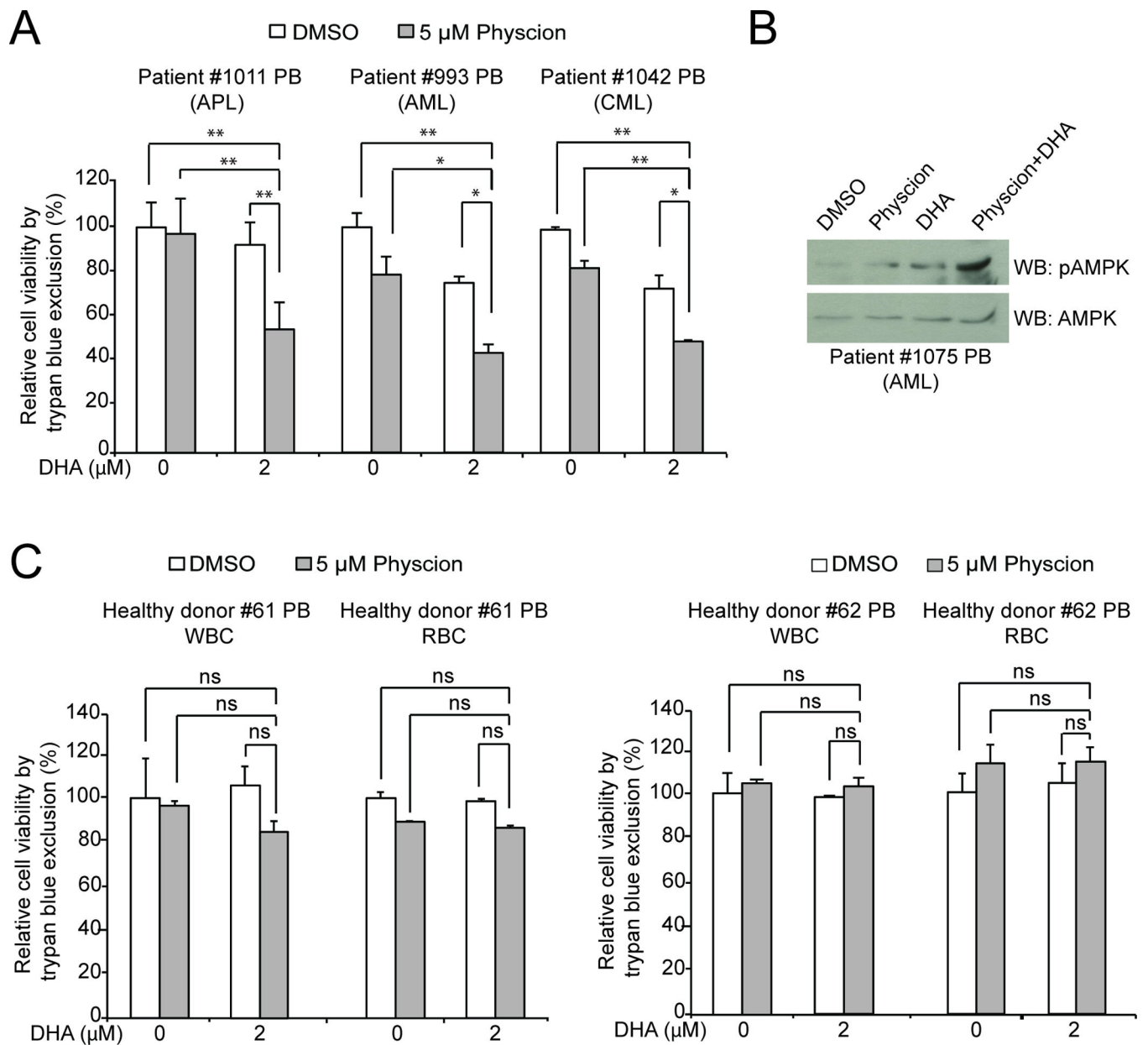
Figure 7. Combined treatment with Physcion + DHA inhibits primary human leukemia cells with minimal toxicity

(**A**) Effects of combined treatment with Physcion + DHA for 48 hours on cell viability were examined in human primary leukemia cells isolated from the PB of representative APL, AML and CML patients. (**B**) Effect of Physcion + DHA treatment on phosphorylation levels of AMPK (T172) was examined by Western blot using primary leukemia cells isolated from the PB of a representative AML patient. (**C**) Combined treatment with Physcion + DHA shows no toxicity in treatment (48 hours) of isolated mononucleocytes (*left panels*) and red blood cells (*right panels*) from peripheral blood samples of two representative healthy human donors (#61 on the left and #62 on the right). The error bars represent mean values

±SD from three technical replicates of each sample. p values were determined by two-tailed Student's *t* test (*0.01 < p < 0.05; (**0.001 < p < 0.01; ns, not significant).

Table 1

Athymic nude mouse hematology. Nude mice were treated with vehicle control, S3 alone (5 mg/kg/day), DHA alone (2.5 mg/kg/day), or S3+DHA for 30 days. CBC analysis shows no significant difference in the hematopoietic properties among the four groups of mice.

Test name	Normal Range	DMSO (mean)	5 mg/kg/day S3 (mean)	2.5 mg/kg/day DHA (mean)	S3+DHA (mean)
WBC (K/µL)	1.8–10.7	6.5±0.53	6.6±0.69	7.2±1.37	9.4±1.12
LYM (K/µL)	0.9–9.3	3.2±0.86	3.6±0.04	4.3±1.27	4.8±1.14
MONO (K/µL)	0.0–0.4	0.24±0.16	0.3±0.13	0.2±0.03	0.3±0.15
GRAN (K/µL)	0.1–2.4	2.8±0.81	2.6±0.84	2.6±0.54	4.1±0.23
HCT (%)	35.1–45.4	49.7±4.17	42.6±8.08	52.9±4.2	46.6±5.72
MCV (fl)	45.4–60.3	59.0±1.57	59.3±1.1	59.7±2.13	60.4±2.23
RDWa (%)	12.4–27.0	15.6±0.26	15.5±0.21	15.7±0.49	17.3±0.26
Hb (g/dL)	11.0–15.1	12.9±1.25	11.1±2.48	13.5±0.85	11.8±1.70
MCHC (g/dL)	30.2–34.2	26.1±0.62	25.8±1.04	25.5±0.52	25.3±1.50
RBC (M/mL)	6.36–9.42	8.4±0.50	7.2±1.27	8.9±0.92	7.7±0.67
MCH (pg)	14.1–19.3	15.4±0.52	15.3±0.89	15.2±0.67	15.3±1.00
PLT (K/µL)	592–2972	905.3±198	991.7±117	985±38.6	1120±488.09
MPV (fl)	5.0–20.0	5.5±0.26	5.7±0.26	5.2±0.06	6.2±0.72