Targeting intermediary metabolism enhances the efficacy of BH3 mimetic therapy in haematological malignancies

Running title: Modulation of intermediary metabolism in cancer therapy

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Abstract

BH3 mimetics are novel targeted drugs with remarkable specificity and potency and enormous potential to improve cancer therapy. However, acquired resistance is an emerging problem. We report the rapid development of resistance in chronic lymphocytic leukemia cells isolated from patients exposed to increasing doses of Navitoclax (ABT-263), a BH3 mimetic. To mimic such rapid development of chemoresistance, we have developed simple resistance models to three different BH3 mimetics, targeting BCL-2 (ABT-199), BCL-X_L (A-1331852) or MCL-1 (A-1210477), in relevant haematological cancer cell lines. In these models, resistance could be attributed neither to consistent changes in expression levels of the anti-apoptotic proteins nor interactions among different pro- and anti-apoptotic BCL-2 family members. Using genetic silencing, pharmacological inhibition and metabolic supplementation, we report that targeting of glutamine uptake and its downstream signalling pathways, namely glutaminolysis, reductive carboxylation, lipogenesis, cholesterogenesis and mTOR signalling result in marked sensitisation of the chemoresistant cells to BH3 mimetic-mediated apoptosis. Furthermore, our findings highlight the possibility of repurposing widely used drugs, such as statins, to target intermediary metabolism and improve the efficacy of BH3 mimetic therapy.

Introduction

Failure to undergo apoptosis is a cardinal feature of cancer and several targeted therapies, such as the small molecule inhibitors targeting specific members of the anti-apoptotic BCL-2 family - navitoclax/ ABT-263 (targeting BCL-2, BCL-X_L and BCL-w) and venetoclax/ ABT-199 (BCL-2 specific) - are aimed at facilitating cancer cell clearance by enhanced apoptosis.[1-4] Recently, selective inhibitors of BCL-X_L (A-1331852) and MCL-1 (A-1210477 and S63845) have also been synthesised.[5-7] Despite their selectivity in targeting distinct anti-apoptotic BCL-2 family members, and remarkable potency in inducing rapid and extensive apoptosis in a wide variety of malignancies, resistance to BH3 mimetics, in particular venetoclax, is starting to be reported in the clinic. Elevated levels of multiple members of the anti-apoptotic BCL-2 family proteins, including BCL-X_L and MCL-1, are often implicated in such chemoresistance.[8-13] Although it may be possible to target these proteins with a combination of selective BH3 mimetics, the potential toxicities associated with such combination therapy may be problematic.

Altered metabolism is a promising approach to enhance the efficacy of chemotherapeutic agents, as a requirement for intermediary metabolites, such as glucose and glutamine, for the survival and proliferation of cancer cells is well documented.[1,14-19] This is a promising approach, as drugs targeting different stages of intermediary metabolism are already approved or in trials for treating different malignancies.[20,21] In this study, we report a low level of resistance that developed in chronic lymphocytic leukemic (CLL) cells from patients exposed to Navitoclax. To mimic this modest resistance, we have developed simple resistance models to different BH3 mimetics and demonstrate that downregulating glutamine uptake or metabolism as well as its downstream signalling cascades, such as reductive carboxylation, lipogenesis and cholesterogenesis, result in enhanced apoptosis of cancer cells resistant to different BH3 mimetics, thus highlighting the possibility that inhibition of key regulatory enzymes of these metabolic pathways may enhance sensitivity to BH3 mimetic therapy.

Methods

Reagents and Antibodies

ABT-263, A-1331852 and A-1210477 were from AbbVie (North Chicago, IL, USA). ABT-199, epigallocatechin gallate (EGCG), CB-839, simvastatin, rapamycin and torin-1 from Selleck Chemicals (Houston, TX, USA), Gamma-L-glutamyl-p-nitroanilide (GPNA) from Insight Biotechnology (Wembley, Middlesex, UK), Azaserine from Cambridge Bioscience (Cambridge, UK), aminooxyacetate (AOA), sodium palmitate, filipin, dimethyl α-ketoglutarate, oxaloacetate and citrate from Sigma-Aldrich (Gillingham, UK), L-glutamine from Life Technologies (Paisley, UK) and GSK2194069, SB204990, atorvastatin, pitavastatin and bafilomycin A1 from Tocris (Abingdon, UK) were used. Antibodies against PARP, BCL-2, MCL-1, BAX, BAK and GAPDH from Santa Cruz Biotechnology (Santa Cruz, CA, USA), caspase-3, caspase-9, BCL-X_L, BCL-w, BIM, PUMA, BAD, IDH2, ACL, ACO2, ATG5 and ATG7 from Cell Signalling Technology (MA, USA), BID from Prof. J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands), NOXA from Millipore (Watford, UK) and SLC1A5, GLS, GFAT, GLUD1, IDH3, FASN and HMGR from Abcam (Cambridge, UK) were used.

Primary CLL cells and Cell lines

Peripheral blood samples from CLL patients were obtained with patient consent and ethics committee approval (06_Q2501_122) from Leicester Haematological Tissue Bank and cultured as described.[38] CLL samples were obtained from patients enrolled in a Phase 1/2a Study of ABT-263 (Navitoclax) in patients with relapsed or refractory CLL (NCT00481091). Lymphocytes were purified and cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) (Life Technologies Inc.). Alternatively, blood from patients was incubated at 37°C in 48-well plates and apoptosis assessed as described previously.[39,40] Blood samples were collected prior to the first *in vivo* dose of navitoclax or 4 h after dosing during the lead-in-period (L1D1; Day 1 of lead-in-period), or day 1 of cycle 1 (C1D1), cycle 3 (C3D1) or cycle 5 (C5D1). Samples were collected 4 h after dosing as blood concentrations of ABT-263 were maximal at this time.[41] For culture of CLL cells, mouse fibroblast L cells were irradiated with 75 Gray and seeded in 24-well plates (3 $x10^5$ cells/ well). CLL cells were cultured at 1.5 x 10^6 cells / well on the L cells and removed when required by gentle washing with RPMI before treatment. Mantle cell lymphoma (MAVER-1), chronic myeloid leukemia (K562) and multiple myeloma (NCI-H929) cell lines were cultured in RPMI 1640 medium but the medium was supplemented with 0.02% 2-mercaptoethanol for culturing H929 cells. Cell lines were either from DMSZ (Braunshweig, Germany) or ATCC (Middlesex, UK) and subjected to short tandem repeat (STR) profiling to confirm identity.

Resistance models

The different resistance models to relevant BH3 mimetics were developed by treating control cells (represented as A in all schemes) of MAVER-1, K562 and H929 to the relevant BH3 mimetics, ABT-199 (10 nM), A-1331852 (10 nM) or A-1210477 (5 µM), respectively. In the first resistance model, cells were exposed to their appropriate BH3 mimetic for 24 h followed by 2 weeks (2 w) without drug resulting in the cells depicted as B. These cells were further exposed to their appropriate BH3 mimetic for a further 24 h followed again by 2 weeks without drug resulting in C. This procedure was repeated a further twice resulting in E. In the second resistance model, cells were exposed to their appropriate BH3 mimetic for 24 h followed by 8 weeks without drug, resulting in the cells depicted as A4. The cells were collected every 2 weeks and labelled as A1, A2 and A3, respectively. In the third resistance model, cells were exposed to increasing concentrations of the appropriate BH3 mimetic, every 5 days (5 d) resulting in cells depicted as A-a, A-b, A-c and A-d. The fourth model of resistance was made in a similar manner, but the 5 d treatment period was split into a 2 d treatment, followed by 3 d without drug, resulting in cells depicted as A-i, A-ii, A-iii and A-iv.

Metabolic deprivation, supplementation and apoptosis measurements

For glutamine deprivation experiments, cells were washed with PBS and re-suspended in SILAC RPMI 1640 Flex Media (Life Technologies Inc.), supplemented with glucose (2 mg/mL) and 10% FBS, for 16 h. For supplementation studies, the indicated concentrations of metabolites were added to the glutamine free media, immediately before glutamine deprivation. For lipid supplementation studies, sodium palmitate (dissolved in water at 70 °C for a stock concentration of 100 mM and added dropwise into fatty acid-free BSA (10%) to produce a final concentration of 10 mM) was supplemented in the culture media. The extent of apoptosis in cells following different treatments was quantified by FACS following staining of the cells with Annexin V-FITC and propidium iodide to measure phosphatidylserine externalisation, as previously described.[42]

siRNA knockdowns, immunoprecipitation and western blotting

Cells were transfected with 10 nM of siRNAs against SLC1A5 (SI00079730), GLS (SI03155019), GFAT (SI03246355), GLUD1 (SI02654743), IDH2 (SI02654820), IDH3 (SI00300524), ACO2 (SI03019037), ACLY (SI02663332), FASN (SI00059752), HMGR (SI00017136), ATG5 (SI02633946) and ATG7 (SI04344830) from Qiagen Ltd. (Manchester, UK) using Interferin (Polyplus Transfection Inc, NY), according to the manufacturer's protocol and processed 72 h after transfection. Immunoprecipitation and western blotting were carried out according to standard protocols.[43]

Statistical Analysis

One-way ANOVA multiple comparisons and Fishers LSD test $P \leq 0.01$ were performed for comparing sensitive and resistant cells. For CLL patient samples, one-way repeated measures ANOVA with Fishers LSD test $P \leq 0.01$ was used and statistics analysed using GraphPad Prism 6 software (La Jolla, CA).

Results

Haematological malignancies acquire rapid resistance to BH3 mimetics

The potential of BH3 mimetic therapy in cancer was first demonstrated in the treatment of BCL-2-dependent chronic lymphocytic leukemia (CLL) using navitoclax/ ABT-263. In a phase 1/2 clinical trial of navitoclax, CLL patients were treated for an initial lead-in-period of 7 days with a low dose of navitoclax (100 mg daily) followed by 5-7 treatment cycles, with each cycle lasting 21 days during which the patients received 250 mg navitoclax daily. Analysis of the blood samples collected from these patients, either prior to the first *in vivo* dose of navitoclax or 4 h after dosing, during the different cycles of therapy revealed marked changes in the ability of navitoclax to induce apoptosis in the CLL cells (Fig. 1A). The first *in vivo* dose of navitoclax on day 1 of the lead-in period (L1D1) resulted in a time-dependent induction of apoptosis, as assessed by phosphatidylserine (PS) externalisation and ultrastructural changes, in comparison to the CLL cells from the same patients prior to treatment (Fig. 1A and Supplemental Fig. 1). A progressive increase in resistance to navitoclax was observed in CLL cells *in vivo* during the different cycles of treatment (Fig. 1A). Since these studies were carried out in whole blood, we wished to ascertain whether the decrease in ABT-263-induced apoptosis in CLL cells could be attributed to chemoresistance. To test this, CLL cells were isolated from these patients at the beginning of each treatment cycle and exposed to increasing concentrations of ABT-263. A significant decrease (3-fold difference in the IC50 values between the lead-in period and cycle 5) was observed in their ability to undergo ABT-263-induced apoptosis (Fig. 1B), demonstrating that continued dosing of patients with ABT-263 resulted in a modest, yet significant increase in chemoresistance.

Since chemoresistance is an emerging problem in BH3 mimetic therapy, we wished to extend these studies to more selective BH3 mimetics, such as ABT-199, which has replaced navitoclax owing to the dose-limiting thrombocytopenia associated with BCL-X_L inhibition.[3] Moreover, other selective BH3 mimetics that target BCL-X_L (A-1331852) and MCL-1 (A-1210477 and S63845) have been introduced for use in several other malignancies.[5-7] Using these BH3 mimetics and relevant cancer cell lines, we wished to mimic the rapid resistance observed in CLL patients following navitoclax (Fig. 1A and B), in order to identify ways to tackle chemoresistance, as it emerges. For this, we chose the BCL-2-dependent MAVER-1, BCL-X_L-dependent K562 and MCL-1-dependent H929 cell lines and exposed them to ABT-199, A-1331852 and A-1210477, respectively, to generate different resistant models (Fig. 1C and Supplemental Fig. 2). Initial exposure of the relevant cell lines to the corresponding BH3 mimetic resulted in a rapid, time-dependent induction of apoptosis as assessed by the activation of caspase-9 and -3 as well as the cleavage of the canonical caspase substrate, PARP (Supplemental Fig. 2A). Resistance to BH3 mimetics in these cells was generated by following the scheme presented (Fig. 1C), when the initially sensitive cells (A) became relatively resistant (E), after four exposures (within 8 weeks) to their respective BH3 mimetic (Fig. 1C). Similarly, a rapid resistance to the different BH3 mimetics was also observed using the other three resistance models (Supplemental Fig. 2B-D). The rapid and modest resistance to the different BH3 mimetics in these cell lines was comparable to the extent of resistance observed in CLL cells during navitoclax therapy (Fig. 1B).

Resistance to BH3 mimetics can be overcome by inhibiting multiple BCL-2 family members

Since resistance to BH3 mimetics has often been attributed to elevated expression levels of one or more anti-apoptotic BCL-2 family members, we wished to identify whether such changes could be responsible for the observed resistance. Comparison of the sensitive (A), intermediate (C) and resistant (E) cells from the different cell lines did not reveal any consistent differences in BCL-2 family expression to explain the resistance (Supplemental Fig. 3). Therefore, we sought to identify whether changes in protein-protein interactions among different pro-apoptotic BH3-only members and their anti-apoptotic counterparts could explain the resistance to BH3 mimetics. For this, we performed immunoprecipitation studies to isolate the anti-apoptotic proteins bound to BIM and PUMA, which were abundantly expressed in the three different cell types. However, in the sensitive (A) and resistant (E) MAVER-1 cells, immunoprecipitation of BIM and PUMA revealed similar binding of BCL-2 and BCL-X_L and little or no binding to MCL-1 (Supplemental Fig. 4). Similarly, no differences were observed in the binding of BIM and PUMA to BCL-X_L and MCL-1 in sensitive and resistant K562 or H929 cells (Supplemental Fig. 4).

Although the protein expression levels and immunoprecipitation studies did not support an involvement of other BCL-2 family proteins in the observed resistance, the resistance to ABT-199 observed in MAVER-1 cells was completely overcome by a combination of ABT-199 with either A-1331852 or A-1210477, but not by either A-1331852 or A-1210477 alone, suggesting that the resistant cells in addition to BCL-2 also depended on BCL-X_L and/or MCL-1 for survival (Fig. 1D). Furthermore, a combination of all three BH3 mimetics induced apoptosis in all the resistant cells, emphasising the importance of all three anti-apoptotic BCL-2 family members in chemoresistance in these cells (Fig. 1D). In K562 and H929 cells, the resistance was overcome by the combination of A-1331852 and A-1210477, but not ABT-199, thus implicating primary roles for BCL-X_L and MCL-1 in chemoresistance (Figs. 1E and F). Similar to the MAVER-1 cells, the chemoresistant K562 cells also exhibited enhanced apoptosis following a combination of all three BH3 mimetics (Fig. 1E), suggesting that some contribution of BCL-2 could not be totally excluded in these cells. These observations were almost entirely reproducible in the other three resistant models (Supplemental Fig. 5), supporting the notion that BCL-X_L and/or MCL-1 significantly contributed to the observed chemoresistance in the different resistance models.

Modulation of glutamine uptake and/or metabolism enhances sensitivity to BH3 mimetics

Although the above results demonstrate that a combination of BH3 mimetics can overcome the resistance, such an approach targeting multiple members of the BCL-2 family requires careful evaluation of the therapeutic index, as these proteins perform redundant functions in the maintenance of normal cellular homeostasis. An alternative strategy to overcome chemoresistance to BH3 mimetics could be achieved by altered metabolism, as depriving cells of glutamine has recently been shown to overcome MCL-1-mediated chemoresistance in multiple myeloma.[19] In our experiments, glutamine deprivation for 16 h alone did not exhibit any effect on overall cell survival and yet, sensitised both the sensitive (A) and resistant (E) cells to BH3 mimetic-mediated apoptosis (Fig. 2A). The increase in apoptosis observed in both sensitive (A) and resistant (E) cells indicates that glutamine deprivation most likely provides an additional cytotoxic cue that induces apoptosis in the sensitive and resistant cells, but could also bypass the resistance mechanism in the resistant cells.

Nevertheless, our results suggest that targeting the glutamine metabolic pathway could enhance apoptosis and circumvent chemoresistance to BH3 mimetics in all our resistance models (Fig. 2A and Supplemental Fig. 6). To investigate the therapeutic potential of this approach, we wished to further understand how changes in glutamine metabolism might alter BH3 mimetic-mediated apoptosis.

Glutamine is transported into cells primarily *via* the SLC1A5 transporter and metabolised to glutamate, primarily via glutaminase (GLS)-mediated glutaminolysis.[22] Alternatively, glutamate can be generated from glutamine as a by-product of the hexosamine biosynthetic pathway, during the conversion of fructose-6-phosphate to glucosamine-6-phosphate, catalysed by the enzyme, glutamine:fructose-6-phosphate-amidotransferase (GFAT) (Fig. 2B).[22] Glutamate can then generate α-ketoglutarate (α-KG) either via glutamate dehydrogenase (GLUD)-mediated oxidative deamination or a series of aminotransferase reactions (Fig. 2B).[14,22] Downregulation by RNA interference or pharmacological inhibition of key players involved in both glutamine uptake and its subsequent metabolism restored sensitivity of chemoresistant K562 cells to A-1331852-mediated apoptosis, albeit to varying degrees (Figs. 2C and D). While downregulation of SLC1A5 and GLS resulted in enhanced sensitivity to A-1331852-mediated apoptosis in the different cell lines tested, inhibition of other enzymes in the glutamine metabolic pathway produced more modest effects (Figs. 2C, D and Supplemental Fig. 7).

Targeting reductive carboxylation enhances sensitivity to BH3 mimetics

Metabolic supplementation of the glutamine-deprived cells with either glutamine or α-KG restored the resistance of K562 cells to A1331852-induced apoptosis (Fig. 3A). Since glutamine-derived α-KG feeds into the tricarboxylic acid (TCA) cycle, we wished to explore the functions of the TCA cycle and its intermediates in chemoresistance to BH3 mimetics. For this, we supplemented glutamine-deprived cells with TCA intermediates, such as oxaloacetate and citrate. Strikingly, supplementation with citrate, but not oxaloacetate, restored the resistance of K562 cells to A1331852-induced apoptosis (Fig. 3A). These results suggested that conversion of α-KG to citrate *via* reductive carboxylation may play a role in regulating sensitivity to BH3 mimetics.

Reductive carboxylation involves the conversion of α-KG to isocitrate (catalysed by isocitrate dehydrogenases 1 and 2; IDH1/2), which then generates citrate (catalysed by aconitase) (Fig. 3B).[23] While IDH1/2 catalyse reductive carboxylation of α-KG, another isoform of isocitrate dehydrogenase, IDH3 catalyses the reverse-conversion of isocitrate to α-KG.[23] Silencing the expression of IDH2 and aconitase, but not IDH3, restored the sensitivity of chemoresistant K562 cells to A-1331852-mediated apoptosis (Figs. 3C and D), suggesting that the availability of citrate could be associated with the chemoresistance phenotype. To test this, IDH2-downregulated K562 cells were supplemented with citrate to identify whether addition of citrate could overcome the inhibition of reductive carboxylation and revert the associated increase in A-1331852-induced apoptosis. Indeed, cells supplemented with citrate, but not glutamine or α-KG, restored the chemoresistance of IDH2 downregulated cells (Fig. 3E), thus confirming the involvement of reductive carboxylation and the availability of citrate as crucial players for the observed chemoresistance.

Downregulation of lipogenesis and cholesterogenesis enhances sensitivity to BH3 mimetics

Since citrate generated as a consequence of reductive carboxylation of α-KG is a major source of carbon for lipid synthesis, we wished to investigate whether inhibition of lipogenesis could enhance sensitivity to BH3 mimetics (Fig. 4A). Using a complementary approach of genetic and pharmacological inhibition of ATP-citrate lyase (ACLY), which catalyses the conversion of citrate to acetyl-CoA,[24,25] as well as Fatty acid synthase (FASN), which synthesises long chain fatty acids following the condensation of acetyl-CoA and malonyl-CoA,[26,27] we identified that modulation of lipogenesis pathway, either using genetic silencing or pharmacological inhibition of ACLY (using SB204990) or FASN (using GSK2194069) could enhance sensitivity of cells to BH3 mimetics (Fig. 4B, C and Supplemental Figs. 7C and D). Furthermore, metabolic supplementation with palmitate (the product of FASN; Fig. 4A) in cells treated with GSK2194069 reverted the sensitised cells to their original chemoresistant phenotype (Fig. 4D), thus obviating a requirement for FASN. These findings conclusively demonstrated that enhanced lipogenesis was associated with chemoresistance to BH3 mimetics and targeting lipogenesis could circumvent such resistance by enhancing BH3 mimetic-mediated apoptosis.

Acetyl-CoA generated from citrate can also feed into the cholesterol biosynthetic pathway, thus resulting in enhanced cholesterol production in cells. Targeting the rate-limiting step of cholesterol biosynthesis (catalysed by HMG-CoA reductase; HMGR), either by genetic knockdowns (Fig. 4E) or pharmacological inhibition, using three widely used statins, simvastatin, atorvastatin and pitavastatin (Fig. 4F) reversed resistance and restored the sensitivity of cells to BH3 mimetics (Figs. 4E, F and Supplemental Fig. 7E). Taken together, these data demonstrate that inhibition of several key players in lipid synthesis, including ACLY, FASN and HMGR, enhance the sensitivity to BH3 mimetics.

Targeting the mTOR signalling cascade enhances sensitivity to BH3 mimetics

Since glutamine metabolism has been extensively implicated in mTOR signalling,[22,28] we speculated whether targeting mTOR kinases could enhance sensitivity to BH3 mimetics. Inhibition of mTOR kinases with rapamycin and torin-1 resulted in significant sensitisation of cells to BH3 mediated apoptosis (Fig. 5A and Supplemental Fig. 7F). To identify whether torin-1-mediated sensitisation of cells to apoptosis was due to autophagy, we exposed the sensitive and resistant cells to bafilomycin A1 (Baf A1), which blocks the autophagic flux by preventing lysosomal fusion of the autophagosomes. Exposure to bafilomycin A1 failed to revert torin-1-mediated chemosensitisation, suggesting that this effect could be independent of autophagy (Fig. 5B). Furthermore, genetic silencing of autophagy proteins, ATG5 and ATG7, which are critical for autophagy induction, also failed to revert torin-1-mediated sensitisation (Fig. 5C), confirming our finding that mTOR inhibition circumvented resistance and enhanced sensitivity to BH3 mimetics independent of autophagy. In summary, our findings demonstrate that modulation of glutamine metabolism and its downstream signalling pathways, namely reductive carboxylation, lipogenesis and cholesterogenesis, as well as inhibition of mTOR signalling could enhance the therapeutic efficacy of BH3 mimetic therapy thereby circumventing chemoresistance to BH3 mimetics (Fig. 5D).

Targeting intermediary metabolism enhances sensitivity to navitoclax in primary CLL patient samples

Our results indicate that targeting different facets of intermediary metabolism enhanced sensitivity to different BH3 mimetics in cell lines derived from relevant haematological malignancies. To further extend our observations in cell lines to primary patient samples, we used CLL cells isolated from patients during the lead-in period (L1D1) as well as cells from the same patients after 5 cycles of navitoclax therapy (C5D1), as previously detailed in Figure 1. Using these samples, we wished to assess whether modulating glutamine metabolism would enhance apoptosis mediated by navitoclax. For this, we exposed CLL cells to CB-839 and Simvastatin for 24 h followed by navitoclax for 4 h and assessed the extent of apoptosis. In agreement with our cell line data, both CB-839 and statins overcame the resistance to navitoclax-mediated apoptosis in primary CLL cells (Figure 6), supporting the therapeutic translatability of our data from cell lines to patients.

Discussion

Anti-apoptotic BCL-2 family members have been attractive drug targets both because of their high expression levels in several cancers and also their well-characterised pro-survival roles. Even with extensive supportive *in vitro* data, the use of BH3 mimetics in treating cancer patients is still in its infancy, with venetoclax, a BCL-2 specific inhibitor, only recently receiving approval for treatment of refractory chronic lymphocytic leukaemia.[4] The development of BH3 mimetics to target BCL-X_L and MCL-1 in patients will be extremely valuable in the treatment of several cancer types. However potential mechanisms of resistance to BH3 mimetics need to be recognised as they emerge and ways to circumvent resistance identified. Several resistance mechanisms, including mutations of the target site,[29] post-translational modifications,[30,31] and elevated levels of anti-apoptotic BCL-2 family members,[8,11,32,33] have already been identified. While some of these resistance mechanisms could be overcome by co-administration of other specific BH3 mimetics that target BCL-X_L and/or MCL-1,[5-7] such inhibitors are not yet clinically available and the potential toxicities associated with the simultaneous inhibition of multiple BCL-2 family members are not known.

Measures to overcome chemoresistance have resulted in exploring the therapeutic benefits of modulating intermediary metabolism in BH3 mimetic-mediated apoptosis.[19,20,34] Although the mechanisms by which glutamine could regulate cancer cell proliferation have been extensively studied, the interrelationship between glutamine metabolism and apoptosis requires further study. Previous findings have reported a dependence on Myc for glutamine-mediated apoptosis[14] and that c-Myc activates glutaminolysis by upregulating both the glutamine transporter, SLC1A5, and glutaminase, GLS-1.[35,36] However, we were unable to detect an increase in expression levels of Myc, SLC1A5 or GLS-1 in our resistance models (Fig. 3 and data not shown). The ability of glutamine to regulate apoptosis and/or chemoresistance could also be due to its regulatory effect on mitochondrial oxidative phosphorylation.[20] Although we do not entirely understand how glutamine metabolism impinges on apoptosis at this point, our data strongly support the notion that modulating glutamine metabolism and its related signalling pathways, such as reductive carboxylation, lipogenesis, cholesterogenesis and mTOR signalling could enhance BH3 mimetic-mediated apoptosis in several haematological malignancies (Figs. 3-6). This is particularly promising, as glutaminase inhibitors, such as CB-839 and related drugs are already in clinical trials for the treatment of several malignancies[20,37] and other drugs targeting cholesterogenesis, such as statins are the most commonly prescribed drugs to millions of people worldwide. While this manuscript was in preparation, an independent study comparing a large cohort of CLL patients, many of whom were statin users, reported that response to venetoclax/ ABT-199 was enhanced among statin users in three different clinical trials.[44] These findings highlight the possibility of repurposing several drugs targeting the intermediary metabolic pathways in conjunction with BH3 mimetic therapy to enhance the therapeutic effectiveness and overcome the emerging chemoresistance in several cancers.

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Conflict of Interest Disclosure

The authors do not have any conflict of interest.

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Figure Legends

Figure 1. Haematological malignancies acquire rapid resistance to BH3 mimetics. (A) Blood samples collected from CLL patients (n=5), either prior to the first *in vivo* dose of navitoclax or 4 h after dosing during different stages of treatment - day 1 of the initial lead-in-period (L1D1), day 1 of cycle 1 (C1D1), day 1 of cycle 3 (C3D1) or day 1 of cycle 5 (C5D1) - were incubated *ex vivo* and the extent of apoptosis in the $CD19^+$ CLL cells assessed at the indicated time points by phosphatidylserine (PS) externalisation. (**B**) CLL cells isolated from these patients at the beginning of each treatment cycle, as indicated in the figure, were exposed *in vitro* to increasing concentrations of ABT-263, the extent of apoptosis assessed and IC_{50} values are shown. (**C**) Scheme for establishing resistance to specific BH3 mimetics in relevant haematological cell lines, as explained in the methods. Sensitive (A) and resistant (E) cells of MAVER-1, K562 and H929 cells were exposed for 4 h to ABT-199 (10 nM), A-1331852 (10 nM) and A-1210477 (5 μM), respectively, and apoptosis assessed. (**D-F**) Combination with some but not all BH3 mimetics restores the apoptotic sensitivity of resistant (E) MAVER-1, K562 and H929 cells exposed for 4 h to ABT-199 (10 nM), A-1331852 (10 nM) or A-1210477 (5 μM), respectively. ***P $\leqslant$0.001, **P$\leqslant$0.01; Error bars = Mean ± SEM (n=3).

Figure 2. Inhibition of glutamine uptake and metabolism enhances sensitivity to BH3 mimetics. (**A**) Deprivation of glutamine (Gln) for 16 h restores the apoptotic sensitivity of resistant (E) MAVER-1, K562 and H929 cells to a 4 h exposure of the indicated BH3 mimetic. (**B**) Scheme representing the glutamine uptake and metabolism pathway. (**C**) Apoptotic sensitivity of K562 resistant (E) cells exposed to A-1331852 (10 nM) for 4 h was restored following genetic knockdown for 72 h with the indicated siRNA. (**D**) Apoptotic sensitivity of K562 resistant (E) cells exposed to A-1331852 (10 nM) for 4 h was restored following pharmacological inhibition of glutamine uptake or metabolism with GPNA (5 mM) for 48 h, CB-839 (10 μM) for 72 h, azaserine (25 μM) for 16 h and AOA (500 μM) for 24 h but not with EGCG (50 μM) for 24 h. Western blots confirmed the knockdown efficiency of the different siRNAs. ***P $\leqslant$0.001, **P$\leqslant$0.01; Error bars = Mean ± SEM (n=3).

Figure 3. Modulation of reductive carboxylation enhances sensitivity to BH3 mimetics. (**A**) K562 (A and E) cells were cultured in normal RPMI medium or glutamine-free medium with and without the supplementation of glutamine (2 mM), exposed to A-1331852 (10 nM) for 4 h and the extent of apoptosis assessed. Addition of citrate (4 mM) and α-ketoglutarate (α-KG) (4 mM) but not oxaloacetate (4 mM) for 16 h reversed the sensitivity of the resistant (E) cells in glutamine-deprived media. (**B**) Scheme representing the link between tricarboxylic acid (TCA) cycle and reductive carboxylation. (**C**) K562 (A and E) cells were transfected with siRNAs against IDH2, IDH3 and aconitase for 72 h, followed by a 4 h exposure to A-1331852 and apoptosis assessed. (**D**) Western blots confirmed the knockdown efficiency of the different siRNAs. (**E**) K562 (A and E) cells, transfected with a siRNA against IDH2 for 72 h, were glutamine deprived with and without the supplementation of glutamine (2 mM), α-ketoglutarate (α-KG) or citrate (both at 4 mM) for 16 h and the extent of apoptosis following A-1331852 (10 nM) for 4 h was assessed. ***P $\leqslant$0.001. Error bars = Mean ± SEM (n=3).

Figure 4. Inhibition of lipogenesis and cholesterogenesis enhances sensitivity to BH3 mimetics. (**A**) Scheme representing reductive carboxylation, lipogenesis and cholesterogenesis. (**B**) Apoptotic sensitivity of K562 resistant (E) cells exposed to A-1331852 (10 nM) for 4 h was restored following genetic knockdown for 72 h of key enzymes in fatty acid synthesis. Western blots confirmed the knockdown efficiency of the different siRNAs. (**C**) Apoptotic sensitivity of K562 resistant (E) cells exposed to A-1331852 (10 nM) for 4 h was restored following pharmacological inhibition of key enzymes in fatty acid synthesis using SB204990 (1 μM) for 72 h or GSK2194069 (100 nM) for 48 h. (**D**) Metabolic supplementation of K562 (A and E) cells with palmitate (50 μM) for 48 h prior to the exposure of cells to GSK2194069 (100 nM) overcame the sensitisation effect of GSK2194069 on A-1331852-mediated apoptosis. (**E**) genetic knockdown for 72 h of HMGR or (**F**) pharmacological

inhibition of HMGR by simvastatin (250 nM) for 72 h, atorvastatin (10 μM) for 48 h or pitavastatin (1 μM) for 72 h. ***P $\leqslant$0.001; **P$\leqslant$0.01. Error bars = Mean ± SEM (n=3).

Figure 5. Modulation of mTOR signalling enhances sensitivity to BH3 mimetics independent of autophagy. (**A**) Apoptotic sensitivity of K562 resistant (E) cells exposed to A-1331852 (10 nM) for 4 h was restored following pharmacological inhibition of mTOR signalling using rapamycin (100 nM) or torin-1 (10 nM) for 16 h. (**B**) Inhibition of mTOR-regulated autophagy using 3-MA (10 mM) or bafilomycin A1 (100 nM) for 1 h, followed by torin-1 (10 nM) for a further 16 h, resulted in varying effects on A-1331852-mediated apoptosis. (**C**) Genetic knockdown of ATG5 and ATG7 for 72 h failed to revert torin-1 (10 nM)-mediated sensitisation of apoptosis in K562 resistant (E) cells, following A-1331852 (10 nM) for 4 h. Western blots confirmed the knockdown efficiency of ATG5 and ATG7 siRNA. ***P $\leqslant$0.001. Error bars = Mean ± SEM (n=3). (**D**) Scheme representing glutamine uptake by SLC1A5 (inhibited by GPNA), glutaminolysis (inhibited by CB-839) to generate α-ketoglutarate, reductive carboxylation of α-ketoglutarate to generate citrate, which produces acetyl-CoA by a reaction catalysed by ACLY (inhibited by SB204990), which eventually results in lipogenesis (inhibited by GSK2194069) and cholesterogenesis (inhibited by statins). Glutamine uptake, metabolism and its downstream signalling cascade can feed into mTOR signalling (inhibited by torin-1), all of which promote cell growth. In this study, we demonstrate that modulation of these distinct intermediary metabolic pathways could successfully sensitise cancer cells to BH3 mimetic-mediated apoptosis.

Figure 6. Inhibition of GLS and HMGR circumvents resistance to navitoclax-mediated apoptosis in primary CLL cells. CLL cells isolated from 5 patients during the initial lead-in-period (L1D1) or day 1 of cycle 5 (C5D1) were cultured *ex vivo* on feeder layer for 24 h, followed by exposure for a further 24 h to (**A**) CB-839 (50 nM) or (**B**) simvastatin (10 nM), and removed from the

feeder layer for further exposure to navitoclax (50 nM) for 4 h. The extent of apoptosis was assessed as before. *P $\leqslant$0.05. Error bars = Mean ± SEM (n=5).

Figure 1

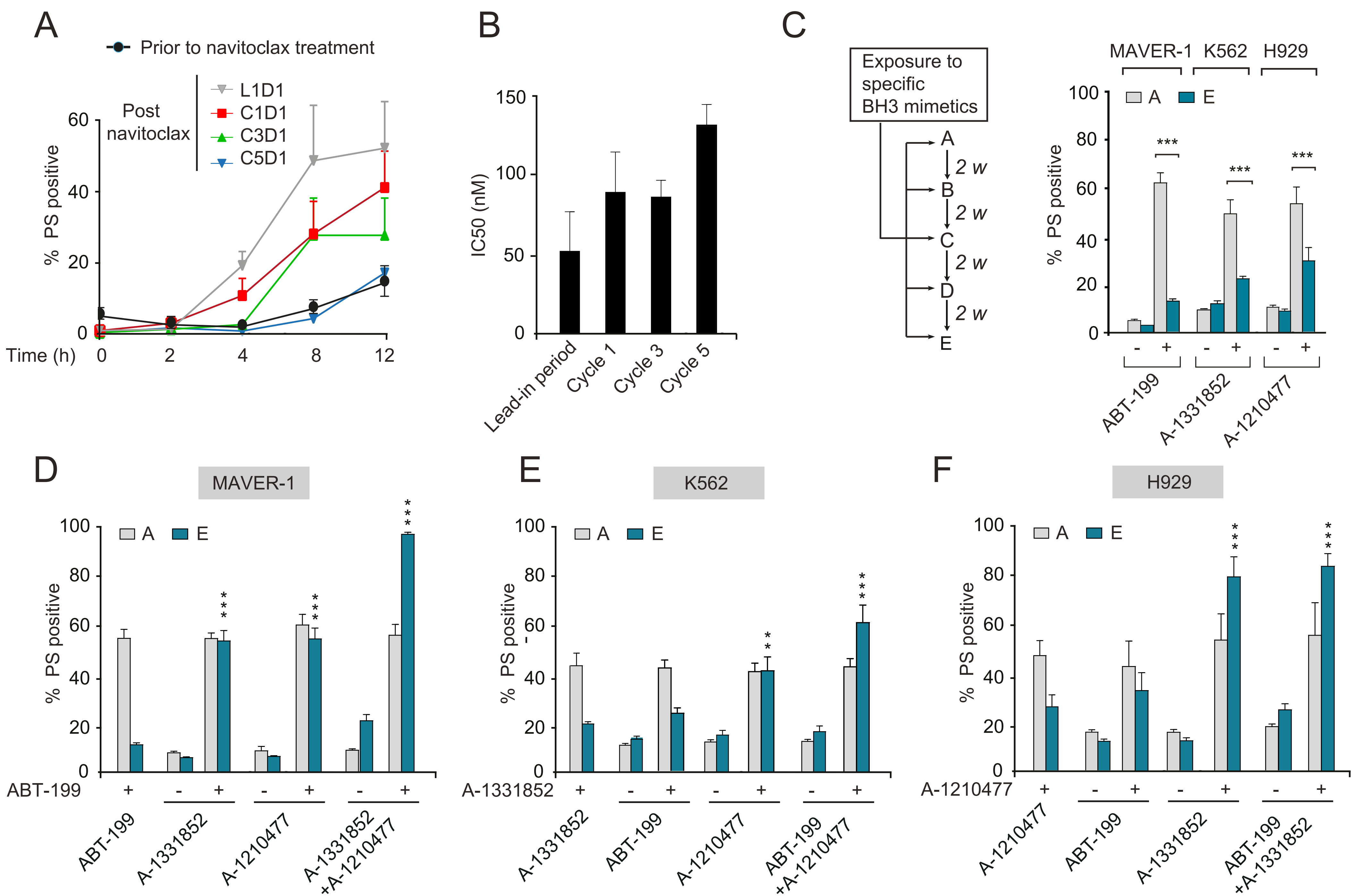
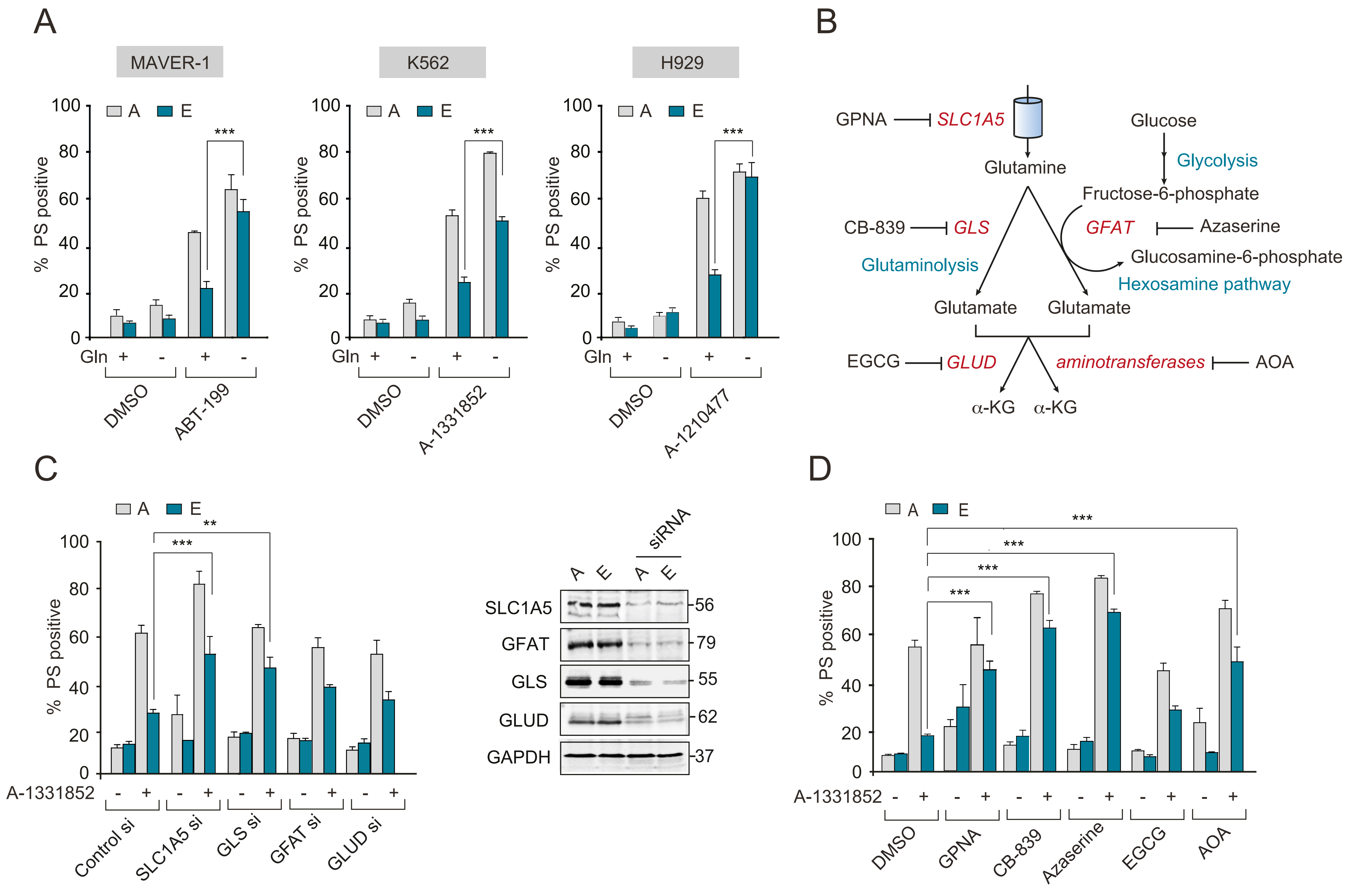
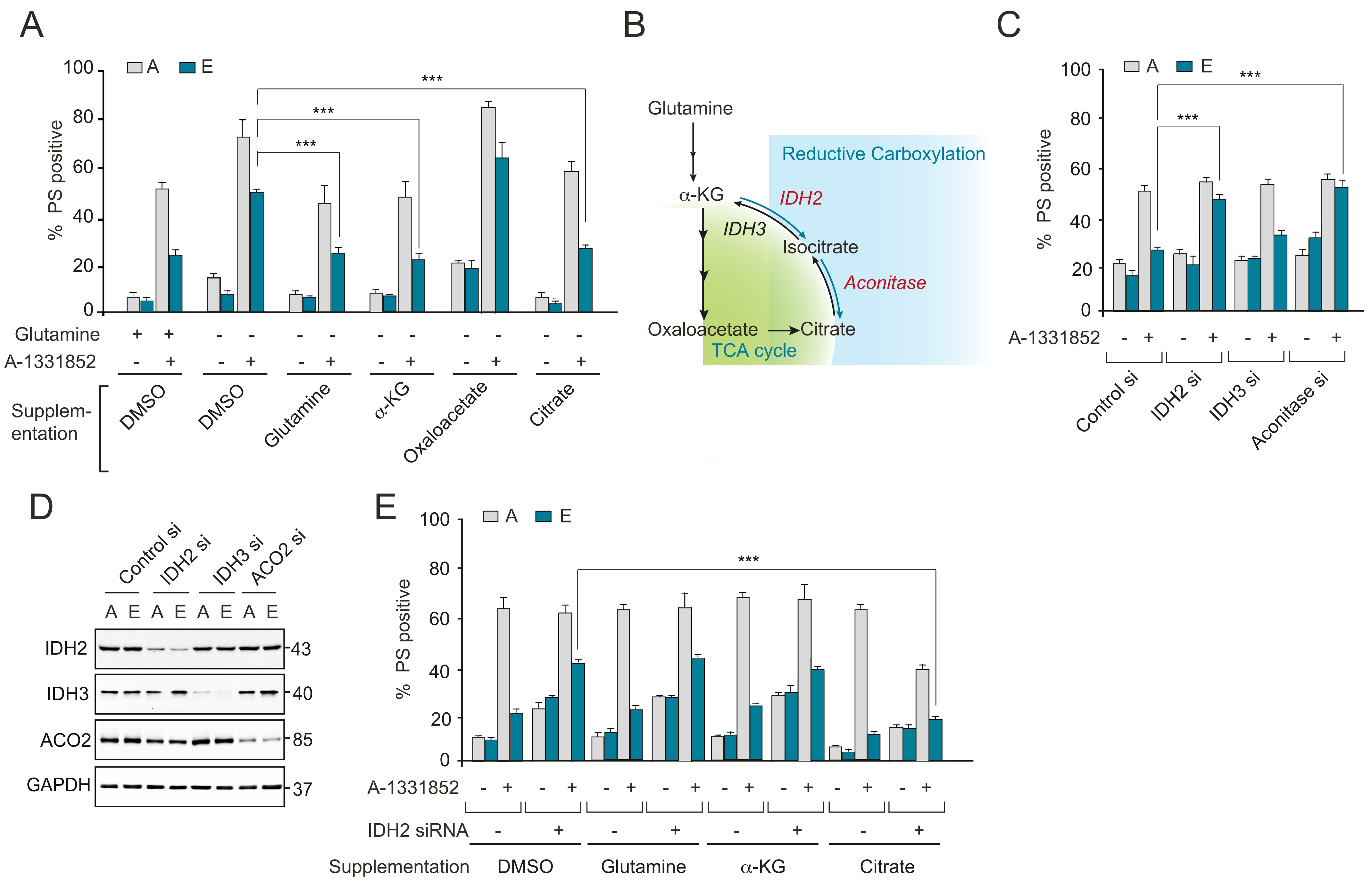

A
Prior to navitoclax treatment
Post navitoclax
L1D1
C1D1
C3D1
C5D1
% PS positive
Time (h)
0
2
4
8
12
20
40
60
B
IC50 (nM)
0
50
100
150
Lead-in period
Cycle 1
Cycle 3
Cycle 5
C
Exposure to specific BH3 mimetics
A
2 w
B
2 w
C
2 w
D
2 w
E
MAVER-1
K562
H929
% PS positive
***
ABT-199
A-1331852
A-1210477
D
MAVER-1
% PS positive
ABT-199
A-1331852
A-1210477
A-1331852 +A-1210477
E
K562
% PS positive
A-1331852
ABT-199
A-1210477
ABT-199 +A-1210477
F
H929
% PS positive
A-1210477
ABT-199
A-1331852
ABT-199 +A-1331852

Figure 2



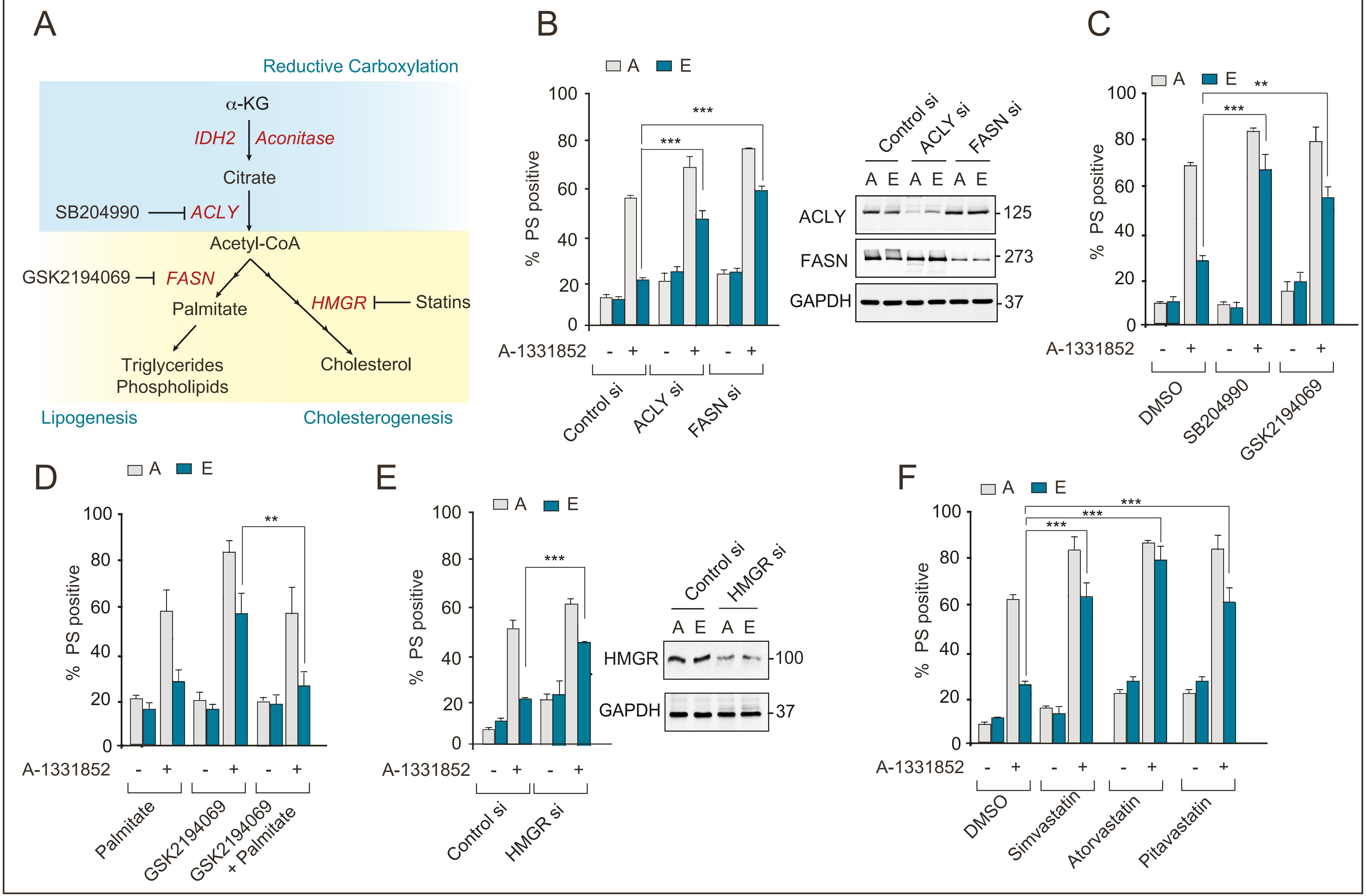
A
MAVER-1
K562
H929
% PS positive
Gln
DMSO
ABT-199
A-1331852
A-1210477
B
GPNA
SLC1A5
Glutamine
Glucose
Glycolysis
Fructose-6-phosphate
CB-839
GLS
GFAT
Azaserine
Glutaminolysis
Glucosamine-6-phosphate
Hexosamine pathway
Glutamate
Glutamate
EGCG
GLUD
aminotransferases
AOA
α-KG
α-KG
C
A
E
Control si
SLC1A5 si
GLS si
GFAT si
GLUD si
siRNA
SLC1A5
GFAT
GLS
GLUD
GAPDH
56
79
55
62
37
D
DMSO
GPNA
CB-839
Azaserine
EGCG
AOA

Figure 3



A
B
C
D
E
% PS positive
Glutamine
A-1331852
Supplem-
entation
DMSO
Glutamine
α-KG
Oxaloacetate
Citrate
***
Reductive Carboxylation
IDH2
IDH3
Isocitrate
Aconitase
Oxaloacetate
TCA cycle
Control si
IDH2 si
IDH3 si
Aconitase si
ACO2 si
IDH3
ACO2
GAPDH
43
40
85
37
IDH2 siRNA
Supplementation

Figure 4



A
Reductive Carboxylation
α-KG
IDH2
Aconitase
Citrate
SB204990
ACLY
Acetyl-CoA
GSK2194069
FASN
Palmitate
HMGR
Statins
Triglycerides
Phospholipids
Cholesterol
Lipogenesis
Cholesterogenesis
B
A E
% PS positive
A-1331852
Control si
ACLY si
FASN si
ACLY
FASN
GAPDH
125
273
37
C
% PS positive
DMSO
SB204990
GSK2194069
D
Palmitate
GSK2194069
GSK2194069 + Palmitate
E
HMGR si
HMGR
100
F
Simvastatin
Atorvastatin
Pitavastatin

Figure 5

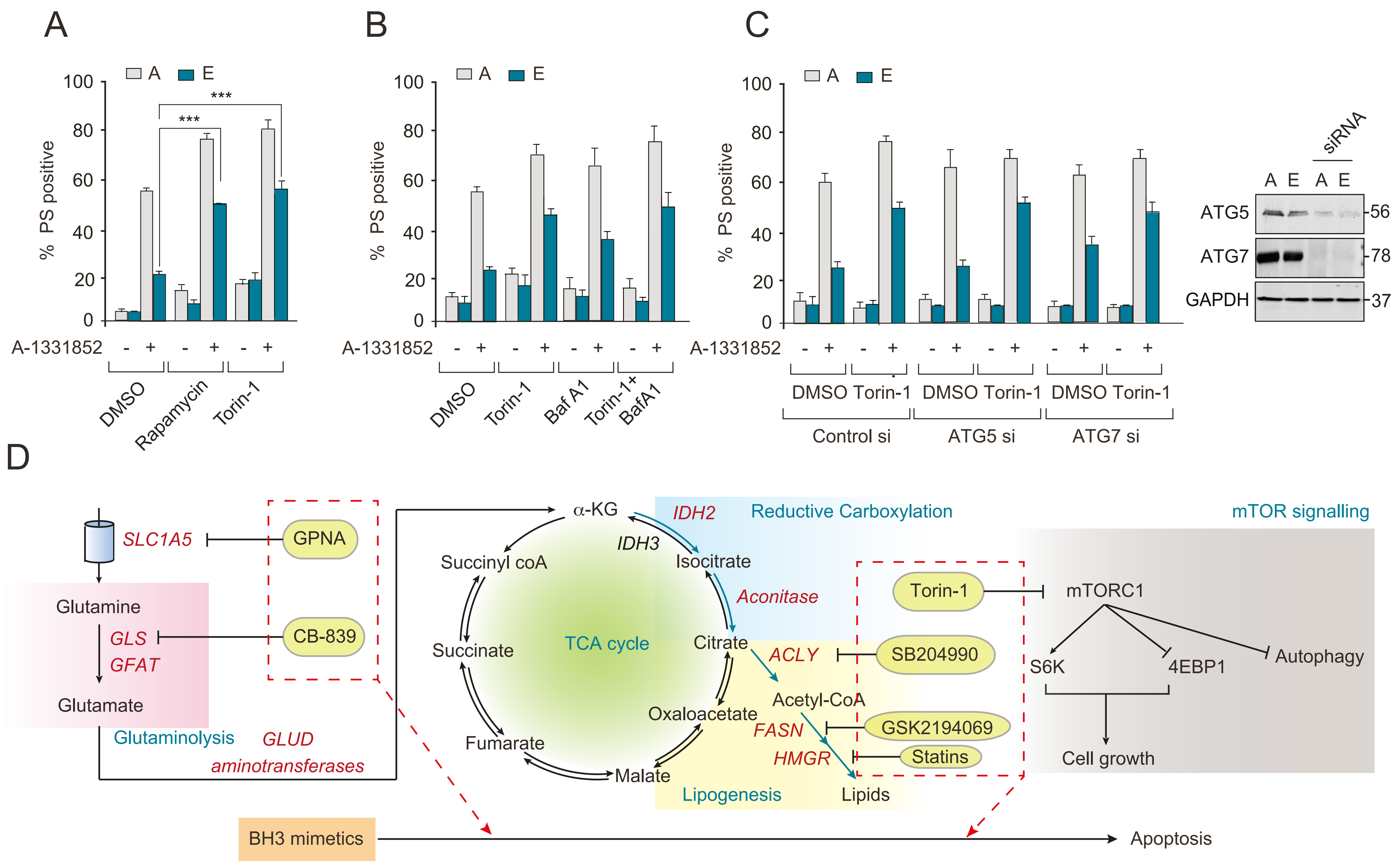
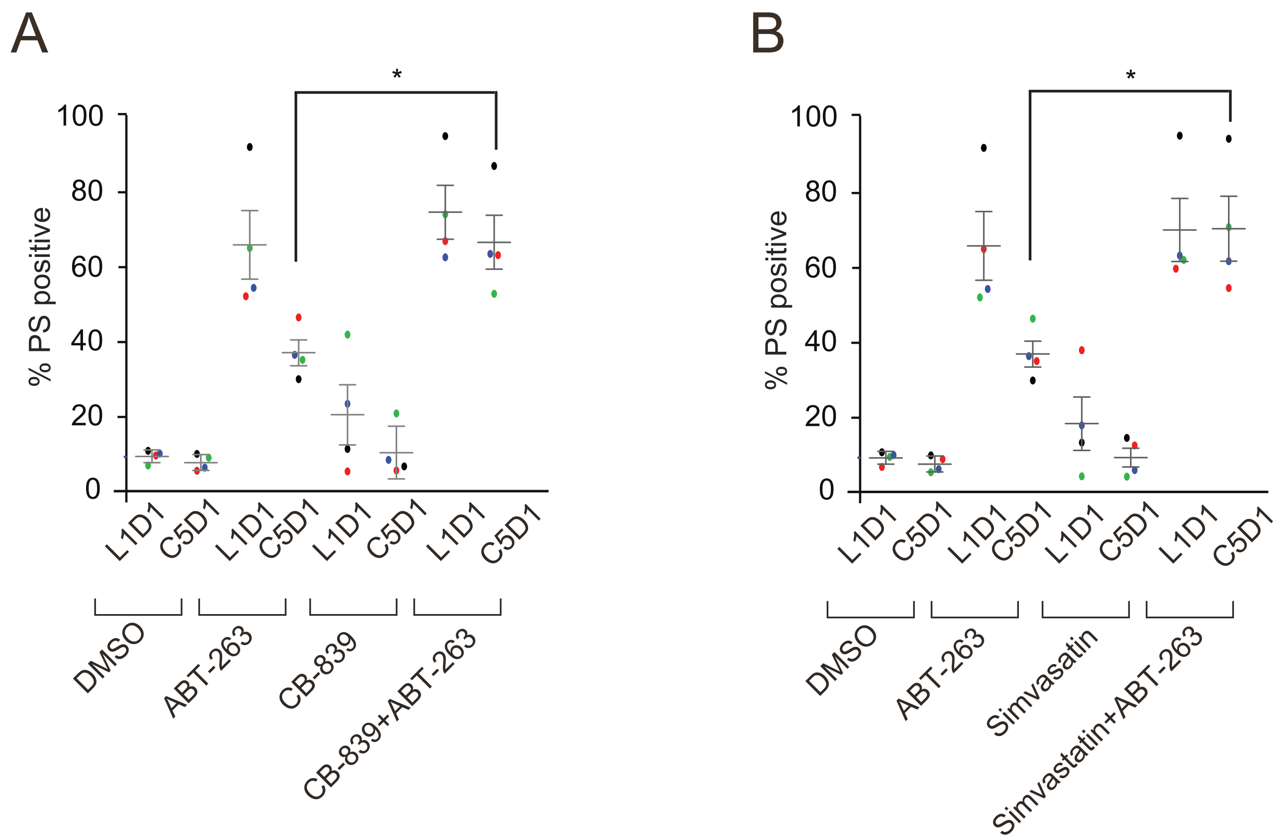

A
B
C
D
A
E
100
80
60
40
20
0
% PS positive
***
***
A-1331852
- + - + - +
DMSO
Rapamycin
Torin-1
DMSO
Torin-1
Baf A1
Torin-1+ BafA1
DMSO Torin-1 DMSO Torin-1 DMSO Torin-1
Control si
ATG5 si
ATG7 si
siRNA
A E A E
ATG5
ATG7
GAPDH
56
78
37
SLC1A5
GPNA
Glutamine
GLS
GFAT
CB-839
Glutamate
Glutaminolysis
GLUD
aminotransferases
α-KG
IDH3
IDH2
Reductive Carboxylation
Succinyl coA
Isocitrate
Aconitase
Succinate
TCA cycle
Citrate
ACLY
SB204990
Acetyl-CoA
Oxaloacetate
FASN
GSK2194069
HMGR
Statins
Fumarate
Malate
Lipogenesis
Lipids
mTOR signalling
Torin-1
mTORC1
S6K
4EBP1
Autophagy
Cell growth
BH3 mimetics
Apoptosis

Figure 6


A
B
% PS positive
100
80
60
40
20
0
*
L1D1
C5D1
DMSO
ABT-263
CB-839
CB-839+ABT-263
Simvasatin
Simvastatin+ABT-263

Supplemental information

Supplemental Figure S1. First *in vivo* dose of navitoclax resulted in marked ultrastructural changes in CLL cells. CLL cells isolated from two different patients prior to receiving navitoclax and 4 h after the first *in vivo* dosing during day 1 of the lead-in-period (L1D1) were incubated *ex vivo* for a further 8 h, before fixation in 2% glutaraldehyde and transmission electron microscopy. CLL cells from patients exposed to navitoclax exhibited chromatin condensation and rupture of the outer mitochondrial membrane. Scale bar – 500 nm.

Supplemental Figure S2. Rapid development of resistance to BH3 mimetic-mediated apoptosis in haematological cell lines. (**A**) Exposure of MAVER-1, K562 and H929 cells to ABT-199 (10 nM), A-1331852 (10 nM) and A-1210477 (10 μM), respectively resulted in a time dependent induction of apoptosis as assessed by western blotting for the indicated proteins. (**B-D**) Schemes used for developing the other three models of drug resistance as described in the Methods. Assessment of apoptosis by PS externalisation showed that resistance developed in the different cellular models following exposure for 4 h to ABT-199 (10 nM), A-1331852 (10 nM) and A-1210477 (5 μM), respectively. ***P ⩽0.001; Error bars = Mean ± SEM (n=3).

Supplemental Figure S3. Resistance to BH3 mimetic-mediated apoptosis could not be attributed to marked changes in the expression levels of BCL2 family members. Immunoblots of BCL-2 family members showed no major changes in the indicated cell lines, during the development of resistance, depicted as A, C and E. A is the sensitive parent line, whereas C and E are relatively more resistant to the BH3 mimetics compared to A. * in the PUMA immunoblot depicts a non-specific band.

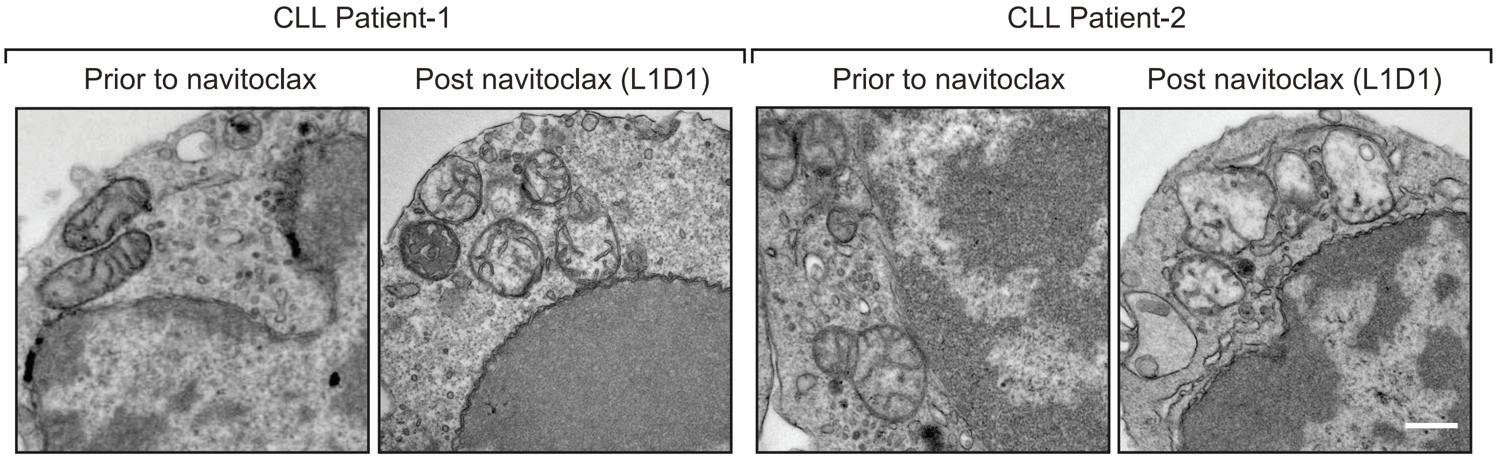
Supplemental Figure S4. Protein-protein interactions among anti- and pro-apoptotic BCL-2 family proteins are similar in both sensitive and resistant cells. Immunoprecipitates of BIM and PUMA in MAVER-1, K562 and H929 (A and E) cells showed no major differences in the binding of the indicated proteins. BC represents the beads control.

Supplemental Figure S5. Resistance to BH3 mimetics in the different resistance models can be overcome by simultaneous inhibition of multiple BCL-2 members. (**A-C**) Sensitive and resistant lines of MAVER-1, K562 and H929 from the different resistance models, as explained in methods and depicted in Supplemental figure 2, were exposed for 4 h to ABT-199 (10 nM), A-1331852 (10 nM) or A-1210477 (5 μM) and the extent of apoptosis assessed by PS externalisation. ***P $\leqslant$0.001, **P$\leqslant$0.01; Error bars = Mean ± SEM (n=3).

Supplemental Figure S6. Glutamine deprivation sensitises the different cell lines to BH3 mimetic-mediated apoptosis. (**A-C**) Sensitive and resistant lines of MAVER-1, K562 and H929 from the different resistance models, as explained in methods and depicted in Supplemental figure 2, were deprived of glutamine for 16 h and the extent of apoptosis assessed following a 4 h exposure to ABT-199 (10 nM), A-1331852 (10 nM) or A-1210477 (5 μM). ***P $\leqslant$0.001; Error bars = Mean ± SEM (n=3).

Supplemental Figure S7. Modulation of intermediary metabolism enhances sensitivity to BH3 mimetics in distinct haematological cell lines Exposure to GPNA (5 mM for 48 h), CB-839 (10 μM for 72 h), SB204990 (1 μM for 72 h), GSK2194069 (100 nM for 48 h), simvastatin (250 nM for 72 h) or torin-1 (10 nM for 24 h) enhances the sensitivity of the chemoresistant MAVER-1 and H929 cells to their respective BH3 mimetics. Apoptosis was assessed by PS externalisation following exposure for 4 h to ABT-199 (10 nM) or A-1210477 (5 μM). ***P $\leqslant$0.001; *P$\leqslant$0.1; Error bars = Mean ± SEM (n=3).

Figure S1



CLL Patient-1
CLL Patient-2
Prior to navitoclax
Post navitoclax (L1D1)
Prior to navitoclax
Post navitoclax (L1D1)

Figure S2

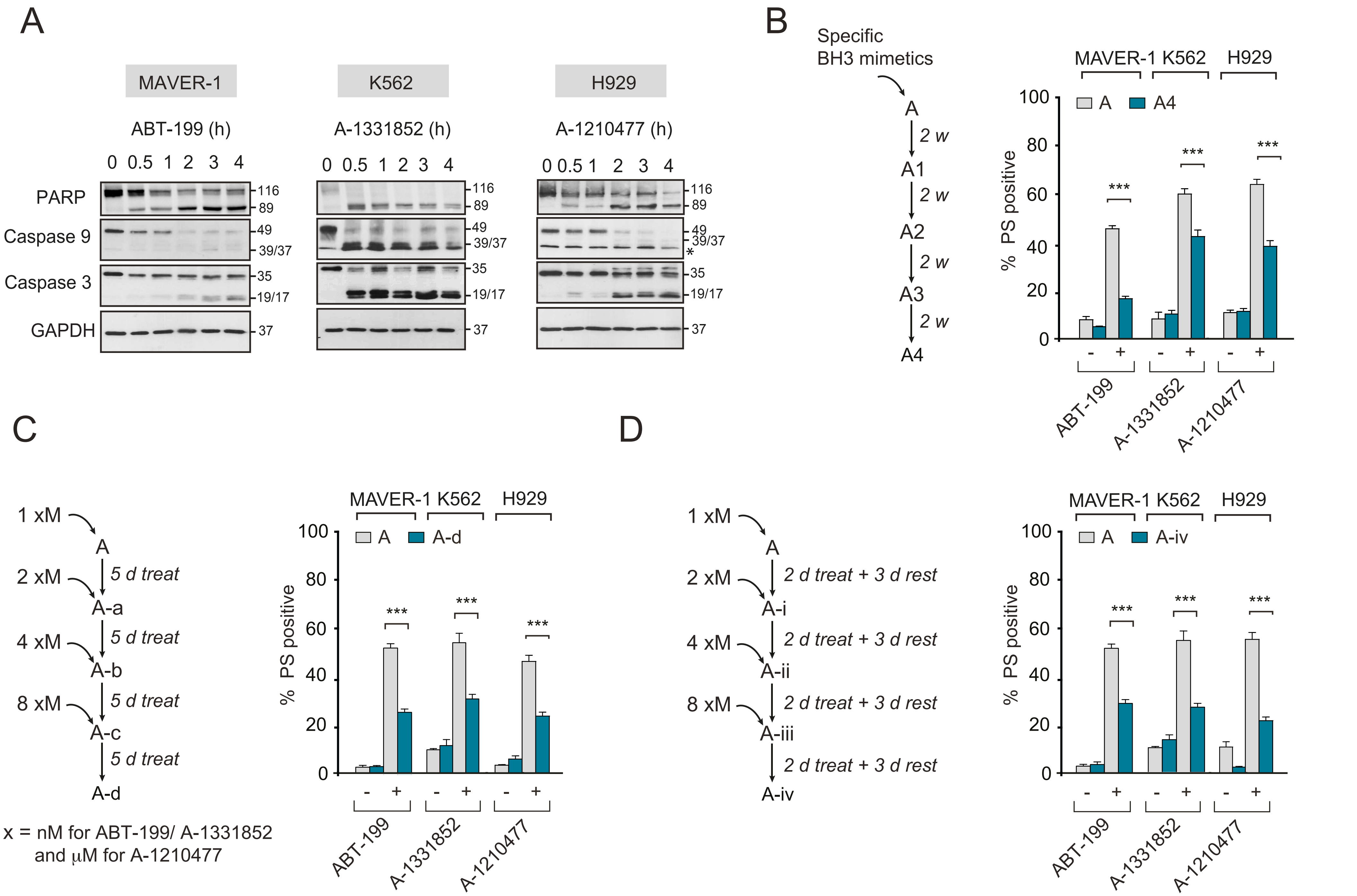
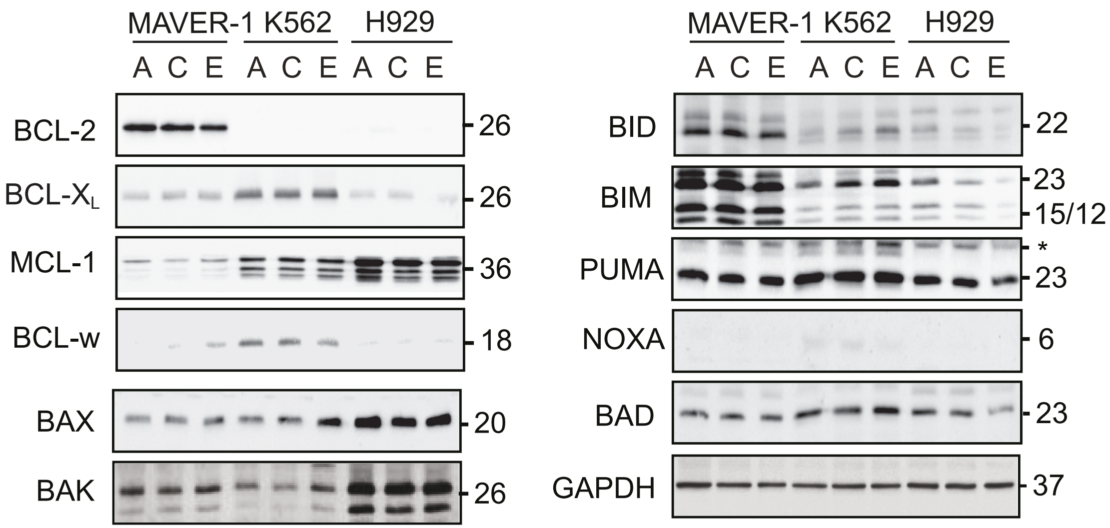
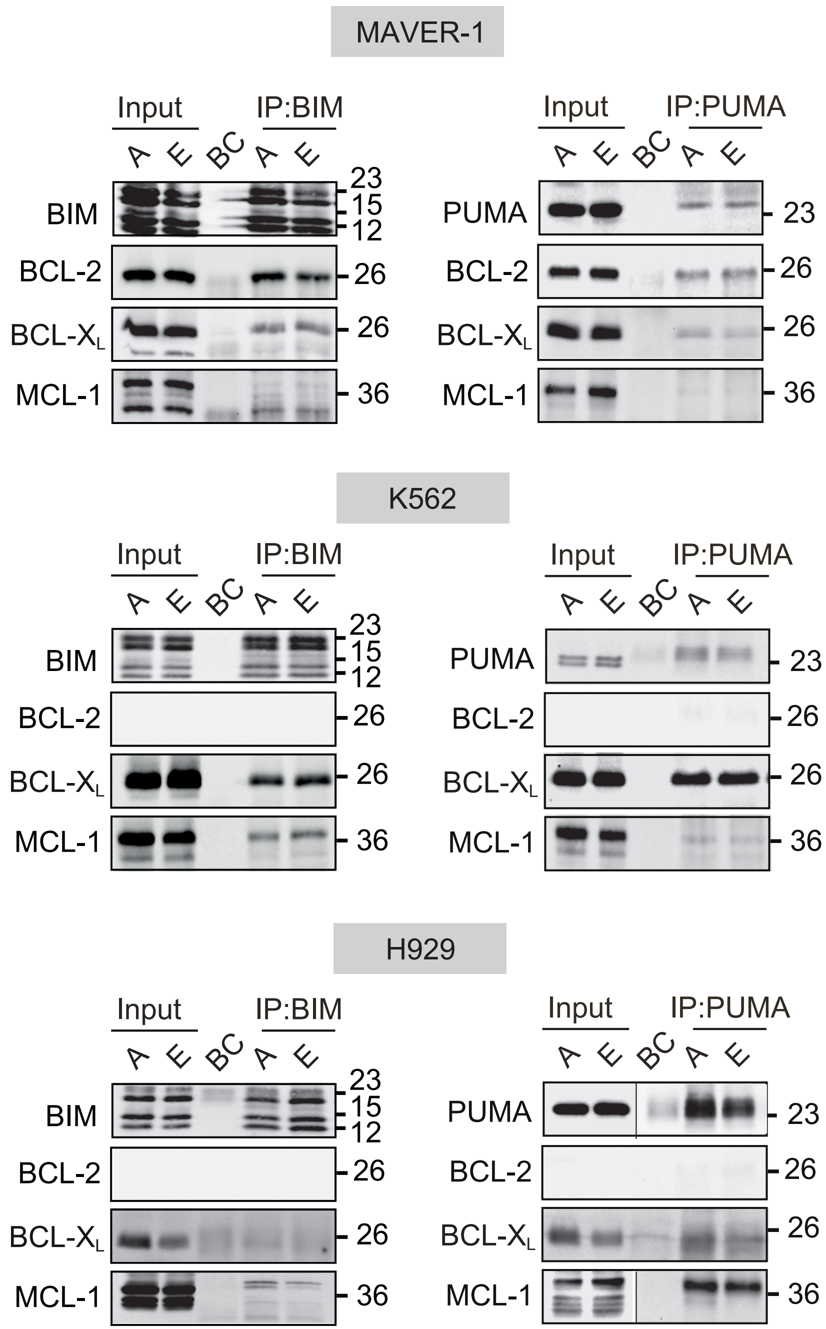

A
MAVER-1
K562
H929
ABT-199 (h)
A-1331852 (h)
A-1210477 (h)
0 0.5 1 2 3 4
PARP
Caspase 9
Caspase 3
GAPDH
116
89
49
39/37
35
19/17
37
*
B
Specific BH3 mimetics
A
2 w
A1
A2
A3
A4
MAVER-1 K562 H929
A A4
% PS positive
***
ABT-199
A-1331852
A-1210477
C
1 xM
2 xM
4 xM
8 xM
5 d treat
A-a
A-b
A-c
A-d
x = nM for ABT-199/ A-1331852 and μM for A-1210477
A A-d
D
2 d treat + 3 d rest
A-i
A-ii
A-iii
A-iv
A A-iv

Figure S3



MAVER-1 K562 H929
A C E A C E A C E
BCL-2
26
BCL-XL
26
MCL-1
36
BCL-w
18
BAX
20
BAK
26
MAVER-1 K562 H929
A C E A C E A C E
BID
22
BIM
23
15/12
PUMA
*
23
NOXA
6
BAD
23
GAPDH
37

Figure S4



MAVER-1
Input
IP:BIM
A
E
BC
A
E
BIM
BCL-2
BCL-XL
MCL-1
23
15
12
26
26
36
Input
IP:PUMA
A
E
BC
A
E
PUMA
BCL-2
BCL-XL
MCL-1
23
26
26
36
K562
Input
IP:BIM
A
E
BC
A
E
BIM
BCL-2
BCL-XL
MCL-1
23
15
12
26
26
36
Input
IP:PUMA
A
E
BC
A
E
PUMA
BCL-2
BCL-XL
MCL-1
23
26
26
36
H929
Input
IP:BIM
A
E
BC
A
E
BIM
BCL-2
BCL-XL
MCL-1
23
15
12
26
26
36
Input
IP:PUMA
A
E
BC
A
E
PUMA
BCL-2
BCL-XL
MCL-1
23
26
26
36

Figure S5

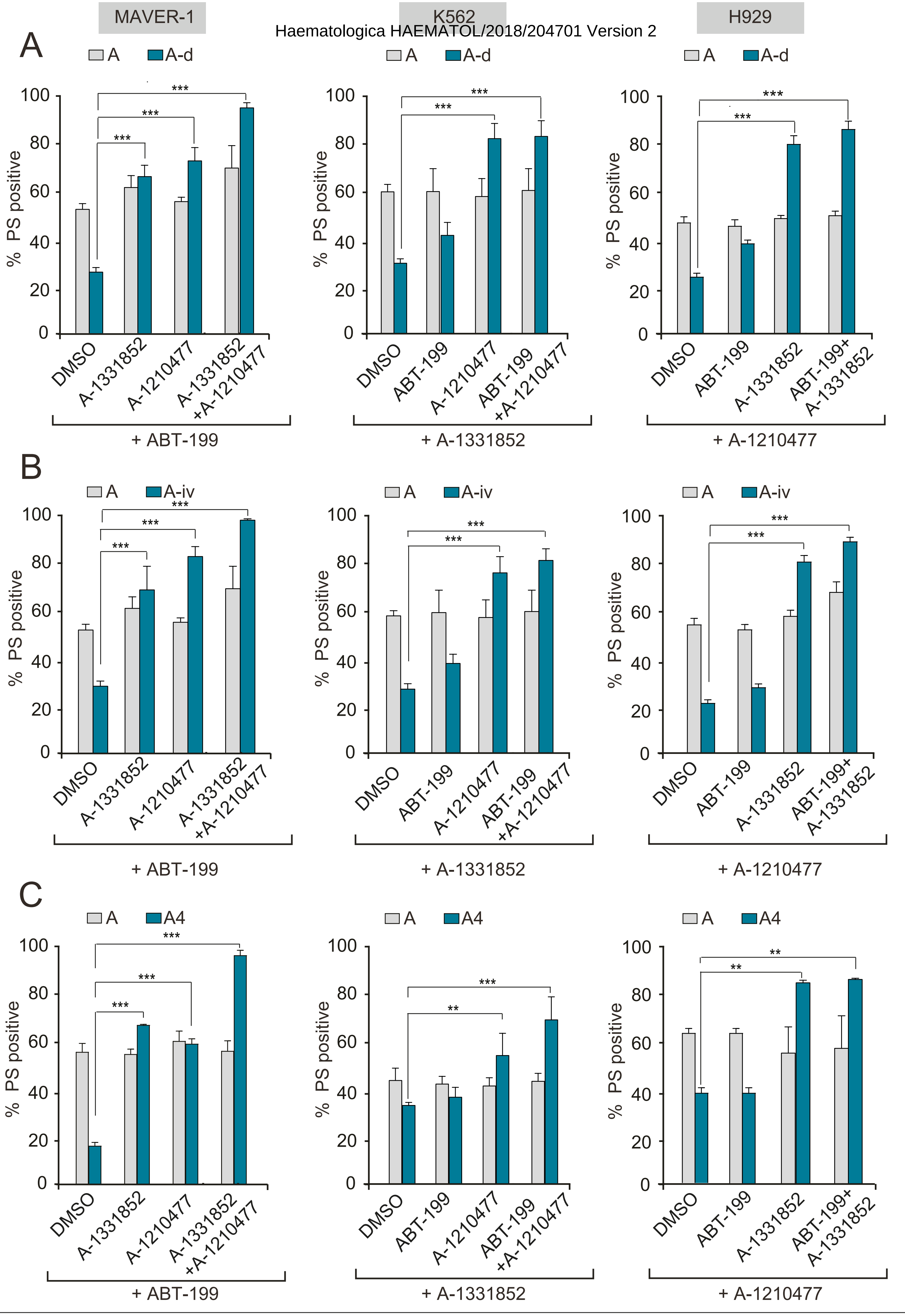
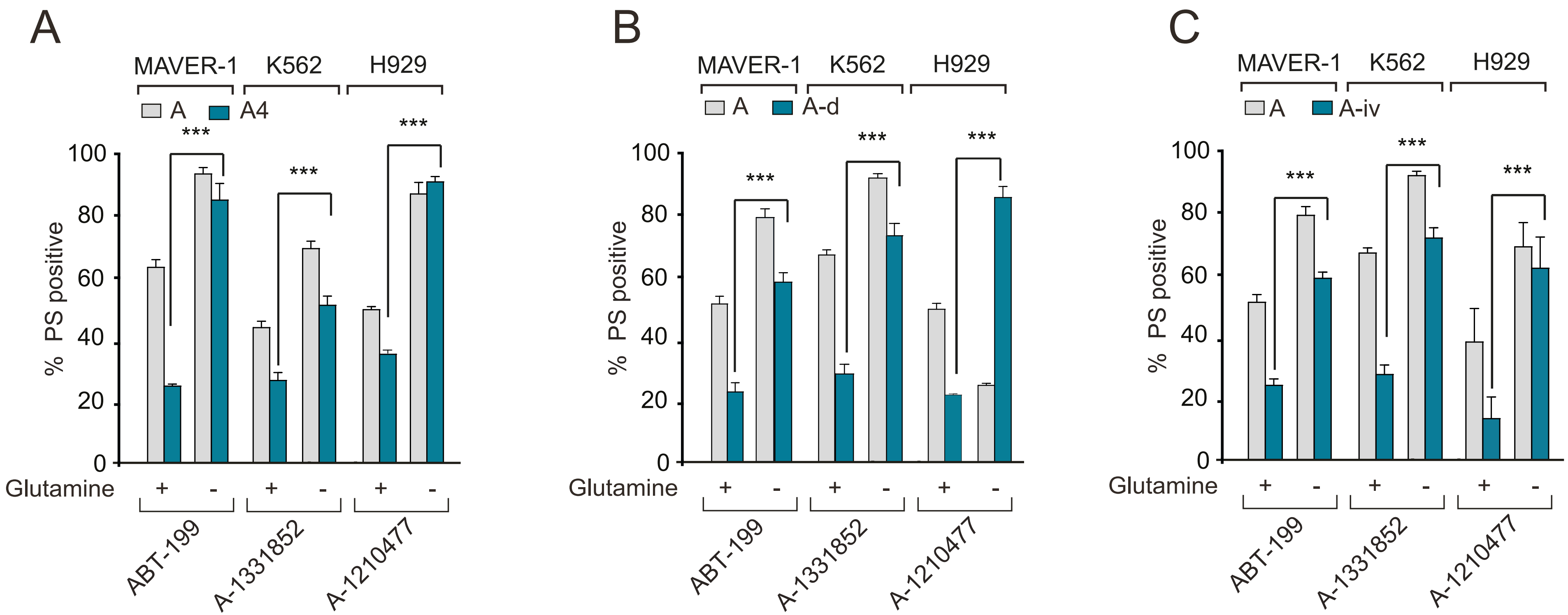
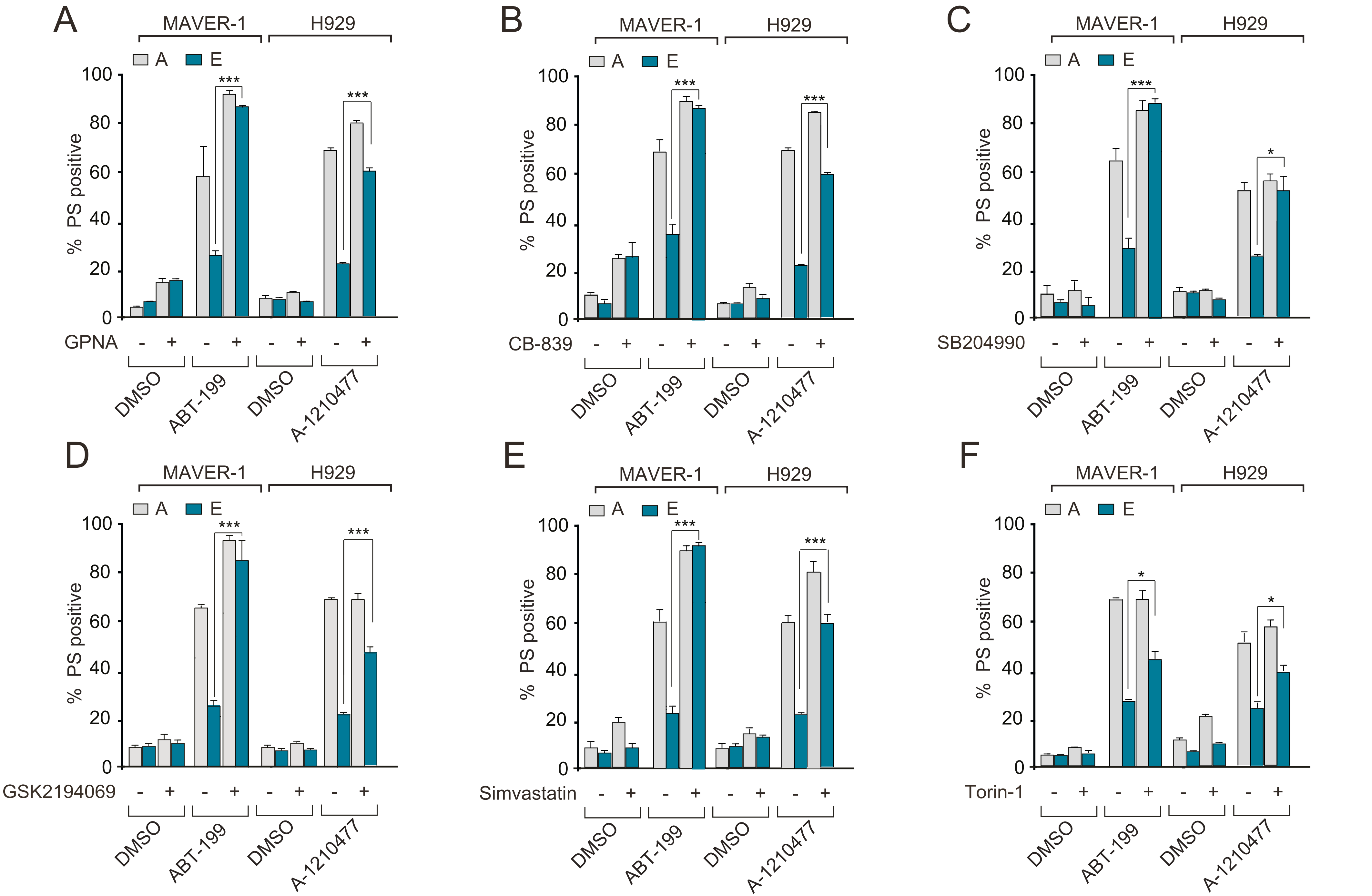

MAVER-1
K562
H929
A
B
C
A
A-d
A-iv
A4
100
80
60
40
20
0
% PS positive
***
**
DMSO
A-1331852
A-1210477
A-1331852 +A-1210477
ABT-199
ABT-199 +A-1210477
ABT-199+ A-1331852
+ ABT-199
+ A-1331852
+ A-1210477

Figure S6



A
B
C
MAVER-1
K562
H929
A
A4
A-d
A-iv
***
100
80
60
40
20
0
% PS positive
Glutamine
+
-
ABT-199
A-1331852
A-1210477

Figure S7


A
B
C
D
E
F
MAVER-1
H929
A
E
% PS positive
0
20
40
60
80
100
***
*
GPNA
CB-839
SB204990
GSK2194069
Simvastatin
Torin-1
-
+
DMSO
ABT-199
A-1210477