

## **SUPPLEMENTAL INFORMATION**

### **Supplemental methods**

#### **Patient's characteristics**

399 patients with high risk DLBCL [age-adjusted international prognostic index score (aaIPI) 2-3] were originally enrolled in the DLCL04 trial [1]. Median follow-up was 72 months. The results of this study did not support the role of first line intensification in DLBCL as 5-year overall survival (OS) rates were similar in the no transplantation group (77%) vs the transplantation group (78%). The overall outcome of the patients analyzed in the DLCL04 cohort of the present study was superimposable to the outcome of the original study (1) (5-year OS 74% vs 78%  $p=ns$ ) (Figure S1). Only cases originally diagnosed as non-otherwise specified (NOS) DLBCL, (including those originally diagnosed as DLBCL-NOS and nowadays included in the HG-BCL provisional category) [2] were considered. 94 stage III-IV DLBCL patients enrolled in the DLCL04 trial with available FFPE tissue were initially considered in this analysis (discovery cohort). T-GEP success rate was 92.6% ( $n=87$ ), with 7 cases not yielding enough high-quality mRNA to undergo successful GEP assessment. Only cases of non-otherwise specified (NOS) histology were considered. Therefore 11 cases classified in different DLBCL categories were excluded. In 7 cases we could not retrieve enough tissue for additional immunohistochemistry studies and these cases were excluded. The “real-life” validation cohort included 91 consecutive DLBCL NOS cases with available FFPE tissue for T-GEP and IHC, treated with R-CHOP/CHOP-like regimens at the Seragnoli Institute of Hematology and Medical Oncology in Bologna. Success rates of T-GEP was 91% (8 failures); 10 cases were excluded for issues related to histologic classification (non-NOS histology) and 7 additional cases for lack of tissue availability for additional IHC studies. 69 NOS-DLBCL FFPE patient samples from the DLCL04 trial were finally included in this study. Patients in the DLCL04 trial were treated according to the

respective treatment protocols, with 30 patients undergoing autologous stem cell transplant (ASCT) consolidation. None of the patients in the real-life cohort underwent ASCT consolidation, according to current treatment guidelines. The OS curve of the real life cohort is depicted in Figure S1. Study algorithm is detailed in figure 1.

## **Immunohistochemistry**

Immunohistochemistry (IHC) was centralized in Bologna for the DLCL04 trial and in Milan for the real-life control group. The IHC results were independently evaluated by 4 expert haematopathologists (CA, SP, SF, VT). Protein expression was recorded as the percentage of positive tumor cells in 10% increments. The antibodies source and dilutions are shown in Table S1.

Tissue microarrays (TMAs) were prepared from paraffin blocks using a precision instrument as previously described [3]. One-mm diameter cores were obtained from representative tumor areas and transferred into a recipient paraffin block, cut to 2-mm thick sections, and transferred on glass slides. At both sites, antigen retrieval was carried on PT-links at 92°C for 5 minutes in EnVision Flex Target Retrieval Solution High pH (Dako Agilent, Glostrup, Denmark). All IHC tests were performed on AutoStainer Plus platforms, using the LSAB+REAL Detection System (Dako Agilent). The IHC preparations were counterstained with Gill's haematoxylin and mounted in Kaiser's glycerin. The IHC results were independently evaluated by 4 expert haematopathologists (CA, SAP, SF, VT). In case of discrepant results among the observers, the IHC preparations were jointly reviewed at a multi-head microscope until consensus was reached. Microphotographs were taken using an Olympus BX53 light microscope with an Olympus DP71 camera. Original magnifications are specified in figure legends.

## Cell lines

The human DLBCL-derived cell lines SUDHL-4, SUDHL-6, OCI-LY-19, BJAB, U-2932 were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures (Braunschweig, Germany); SUDHL-2, SUDHL-10, OCI-LY-1, OCI-LY-18, DOHH2, SUDHL-5, PFEIFFER, DAUDI were obtained from ATCC (American Type Culture Collection). The DLBCL derived cell lines (HBL-1, TMD8) were provided by Dr. A. Younes (Memorial Sloan Kettering Cancer Center, NY). Cell lines were cultured in RPMI 1640 medium or in Iscove Modified Dulbecco medium supplemented with 10 to 20% heat-inactivated fetal bovine serum, 1% L-glutamine, and penicillin-streptomycin in a humid environment of 5% CO<sub>2</sub> at 37°C.

Genomic data cell line annotations are in line with Cancer Cell Line Encyclopedia (CCLE: <https://portals.broadinstitute.org/ccle/about>), CBio Portal for Cancer Genomics ([https://www.cbioportal.org/study/summary?id=lymphoma\\_cellline\\_msk\\_2020](https://www.cbioportal.org/study/summary?id=lymphoma_cellline_msk_2020)) [4], Derenzini et al. Oncotarget 2015 [5], Hicks et al. Haematologica 2018 [6], Juskevicius et al. Leukemia & Lymphoma 2018 [7], Li et al. J Hematol Oncol 2019 [8].

## Inducible Tet-On BCL-2 overexpression system

To overexpress the coding sequence (CDS) of *BCL2* in the SUDHL5 BCL2 negative cell line we used the Collecta InDOXible Tet-Activated cDNA Lentiviral Expression System (custom Collecta).

Two lentiviral Vectors, the inducible cDNA, that is under the control of the responsive promoter (TRE), and the transactivator (rtTA), were transduced separately into the SUDHL5 cells, allowing gene overexpression after the tetracycline analog doxocycline induction. The

transactivator vector contains a puromycin resistance gene (Puro) while the TRE-Empty and TRE-BCL2 vectors contain a green fluorescent protein (GFP) mark (Ubi-GFP).

### **Western blotting**

Preparation of cellular protein lysates was performed by using the Cell Signalling lysis buffer (#9803) according to manufacturer's extraction protocol. A total of 30 ug of protein was denatured in Laemli buffer at 95C for 5 minutes and western immunoblotting was performed using the Biorad system (TGX 4-15% gels). Transfer was performed using the Trans Blot turbo system (Biorad) onto PVDF membranes. Images were acquired by using the BioRad Imaging Chemidoc MP system. Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies were purchased from Biorad (#170-6515, #170-6516).

The following antibodies for western blotting were purchased from cell signalling technology: pCHK1 S345 (#2348),  $\gamma$ H2AX S139 (#2577), Cleaved Caspase 3 (#9664), BCL-2 (#4223).

The following antibodies were purchased from Abcam: c-MYC (#32072), lamin B1 (#16048). Beta-Actin (#A5316) and Vinculin (#V9131) were from SIGMA.

### **Caspase 3/7 activity assay**

Cells were seeded in 96-well plates at 25,000 cell/100  $\mu$ l/well with either vehicle (DMSO 0.1%) or of drugs for 1, 3, 6, 12 and 24 hours. Measurements of caspase activities in cells were performed using the commercially available Caspase-Glo 3/7 Assay (Promega), according to the manufacturer's instructions.

### **qPCR assays**

Total RNA was extracted with the ZYMO RESEARCH Quick-RNA MiniPrep kit protocol. A total of 1 µg of RNA was converted to cDNA using LunaScript RT SuperMix kit (Bio-Labs). Real-time polymerase chain reaction (PCR) was performed using ABI7500 PCR machine (Applied Biosystem). Primers used are *ACTIN*, *MYC*, *BCL-2*.

| Primer          | Sequence             |
|-----------------|----------------------|
| <i>MYC</i> Fw   | TTCGGGTAGTGGAAAACCAG |
| <i>MYC</i> Rv   | CAGCAGCTCGAATTTCTTCC |
| <i>ACTIN</i> Fw | GAACGGTGGTGTGTCGTTC  |
| <i>ACTIN</i> Rv | GCGTCTCGTCTCGTCTCACT |
| <i>BCL-2</i> Fw | GAGTTCGGTGGGGTCATGT  |
| <i>BCL-2</i> Rv | GCCGGTTCAGGTACTCAGTC |

### Cell cycle analysis

Single cell suspensions were prepared and fixed with 70% fresh Ethanol for 2 hrs. After fixation, cells were washed twice with 1x PBS, 1% BSA. Cells were then stained with Propidium Iodide (PI Sigma) 10 µg/ml + RNase overnight, protected from light before analysis. Data Acquisition: Flow cytometric data was acquired on a BD FACS Celesta using BD FACS Diva Software. Analysis was performed using FlowJo 9.3.

### FISH analysis

FISH studies were conducted on paraffin sections using the following probes: Vysis LSI *MYC* dual color break-apart, Vysis LSI *BCL-2* dual color break-apart, Vysis LSI *BCL-6* dual color break-apart and Vysis LSI IGH/*MYC*/CEP8 Tri-color FISH probe kit. In brief, the slides were deparaffinized, co-denatured with probe, hybridized overnight, washed and then mounted with DAPI/Antifade. For each probe, 200 interphase nuclei were analyzed to

detect rearrangement and numerical abnormalities. Cut-off values were established for each probe by assessing 10 normal controls (reactive lymph nodes) and choosing values 3SD above the mean. Gains were considered when a pattern of three or four copies of the gene were identified, whereas more than four copies were considered as amplifications.

### **Additional NanoString methods**

Total RNA was extracted from three sections of 15- $\mu$ m of each FFPE sample using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher). Yield and quality of RNA extracted was assessed. Quantitative RNA analysis was performed using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). RNA quality was scored according to DV200 values (percentage of RNA fragments  $\geq$ 200 nucleotides), utilizing the Agilent 2100 BioAnalyzer.

Gene expression was measured on the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA). Gene expression was measured on the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA).

Gene expression data were analyzed by the NanoString Company using a modified RUO version of the NanoString Lymphoma Subtyping Test (LST) algorithm to determine the Cell-of-Origin molecular subtype of each sample [9]. The system computes the relative abundance of each mRNA transcript of interest, through a multiplexed hybridization assay and digital readouts of fluorescent barcoded probes, which are hybridized to each transcript. An nCounter CodeSet (NanoString Technologies) containing capture and reporter probes (the latter attached to a color barcode) was hybridized to 200 ng of total RNA for 20 hours at 65 °C, according to the manufacturer's instructions. Hybridized samples were loaded into the nCounter Prep Station for post-hybridization processing. Target mRNA was assessed with nCounter Digital Analyzer.

For T-GEP analyses, the quality control and normalization of NanoString nCounter data were performed using R package NanoStringNorm. The Raw NanoString counts for each gene were subjected to a technical normalization considering positive and negative probes. A normalization factor was calculated by obtaining the geometric mean of the positive controls used for each sample and applied to the raw counts of the nCounter output data to eliminate variability that was unrelated to the samples. The resulting data were normalized again with the geometric mean of the housekeeping genes (*ISY1*, *R3HDM1*, *TRIM56*, *UBXN4* and *WDR55*). Normalized data were log2-transformed for further analyses.

#### List of genes and target sequences of the 22- gene panel

| GeneName | ProbeID                   | Comments     | TargetSeq                                                                                                 |
|----------|---------------------------|--------------|-----------------------------------------------------------------------------------------------------------|
| 1        | <i>UBXN4</i> NM_014607.3  | HOUSEKEEPING | CATCGCGACGGCCAAAAGGAGCGGGCGCGGTCTTCGTGGTGTTCGTGGCAGGT<br>GATGATGAACAGTCTACACAGATGGCTGCAAGTTGGGAAGATGATAAA |
| 2        | <i>ISY1</i> NM_020701.2   | HOUSEKEEPING | GGCAAACATCAGTGTCTGTGGGTAGTTGGAATCTTCAGTTCCTGTGAGCGTC<br>GGCGTCTTCTGGGCCTGTGGAGTTTCTTGGACAGGGGCCGCGGGGCT   |
| 3        | <i>R3HDM1</i> NM_015361.2 | HOUSEKEEPING | CCTGTGTTCCCAAGAGAATTACATTATTGACAAAAGACTCCAAGACGAGGATG<br>CCAGTAGTACCCAGCAGAGGCGCCAGATATTTAGAGTTAATAAAGAT  |
| 4        | <i>WDR55</i> NM_017706.4  | HOUSEKEEPING | CTACCTCTTCAATTGGAATGGCTTTGGGGCCACAAGTGACCGCTTTGCCCTGA<br>GAGCTGAATCTATCGACTGCATGGTTCCAGTCACCGAGAGTCTGCTG  |
| 5        | <i>TRIM56</i> NM_030961.1 | HOUSEKEEPING | GTGGAGGCCGAGGACATTTTCCTGAAGGGCAGGGGTTGGCAACTTTTCAACAT<br>GGAGTGCCAAACTGCTAACCCGTCTTCTAGTGTGTGAGAATAGGGAC  |
| 6        | <i>MYC</i> NM_002467.3    | ENDOGENOUS   | TCGGACACCGAGGAGAATGTCAAGAGGCGAACACACAACGTCTTGGAGCGCC<br>AGAGGAGGAACGAGCTAAAACGGAGCTTTTTTGGCCCTGCGTGACCAGA |

7 *PIM2* NM\_006875.2 ENDOGENOUS  
GCCATCCAGCACTGCCATTCCCGTGGAGTTGTCCATCGTGACATCAAGGATGA  
GAACATCCTGATAGACCTACGCCGTGGCTGTGCCAAACTCATTGATT

8 *IRF4* NM\_002460.1 ENDOGENOUS  
GGGCACTGTTTTAAAGGAAAGTTCCGAGAAGGCATCGACAAGCCGGACCCTCC  
CACCTGGAAGACGCGCCTGCGGTGCGCTTTGAACAAGAGCAATGACTT

9 *TNFRSF13B* NM\_012452.2 ENDOGENOUS  
TGCAAAACCATTTGCAACCATCAGAGCCAGCGCACCTGTGCAGCCTTCTGCAG  
GTCACCTCAGCTGCCGCAAGGAGCAAGGCAAGTTCTATGACCATCTCC

10 *S1PR2* NM\_004230.2 ENDOGENOUS  
TCCCGCCAGGTGGCCTCGGCCTTCATCGTCATCCTCTGTTGCGCCATTGTGGT  
GGAAAACCTTCTGGTGCTCATTGCGGTGGCCCGAAACAGCAAGTTCC

11 *MME* NM\_000902.2 ENDOGENOUS  
GGATTGTAGGTGCAAGCTGTCCAGAGAAAAGAGTCCTTGTTCCAGCCCTATTC  
TGCCACTCCTGACAGGGTGACCTTGGGTATTTGCAATATTCCTTTGG

12 *ASB13* NM\_024701.3 ENDOGENOUS  
GGACACGTAGGCGGTACCACTAAGGTTTTGGTAATGAGCCATTCAAACCGACA  
GCAGTGTGAAGGTGTGTCAAGGTGTATATTCTCGTGGCTCGGCATTTC

13 *BCL2* NM\_000657.2 ENDOGENOUS  
GTGAAGCAGAAGTCTGGGAATCGATCTGGAAATCCTCCTAATTTTTACTCCCTC  
TCCCCGCGACTCCTGATTCATTGGGAAGTTTCAAATCAGCTATAAC

14 *CYB5R* NM\_016229.3 ENDOGENOUS  
CCATGTCTTAGGGCTTCCTGTAGGTAACATGTCCAGCTCTTGGCAAAAATCGA  
TAATGAATTGGTGGTCAGGGCTTACACCCCTGTCTCCAGTGATGAT

15 *MAML3* NM\_018717.4 ENDOGENOUS  
TGGAAGCCATCAACAATTTGCCAGTAACATGCCACTGCCTTCAGCTTCTCCTC  
TTCACCAACTTGACCTGAAACCTTCTTTGCCCTTGACAGAACAGTGG

16 *SERPINA9* NM\_001042518.1 ENDOGENOUS  
CCACTAAATCCTAGGTGGGAAATGGCCTGTAACTGATGGCACATTGCTAATG  
CACAAGAAATAACAAACCACATCCCTCTTTCTGTTCTGAGGGTGCAT

17 *MYBL1* XM\_034274.14 ENDOGENOUS  
CTCCTTTTAAGAATGCGCTTGCTGCTCAGGAGAAAAAATATGGACCTCTTAAAA  
TTGTGTCCCAGCCACTTGCTTTCTTGAAGAAGATATTCGGGAAGT

18 *RAB7L1* NM\_001135664.1 ENDOGENOUS  
CATTTGAATTGTCTCCTGACTACTGTCCAGTAAGGAGGCCCATTTGTCACTTAGA  
AAAGACACCTGGAACCCATGTGCATTTCTGCATCTCCTGGATTAGC

19 *LIMD1* NM\_014240.2 ENDOGENOUS  
AAGGCAAGTCTCAGGAACCCATGCAGGTACATCGCTTGACCTGTTTTTAGCT  
TATTTAATGACGGGCTTTTGGGAAGAGCTGCCCGCATACTGAGAGAC

20    *ITPKB*        NM\_002221.3                    ENDOGENOUS  
          GGTTTGCGCCTCTGGGCATGTAGTCTACACAGGACCTGAGAATCTGAGAACT  
          GCAGCCGCACGGTTGTTTATGGAGCTTTGGGCGGGGGCTGAGCCCGC

21    *CREB3L2*    NM\_001253775.1                    ENDOGENOUS  
          CGCACTTCTCAGAACTTCTGGATGAGTTTTCCCAGAACGTCTTGGGTCAGCTC  
          CTGAATGATCCTTTCTCTCAGAGAAGAGTGTGTCAATGGAGGTGGA

22    *CCDC50*      NM\_174908.3                    ENDOGENOUS  
          AGGACATAGCTCGCCTTTTGCAAGAAAAGGAGTTACAGGAAGAGAAAAAGAGA  
          AAGAAACACTTTCCAGAGTTCCCTGCAACCCGTGCTTATGCAGATAG

### **Additional HTS methods and statistical analysis**

For the synergy assessment studies, a compound matrix was prepared in which compound 1 was titrated against compound 2 at 2.5  $\mu$ M in a doubling dilution series and transferred into a 1536-well microtiter assay plate. Internal controls for each assay plate were dispensed as previously described [10]. To start the assay, cells were seeded at 1,000 cells per well in 8  $\mu$ L of complete RPMI medium and incubated for 2 days followed by 1  $\mu$ L Alamar Blue for an additional day at 37°C. Plates were imaged on the LEADseeker™ Multimodality Imaging System (GE Healthcare, Piscataway, NJ) and resulting files were deconvoluted to obtain fluorescence values associated with each drug.

The drugs were tested for activity both as single agents and in combinations on multiple cell lines through the MSKCC high throughput screening core facility (HTSCF). The residual cell viability post treatment with specific drug combinations was assessed in an Alamar Blue (AB) assay using the LEADseeker Multimodality Imaging System (GE Healthcare, Piscataway, NJ). The data were converted into percent inhibitions conferred by each combination relative to both the high (1% DMSO v/v) and the low (1  $\mu$ M killer mix) control averages ( $\mu$ ). “killer mix” consists of a HTSCF proprietary mixture of cytotoxic compounds. The percent inhibitions were defined as:

$$\%inhib_i = (\mu_{high\ control} - value_i) / (\mu_{high\ control} - \mu_{low\ control}) \times 100$$

Since the percent inhibition is derived from data measured with error some of the computed numbers can fall outside the [0, 100]% interval. We replaced such values by the appropriate boundary value. We used the average percent inhibition of the replicates at a dose level (single agent or combination) as the activity at that dose level.

In order to evaluate whether a drug combination shows synergy we compared the observed activity at the combination at that level to the expected activity under Bliss independence model. The expected activity can be written as

$$\begin{aligned} P(Inh|A, B) &= 1 - P(\overline{Inh}|A, B) = 1 - P(\overline{Inh}|A) \times P(\overline{Inh}|B) \\ &= 1 - [1 - P(Inh|A)] \times [1 - P(Inh|B)] \end{aligned}$$

where A and B are the two drugs, *Inh* and  $\overline{Inh}$  denote inhibited and not inhibited respectively [11,12]. The observed can be compared to the expected activity using a simple difference where values around 0 represent additive relationship, large positive values represent synergy and large negative values antagonism. However the same magnitude of the difference represents different relative change depending on the expected activity. Thus we also use a log-odds measure given as  $\log\{[P_o(1 - P_e)]/[P_e(1 - P_o)]\}$  where  $P_o$  and  $P_e$  are observed and expected activities. For each drug combination we generated a heatmap for the observed inhibition, the difference between observed and expected inhibition and the log-odds of the observed to expected inhibition. The observed and expected inhibition values were rescaled prior to calculating the log-odds by the function  $(0.8 * \text{observed/expected} + 0.1)$ , this was done in order to adjust for instances in which the observed or expected inhibition was 0 or 1. These numbers are binned into intervals suitable for each scale and color coded for simple visualization of the combined activity. For comparing drug combinations we combined the log-odds data across all cell lines and used the medians to rank the combinations. The data are shown as boxplots. All analyses were done using R programming language [13].

### **8-OHdG ELISA assay**

Determination of 8-hydroxy-2 deoxyguanosine (8-OHdG) levels on purified cellular DNA was performed by using the competitive enzyme-linked immunosorbent assay (ELISA) kit (ab201734, Abcam) following the manufacturer's protocol.

Cells purified DNA was digested using nuclease P1, treated with alkaline phosphatase and added to an 8-OHdG-coated 96-well plate that was detected with horseradish peroxidase conjugated 8-OHdG antibody. A colored signal was obtained after incubation with tetramethylbenzidine substrate, and the absorbance was measured at 450 nm, using a multiplate reader (GloMax® Discover Microplate Reader). The concentrations were calculated according to standard curve obtained from the OD values and final values were then normalized to the control sample concentration (ng/ml).

### ***In vitro* proliferation assay and combination index calculation.**

Cells were seeded in 96-well plates at 25,000 cell/100 µl/well with either DMSO 0.1% or increasing concentrations of drugs for the indicated time. Cell viability was assessed by adding Cell Titer Glo reagent (Promega) to the culture medium, according to manufacturer`s instructions. Replicates were averaged for the combination index analysis. The selected combinations were tested for nature of effect using the Chou-Talalay (CT) method [14], which enables quantification of drug outcomes as a combination index (CI); CI=1 shows additivity, CI<1 shows synergism, and CI>1 shows antagonism.

### ***In vivo* studies**

Cells ( $10^6$ ) were xenografted via tail vein injection into 6- to 8-week-old female NSG mice (Charles River, Italy). Tumour growth was monitored three times per week by whole-body imaging on an IVIS Lumina III platform.

Mice were bred and housed under pathogen-free conditions in the animal facilities at the European Institute of Oncology–Italian Foundation for Cancer Research (FIRC) Institute of Molecular Oncology (IEO–IFOM, Milan, Italy) campus.

Briefly, mice were IP injected with 150 mg/kg of XenoLight D-Luciferin - K<sup>+</sup> Salt Bioluminescent Substrate (PerkinElmer). After 10 minutes, animals were anaesthetized with isofluorane apparatus and images acquired using Living Image Software, version 4.2 (Caliper Life Sciences). Radiant efficiency was calculated on the basis of the epifluorescence signal, as indicated in the user manual. Treatment started about 20 days from tumor injection, when a bioluminescence signal was detectable in each individual mouse.

In the first set of experiments, 5 mice per group were randomized into control and treatment arms receiving either a vehicle (control group) or different dose levels of the compounds, specifically: i) Prexasertib 5 and 10 mg/kg, subcutaneous, BID, 3 times a week, and ii) Venetoclax 25, 50 and 75 mg/kg, by oral gavage, for 5 days on- 2 days off for a total of 21 days (venetoclax data not shown). In another set of experiments, combination studies were performed as follows: Vehicle, Prexasertib 2,50 and 1,25 mg/kg, Venetoclax 50 mg/kg, and the combinations (Prexasertib 1.25 mg/kg BID 3 days/week + Venetoclax 50 mg/kg 5 days on - 2 days off; Prexasertib 2.5 mg/kg BID 3 days/week + Venetoclax 50 mg/kg 5 days on- 2 days off). Five mice per group were used. For combination experiments, with a sample size of 5 mice per group, we can achieve 90% power to detect a difference of overall survival at a significance level of 0.05 with log-rank test, assuming a survival rate of 20% at day 80 in the control groups, and 100% in the experimental group.

Prexasertib was prepared in 20% Captisol™, venetoclax was dissolved in 60% Phosal 50 PG, 30% polyethylene glycol, and 10% ethanol. Solutions were freshly prepared from dry powder just before each injection. Tumors growth and body weight were assessed three times weekly. After 3 weeks of treatment and after 6 hours from last dose tumor samples were collected for immunoblotting.

Mice were observed daily throughout the treatment period for signs of morbidity/mortality.

### Supplementary tables

Table S1.

| <b>Antibody</b> | <b>Source</b>       | <b>Clone</b> | <b>Dilution</b> |
|-----------------|---------------------|--------------|-----------------|
| γH2AX S139      | Cell Signaling 2577 | -            | 1:25            |
| 8-OhDG          | Abcam               | N45.1        | 1:200           |
| BCL-2           | Dako                | 124          | 1:100           |
| c-MYC           | Abcam               | Y69          | 1:100           |

Table S2.

| <b>DLCL04 (N=69)</b>           | <b>Patients N°</b> | <b>Hazard Ratio (95%CI)</b> | <b>P-value</b> |
|--------------------------------|--------------------|-----------------------------|----------------|
| MYC/BCL-2 DE Nano              |                    |                             |                |
| DE                             | 18                 | 1                           | 0.02           |
| Non-DE                         | 51                 | 0.35 (0.11-1.08)            |                |
| MYC/BCL-2 DE IHC               |                    |                             |                |
| DE                             | 21                 | 1                           | 0.11           |
| Non-DE                         | 48                 | 0.48 (0.17-1.35)            |                |
| <b>Real-Life Cohort (N=66)</b> | <b>Patients N°</b> | <b>Hazard Ratio (95%CI)</b> | <b>P-value</b> |
| MYC/BCL-2 DE Nano              |                    |                             |                |
| DE                             | 17                 | 1                           | 0.01           |
| Non-DE                         | 49                 | 0.39 (0.14-1.09)            |                |
| MYC/BCL-2 DE IHC               |                    |                             |                |
| DE                             | 17                 | 1                           | 0.39           |
| Non-DE                         | 49                 | 0.77 (0.28-2.12)            |                |

MYC/BCL-2 Nano (MYC and BCL-2 mRNA levels assessed by NanoString profiling)

DE (double expresser), non-DE (non-double expresser)

Supplementary Figures

Figure S1

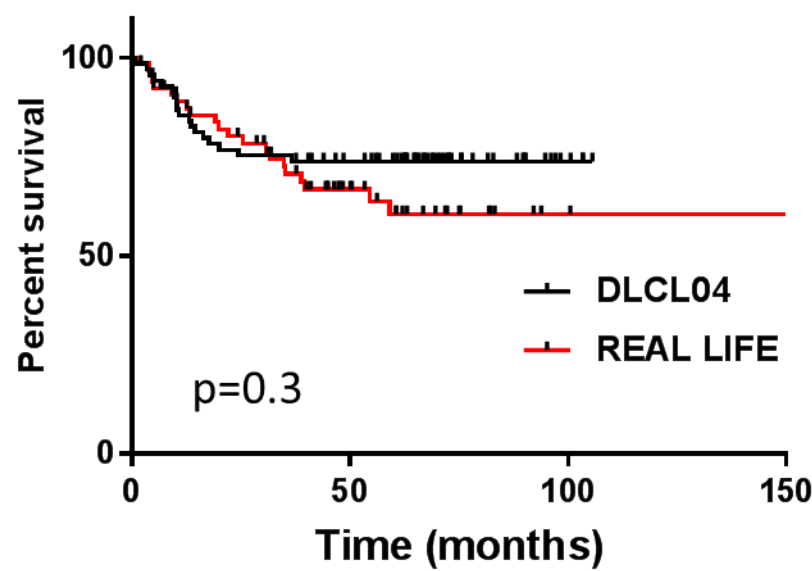
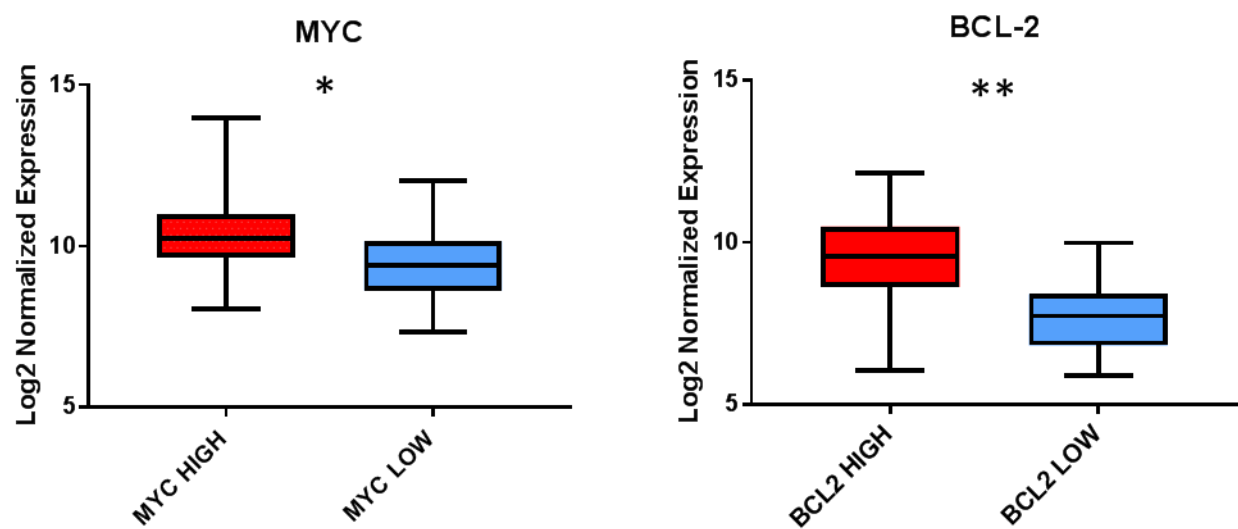


Figure S2

A



B

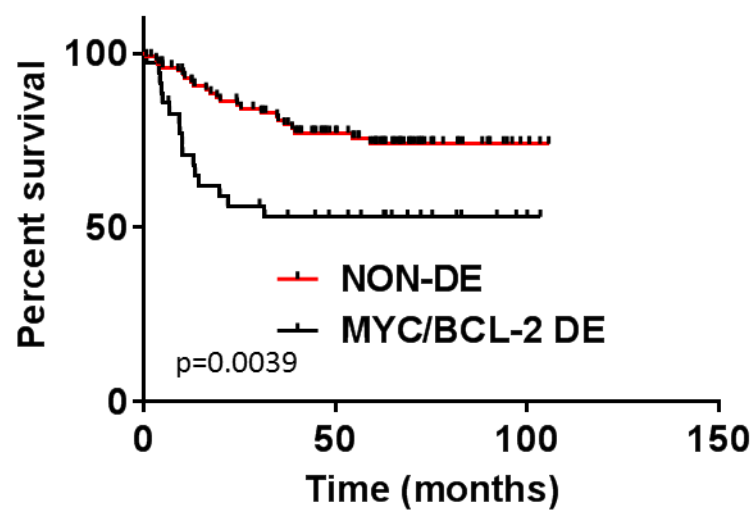
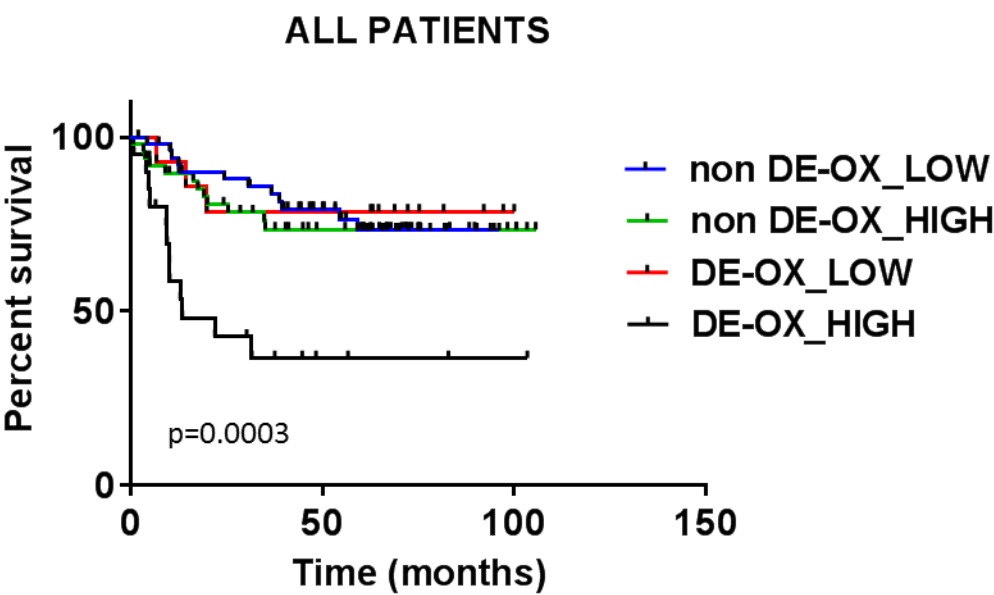


Figure S3

A



B

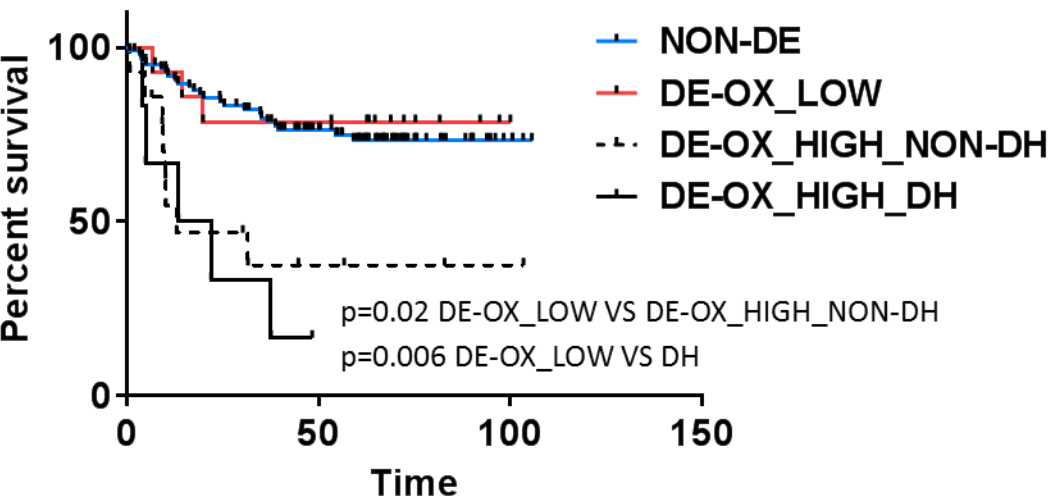
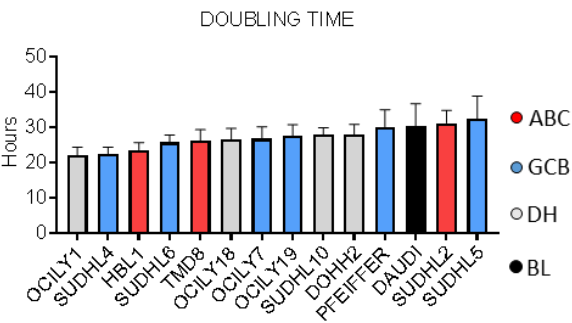
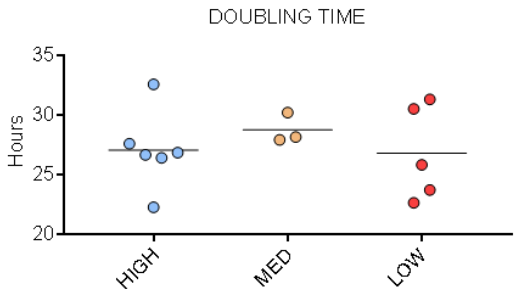


Figure S4

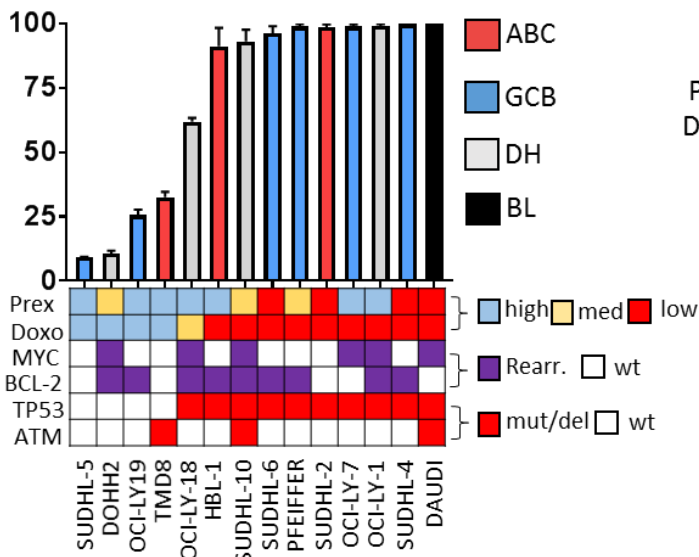
A



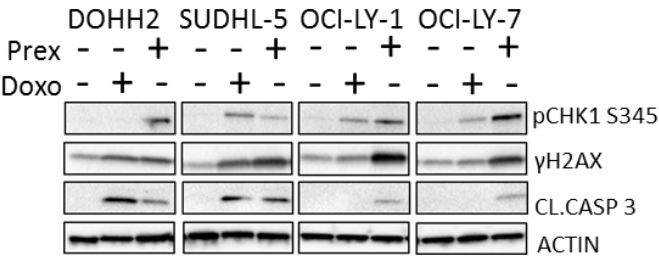
B



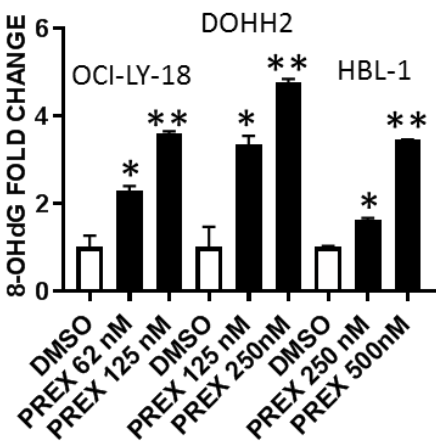
C



D



E



F

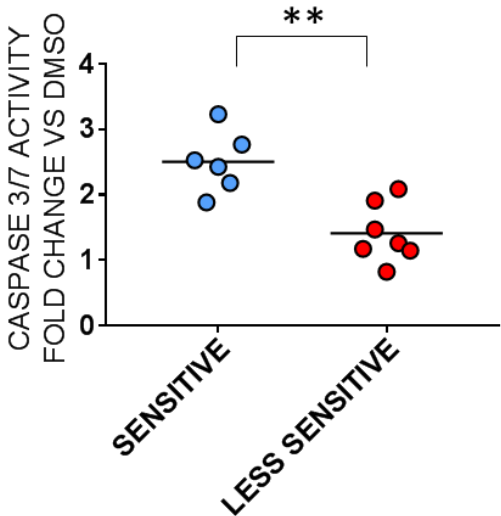


Figure S5

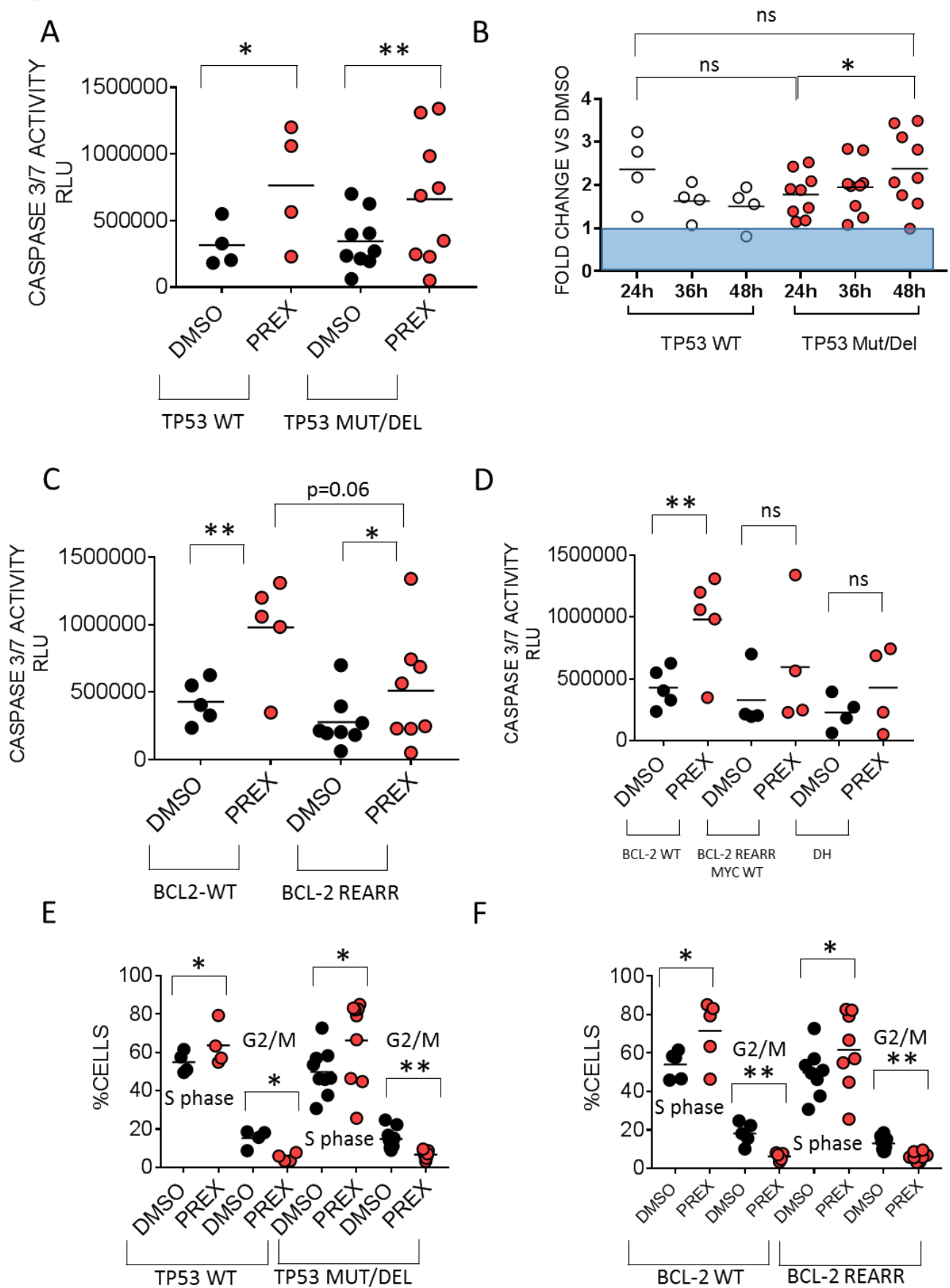
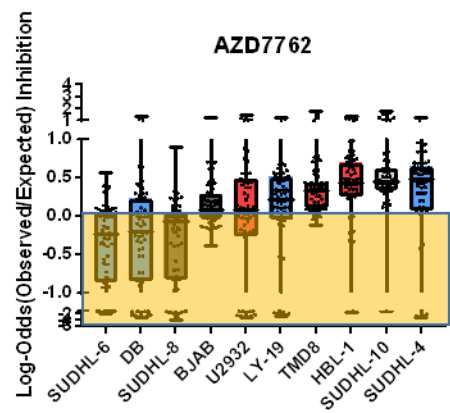
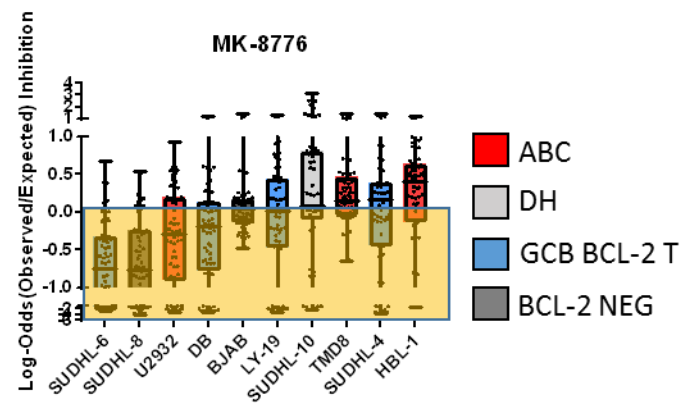


Figure S6

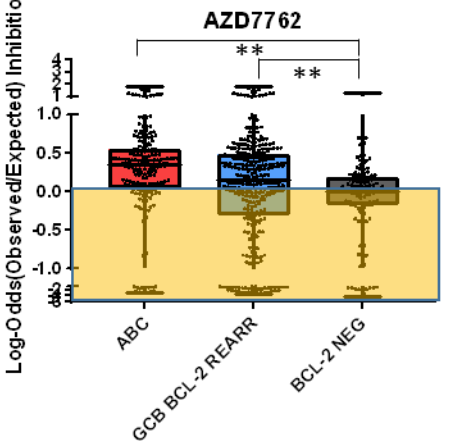
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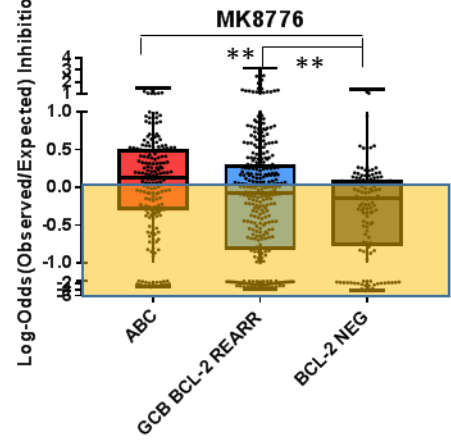
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C



D



E

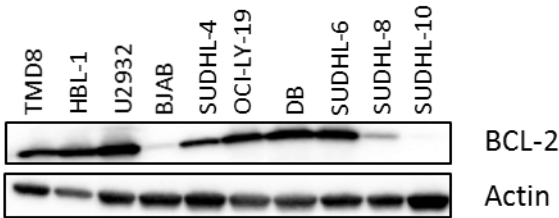


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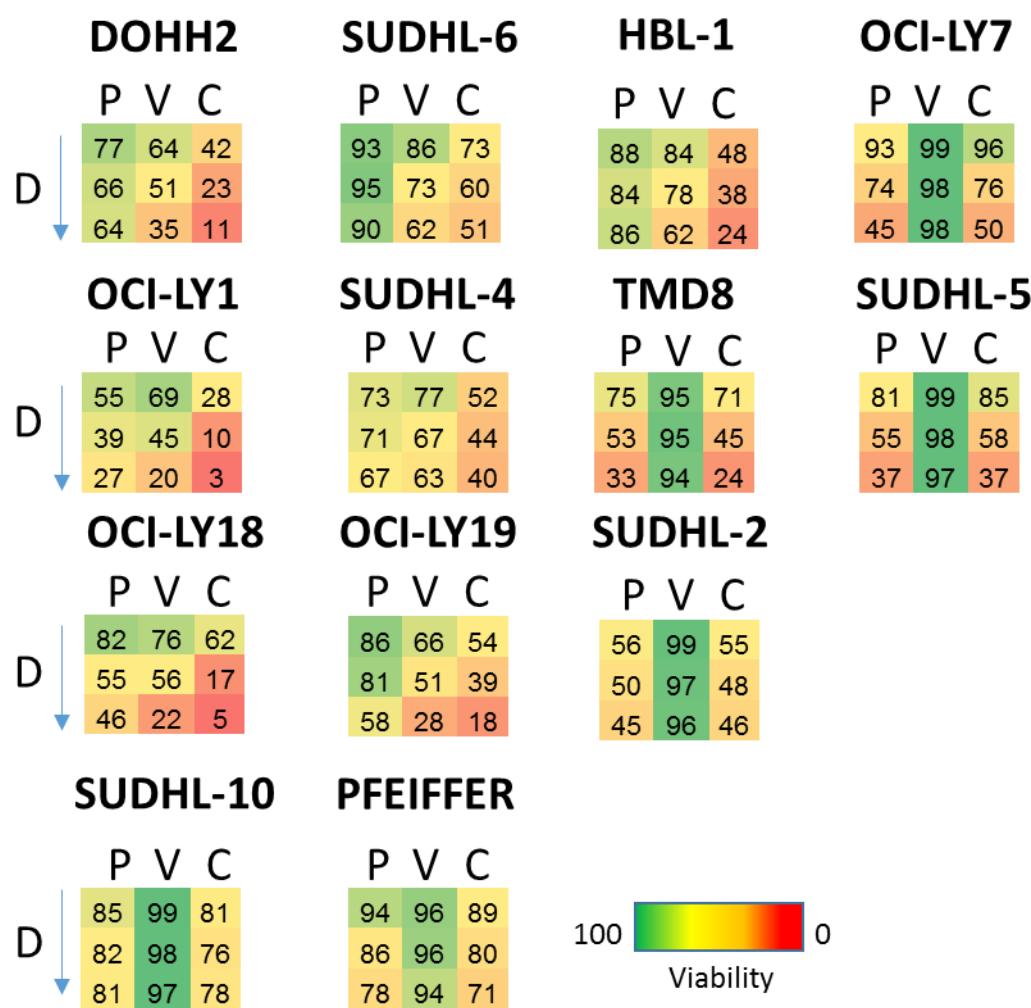


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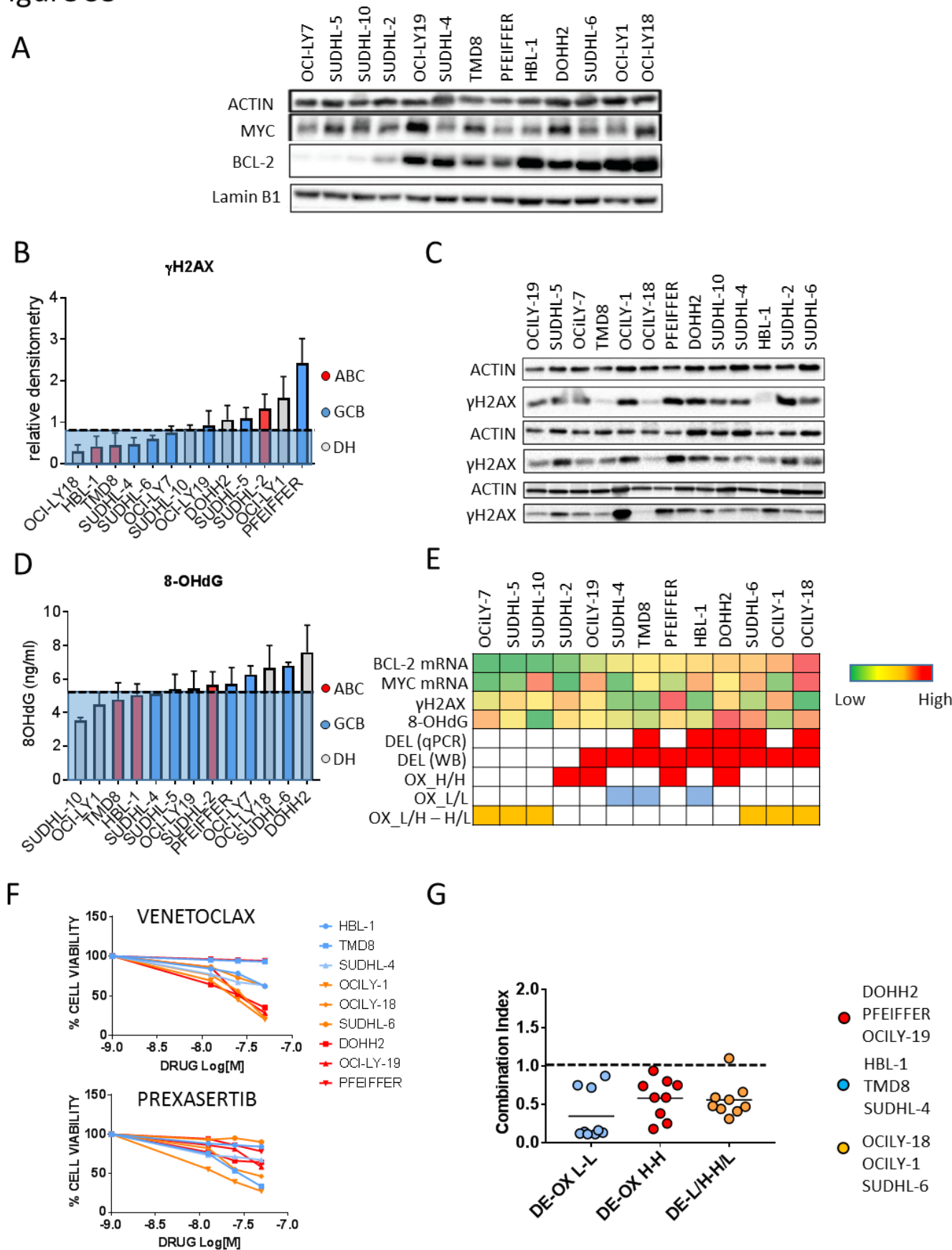
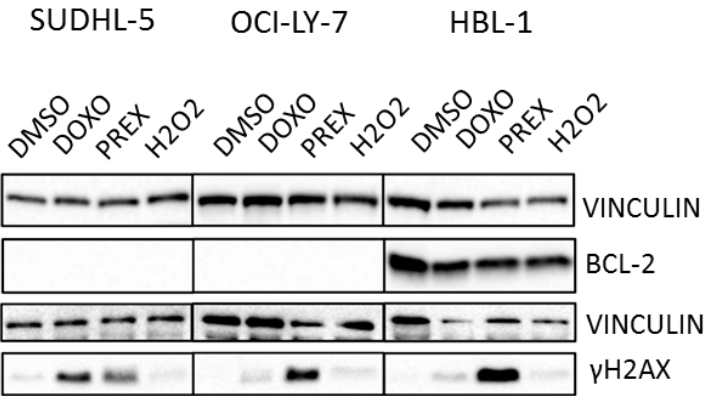


Figure S9

A



B

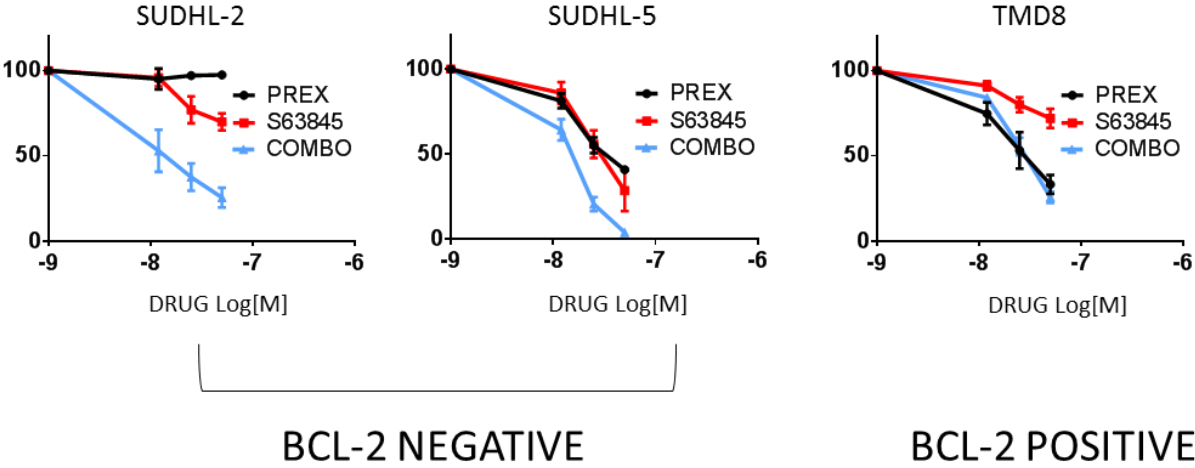
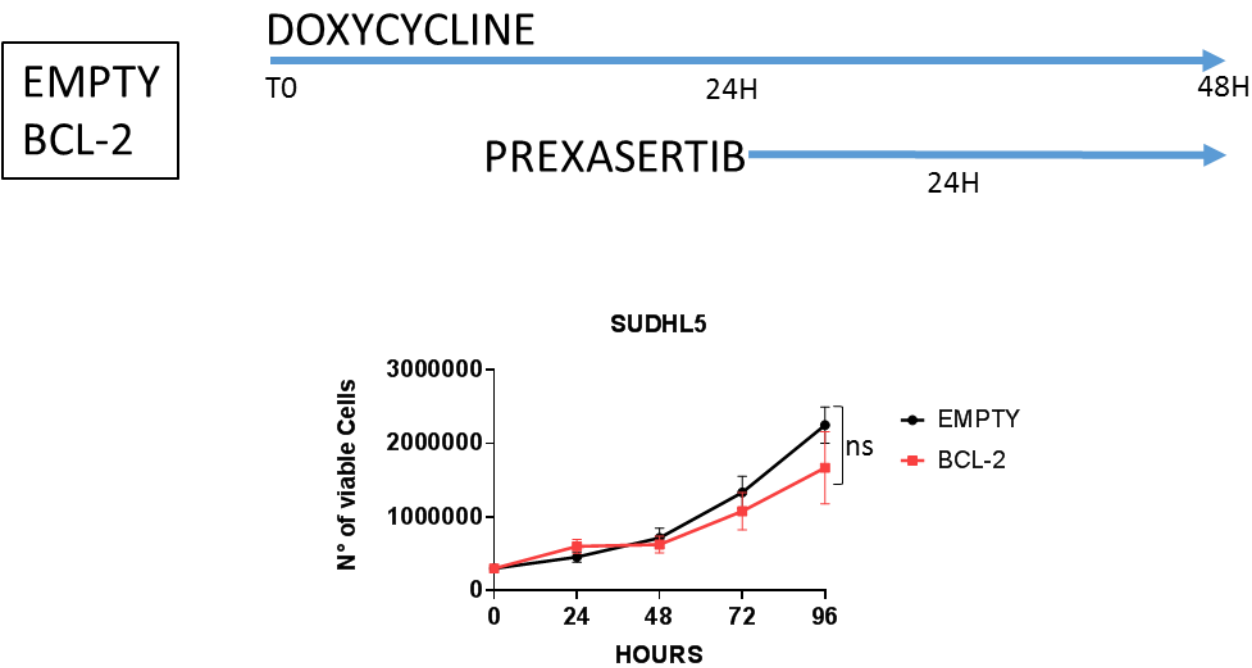


Figure S10

A



B

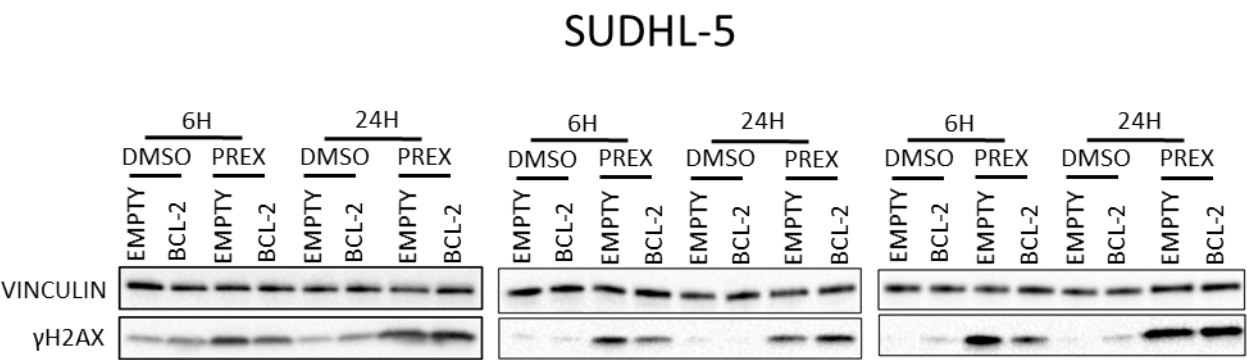
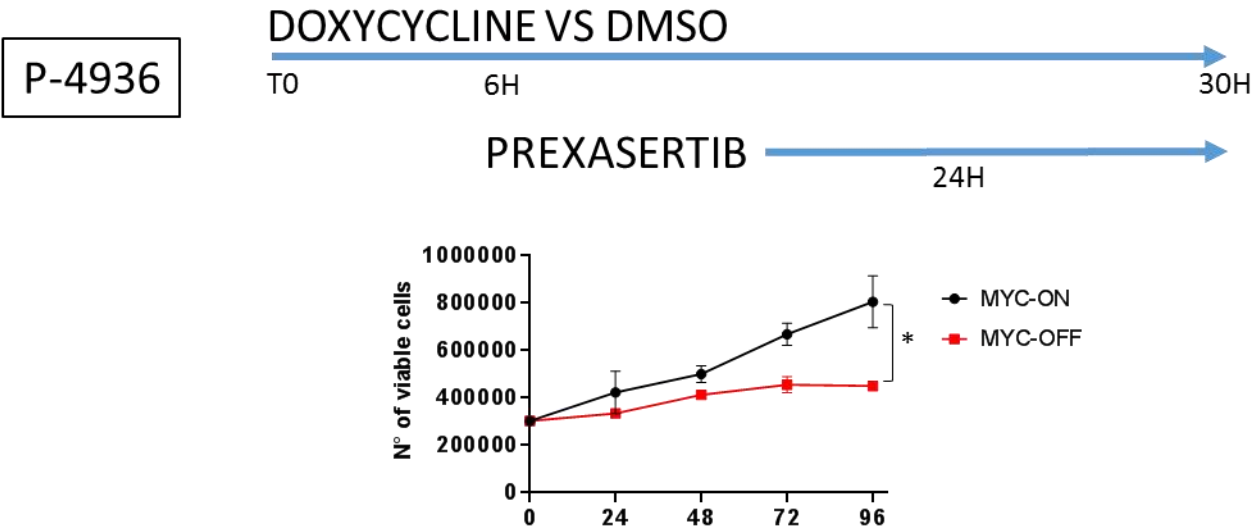
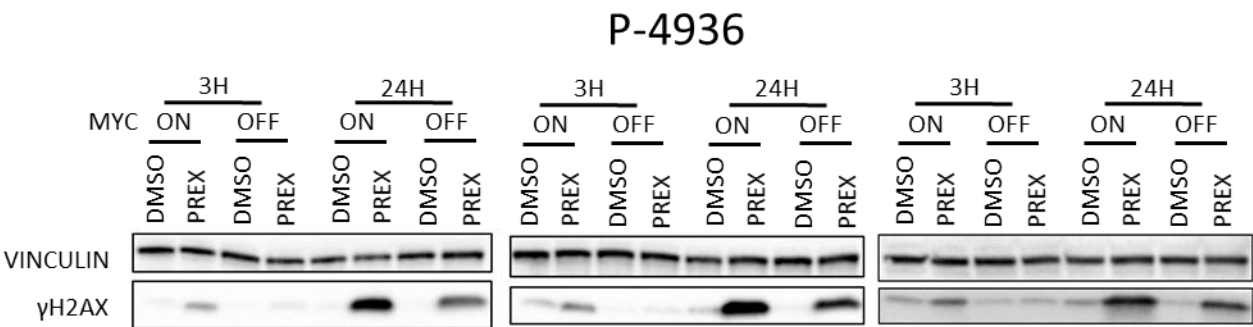


Figure S11

A



B



C

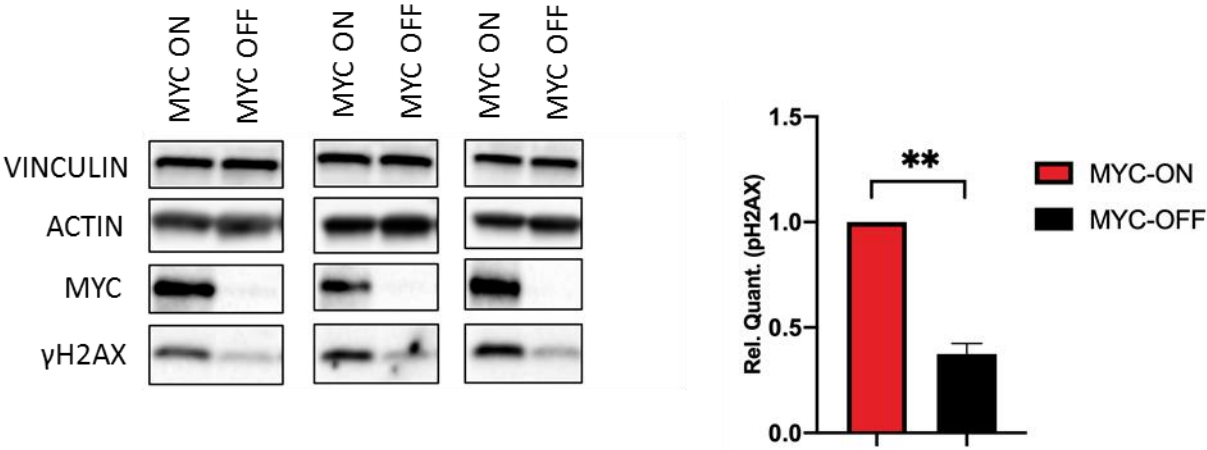
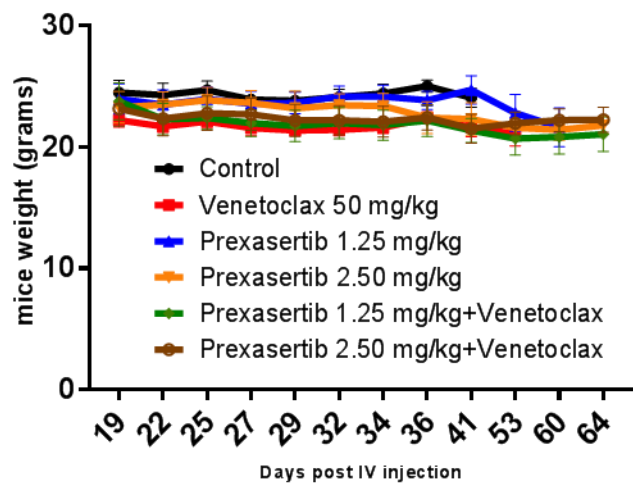
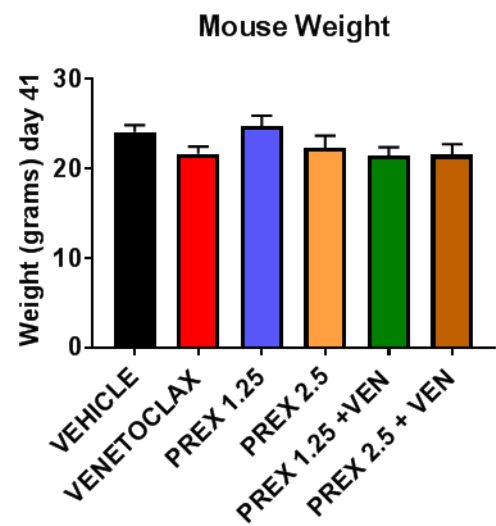


Figure S12

A



B



## Supplementary Figures Legends

Figure S1. Overall survival of the discovery and validation cohorts.

Overall survival curves of the discovery and validation cohorts, showing similar outcomes in the 2 cohorts. P values were calculated with the log rank test.

Figure S2. MYC and BCL-2 status and outcome

- A) Box plot graphs showing significant correlation and concordance between NanoString and immunohistochemistry in the determination of BCL-2 and MYC levels. mRNA levels detected by NanoString in the BCL-2 and MYC negative and positive subgroups as classified by immunohistochemistry (applying a standard 50% and 40% cut-off for BCL-2 and MYC respectively) are represented here. P value were calculated with the Student's T test, \*  $p < 0.05$ , \*\*  $p < 0.01$ .
- B) Overall survival curve of MYC/BCL-2 double expressers as assessed by T-GEP vs non-double expressers in the whole patients cohort (discovery+validation). P values were calculated with the log rank test.

Figure S3. Integration of MYC/BCL-2 status with  $\gamma$ H2AX and 8-OHdG for outcome prediction.

- A) Survival analysis integrating MYC/BCL-2 status assessed by T-GEP with  $\gamma$ H2AX and 8-OHdG levels assessed by immunohistochemistry in the whole patient cohort (discovery+validation). P values were calculated with the log rank test.

- B) OS curves of non-DE (non-double expresser DLBCL) cases vs DE-OX\_low vs DE-OX\_high\_non-DH (DE-OX-high non double hit), vs DE-OX\_high\_DH (DE-OX\_high double hit) are compared. P values were calculated with the log rank test.

Figure S4. Differential activity of Prexasertib and Doxorubicin in DLBCL cell lines and effects of Prexasertib on oxidative DNA damage.

- A) Bar graph showing average cell doubling time in our panel of aggressive B-cell lymphoma cell lines. Error bars represent standard error of the mean (SEM) of triplicate measurements.
- B) Scatter plot showing cell doubling time according to Prexasertib sensitivity (high vs med vs low) in our panel of B-cell lymphoma cell lines. Each point represents the mean of 3 independent experiments.
- C) Bar graph showing percentages of cell viability of different B-cell lymphoma cell lines treated with Doxorubicin 125 nM for 24 hours. Below the graph, a heat map showing 1- information on Prexasertib (Prex) and Doxorubicin (Doxo) sensitivity defined as: high > 50% inhibition of cell viability; med 25-50% inhibition; low < 25% inhibition. 2- annotation regarding *MYC*, *BCL-2*, *TP53*, *ATM* status in all cell lines (rearr.: rearrangement; wt: wild type; mut/del: mutation/deletion). See supplemental methods for detailed references of genomic annotations. Error bars represent standard error of the mean (SEM) of triplicate experiments.
- D) Representative western blot assays depicting the effects of 24-hour incubation with DMSO, 125 nM Prexasertib or 125 nM Doxorubicin, on  $\gamma$ H2AX, pCHK1 S345 and cleaved caspase 3 (CL. CASP 3) expression levels in DLBCL cell lines showing high and intermediate sensitivity to both Prexasertib and Doxorubicin (SUDHL-5

and DOHH2) vs cell lines showing high sensitivity to Prexasertib but low sensitivity to Doxorubicin (OCI-LY-1, OCI-LI-7).

- E) Bar graph showing 8-OhDG levels in DNA extracted from OCI-LY-18, DOHH2 and HBL-1 cells treated with DMSO or the indicated doses of Prexasertib (PREX) for 6 hours. Differences between groups were calculated with the Student T test. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Error bars represent standard error of the mean (SEM) of triplicate experiments.
- F) Scatter plot representing the effects of 125 nM Prexasertib on apoptosis in DLBCL cell lines: highly sensitive (high sensitivity) vs less sensitive (medium or low sensitivity). Cells were incubated with Prexasertib for 24 hours and caspase 3/7 activation assessed with the Caspase Glo 3/7 assay system (Promega™). Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test.

Figure S5. Effects of Prexasertib on apoptosis and cell cycle in DLBCL cell lines according to *BCL-2* and *TP53* status.

- A) Scatter plot representing the effects of 125 nM Prexasertib on apoptosis in DLBCL cell lines according to *TP53* status (WT vs Mut/Del). Cells were incubated with Prexasertib for 24 hours and caspase 3/7 activation assessed with the Caspase Glo 3/7 assay system (Promega™). Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test. RLU (relative light units).
- B) Graph showing fold changes over time in caspase 3/7 activity in DLBCL cell lines treated with Prexasertib according to the *TP53* status. *TP53* mut/del cell lines showed a tendency towards increased caspase 3/7 activation at late time points (48 hrs).

Error bars represent standard error of the mean (S.E.M) of triplicate experiments. Differences between groups were calculated with the Student T test. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

- C) Scatter plot representing the effects of 125 nM Prexasertib on apoptosis in DLBCL cell lines according to *BCL-2* status (WT vs Rearranged). Cells were incubated with Prexasertib for 24 hours and caspase 3/7 activation assessed with the Caspase Glo 3/7 assay system (Promega™). Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test. RLU (relative light units).
- D) Scatter plot representing the effects of 125 nM Prexasertib on apoptosis in DLBCL cell lines according to *MYC* and *BCL-2* status [*BCL-2* WT vs *BCL-2* Rearranged vs *MYC/BCL-2* rearranged (DH)]. Cells were incubated with Prexasertib for 24 hours and caspase 3/7 activation assessed with the Caspase Glo 3/7 assay system (Promega™). Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test. RLU (relative light units).
- E) Scatter plot representing the effects of 125 nM Prexasertib on cell cycle in DLBCL cell lines according to *TP53* status (WT vs Mut/Del). Cells were incubated with Prexasertib for 24 hours and changes in cell cycle fractions assessed by flow cytometry with propidium iodide staining. Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test.
- F) Scatter plot representing the effects of 125 nM Prexasertib on cell cycle in DLBCL cell lines according to *BCL-2* status (WT vs Rearranged). Cells were incubated with Prexasertib for 24 hours and changes in cell cycle fractions assessed by flow cytometry with propidium iodide staining. Each point represents the mean of 3 independent experiments. P value was calculated with the paired Student's T test.

Figure S6. HTS data analysis

- A) Boxplot graph summarizing the results of the combinatorial drug screening (AZD7762 + Venetoclax) analyzed with the Bliss model in individual cell lines categorized according to the cell of origin and MYC/BCL-2 status. Error bars represent minimum and maximum measured values.
- B) Boxplot graph summarizing the results of the combinatorial drug screening (MK8776 + Venetoclax) analyzed with the Bliss model in individual cell lines categorized according to the cell of origin and MYC/BCL-2 status. Error bars represent minimum and maximum measured values.
- C) Boxplot graph summarizing the results of the combinatorial drug screening (AZD7762 + Venetoclax) analyzed with the Bliss model according to the cell of origin and BCL-2 status. Error bars represent minimum and maximum measured values. Differences between groups (ABC, GCB *BCL-2 rearranged*, GCB *BCL-2 negative*) were calculated with the Student's t test. \* $p < 0.05$ , \*\* $p < 0.01$ .
- D) Boxplot graph summarizing the results of the combinatorial drug screening (MK8776 + Venetoclax) analyzed with the Bliss model according to the cell of origin and BCL-2 status. Error bars represent minimum and maximum measured values. Differences between groups (ABC, GCB *BCL-2 rearranged*, GCB *BCL-2 negative*) were calculated with the Student's t test. \* $p < 0.05$ , \*\* $p < 0.01$ .
- E) Western blot assay showing levels of BCL-2 protein expression in the cell line panel used for HTS experiments.

Figure S7. Validation combinatory experiments of Prexasertib and Venetoclax in DLBCL cell lines.

Heat maps showing viability following treatment for 24 hours with increasing doses (D) (12.5-25-50 nM) of Prexasertib (P), Venetoclax (V), and the combination (C), in DLBCL cell lines. Viability values (mean of 3 independent experiments) are depicted in a colorimetric scale from red (low) to yellow (medium) to green (high) with respect to DMSO (control).

Figure S8. Baseline  $\gamma$ H2AX and 8-OHdG levels (OX\_status), MYC/BCL-2 status, and the activity of combined DDR and BCL-2 inhibition in DLBCL cell lines.

- A) Western blot assay showing levels of MYC and BCL-2 protein expression in the cell line panel used for validation experiments.
- B) Bar graph showing quantitative densitometry analyses (ImageJ software) of baseline  $\gamma$ H2AX levels normalized vs actin (3 independent western blots assays shown in Figure S8C) in our panel of DLBCL cell lines. Error bars represent standard error of the mean (SEM) of triplicate measurements.
- C) Three independent western blot assays showing  $\gamma$ H2AX protein expression (and actin) in the cell line panel used for validation experiments.
- D) Bar graph showing baseline 8-OHdG levels assessed with a dedicated ELISA assay (3 independent measurements) in our panel of DLBCL cell lines. Error bars represent standard error of the mean (SEM) of triplicate measurements.
- E) Heat map showing *MYC* and *BCL-2* mRNA levels assessed by qPCR in our cell line panel,  $\gamma$ H2AX levels as depicted in panel B, 8-OHdG levels as assessed by ELISA and depicted in panel D [in a colorimetric scale from green (low) to yellow (medium) to red (high)], DE lymphoma (DEL) status based on qPCR or western blot data (in red), and OX\_status based on  $\gamma$ H2AX/8-OHdG levels. For each individual variable values below the median were considered as “low”, whereas values equal or above

the median were considered as “high”. OX\_status was defined as a) OX\_H-H (high  $\gamma$ H2AX/high 8-OHdG, in red, corresponding to the OX\_high status defined in DLBCL tissues by IHC), OX\_H-L/L-H (either high  $\gamma$ H2AX/low 8-OHdG or low  $\gamma$ H2AX/high 8-OHdG, in orange) and OX\_L-L (low  $\gamma$ H2AX/low 8-OHdG, in blue). The latter two categories correspond to the OX\_low status defined by IHC.

F) Cell titer glo assays of DLBCL cell lines treated with single agent Venetoclax and Prexasertib and classified by combining DE status evaluated by western blot (MYC and BCL-2 protein levels) with  $\gamma$ H2AX/8-OHdG status (based on the heat map showed in panel D): cell lines were classified as a) DE-OX\_H-H (high MYC/BCL-2, high  $\gamma$ H2AX/high 8-OHdG, in red), DE-OX\_H-L/L-H (high MYC/BCL-2, and either high  $\gamma$ H2AX/low 8-OHdG or low  $\gamma$ H2AX/high 8-OHdG, in orange) and DE-OX\_L-L (high MYC/BCL-2, low  $\gamma$ H2AX/low 8-OHdG, in blue). Cells were incubated with increasing doses of Prexasertib or Venetoclax for 24 hours (12.5-25-50 nM).

Viability values represent the mean of 3 independent experiments.

G) Scatterplot representing combination index data of Prexasertib combined with Venetoclax in 9 DE DLBCL cell lines (as defined by MYC and BCL-2 protein levels) according to the baseline  $\gamma$ H2AX/8-OHdG (OX) status (H-H in red vs L-L in blue vs L-H/H-L in orange). 3 doses of Prexasertib (12.5, 25, 50 nM) were combined with 3 doses of Venetoclax (12.5, 25, 50 nM) for 24 hours, allowing 3 combinatory values for each cell line. Values below 1 indicate synergistic interactions. See also figure 4C.

Figure S9. DDR inhibition and DDRi-based combinations in BCL-2 negative cell lines

- A) Western blot assays showing the effects of 24 hours incubation with Prexasertib 125 nM, Doxorubicin 125 nM and H<sub>2</sub>O<sub>2</sub> 150  $\mu$ M, on BCL-2 and  $\gamma$ H2AX levels in 2 BCL-2 negative (SUDHL-5 and OCILY-7) and one BCL-2 positive cell line (HBL-1).
- B) Cell Titer Glo experiments in 2 representative BCL-2 negative (SUDHL-5, SUDHL-2), and one BCL-2 positive (TMD8) cell line. Cells were incubated with increasing concentrations of Prexasertib and the MCL-1 inhibitor S63845 (12.5, 25, 50 nM), and cell viability was assessed after 24 hours. Error bars represent standard error of the mean (S.E.M) of triplicate experiments.

Figure S10. Role of BCL-2 in modulating sensitivity to DDR inhibition.

- A) Experimental design of the experiments represented in Figure 5 A-D. SUDHL-5 cells (transfected with EMPTY VECTOR or a BCL-2 TET-ON inducible system (BCL-2 EXP) were preincubated with doxycycline 1  $\mu$ g/ml for 24 hours and then treated with Prexasertib at the indicated doses. Lower panel: Growth curves of SUDHL-5 cells cultured in the presence or absence of BCL-2 (EMPTY vs BCL-2) for up to 96 hrs.
- B) Western blot assays from triplicate experiments depicting the effects of Prexasertib treatment (6nM) on  $\gamma$ H2AX induction in the presence or absence of BCL-2 in SUDHL-5 cells. Densitometry analyses were performed with the ImageJ software and are represented in Figure 5B.

Figure S11. Role of MYC in modulating sensitivity to DDR inhibition.

- A) Experimental design of the experiments represented in Figure 5 E-H. P-4936 cells (carrying a tetracycline inducible promoter, TET-OFF MYC) were preincubated with doxycycline 1  $\mu$ g/ml for 6 hours and then treated with Prexasertib at the indicated

doses. Lower panel: Growth curves of P-4936 cells cultured in the presence or absence of MYC (MYC-ON vs MYC-OFF) for up to 96 hrs.

B) Western blot assays from triplicate experiments depicting the effects of Prexasertib treatment (25 nM) on  $\gamma$ H2AX induction in the presence or absence of MYC in P-4936 cells. Densitometry analyses were performed with the ImageJ software and are represented in Figure 5F.

C) Left panel: Western blot assays from triplicate experiments depicting the effects of MYC depletion on baseline  $\gamma$ H2AX levels in P-4936 cells. Densitometry analyses of  $\gamma$ H2AX (based on the blots represented in the left panel) were performed with the ImageJ software and are represented in the right panel.

Figure S12. Variations in body weight over time in mice treated with Prexasertib, Venetoclax and the combinations in a PDX DH-DLBCL mouse model.

A) Graph showing variations of body weight over time in mice treated with vehicle (n=5) Prexasertib 1.25 and 2.5 mg/kg BID 3 times/week (n=5), Venetoclax 50 mg/Kg 5 days a week (n=5), and the combinations (n=5).

B) Columns graphs showing body weights at day 41 in mice treated with vehicle, prexasertib (1.25 and 2.5 mg/Kg 3 times/week), venetoclax 50 mg/Kg 5 days a week, and the combinations.

## References

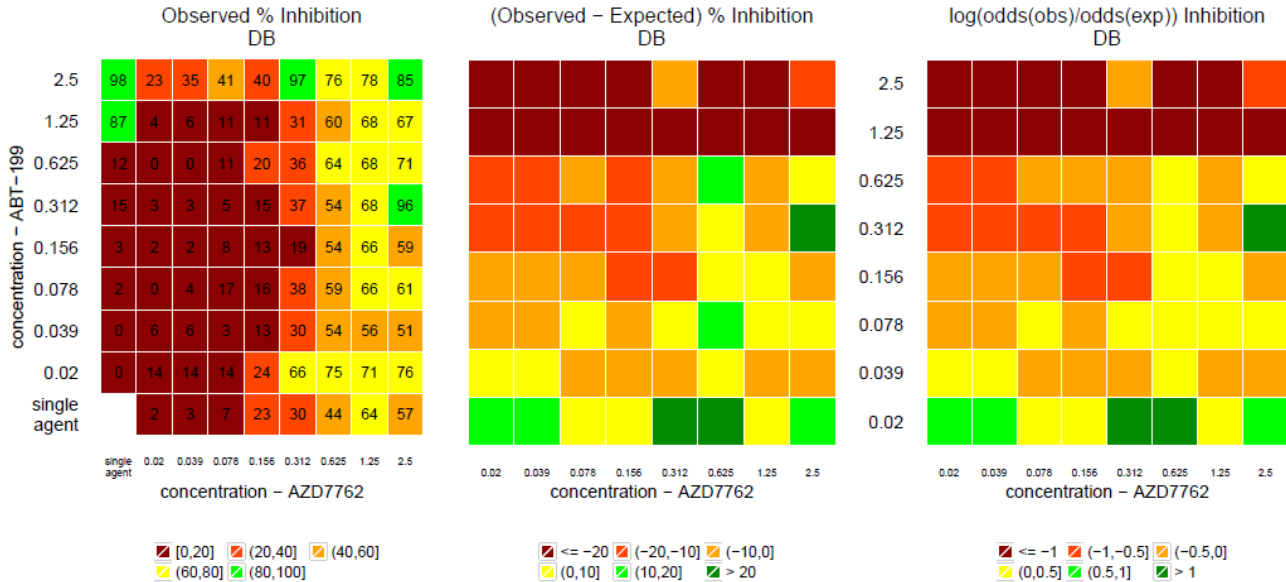
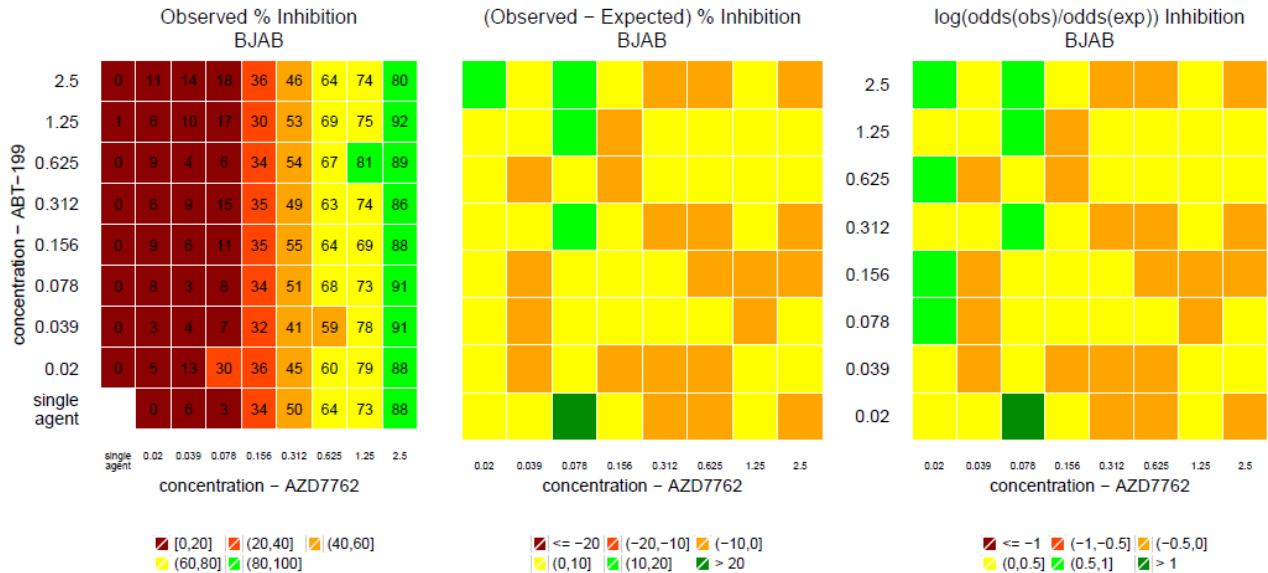
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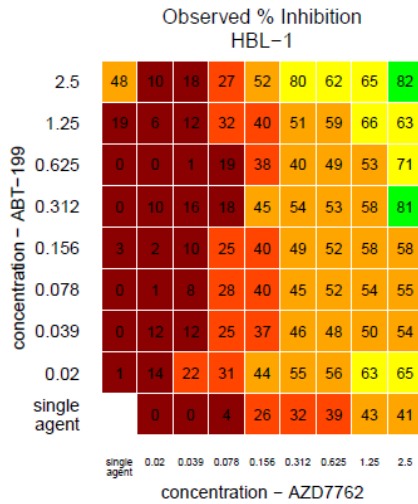
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# **HIGH THROUGHPUT DRUG SCREENING (HTS)**

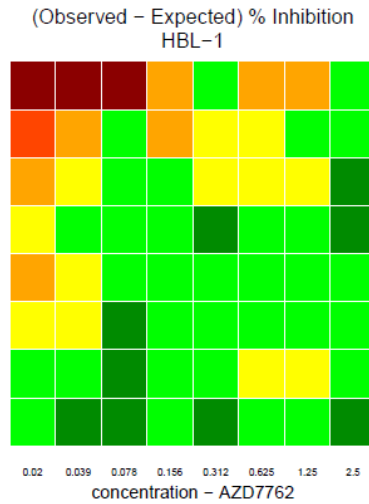
## **DATA SUPPLEMENT**

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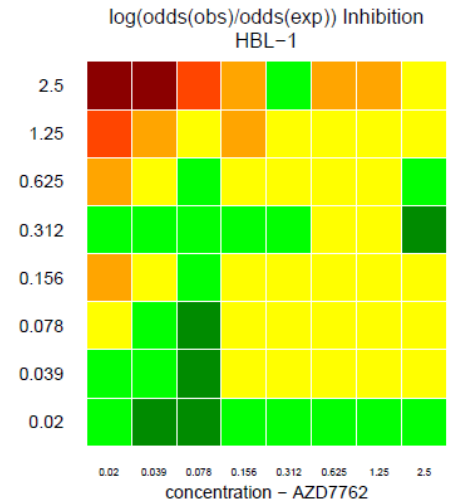




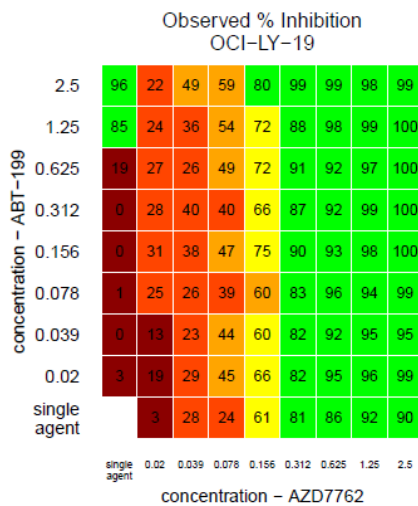
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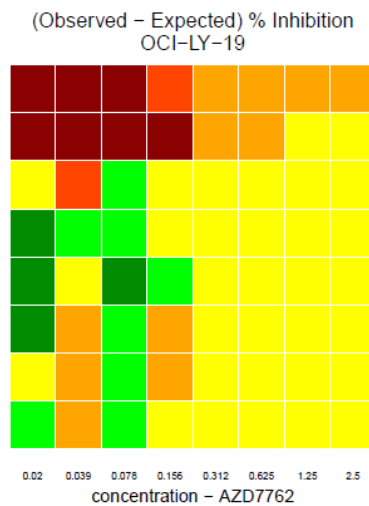
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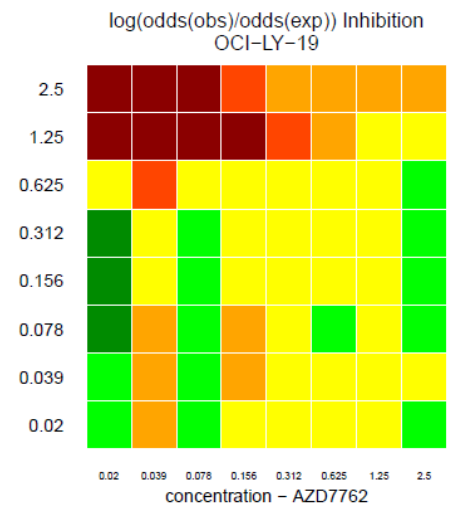
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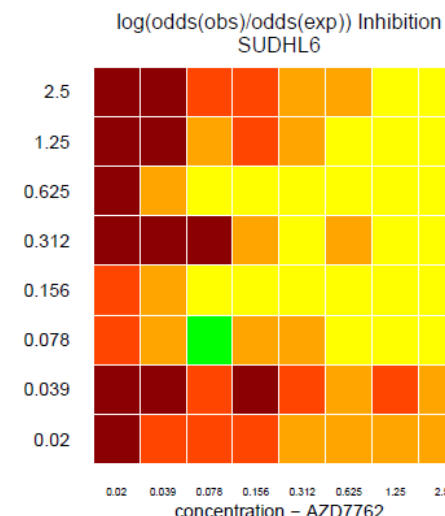
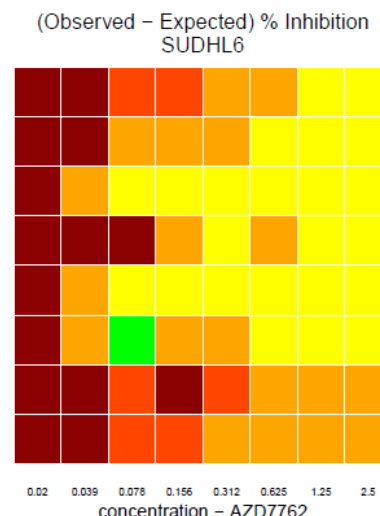
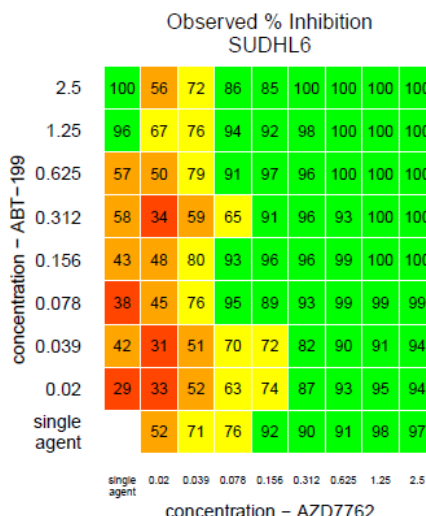
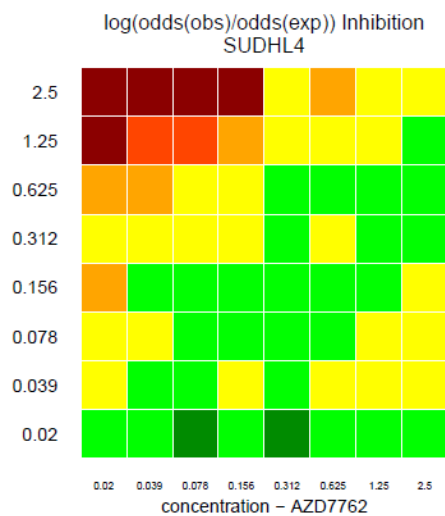
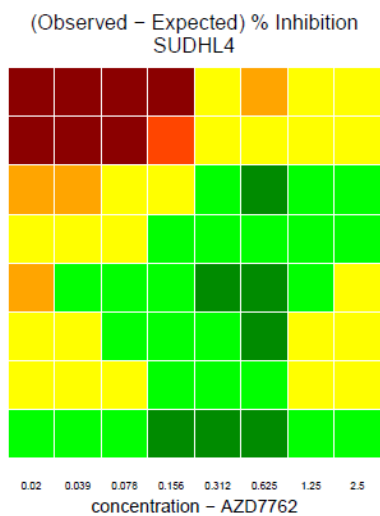
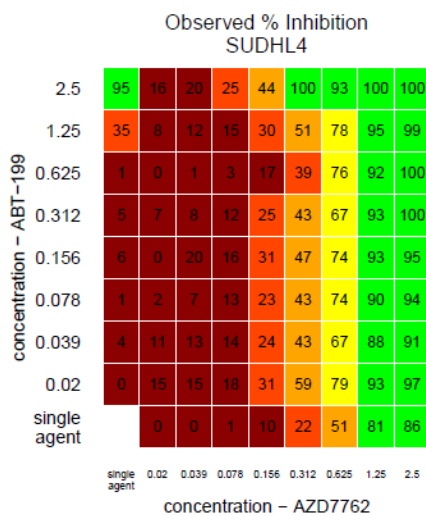
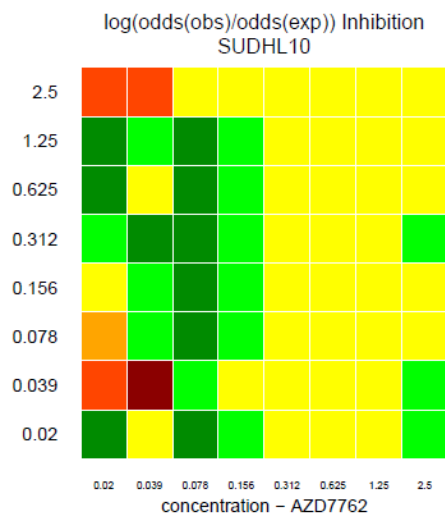
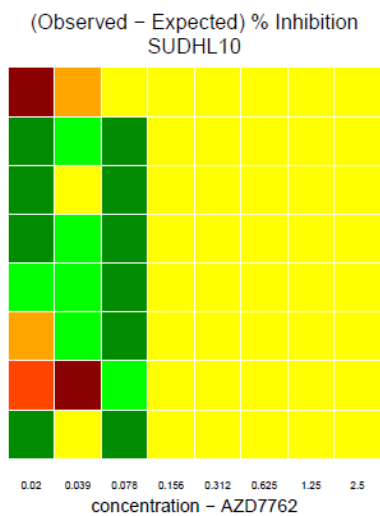
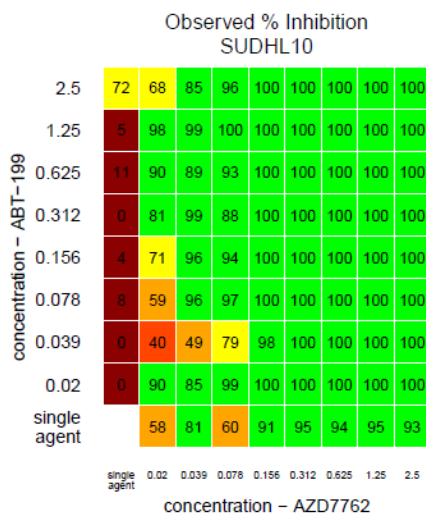
■ [0,20] ■ (20,40] ■ (40,60]  
■ (60,80] ■ (80,100]

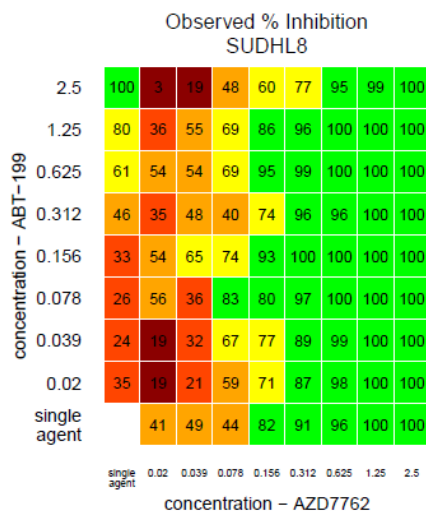


■ ≤ -20 ■ (-20,-10] ■ (-10,0]  
■ (0,10] ■ (10,20] ■ > 20

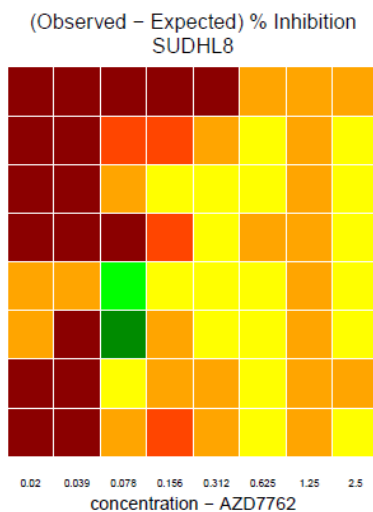


■ ≤ -1 ■ (-1,-0.5] ■ (-0.5,0]  
■ (0,0.5] ■ (0.5,1] ■ > 1

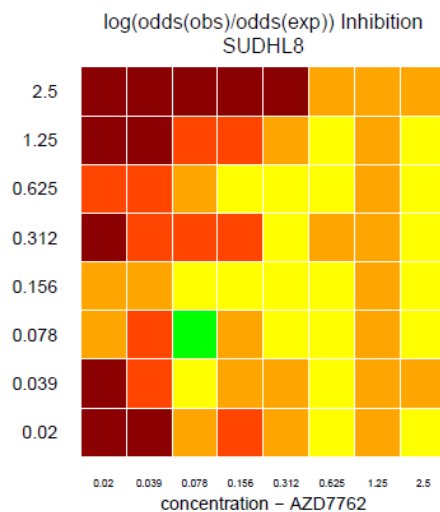




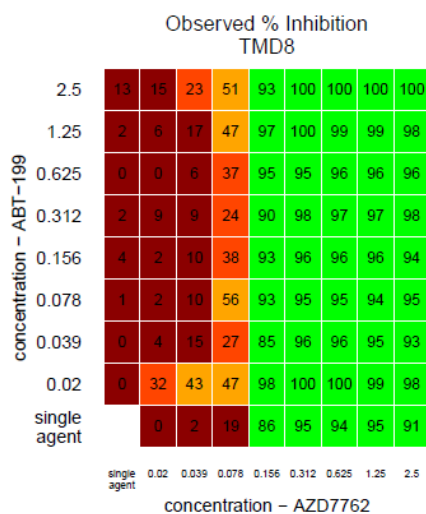
[0,20] [20,40] [40,60]  
 [60,80] [80,100]



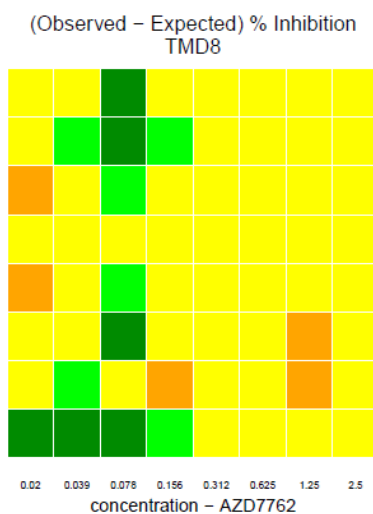
<= -20 (-20,-10] (-10,0]  
 (0,10] (10,20] > 20



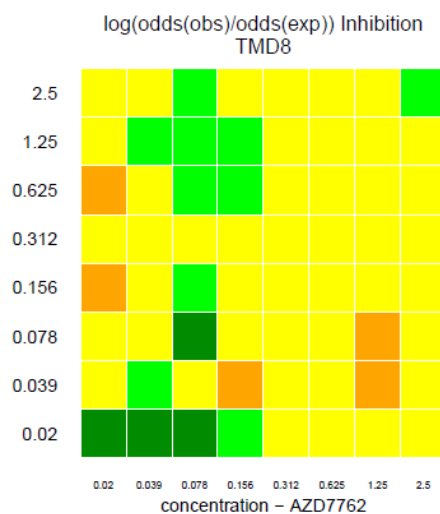
<= -1 (-1,-0.5] (-0.5,0]  
 (0,0.5] (0.5,1] > 1



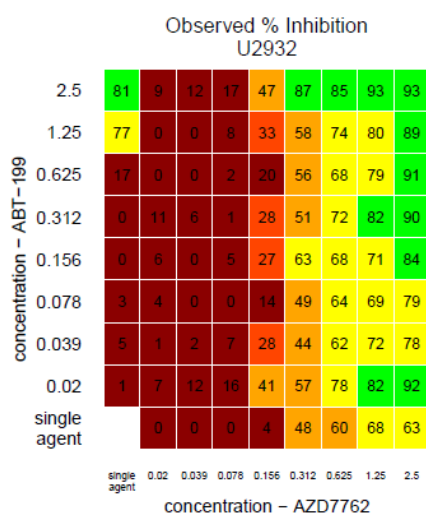
[0,20] [20,40] [40,60]  
 [60,80] [80,100]



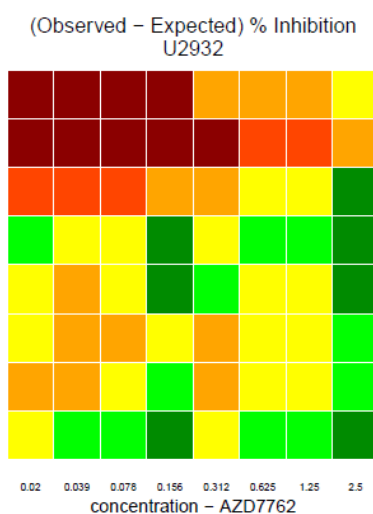
<= -20 (-20,-10] (-10,0]  
 (0,10] (10,20] > 20



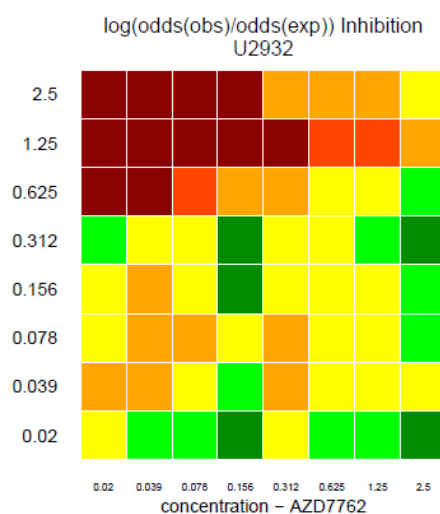
<= -1 (-1,-0.5] (-0.5,0]  
 (0,0.5] (0.5,1] > 1



[0,20] [20,40] [40,60]  
 [60,80] [80,100]

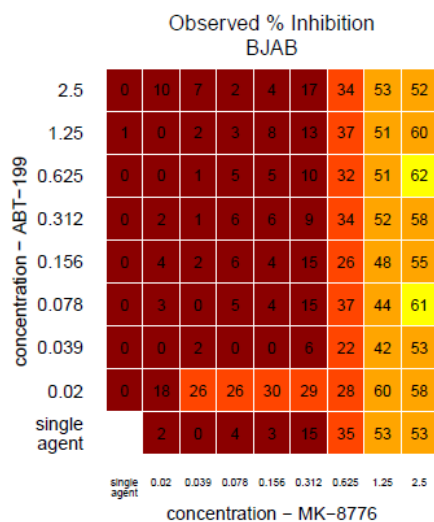


<= -20 (-20,-10] (-10,0]  
 (0,10] (10,20] > 20

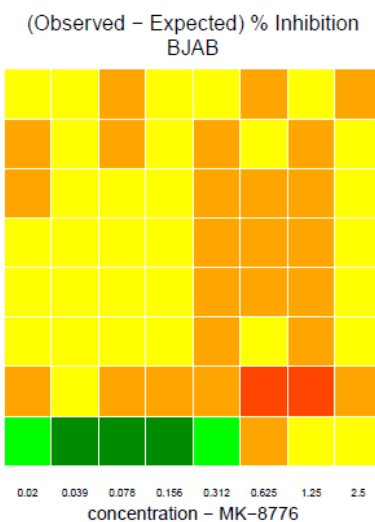


<= -1 (-1,-0.5] (-0.5,0]  
 (0,0.5] (0.5,1] > 1

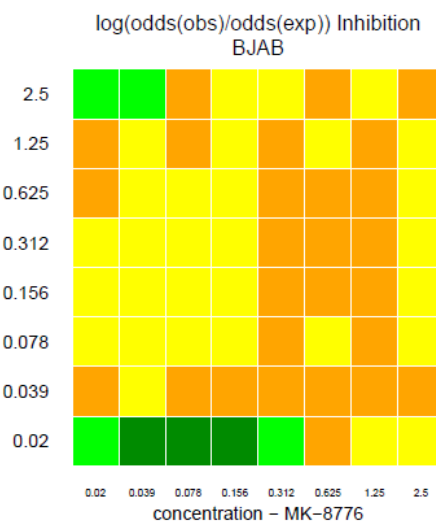
# MK8776 + ABT-199 (VENETOCLAX)



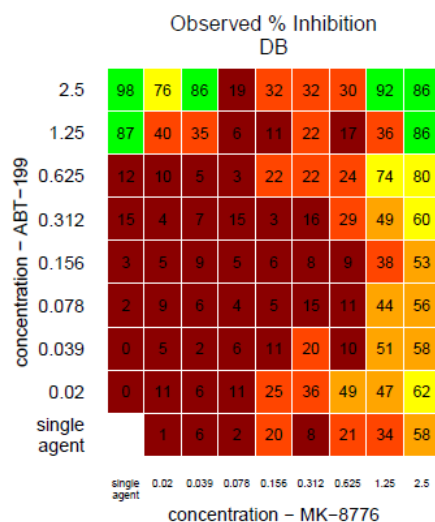
[0,20] [20,40] [40,60]  
 [60,80] [80,100]



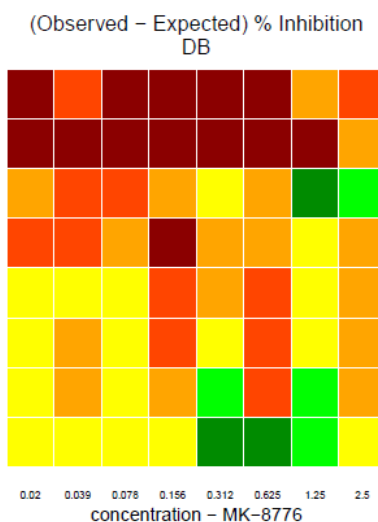
<= -20 (-20,-10) (-10,0)  
 (0,10] (10,20] > 20



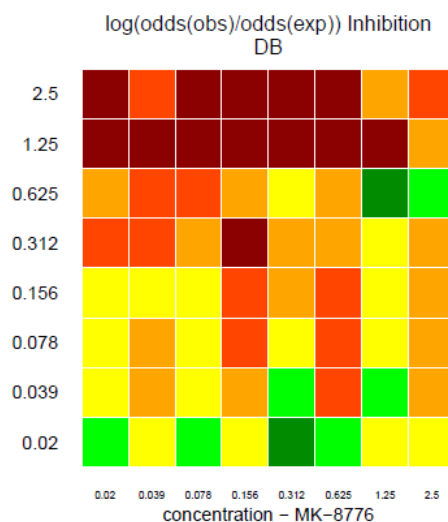
<= -1 (-1,-0.5] (-0.5,0]  
 (0,0.5] (0.5,1] > 1



[0,20] [20,40] [40,60]  
 [60,80] [80,100]

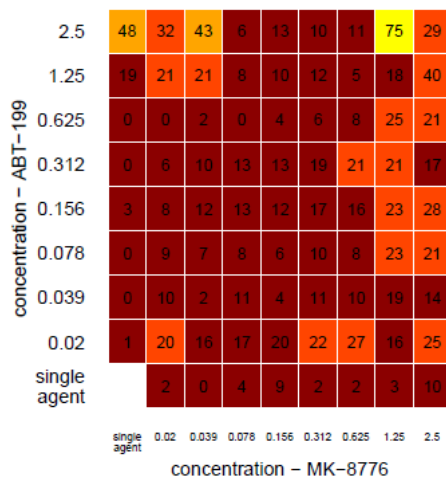


<= -20 (-20,-10) (-10,0)  
 (0,10] (10,20] > 20



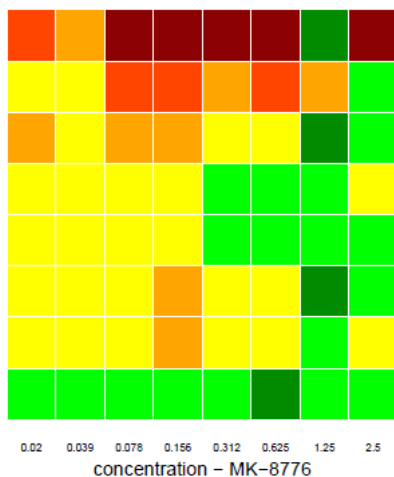
<= -1 (-1,-0.5] (-0.5,0]  
 (0,0.5] (0.5,1] > 1

Observed % Inhibition  
HBL-1



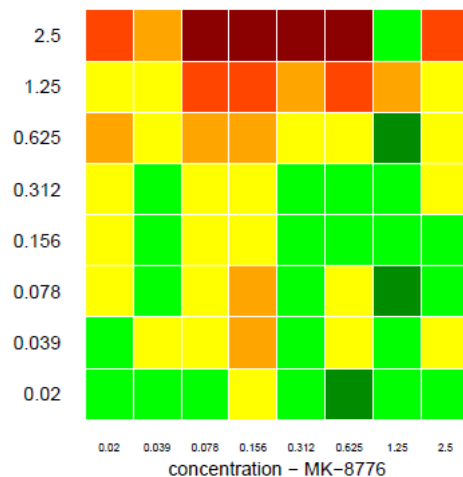
[0,20] [20,40] [40,60]  
 [60,80] [80,100]

(Observed - Expected) % Inhibition  
HBL-1



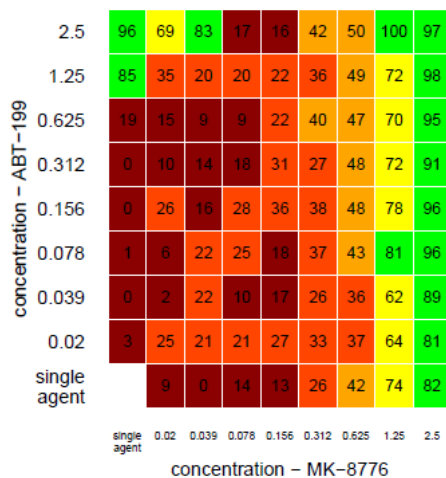
<= -20 (-20,-10) (-10,0)  
 (0,10] (10,20] > 20

log(odds(obs)/odds(exp)) Inhibition  
HBL-1



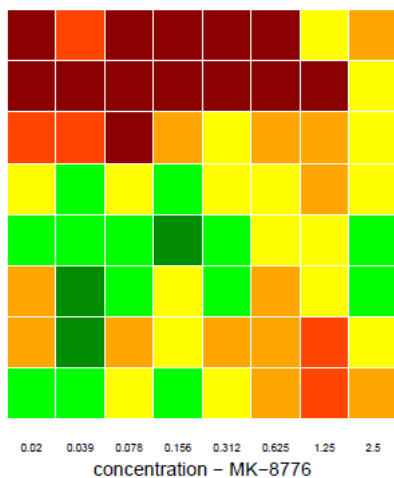
<= -1 (-1,-0.5) (-0.5,0)  
 (0,0.5] (0.5,1] > 1

Observed % Inhibition  
OCI-LY-19



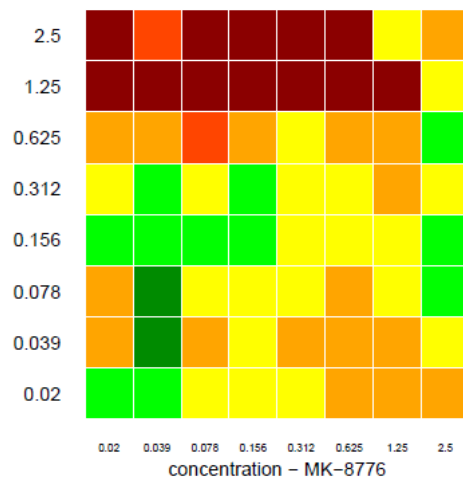
[0,20] [20,40] [40,60]  
 [60,80] [80,100]

(Observed - Expected) % Inhibition  
OCI-LY-19



<= -20 (-20,-10) (-10,0)  
 (0,10] (10,20] > 20

log(odds(obs)/odds(exp)) Inhibition  
OCI-LY-19



<= -1 (-1,-0.5) (-0.5,0)  
 (0,0.5] (0.5,1] > 1

