Title: The caspase-8 inhibitor emricasan combines with the Smac-mimetic birinapant to induce necroptosis and treat Acute Myeloid Leukemia.

Authors: G. Brumatti[1, 2#], C. Ma[1, 2#], N. Lalaoui[1, 2], N. Nguyen[3], M. Navarro[4], J. Richmond[5], M. Ghisi[6], J. M. Salmon[6], N. Silke[1, 2], S. P. Glaser[1, 2], E. de Valle[7, 8], R. Gugasyan[7, 8], M. A. Gurthridge[3], S. M. Condon[9], R. W. Johnstone[6], R. Lock[5], G. Salvesen[4], A. Wei[3], D. L. Vaux[1, 2], P. G. Ekert[1, 2, 10*‡] and J. Silke[1, 2*‡]

Affiliations:

[1]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia

[2]Dept. of Medical Biology, University of Melbourne, Parkville, Victoria 3050, Australia

[3]Dept. of Clinical Hematology, The Alfred Hospital, Prahan, Victoria 3004, Australia

[4]Sanford-Burham Medical Research Institute, La Jolla, California 92037, USA

[5]Children's Cancer Institute, Lowy Cancer Research Centre, UNSW, NSW 2031, Australia

[6]Peter MacCallum Cancer Centre, East Melbourne, Victoria 3002, Australia

[7]The Burnet Institute, Prahan, Victoria 3004, Australia

[8]Immunology, Central Clinical School, Monash University, Victoria, Australia

[9]TetraLogic Pharmaceuticals Corporation, Malvern, Pennsylvania 19355, USA

[10]Murdoch Children's Research Institute, The Royal Children's Hospital, Parkville, Victoria 3052, Australia

# authors contributed equally to this work

‡ authors contributed equally to this work

* corresponding author: John Silke: silke@wehi.edu.au

Paul Ekert: paul.ekert@mcri.edu.au

One Sentence Summary: The clinically relevant combination of the Smac-mimetic, birinapant, and a caspase inhibitor, emricasan, induces necroptosis to kill AML cells *in vitro* and *in vivo*.

Abstract:

Resistance to chemotherapy is a major problem in cancer treatment and frequently associated with failure of tumor cells to undergo apoptosis. Birinapant, a clinical Smac-mimetic, has been designed to mimic the interaction between Inhibitor of APoptosis proteins (IAPs) and Smac/DIABLO, thereby relieving IAP mediated caspase inhibition and promoting apoptosis of cancer cells. We show that AML cells are sensitive to birinapant induced death and that the clinical caspase inhibitor emricasan/IDN-6556, augments, rather than prevents, birinapant killing. Deletion of caspase-8 sensitized AML to birinapant, whereas combined loss of caspase-8 and the necroptosis effector, MLKL, prevented bir/IDN induced death, showing that inhibition of caspase-8 sensitizes AML cells to birinapant-induced necroptosis. Remarkably however, loss of MLKL alone did not prevent a slower caspase dependent bir/IDN induced death implying that AML will be less likely to acquire resistance to this drug combination. A therapeutic breakthrough in AML has eluded the clinic for decades (*1*). Demonstrated anti-leukemic efficacy and tolerance to the birinapant/emricasan combination *in vivo* suggests that induction of necroptosis warrants clinical investigation as a new therapeutic opportunity in AML.

Introduction

It is a hallmark of cancer cells that they become resistant to apoptosis as they acquire a malignant phenotype (*2*). This has led to the development of novel therapies that resensitize cancers by targeting cell death inhibitor proteins. For example, BH3 mimetics such as venetoclax antagonize

pro-survival Bcl2 family proteins, while Smac-mimetics such as birinapant, antagonize Inhibitor of APoptosis (IAP) proteins (*3*, *4*). Both classes of compounds specifically inhibit protein-protein interactions to activate cell death inducing pathways.

Smac-mimetics were designed to antagonize XIAP and relieve caspases from XIAP-dependent inhibition thereby promoting cancer cell apoptosis (*5*). However these compounds also bind to the BIR domains of cIAPs promoting their auto-ubiquitylation and proteosomal degradation (*6-8*). cIAP degradation has two consequences: in a subset of cancer cells it activates a transcriptional pathway resulting in autocrine production of TNF (*6*, *7*, *9*); and secondly, it sensitizes cells to the cytotoxic activity of TNF, an activity usually kept in abeyance by the cIAPs (*6*, *10*). Several studies have shown that IAPs also play a role in the regulation of the switch between two forms of cell death, apoptosis and necroptosis (*11*, *12*). Upon TNF and TNFR1 interaction, a complex (complex I) containing TNFR-Associated Death Domain (TRADD), RIPK1, TNFR-Associated Factor 2 (TRAF2) and cIAP1/2 is recruited to the receptor. cIAP proteins promote RIPK1 ubiquitylation contributing to canonical NF-κB activation. When cIAP1/2 are depleted by Smac-mimetics, non-ubiquitylated RIPK1 recruits caspase-8 to form an alternative complex, complex IIa, initiating apoptosis (*13*). In the presence of caspase inhibitors, a complex IIb is assembled, consisting of RIPK1, RIPK3 and MLKL, to induce programmed necrosis (necroptosis) (*14*). Thus, Smac-mimetic compounds can induce cell death via two pathways; a caspase-dependent apoptotic pathway and a caspase-independent necroptotic pathway.

AML is an aggressive disease characterized by low survival and high relapse rate (*15*, *16*) and new therapeutic strategies are urgently needed. AML is a genetically heterogeneous disease

however certain chromosomal rearrangements occur commonly (*17*). For example, two subgroups of AML, infant AML and secondary AML associated with prior treatment with DNA topoisomerase II inhibitors, have a high frequency of 11q23 aberration and are associated with a very poor prognosis. 11q23 rearrangements fuse the Lysine (K)- Specific Methyltransferase 2A (*KMT2A*) gene encoding MLL to one of at least 64 different partners. The translocation partners *MLLT3* (encoding AF9), *MLLT1* (encoding ENL) and *AFF4* (encoding AF4) are the most common translocations (*18*). These, and other translocation-driven leukemias, are modeled in mice using retroviral expression of the fusion protein in hematopoietic stem cells (*19, 20*).

We have developed clinically relevant models of AML to test *in vitro* and *in vivo* whether targeting IAPs with the clinical Smac-mimetic birinapant, is effective in the treatment of AML and how it can be most effectively used to increase the chances of cure of AML. Using these models we are able to show that a clinical caspase-8 inhibitor, emricasan, is able to sensitize AML to birinapant and even overcomes birinapant resistance.

Results

Sensitivity of AML to birinapant

To determine the efficiency of birinapant in inducing apoptosis in AML cells, we tested four primary mouse AML models for their sensitivity to birinapant (**Fig. 1A**). In survival assays, MLL-AF9 and MLL-ENL cells were sensitive and killed by birinapant, while Nup98-HoxA9 and HoxA9+Meis1 leukemias were resistant (**Fig. 1B**). Birinapant killed MLL-ENL cells more effectively than a standard AML chemotherapeutic, ara-C (**Fig. 1C**). Leukemic cells pretreated with TNFR1 blocking antibody or generated on a *Tnfr1*$^{-/-}$ background were resistant to birinapant induced cell death (**Fig. 1D & Fig. S1A-E**), demonstrating that birinapant induced TNF/TNFR1-

dependent cell death in MLL-ENL leukemias. To determine the mechanism of cell death, AML cells were treated with birinapant and the pan-caspase inhibitors; Z-VAD-FMK and Q-VD-OPh (**Fig. 1e**). Unexpectedly, rather than inhibiting birinapant induced cell death, Z-VAD-FMK, but not Q-VD-OPh, significantly sensitized MLL-ENL and MLL-AF9, but not HoxA9+Meis1 cells, to birinapant killing (**Fig. 1E**).

Caspase inhibitors sensitizes AML cells to birinapant

Caspase inhibitors could sensitize cells to Smac-mimetics by inhibiting caspase-8 and promoting necroptotic cell death (*21- 23*) (**Fig. 2A**). To test this hypothesis, we generated MLL-ENL AMLs deficient in genes required for necroptosis (**Fig. 2A, B & Fig. S1**). Deleting *Tnfr1*, *Ripk3* or *Mlkl* did not alter the onset or nature of MLL-ENL leukemia (**Fig. 2B**). Loss of *Tnfr1* prevented birinapant/Z-VAD-FMK (bir/ZVAD) induced death, however loss of *Ripk3* or *Mlkl* alone did not protect cells (**Fig. 2C**).

Intrigued, we tested whether the sensitizing effect of caspase-inhibition functioned in birinapant resistant AML cells. To model clinical drug resistance, we generated MLL-ENL AML with acquired resistance to birinapant (MLL-ENL^{R}) by culturing MLL-ENL leukemic cells in increasing doses of birinapant over five weeks (**Fig. S2A**). This AML was 10-fold more resistant than its founder line, or a line cultured without birinapant over five weeks, but still responded to ara-C (**Fig. S2B**). Western blots of resistant and sensitive AML treated with birinapant showed absent caspase activation in MLL-ENL resistant cells compared to sensitive cells (**Fig. S2C**). Nevertheless birinapant resistant AML were strongly sensitized to birinapant killing by Z-VAD-

FMK or Q-VD-OPh (**Fig. 2D**). The human AML cell line MV4-11 is resistant to birinapant but was also sensitized by Z-VAD-FMK to a similar degree as the primary murine AMLs (**Fig. 2E**).

Neither Z-VAD-FMK nor Q-VD-OPh can be used clinically so we tested whether birinapant in combination with a pre-clinical caspase-inhibitor could kill AML cells. IDN-6556/emricasan is a caspase inhibitor that inhibits apoptosis in the liver and is well tolerated in humans (*24*). IDN-6556 was better at promoting birinapant-induced cell death than either Z-VAD-FMK or Q-VD-OPh (compare **Fig. 2E & 3A**), and very few AML cells from different models survived the combined birinapant/IDN-6556 (bir/IDN) treatment (**Fig. 3A, B & Fig. S3A-C)**. Importantly MLL-ENL cells with acquired resistance to birinapant or which co-express an activating Ras-mutation and are resistant to standard chemotherapy (*25, 26*), were all dramatically sensitized to birinapant killing by IDN-6556 (**Fig. 3B & Fig. S3B**). Finally, bir/IDN killing is independent of Bax and Bak mediated apoptosis (**Fig. S3C**).

IDN-6556 combines with birinapant to induce necroptosis in AML

Genetic deletion of *Tnfr1* or TNFR1 inhibition prevented bir/IDN killing, thus the fundamental mechanism of killing by birinapant and bir/IDN is the same (**Fig. 3C & Fig. S4A-B**). *Ripk3* and *Mlkl* deficiency provided a greater degree of protection to bir/IDN than in cells treated with bir/ZVAD (Compare **Fig. 3C** & **2C**), however loss of these necroptotic effectors did not completely prevent bir/IDN induced cell death. Conversely, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ MLL-ENL leukemias were completely resistant to bir/IDN treatment (**Fig. 3C & Fig. S5A-C**), suggesting residual caspase-8 activity was responsible for the cell death observed in *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ cells treated with bir/ZVAD or bir/IDN. This leads to two predictions: firstly that in

necroptosis deficient cells, bir/IDN treated cells will die more slowly than wild type cells and secondly, that the slower cell death might be blockable by additional caspase inhibitor. Consistent with this, cell death mediated by bir/IDN was significantly delayed in *Ripk3*$^{-/-}$ or *Mlkl*$^{-/-}$ knock-out cells, compared to wild-type cells (**Fig. 3D**), and high dose Q-VD-OPh prevented bir/IDN cell death in *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ but not wild type AML (**Fig. 3E**).

Caspase-8 inhibition as the key target of birinapant mediated necroptosis

To test whether genetic loss of caspase-8 would sensitize AML cells to birinapant killing, we attempted to generate *Casp8*$^{-/-}$ AML cells using conditional *Casp8*$^{lox/lox}$ fetal liver progenitors. Unfortunately the single deletion of caspase-8 induced killing of AML cells (data not shown). We instead re-expressed inducible MLKL (MLKL^{i}) in *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ MLL-ENL leukemias (**Fig. S5D**). Birinapant was very potent at inducing cell death in *Casp8*$^{-/-}$*Mlkl*$^{-/-}$+MLKL^{i} AMLs to a level comparable to wild type AMLs treated with bir/IDN (**Fig. 3F**).

The differential ability of IDN-6556, Z-VAD-FMK and Q-VD-OPh to synergize with birinapant to induce cell death may correlate with their respective ability to inhibit caspase-8. The caspase-8/cFLIP$_L$ heterodimer is catalytically active and has been proposed to be the form of caspase-8 that inhibits necroptosis {Pop, 2011 #18;Oberst, 2011 #18}. We therefore compared the ability of these caspase inhibitors to inhibit the caspase-8 homodimer or the caspase-8/cFLIP$_L$ heterodimer. We generated caspase-8 homodimers or caspase-8/cFLIP$_L$ heterodimers *in vitro* as described (*27*), and incubated them with a flourogenic substrate in the presence of each inhibitor. Inhibition rate constant (k_{obs}/I) values for both protease species show that IDN-6556 is a better caspase-8 inhibitor than either Z-VAD-FMK or Q-VD-OPh (**Fig. 3G & Fig. S6A-B**). Strikingly,

IDN-6556 and Z-VAD-FMK inhibited the caspase-8/cFLIP$_L$ heterodimer more efficiently than Q-VD-OPh. These results fit a model whereby inhibition of the caspase-8/cFLIP$_L$ heterodimer efficiently activates birinapant-induced necroptosis. However neither Z-VAD-FMK nor IDN-6556 are able to restrain homodimeric caspase-8 sufficiently to prevent birinapant-induced apoptosis.

Necroptosis mediated by bir/IDN eliminates AML in vivo

To determine if the combination of bir/IDN was tolerated and effective *in vivo*, mice were transplanted with MLL-ENL cells bearing a luciferase transgene. Dosing commenced when the disease burden in these animals reached an emission of 10^7 – 10^8 photons/second (**Fig. 4A, B**). Bir/IDN treatment was well tolerated *in vivo* (**Fig. S7E-G**) and reduced disease burden and increased long-term survival in the majority of animals, with the disease-free state extending 80-100 days after treatment began (**Fig. 4B-C**). A smaller proportion of animals responded to birinapant alone, whereas untreated mice and those receiving IDN-6556 alone rapidly succumbed to AML. Histological examination confirmed remission in combination treated animals (**Fig. 4D**). IDN-6556 accumulates in the liver thus it was noteworthy that combination treated mice had less leukemia in the liver than those treated with birinapant (**Fig. 4D**).

Tolerability and efficacy of bir/IDN therapy in human primary cells

Finally, we tested the bir/IDN combination on primary human hematopoietic cells and Patient Derived Xenografts (PDX) from AML and ALL MLL-harboring leukemias and patient AML bone marrow and peripheral blood samples (**Fig. 4E-F & Fig. S7A-D**). The combined treatment was less toxic to CD34$^+$, PBMC (peripheral blood mononuclear cell), T cells, B cells and NK

cells than standard chemotherapy (**Fig. S7A-D**) and effective in 3 out of 4 PDX samples tested (**Fig. 4E**). To extend our analysis, we compared the response of AML bone marrow and peripheral blood samples to bir or bir/IDN treatment (**Fig. 4F**). Overall four out of eight samples were clearly sensitized to birinapant killing by IDN-6556 (patient 1, 2, 3 & 6). Importantly, chemoresistant AMLs, from patients with relapsed or secondary disease, were sensitive to bir/IDN (patient 1&2, **Fig. 4F**). Four other AML samples responded to birinapant alone (4, 5, 7 & 8) and in three of these birinapant induced death was inhibited by the addition of IDN-6556. These results show that combined IAP/caspase-8/cFLIP$_L$ inhibition is likely to be an effective therapeutic combination for many patients that have failed standard chemotherapy.

Discussion

Despite over three decades of research, the survival rates in Acute Myeloid Leukemia have only improved 24%, with over 40% of the patients relapsing within 12 months after intensive chemotherapy (*16*). In most AML cases, chemoresistance is strongly associated with increased expression of anti-apoptotic proteins such as Bcl-2 and XIAP, and failure of leukemic cells to undergo apoptosis (*3*, *28-30*). Therefore, novel therapies are more likely to succeed if the proposed mechanism of action is distinct from factors typically linked to chemoresistance.

Smac-mimetics, such as birinapant (*31*), are in clinical trials, but like other targeted anti-cancer drugs have limited effectiveness as a single drug. In this study, using two clinical relevant compounds, we show that the combination of birinapant plus the caspase inhibitor emricasan/IDN-6556 is well tolerated and efficiently kills AML cells *in vitro* and *in vivo* by activation of the necroptotic pathway. Together these findings validate the potential of

necroptosis targeting as a new therapeutic strategy to overcome chemoresistance and treat AML.

In our *in vitro* assays we show that AML subtypes, from murine models and human patient cells, differ in their sensitivity to birinapant treatment. However, addition of caspase inhibitors, and particularly emricasan, strongly sensitized all AML types that we tested to birinapant induced cell death. Even AMLs with natural or acquired resistance to birinapant, such as might occur clinically, were killed by the combination of birinapant plus caspases inhibitors. Because caspase inhibition in the presence of IAP antagonist (birinapant) can lead to necroptosis, this result implies that activation of necroptosis is a means to overcome chemoresistance.

Previous studies have shown that necroptosis induced by an experimental Smac-mimetic, BV6, and an experimental caspase inhibitor Z-VAD-FMK, can sensitize AML cell lines to chemotherapy (*32*). Our studies extend this concept. Firstly we show that the combination of a clinical Smac-mimetic and a clinical caspase inhibitor is able induce necroptosis without the addition of chemotherapy and more effectively than the experimental compounds. Furthermore this combination is well tolerated and effective *in vivo*, despite the pharmacodynamic limitations of emricasan. Analysis of the inhibitory profile of emricasan suggests that the reason for the much greater efficacy of this compound compared to Q-VD-OPh in synergizing with birinapant is due to its ability to inhibit the caspase-8 cFLIP heterodimer. It has been suspected that the caspase-8 cFLIP heterodimer is particularly important for inhibiting necroptosis and the fact that emricasan is so effective at inducing necroptosis, while Q-VD-OPh is not, strongly supports this notion (*27, 33*). Our genetic experiments however reveal an unexpected twist: while *Casp8*$^{-/-}$ *Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ AML are completely resistant to birinapant/emricasan induced death

Ripk3$^{-/-}$ and *Mlkl*$^{-/-}$ AML are completely sensitive in long-term survival assays. In short term survival assays this death occurs more slowly than in wild type cells and can be blocked by addition of Q-VD-OPh. These data therefore indicate that indicate that while birinapant and emricasan are strong activators of necroptotic cell death they nevertheless induce an apoptotic cell death too. These results imply that it will be very difficult for leukemic cells to acquire resistance to this therapy.

While our results hold great promise for translation emricasan is not a perfect drug for AML treatment because it has a half life of only 50 minutes in plasma due to a first pass effect in the liver (*24*). The strong inhibition of AML disease in the liver that we observed with birinapant/emricasan is entirely consistent with these pharmacokinetics. It is clear however that for caspase inhibition to be used in the clinic together with birinapant that either emricasan will have to be used with an alternative dosing schedule or a caspase inhibitor with the same inhibitory profile as emricasan but better pharmacokinetics needs to be developed. At the very least, our work should reinvigorate interest in developing such inhibitors with a focus on developing drugs that inhibit the caspase-8/cFLIP heterodimer.

Materials and Methods

Study design

In hypothesis-driven experimental designs, we addressed the therapeutic potential of the relevant clinical drug combination birinapant plus IDN-6556 (bir/IDN) in human AML cell lines, primary human AML cells and murine primary AML cells. The effect of the combination therapy on cell death signaling (apoptosis and necroptosis) and cell growth was assessed by flow cytometry

analysis of cell death markers and colony formation assays. This study was extended to a murine model of AML to analyze the effects of combination therapy *in vivo*. Tolerability and biosafety of the combined therapy bir/IDN was tested *in vivo* in healthy murine specimens, and *in vitro* using $CD34^+$ stem cells and blood cells from healthy patients. All *in vivo* experiments were performed in 8 to 10-week-old mice housed in individually ventilated cages and accordingly to The Walter and Eliza Institute Animal Ethics Committee (AEC) for animal welfare and ethical approach of animals in experimental procedures. *In vivo* experiments were performed two to three times, conducted and analysed blinded. Details on replicates and statistical analyses of *in vivo* and *in vitro* experiments are indicated in the figure legends.

Reagents

Inhibitors Z-VAD-FMK and Q-VD-OPH, and fluorescent substrate Ac-IETD-afc were purchased from SM Biochemicals (Anaheim, Ca). Inhibitor IDN-6556 was purchased from Medchemexpress LLC (Monmouth Junction, NJ). A/C heterodimerizer was purchased from Clontech (Mountain View, Ca). Fluorescence measurements were performed in 96-well white plates using the Gemini EM fluorimeter from Molecular Devices (Sunnyvale, CA). The Smac-mimetic birinapant and the caspase-8 inhibitor, IDN-6556, used in *in vitro* and *in vivo* experiments with AML cells were provided by TetraLogic Pharmaceuticals. Cytarabine (ara-C) and Daunorubicin were purchased from Selleckchem Pharma. Blocking TNF antibodies, murine anti-TNFR-1 and human anti-TNF-alpha were purchased from R&D systems.

Generation and culture of murine AML cells

All *in vivo* experiments were conducted in accordance with The Walter and Eliza Hall Institute Animal Ethics Committee guidelines. MLL-ENL, MLL-AF9, HoxA9/Meis1 and Nup98-HoxA9 retroviral constructs were previously described (*34- 37*). Viral supernatants were produced in 293T cells by co-transfection of oncogenes constructs and packaging plasmids. Progenitor/stem cells derived from fetal liver (E14.5) of C57BL/6 Ly5.2 *Wt*, *Tnfr1*$^{-/-}$, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Casp8*$^{-/-}$ *Mlkl*$^{-/-}$, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Bax*$^{-/-}$*Bak*$^{-/-}$ mice (*38, 39*) were cultured in alpha-MEM medium (Invitrogen) supplemented with 10% FCS, 2mM L-glutamine, 100ng/mL murine stem-cell-factor (Peprotech), 10ng/mL IL-6, 50ng/ml TPO and 10ng/ml Flt3 (WEHI) and transduced with viral supernatant during two consecutive days using the retronectin protocol. Retronectin (30µg/ml, WEHI) pre-coated 24 well plates were spin with viral supernatant at 3000rpm for 1h at 30°C, followed by seeding of 1- 1.5 × 10^6 cells/well in supplemented alpha-MEM medium. After infection, transduced cells were injected into sub-lethally g-irradiated (7.5 Gy) C57BL/6 Ly5.1 mice. Leukemia development was determined by weight-loss, enlarged spleen, anemia, lethargy and hunched posture. Leukemic cells were obtained from bone marrow of sick mice and cultured at 37°C in a 10% CO_2 humidified atmosphere in IMDM media supplemented with 10% fetal calf serum and 3ng/ml IL-3 (R&D).

Human leukemic cells

Human leukemic cell lines MV4-11, U937 and OCI-AML3 were cultured at 37°C in a 10% CO_2 humidified atmosphere in RPMI media supplemented with 10% fetal calf serum. Patient derived leukemic cells were cultured in Stemspan SFEM II media (Stem Cell Technologies)

supplemented with 100ng/ml rhFlt-3L, 100ng/ml rhSCF, 20ng/ml rhIL-3, 20ng/ml rhIL-6 (R&D) and 100IU/ml DNaseI (Roche) and penicillin/streptomycin (Gibco) in 10% CO_2 at 37°C.

Western Blot and reagents

Total cell lysates were prepared by lysis of 1.10^7cells/ml in sodium dodecyl sulfate (SDS) buffer (50mM Tris-HCL, pH 6.8, 2% SDS, 10% glycerol and 2,5% β-mercaptoethanol) and boiling for 10 min. The antibodies used were; anti-mouse Caspase-8 pAb, anti-cleaved caspase-8 (Asp387) pAb and anti-cleaved caspase-3 (Asp175) pAb (Cell Signaling), anti-c-Flip (Dave) mAb and anti-Ripk3 pAb (ProScience), anti-Ripk1 mAb (BD), anti-cIAP-1 mAb (in house) and anti-Actin mAb (Sigma).

Protein expression, Purification and Nomenclature

Genes of human His-tagged caspase-8 (ΔCARD) and $FLIP_L$ (ΔDEDs) were expressed in *E. coli* BL21 (DE3). Protein production was induced with 1mM IPTG and proteins were purified by Ni^{2+}affinity chromatography. For caspase-8 affinity chromatography was followed by further purification on a mono-Q anion-exchange Sepharose column using the AKTA system. Generation of the caspase-8 homodimer (Casp8/Casp8) and the caspase 8/c$FLIP_L$ heterodimer (Casp8/FLIP) has been described elsewhere (*27*). Briefly, the caspase-8 monomer forming the Casp8/Casp8 dimer consists of the wild type sequence of the large and small subunits. Two amino acids, Asp374 and Asp384, located in the linker between the large and small subunits, in the caspase-8 that forms Casp8/FLIP were mutated to Ala to prevent autocleavage. This double mutant caspase-8 was fused C-terminally to the dimerization domain FKBP rendering the working species FKBP-Csp8D2A.

Dimer Activation and Kinetic Characterization

Activity of commercial inhibitor preparations was determined by back titration with caspase-3 using 200µM Ac-IETD-afc and different dilutions of the inhibitors. Residual enzymatic activity was plotted against concentration of inhibitor. In reactions with Csp8/Csp8 proteins, inhibitors, and substrate were prepared in a kosmotropic solution (30mM Tris/HCl (pH 7.4), 1M sodium citrate, 5mM DTT and 0.05% CHAPS). Reactions with Csp8/FLIP proteins, inhibitors, and substrate were prepared in caspase buffer (20mM Pipes (pH 7.2), 0.1M NaCl, 1mM EDTA, 10% sucrose, 0.1% CHAPS and 5mM DTT). Activation of the Csp8/Csp8 homodimer was achieved by incubating Caspase-8 at a final concentration of 10nM in kosmotropic solution for 20 minutes at 37°C. Upon activation the homodimer was reacted in 100µl with a mixture of 200µM Ac-IETD-afc and inhibitor at different concentrations. Reaction progress was monitored by fluorescence emission of the -afc group every 2 seconds for 15 minutes at 37°C. For activation of the Casp8/FLIP heterodimer FKBP-Csp8D2A at a final concentration of 50nM, FRB-FLIP at 250nM and A/C heterodimerizer at 250nM were incubated in caspase buffer for 20 minutes at 37°C. Upon activation, the heterodimer was reacted in 100µl with a mixture of 200µM Ac-IETD-afc and inhibitor at different concentrations. Reaction progress was monitored by fluorescence emission of the -afc group every 2 seconds for 15 minutes at 37°C. Km of Casp8/Casp8 and Casp8/FLIP for Ac-IETD-afc was determined by activating each species with the protocol described above but substrate was reacted in a concentration gradient from 1nM-128nM for the homodimer and 10nM-320nM for the heterodimer. Reaction progress was monitored by fluorescence emission of the -afc group every 2 seconds for 15 minutes at 37°C.

Data Analysis

Data analysis of caspases biochemical assays was performed using Prism 6 Software. Calculations performed with the linear segment of each progress curve (~ first 200 seconds). K_{obs}/I of Z-VAD-FMK, Q-VD-OPH and IDN-6556 for Casp8/Casp8 for were obtained by fitting reaction rates to the pseudo first order model:

$$V_{obs}=Vs*[S]+(Vo-Vs)*(1-exp(-k_{obs}*[S]))/k_{obs}$$

Where S is substrate concentration, Vo is initial velocity and Vs the steady state rate of substrate hydrolysis in the presence of inhibitor. To obtain K_{obs}/I of Q-VD-OPH and IDN-6556 for Casp8/FLIP we applied the same model, however for Z-VAD-FMK, k_2/k_1 was calculated instead. To derive k_2/k_1 rates were fitted to a hyperbola using the following model:

$$K_2/K_1=k_2*[E]/(K_1+[E])$$

Km of Casp8/Casp8 and Casp8/FLIP for Ac-IETD-afc was calculated using the following equation:

$$Vobs=[E]*kcat*[S]/(Km + [S])$$

Statistical analysis of leukemic cells *in vitro* and *in vivo* was performed using Prism 6.0 software. All experiments with data from $n\leq 3$ are represented as means ± SEM. Data from in vitro assays were analyzed using Student's t test (two-tailed) and Kaplan-Meier survival curves was analysed using the log-rank (Mantel-Cox) test and log-rank test for trend. P values are

indicated in figure legends, considering $P < 0.05$ significant.

Clonogenic assays

Colony formation assays of AML cells were performed as previously described (*40*).

Supplementary Materials

Fig. S1. Generation of single gene knockout leukemias

Fig. S2. Generation of MLL-ENL birinapant resistant cells

Fig. S3. IDN-6556 mediated necroptosis in different AML subtypes

Fig. S4. IDN-6556 mediated necroptosis in human cells is impaired by TNFRI deletion and blockage

Fig. S5. Generation of double gene knockout leukemias

Fig. S6. Comparison of caspase inhibitors IDN-6556, Z-VAD and Q-VD

Fig. S7. Biosafety of combined treatment bir/IDN *in vivo* and *in vitro*

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Acknowledgments

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Figures

Fig. 1. AML driven by different oncogenes show varying sensitivity to birinapant

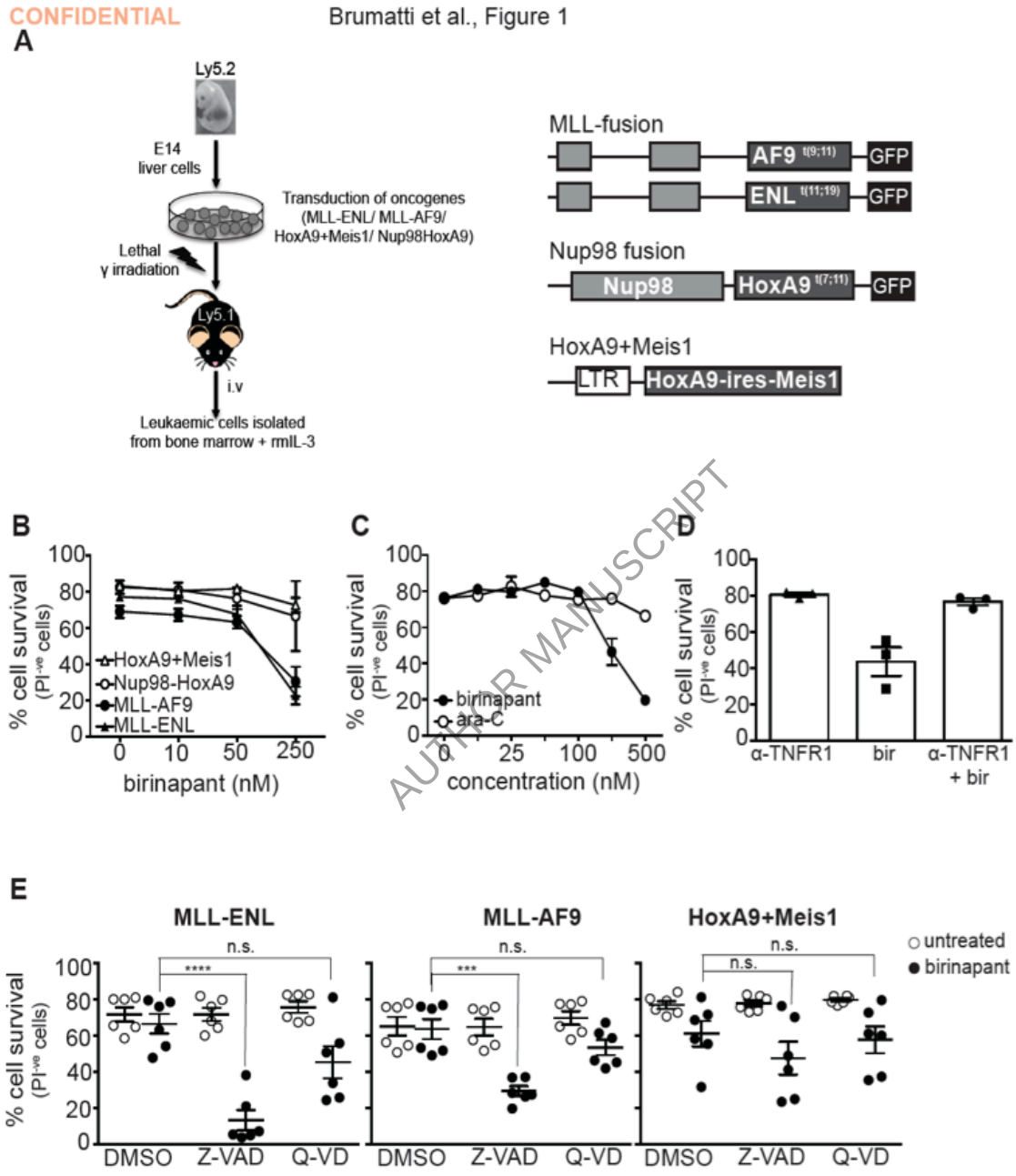


Fig. 1. (**A**) Schematic of oncogenes and AML derivation protocol. (**B**) Survival of different AML in response to birinapant. Primary leukemic cells from four AML models were treated with birinapant for 16h. Cell survival data represents mean ± SEM of 3-6 independent tumors per model. (**C**) Cell viability of MLL-ENL leukemic cells 16h after treatment with different concentrations of birinapant or Cytarabine (Ara-C). Mean ± SEM, n=6 independent tumors. (**D**) TNFR1 is required for cell death mediated by birinapant in MLL-ENL leukemias. Cells were treated with 100nM of birinapant ± TNFR-1 antibody (10μg/ml) for 16h. Data represents mean ± SEM, n=3 independent tumors. (**E**) Cells from primary MLL-ENL, MLL-AF9 and HoxA9+Meis1 leukemias were pre-treated for 15-30min with the pan-caspase inhibitors Z-VAD-FMK (5μM) or Q-VD-OPh (10μM) followed by 100nM birinapant treatment for 16h. Data represents mean ± SEM, n=6.

Fig. 2. Caspase inhibition sensitizes leukemic cells to birinapant.

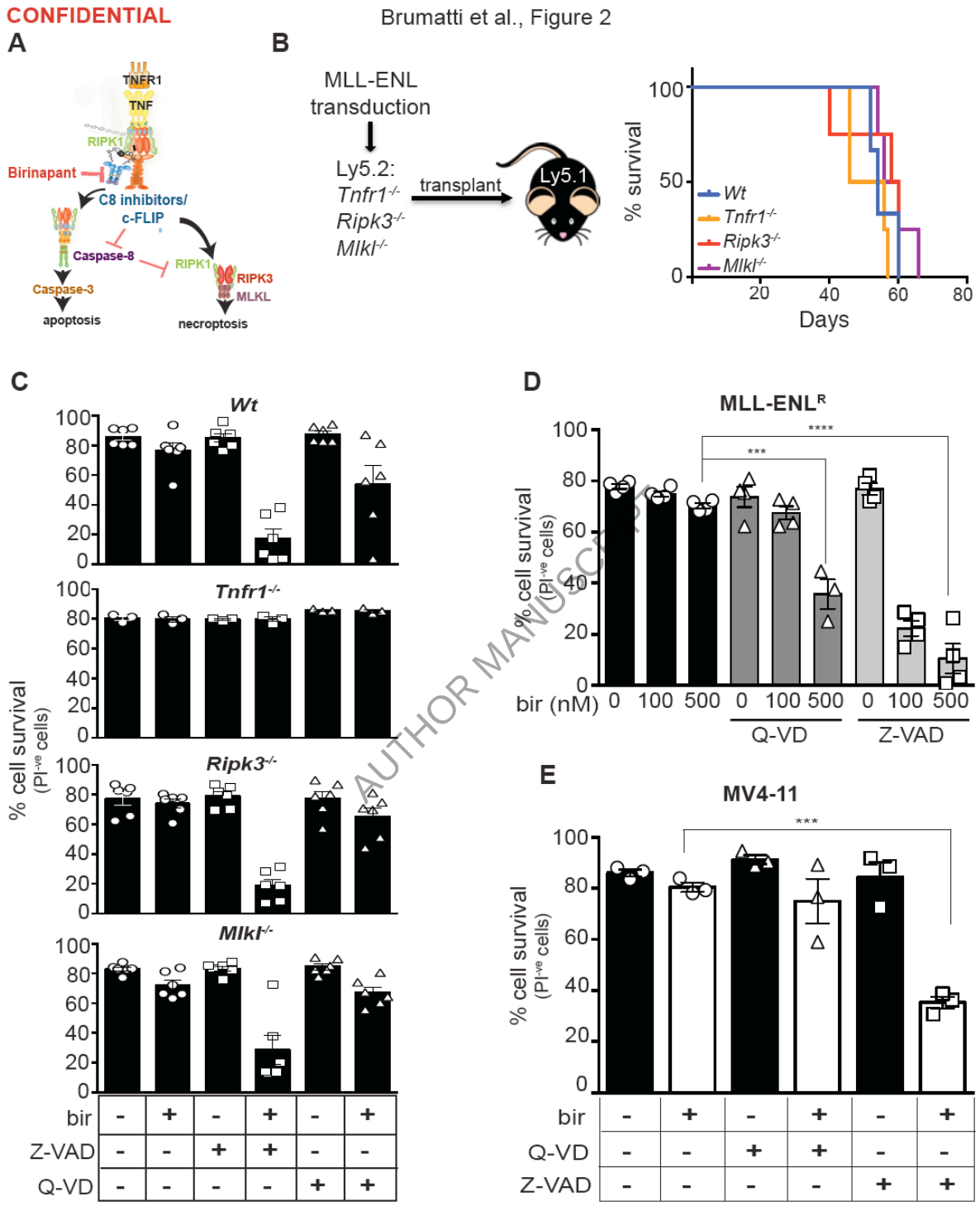
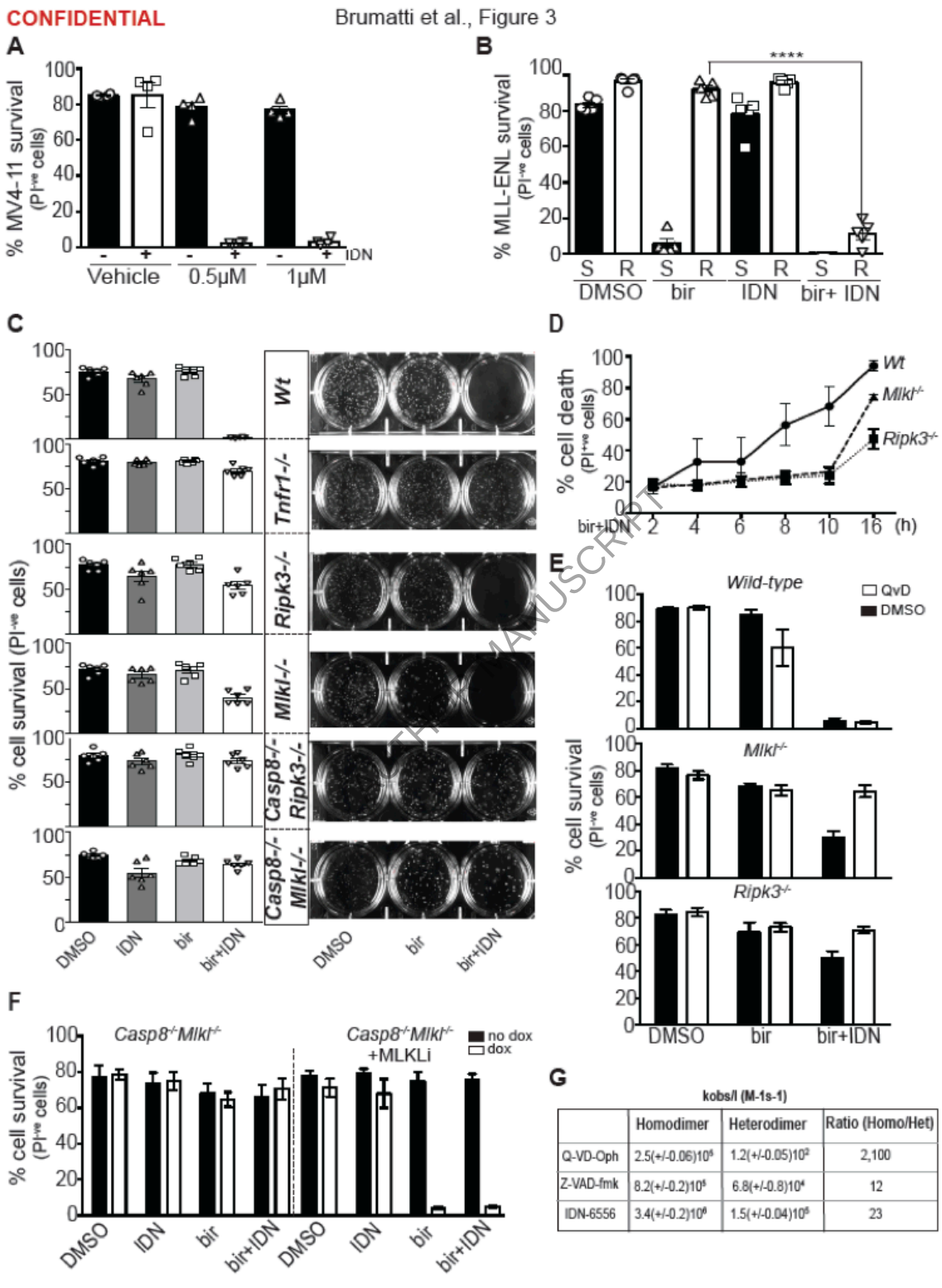


Fig. 2. (**A**) Schematic of TNF death signaling pathways, apoptosis and necroptosis. (**B**) Kaplan-Meier survival curve of mice transplanted with MLL-ENL transduced E14 liver cells of the indicated genotype. (**C**) MLL-ENL cells from wt, *Tnfr1*$^{-/-}$, *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice were pre-treated (15-30min) with the caspase inhibitors Z-VAD-FMK (5μM) or Q-VD-OPh (10μM) followed by birinapant (100nM). Cell viability was determined 16h post treatment. Mean + SEM, n=6. (**D**) Birinapant resistant MLL-ENL leukemic cells are sensitized to cell death induced by birinapant plus pan-caspase inhibitors. Cells were pre-treated (15-30min) with the pan-caspase inhibitors Z-VAD-FMK (10μM) or Q-VD-OPh (10μM) followed by 100 or 500nM of birinapant. Cell viability was determined 16h post treatment. Data represents mean ± SEM of two independent leukemias, each tested in 2 independent experiments (n=4). (**E**) Human AML cell line MV4-11 was treated with birinapant ± Z-VAD-FMK (10μM) or Q-VD-OPh (10μM). Data represents mean ± SEM, n=3. (**F**) Cell survival was determined by PI uptake and flow cytometry throughout. (***) P< 0.0005; (****) P< 0.0001.

Fig. 3. Caspase-8 inhibitors are potent inducers of necroptosis in leukemic cells.



	Homodimer	Heterodimer	Ratio (Homo/Het)
Q-VD-Oph	2.5(+/-0.06)10^5	1.2(+/-0.05)10^2	2,100
Z-VAD-fmk	8.2(+/-0.2)10^5	6.8(+/-0.8)10^4	12
IDN-6556	3.4(+/-0.2)10^6	1.5(+/-0.04)10^5	23

Fig. 3. The caspase-8 inhibitor IDN-6556 sensitises leukemic cells to birinapant killing. (**A**) MV4-11 cells were pre-treated (15-30min) with the caspase inhibitor IDN-6556 (5μM) followed by birinapant for 48h. Data represents mean ± SEM, n=5. (**B**) MLL-ENL birinapant sensitive (S) and MLL-ENL birinapant resistant (R) leukemic cells were pre-treated (15-30min) with IDN-6556 (5μM) followed by birinapant (100nM). Cell viability was determined after 16h. Mean ± SEM, n=5. (**C**) MLL-ENL leukemias derived from wt, *Tnfr1*$^{-/-}$, *Ripk3*$^{-/-}$, *Mlkl*$^{-/-}$, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ progenitors were pre-treated with IDN-6556 (5μM) followed by birinapant (50nM). Cell viability was determined 16h after treatment. Mean ± SEM, n=6. Clonogenic assay to determine long-term survival and proliferation of MLL-ENL leukemia cells treated with birinapant (bir- 250nM) in the presence or absence of IDN-6556 (5μM). Cells were pre-treated for 30min with IDN-6556 followed by birinapant (bir). Image representative of 500 cells were plated in 0.3% agar containing bir or bir+IDN. Clonogenic survival determined 15 days after treatment. (**D**) Time course of killing of MLL-ENL leukemias derived from wt, *Mlkl*$^{-/-}$ and *Ripk3*$^{-/-}$ progenitors pre-treated with IDN-6556 (5μM) followed by birinapant (50nM). (**E**) MLL-ENL leukemias derived from wt, *Mlkl*$^{-/-}$ and *Ripk3*$^{-/-}$ progenitors were pre-treated with IDN-6556 (5μM) followed by birinapant (50nM) ± Q-VD-OPh (10μM). (**F**) MLL-ENL *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ leukemias were transduced with a lentiviral construct expressing MLKL from a doxycycline inducible promoter. Leukemic cells were treated with birinapant ± IDN-6556 in the presence or absence of doxycycline (0.5μg/ml) to induce MLKL. Cell viability was determined 16h after treatment. Mean ± SEM, n=4. (**G**) Comparison of the ability of Z-VAD-FMK, Q-VD-OPh and IDN-6556 to inhibit recombinant caspase-8 homodimer and caspase-8/cFLIP heterodimer. Values are means ± SD of duplicate experiments. Cell survival was determined by PI uptake and flow cytometry throughout.

Fig. 4. Combination of birinapant and clinical caspase-8 inhibitor IDN-6556, prolongs survival of AML.

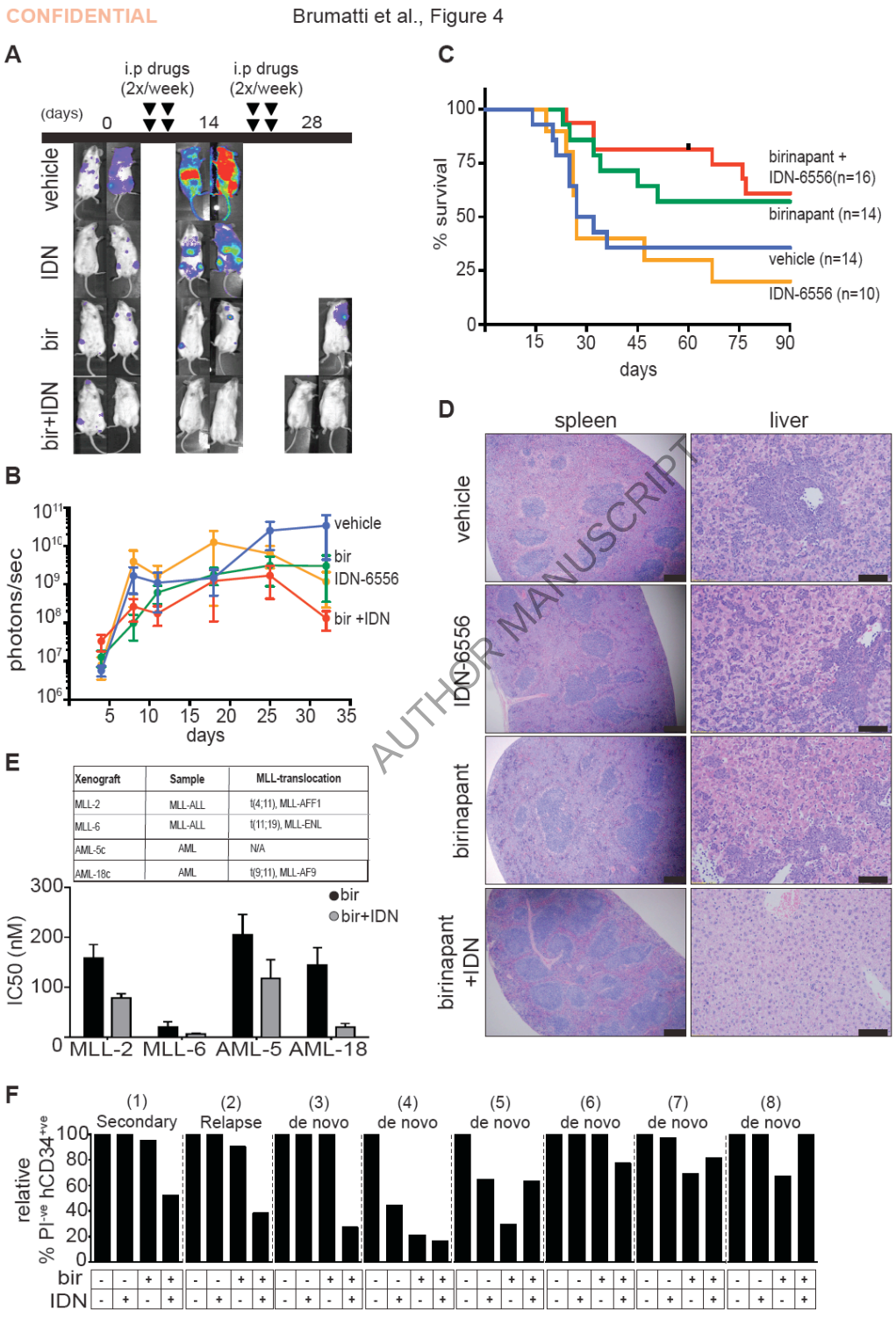


Fig. 4. (**A**) In vivo imaging of tumor progression in mice treated with vehicle, birinapant, IDN-6556 or the combination. Mice were administered drugs twice a week, for 4 weeks, starting treatment 7-10 days after retransplant of tumor cells. (**B**) Graphs represent average photon values from mice treated with birinapant, IDN-6556 or combination. Bars represent SEM of n=14 for vehicle, n=10 for IDN-6556, n=14 for birinapant and n=16 for birinapant+ IDN-6556. (**C**) Kaplan-Meier survival curve of C57BL/6 albino mice treated with vehicle, birinapant, IDN-6556 or combination. (**D**) Histological comparison of spleen and liver from mice burdened with MLL-ENL AML after treatment with birinapant, IDN-6556 or combined therapy**.** Spleen images were taken at magnification of 4x, scale bar 200μm. Liver images were taken at magnification of 20x, scale bar 100μm. (**E**) bir/IDN treatment is effective in ALL and AML PDX samples. IC50 of bir or bir/IDN treatment was determined in PDX samples. Cells were treated for 48h with bir (10-1000nM) ± IDN-6556 (5μM) and cell viability was determined by annexin V/7AAD staining. Data represent means of 4 independent experiments. (**F**) Combined therapy kills patient leukemia samples. Primary leukemic cells from derived from patients of the indicated treatment status, were treated for 24h with birinapant (500nM) ± IDN-6556 (5μM). Cell viability was determined by flow cytometry of $CD34^+$ PI negative cells. Data represent means of 2 independent experiments.

Supplementary Materials:

Fig.S1.

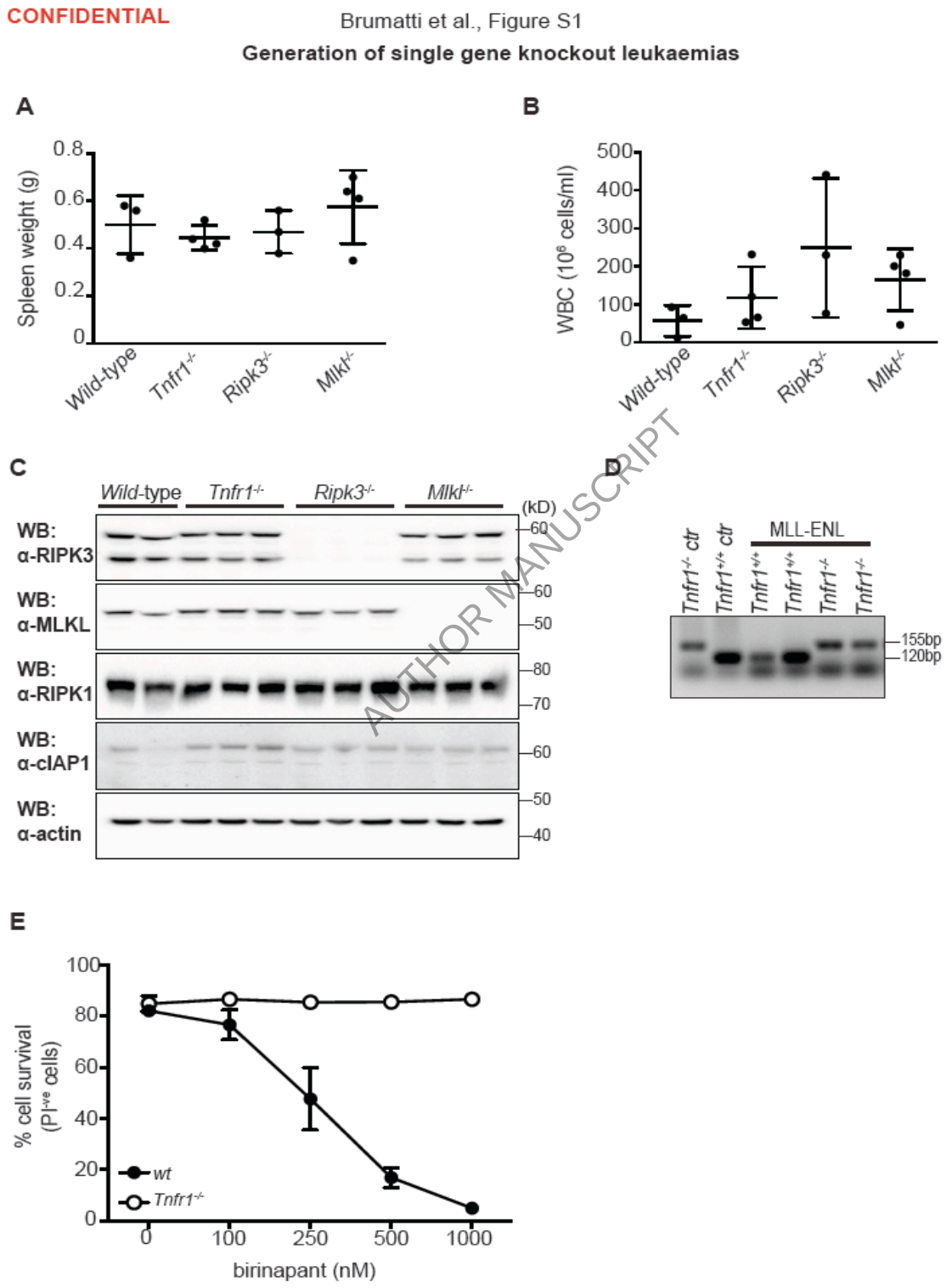


Fig. S1. Generation of MLL-ENL single-gene KO leukemias. (**A-B**) All mice transplanted with MLL-ENL transformed cells presented enlarge spleen **(s**plenomegaly) and high white blood cell count (WBC) as indicators of AML development. (**C-D**) Genotype of MLL-ENL leukemias generated from knock-out mice confirmed by protein and RNA analysis. Western blots probed with antibodies against RIPK3, MLKL, RIPK1 and c-IAP-1. Actin was used as a loading control. (**E**) MLL-ENL leukemic cells derived from wt and *Tnfr1*$^{-/-}$ cells were treated with indicated concentrations of birinapant for 16h. Mean ± SEM, n=3 independent tumors per genotype. Cell survival was determined by PI uptake and flow cytometry throughout.

Fig. S2.

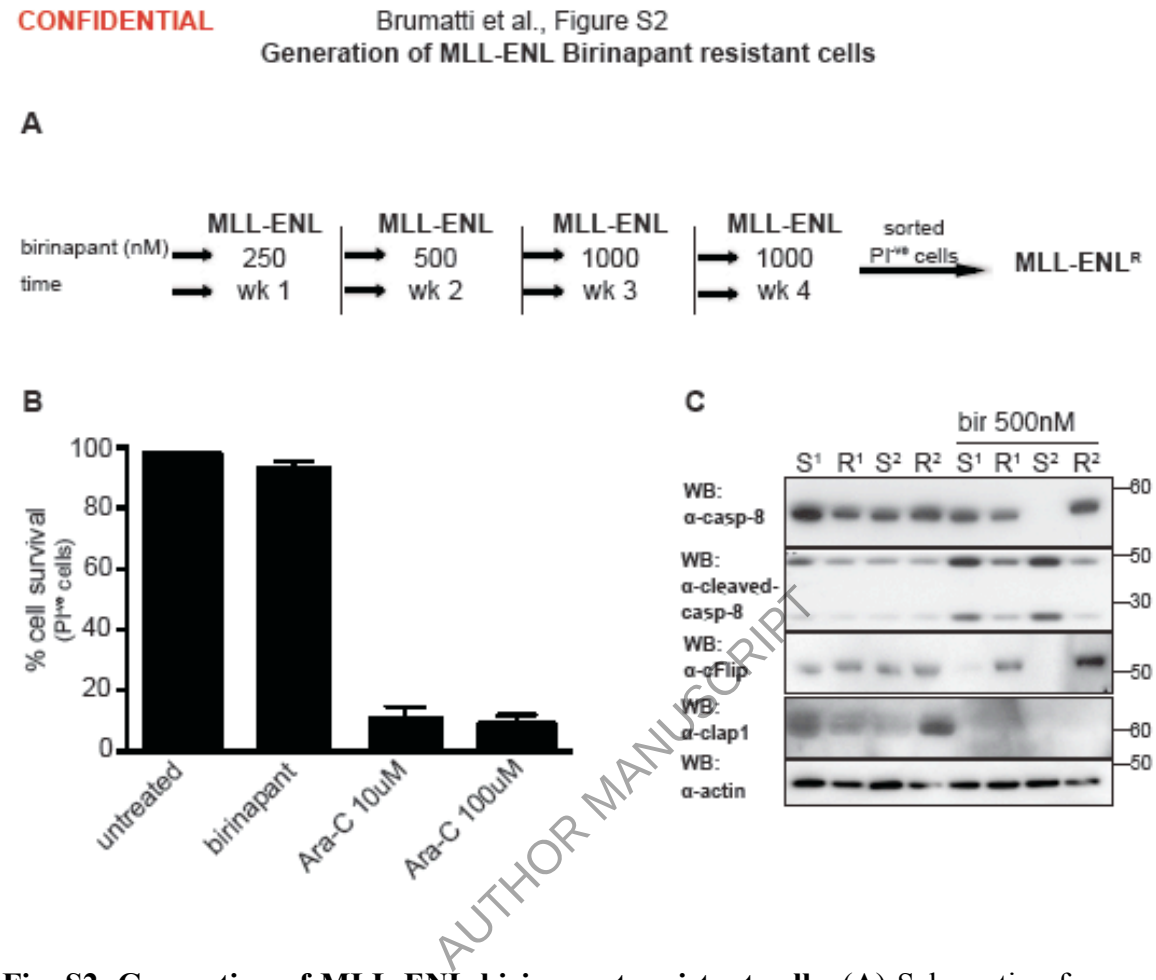


Fig. S2. Generation of MLL-ENL birinapant resistant cells. (**A**) Schematic of generation of MLL-ENL birinapant resistant cells. MLL-ENL sensitive cells were treated for 4 weeks with increasing concentrations of birinapant. (**B**) Birinapant treated PI negative MLL-ENL cells were tested for resistance to birinapant. Cells were cultured with 1000nM birinapant or 10-100µM Ara-C for 18h. Cell survival was determined by PI staining. Data represents Mean ± SEM of n=4 from 2 independent tumors. (**C**) Comparison of protein expression of MLL-ENL sensitive and resistant leukemias after 8h treatment with birinapant (500nM).

Fig. S3.

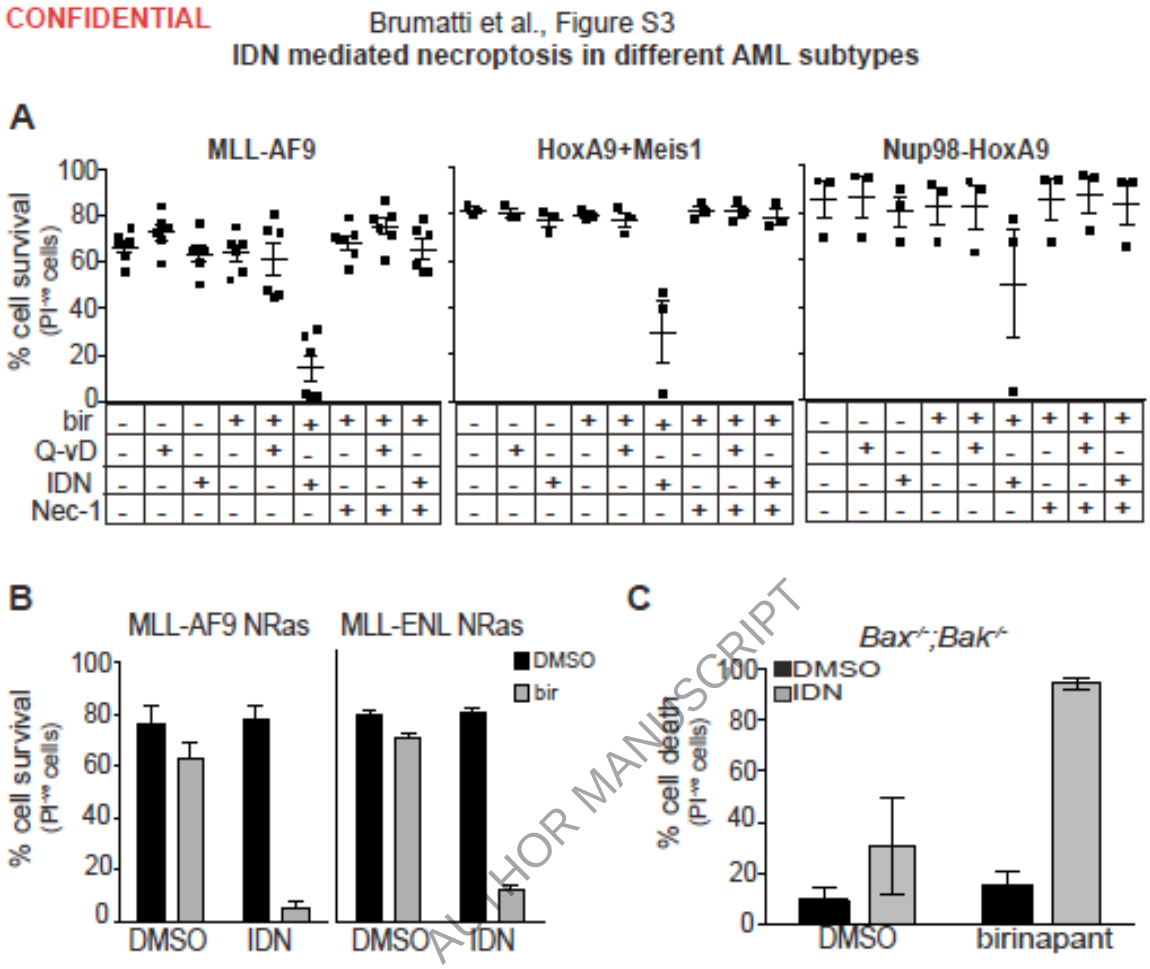


Fig. S3. IDN mediated necroptosis in different AML subtypes. (**A**) Survival of MLL-AF9, HoxA9+Meis1 and Nup98-HoxA9 transformed AML cells. AML cells were pre-treated with the pan-caspase inhibitors IDN-6556 (5μM) or Q-VD (10μM) followed by birinapant (100nM for MLL-AF9 cells and 500nM for HoxA9+Meis1 and Nup98-HoxA9) in the presence or not of Nec-1 (50μM) for 16h. (**B**) MLL-AF9 and MLL-ENL leukemias constitutively expressing activated NRas, were pre-treated for 30 min with IDN (5μM) followed by birinapant (bir; 500nM). Cell survival was determined after 16-18h treatment. Data represents mean ± SEM n=6. (**C**) *Bax*$^{-/-}$*Bak*$^{-/-}$ hematopoietic cells transduced with MLL-ENL were pre-treated or not

with IDN followed by addition birinapant (100nM). Cell survival was determined 16h after treatment. Data represents mean ± SEM n=3.

Fig. S4.

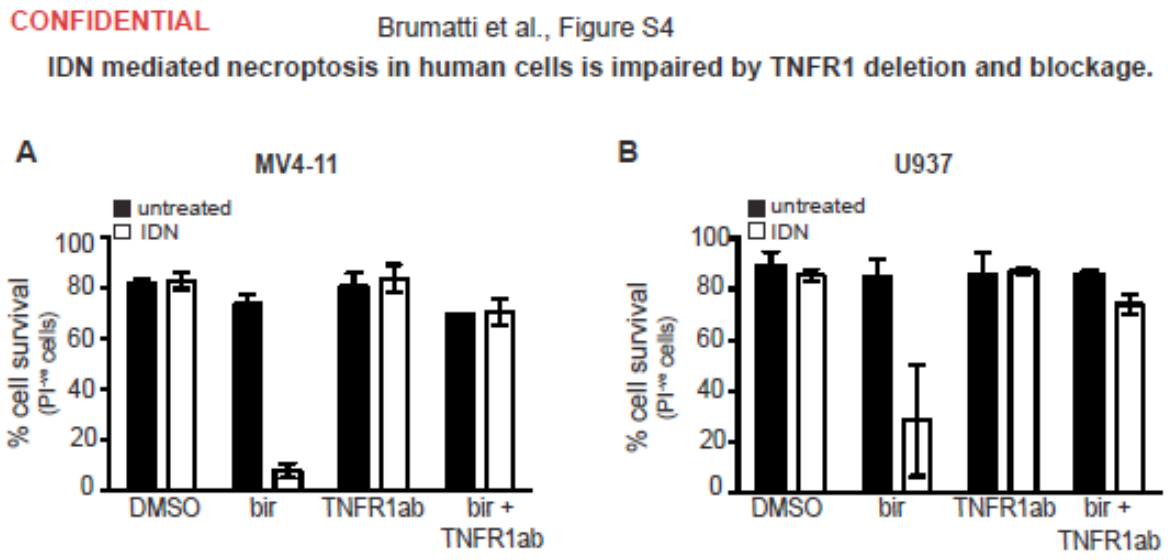


Fig. S4. IDN-6556 induced cell death in human AML cells is impaired when TNFR1 signalling is blocked. (**A-B**) Survival of human leukemic cells MV4-11 and U937 24h after birinapant (500nM) ± IDN (5μM) treatment ± TNFR1 blocking antibody (100ng/ml). Cell survival was determined by PI uptake and flow cytometry throughout.

Fig. S5.

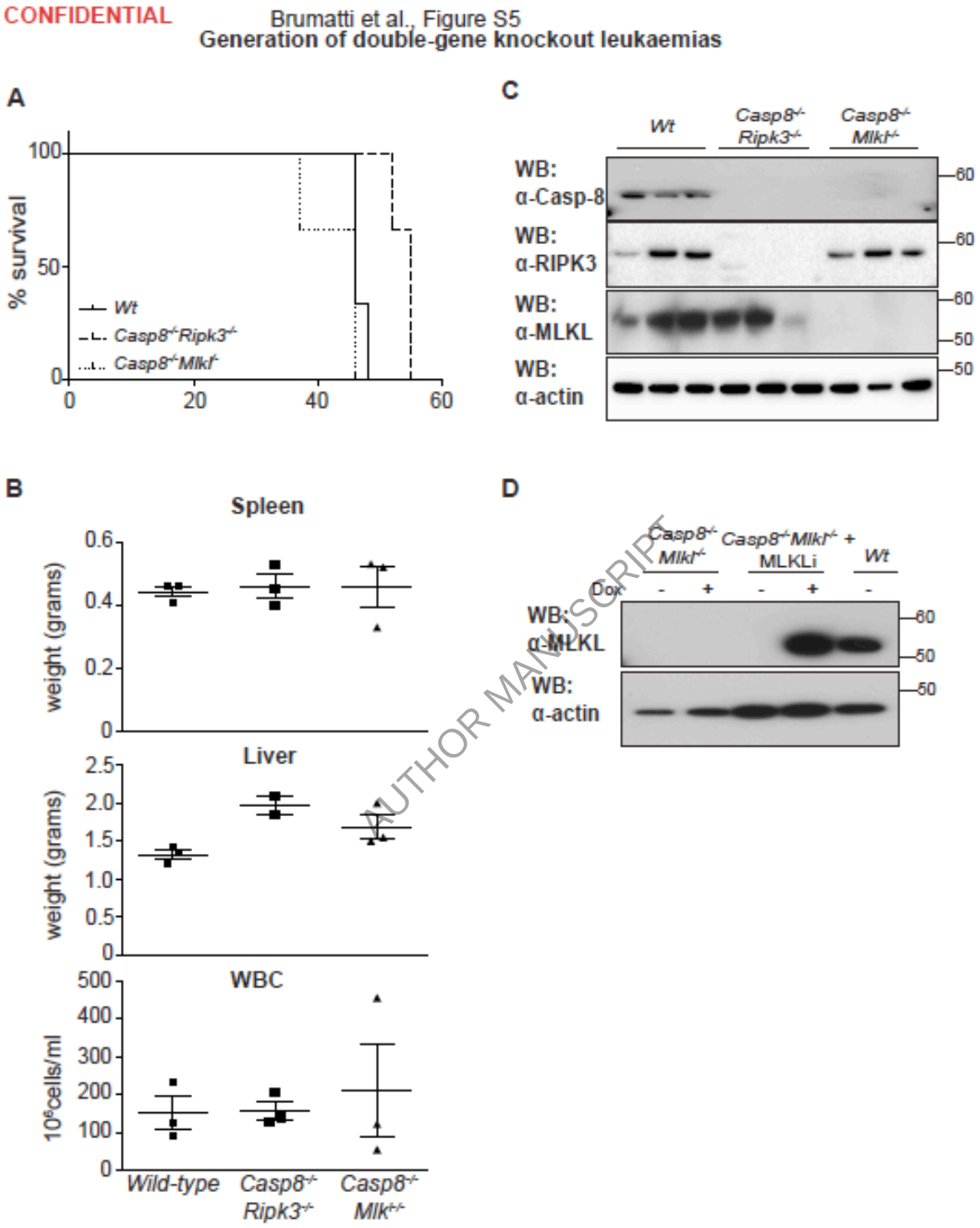


Fig. S5. Generation of MLL-ENL double-gene KO leukemias. (**A**) Kaplan-Meier survival of MLL-ENL leukemias generated from *Wild-type*, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ hematopoietic cells, n=3 from each genotype. (**B**) Spleen, liver and white blood cell count from MLL-ENL wt, *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ leukemic cells. (**C**) Expression of Caspase-8, RIPK3 and MLKL in MLL-ENL leukemias derived from specific double-knock-out mice. (**D**) Representative Western blot of MLKL re-expression in *Casp8*$^{-/-}$*Mlkl*$^{-/-}$ MLL-ENL leukemic cells. Cells were treated or not with 0.5µg/ml doxycycline for 16h. Blots were probed with antibodies against MLKL and Caspase-8. Actin was used as a loading control.

Fig. S6.

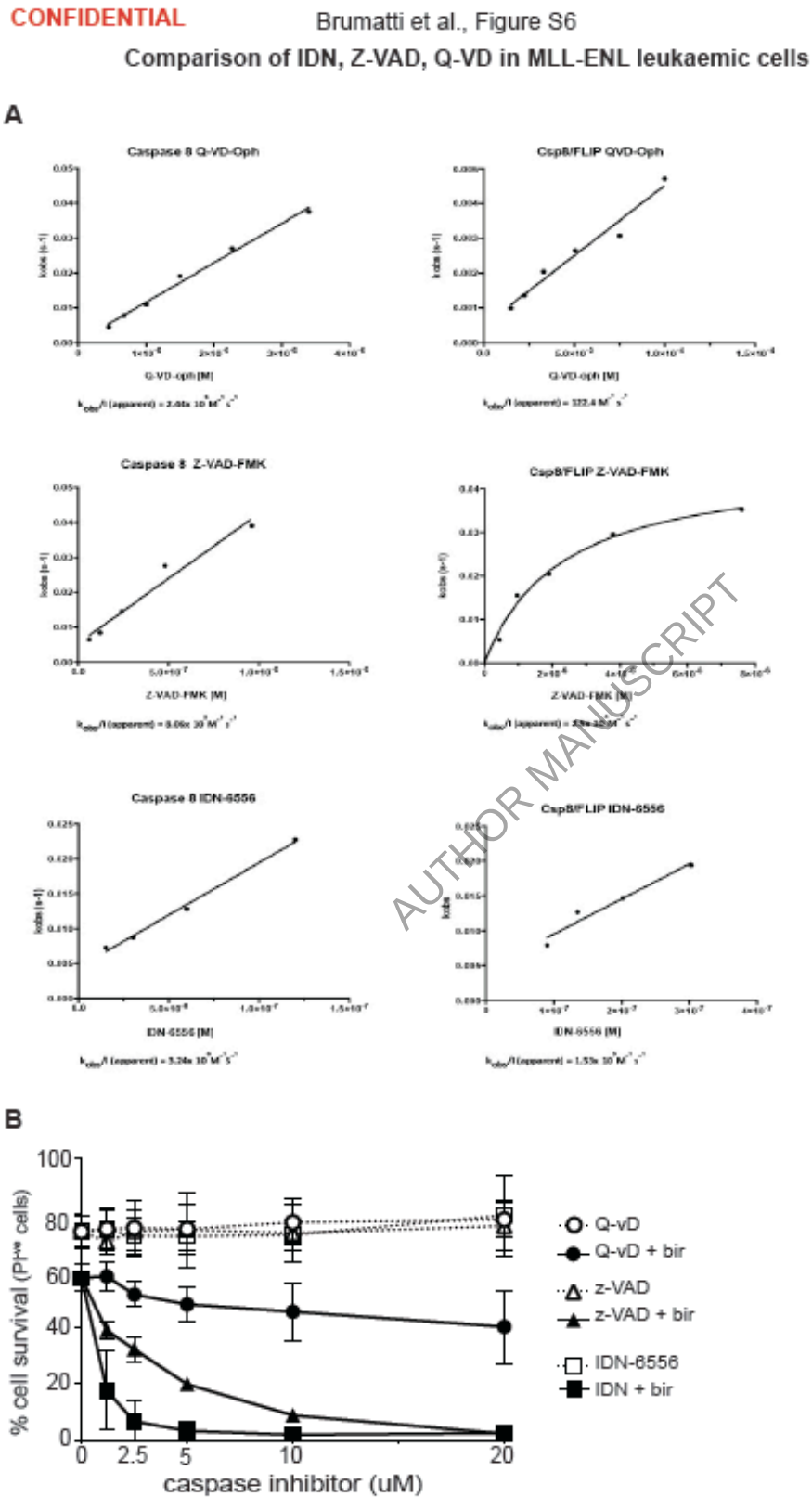


Fig. S6. Comparison of the ability the caspase inhibitors; IDN-6556, Q-VD-OPh and Z-VAD-FMK to inhibit caspase-8 homodimers and caspase-8/cFLIP heterodimers inhibition and promote cell death of MLL-ENL leukemias. (**A**) $K_{obs}/[I]$ for pan-caspase inhibitors Q-VD, Z-VAD and IDN-6556 vs caspase-8 homodimers or caspase-8/cFLIP heterodimers. (**B**) Comparison of the ability of pan-caspase inhibitors to synergize with birinapant to induce cell death in leukemia cells. MLL-ENL leukemic cells were pre-treated (30min) with the indicated concentrations of Q-VD, Z-VAD or IDN-6556 followed by birinapant (100nM) treatment. Cell survival was assessed after 16h by PI staining and flow cytometry. Data represent Mean ± SEM, n=6.

Fig. S7.

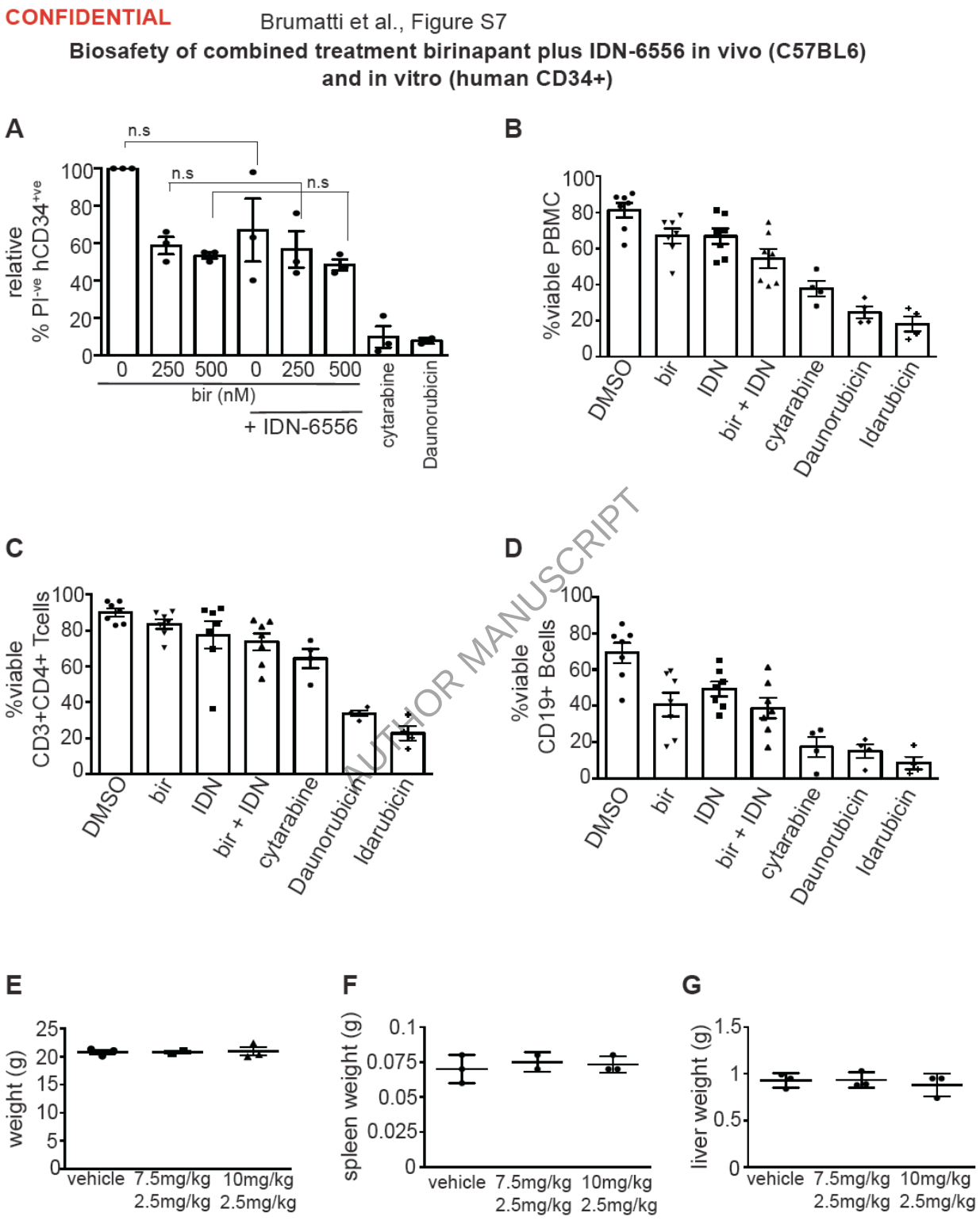


Fig. S7. Biosafety of birinapant plus IDN-6556. (**A-D**) Combined therapy in human healthy patient samples. CD34+ stem cells, PBMC, CD3+CD4+ T cells and B cells from 3 independent control patients were treated for 48h with birinapant (250nM-1000nM) ± IDN-6556 (5μM) or conventional chemotherapies, Cytarabine (10 μM), Daunorubicin (0.4 μM) or Idarubicin (0.4 μM). Cell viability was determined by flow cytometry of PI negative cells. Data represent means of 3 independent experiments. (**E-G**) No toxicity observed *in vivo* for combined therapy birinapant plus IDN-6556. Measurements of body weight, spleen and liver of C57BL6 female mice treated for 4 weeks (i.p injections 2x/week) with birinapant (7.5mg/kg or 10mg/kg) + IDN-6556 (2.5mg/kg).