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High Throughput Screen to Evaluate Combinations With Ibrutinib in Various B-Cell Malignancies

Shalini Chaturvedi,[1] Michael Schaffer,[1] Cuc Davis,[1] Regina Aquino,[1] Emily Stepanchick,[1] Matthias Versele,[2] and Sriram Balasubramanian[1]

[1]Janssen Research & Development, Spring House, PA, USA; [2]Janssen Research & Development, Beerse, Belgium

INTRODUCTION

- Multitargeted drug combinations are evaluated in vitro and preclinically to establish anticancer efficacy and standard of care.
- In B-cell malignancies, Bruton's tyrosine kinase (BTK) has emerged as a crucial therapeutic target, as it is required for B-cell receptor (BCR) signaling, plays a key role in B-cell maturation, and is overexpressed in a number of B-cell malignancies.[1,2]
- Constitutive B-cell activation via the BTK BCR has been implicated in various hematological malignancies, including the activated B-cell (ABC)-like subtype of diffuse large B-cell lymphoma (DLBCL).[3]
- Ibrutinib is a first-in-class, once-daily, oral, covalent inhibitor of BTK with inhibitory activity demonstrated in DLBCL cell lines that overexpress BTK and in animal models of B-cell malignancy.[2,4,5]
- A phase 1 study in patients with relapsed/refractory non-Hodgkin's lymphoma (NHL) demonstrated complete or near-complete BTK occupancy with a clinically active dose of ibrutinib,[5] while subsequent phase 2 and 3 single-agent and combination studies have demonstrated benefits across multiple B-cell malignancies.[6-8]
- Ibrutinib is currently approved in the USA, the EU, and many other countries for patients with:
  - Mantle-cell lymphoma (MCL) or chronic lymphocytic leukemia (CLL) who have received ≥ 1 prior therapy.
  - CLL and deletion of chromosome 17p13.1, as initial therapy.
  - Waldenström's macroglobulinemia.
- Further clinical development of ibrutinib in B-cell malignancies could be aided by establishing ibrutinib combination treatments with maximum synergistic effects.
- We report on the use of a combination high throughput screening (cHTS) platform to identify ibrutinib combinations, doses, and sequence of administration for maximum synergistic effects in cell lines from different B-cell malignancies, with a focus on DLBCL.

METHODS

Cell Lines

- A total of 30 cell lines from various B-cell malignancies (acute myeloid leukemia [AML], acute B-lymphoblastic leukemia [B-ALL], Burkitt's lymphoma [BL], follicular lymphoma [FL], MCL, multiple myeloma [MM], and DLBCL) were examined.
  - The DLBCL cell lines examined were HBL-1, TMD8, OCI-Ly3, OCI-Ly10, and SU-DHL-6-epst.
  - All cell lines were provided by Horizon Discovery Group (Cambridge, UK).
- Cell lines were screened with conditioned media from a human bone marrow stromal cell line to encourage the type(s) of growth stimuli typically present in the tumor microenvironment.
  - Bone marrow stromal cells were provided by Asterand Bioscience (Royston, UK).

Compounds and Antibodies

- Media were supplemented with the following antibodies that bind to the BCR: goat anti-human IgG (#2040-01, 10 μg/mL in assay) and goat anti-human IgM (#2020-01, 10 μg/mL in assay).
  - Antibodies provided by Southern Biotech (Birmingham, AL, USA).
- Ibrutinib was combined with inhibitors of the BCR pathway and regulators of apoptosis.
  - Targets (agents) included BCL-2 (ABT-199 [venetoclax]), isoforms of PI3K (GDC-0941, IPI-145 [duvelisib]), XPO1 (selinexor), BET bromodomain ((+)-JQ1), IRAK4 (aminopyrimidine-1), and MCL-1 (A-1210477).
  - All compounds were sourced by Horizon Discovery Group, except JQ1 (Selleck Chemicals, Houston, TX, USA).
- To identify antiproliferative combination effects at on-target concentrations, the single-agent dose axis for ibrutinib was limited to a concentration of 200 nM.

Antiproliferation Assay

- Screening began once the cells had been expanded and were dividing at their expected doubling times.
  - Cells were seeded (1500 cells/well), equilibrated in assay plates via centrifugation, and placed in incubators attached to dosing modules at 37°C for 24 hours before treatment.
  - End point readout was based upon quantitation of adenosine triphosphate (ATP) as an indicator of viable cells.
  - At time of treatment, a set of untreated assay plates were collected (T_0) and ATP levels were measured by adding ATPlite (Perkin Elmer, Boston, MA, USA).
  - T_0 plates were read using ultrasensitive luminescence and served as untreated controls.
  - Treated assay plates were incubated with compound for 72 hours and then developed for end point analysis using ATPlite.
  - All data points were collected via automated processes, quality controlled, and analyzed using Horizon Discovery proprietary software.

Growth Inhibition

- Growth inhibition (GI) was utilized as a measure of cell viability and measured at the time of dosing (T_0) and after 72 hours (T_{72}).
- A GI reading of 0% represented no growth inhibition, a GI of 100% represented complete growth inhibition, and a GI of 200% represented complete death of all cells in the culture well.
- Horizon Discovery calculated GI by applying the following test and equation derived from the GI calculation used in the National Cancer Institute's NCI-60 high throughput:[9]

$$\text{If } T < V_0: 100 * \left(1 - \frac{T - V_0}{V_0}\right)$$

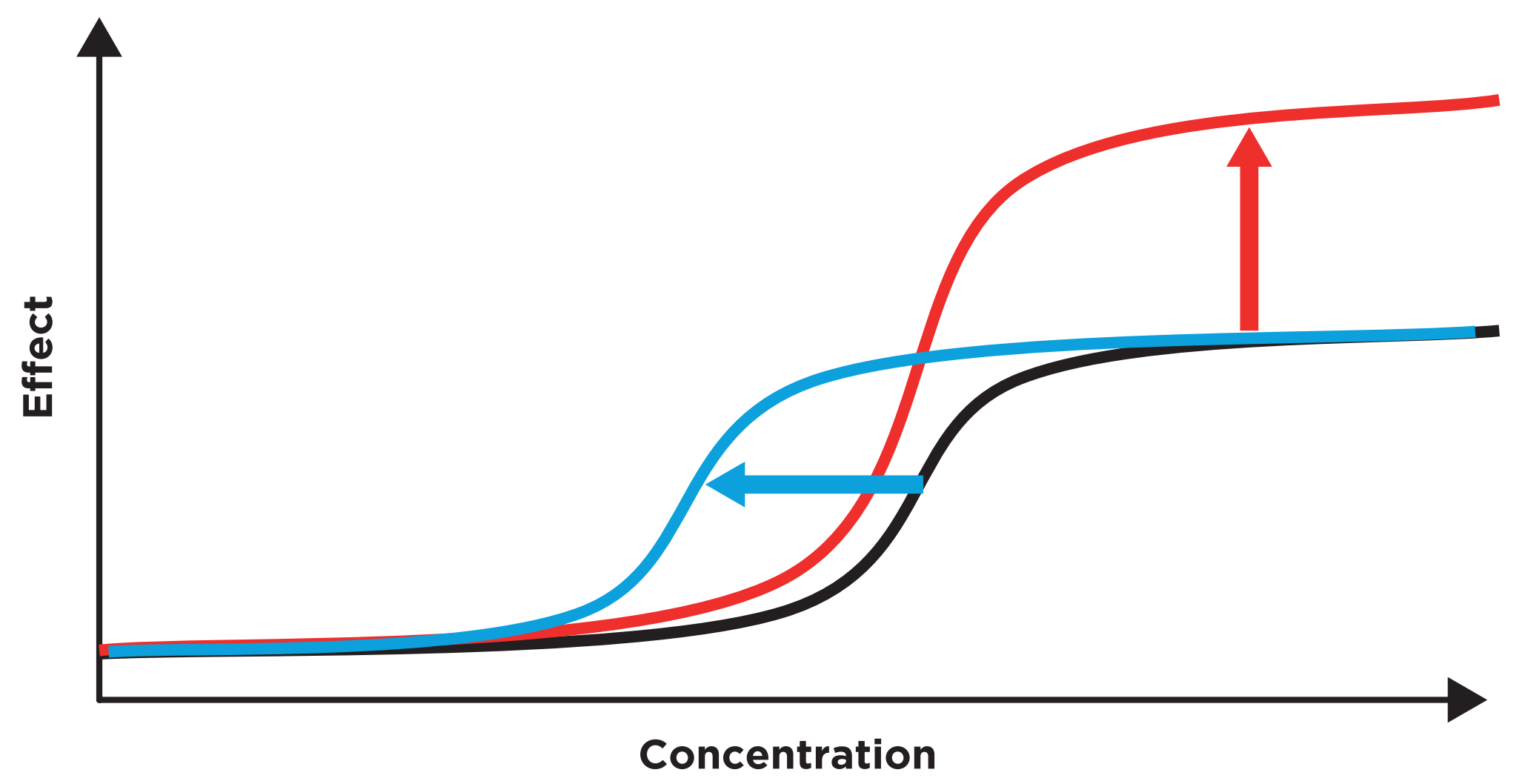
$$\text{If } T \geq V_0: 100 * \left(1 - \frac{T - V_0}{V - V_0}\right)$$

where T is the signal measure for a test article, V is the vehicle-treated control measure, and V_0 is the vehicle-treated control measure at time 0.

Analysis of Combination Activity

- Dose-response matrix screening (9 × 9 optimized matrix, 4 replicates) was used to measure combination effects, which manifest as potency shifts or efficacy boosts (**Figure 1**).

Figure 1. Schematic of Potency Shifts and Efficacy Boosts Used in the Identification of Combination Activity in Dose-Response Matrix Screening



Potency shifts in blue; efficacy boosts in red.

- Combination activity was characterized by comparing each data point with a combination reference model derived from single-agent curves using the Loewe Additivity Model,[10] where synergy was determined relative to Loewe additivity.
- Matrices with moderate to high scores were reviewed to select a cutoff using the histogram analysis feature of the Chalice Analyzer,[11] enabling potentially valid moderate synergies to be identified.
- A scalar measure, the synergy score, was used to characterize the strength of the synergistic interaction and calculated as follows:

  $$\text{Synergy score} = \log f_X \log f_Y \sum \max(0, I_{data})(I_{data} - I_{Loewe})$$

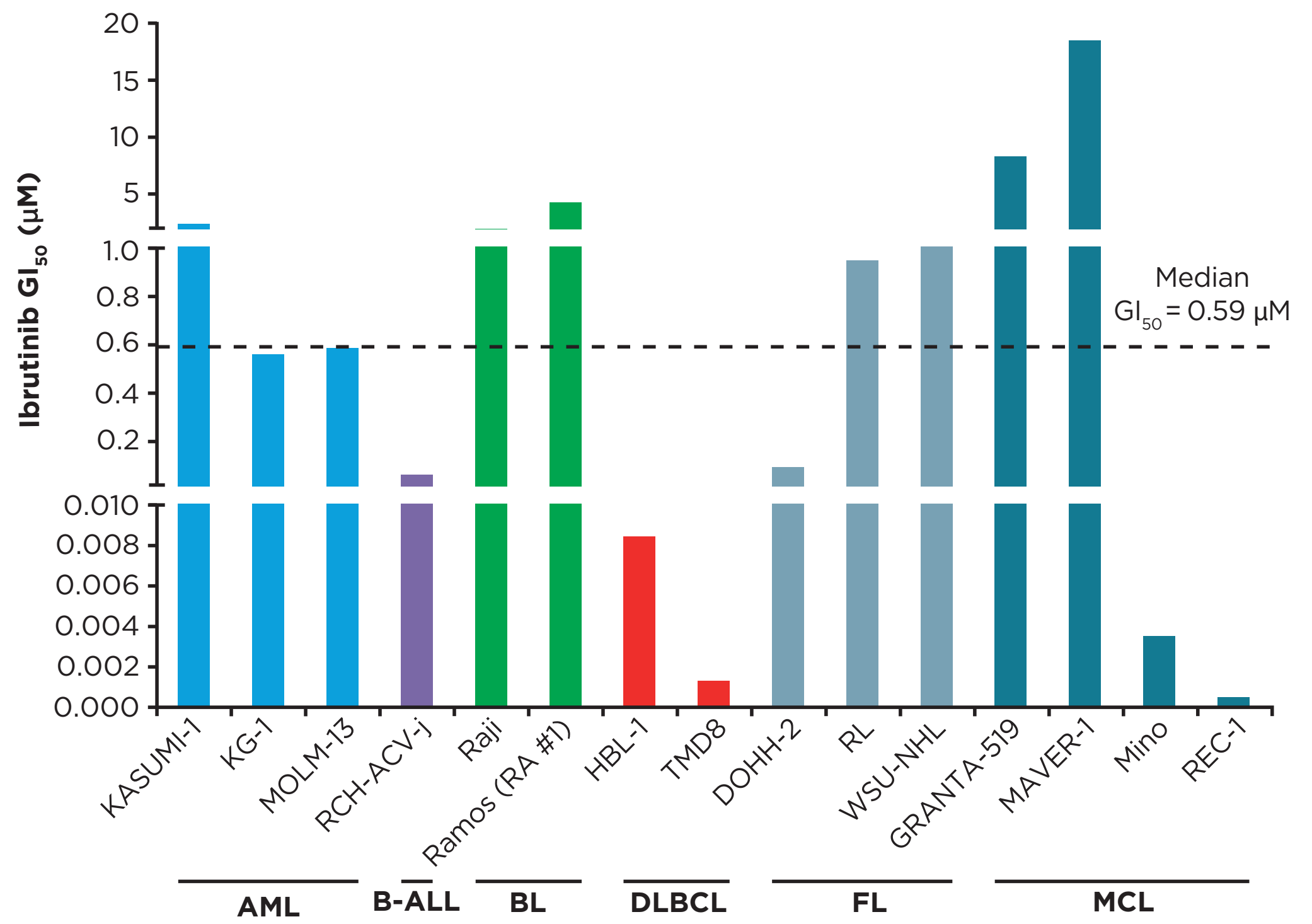
- The fractional inhibition for each component agent and combination point in the matrix was calculated relative to the median of all vehicle-treated control wells.
- Potency shifting was evaluated using an isobologram; this demonstrated how much less drug was required in combination to achieve the desired effect level when compared with single-agent doses needed to reach that same effect.

RESULTS

Single-Agent Dose-Response Analysis

- Single-agent ibrutinib demonstrated varying degrees of activity and potency across the 30 cell lines evaluated within the combination screen.
- Of the DLBCL cell lines tested, the ABC-type cell lines HBL-1 and TMD8 showed the greatest response, with TMD8 being the most sensitive to ibrutinib (GI_{50} of 0.001 μM) (**Figure 2**).
- The MCL cell line REC-1 also showed a GI_{50} value in the picomolar range.
- The median GI_{50} across the entire cell-line panel was 0.59 μM.

Figure 2. Ibrutinib Single-Agent Dose-Response Analysis Showing GI_{50} Values by Type of Hematological Malignancy



Ibrutinib GI_{50} data were extracted from the combination screen and values derived from a fitted curve. Data not shown for 15 cell lines that did not achieve a GI_{50} level.

Combination Activity Based on Synergy Score Analysis

Global analysis

- Cell-line–specific synergistic activity was demonstrated when ibrutinib was combined with compounds from several molecular classes targeting the BCR pathway, at clinically achievable concentrations.
- **Table 1** summarizes synergy scores with all ibrutinib combinations tested across the DLBCL, FL, MCL, AML, and BL cell lines included in the cHTS. Synergy scores shown in **Table 1** have no concentration gating.
- Ibrutinib combinations with BCL-2 and PI3K inhibitors were the most active in the DLBCL cell lines and in 1 FL cell line (DOHH-2).
- Ibrutinib combinations with a BCL-2 inhibitor, along with inhibitors of BET bromodomain, XPO1, and IRAK4, were identified as the most active in 1 MCL cell line (REC-1).
- The highest activity, with a synergy score of 63.9, was seen in the DLBCL cell line TMD8 with the PI3K inhibitor IPI-145.
- Synergy score values for B-ALL and MM cell lines were all below the threshold for synergistic activity.

Table 1. Synergy Scores With Ibrutinib Drug Combinations, Showing Breadth of Activity Across the DLBCL, FL, MCL, AML, and BL Cell Lines Tested in the cHTS

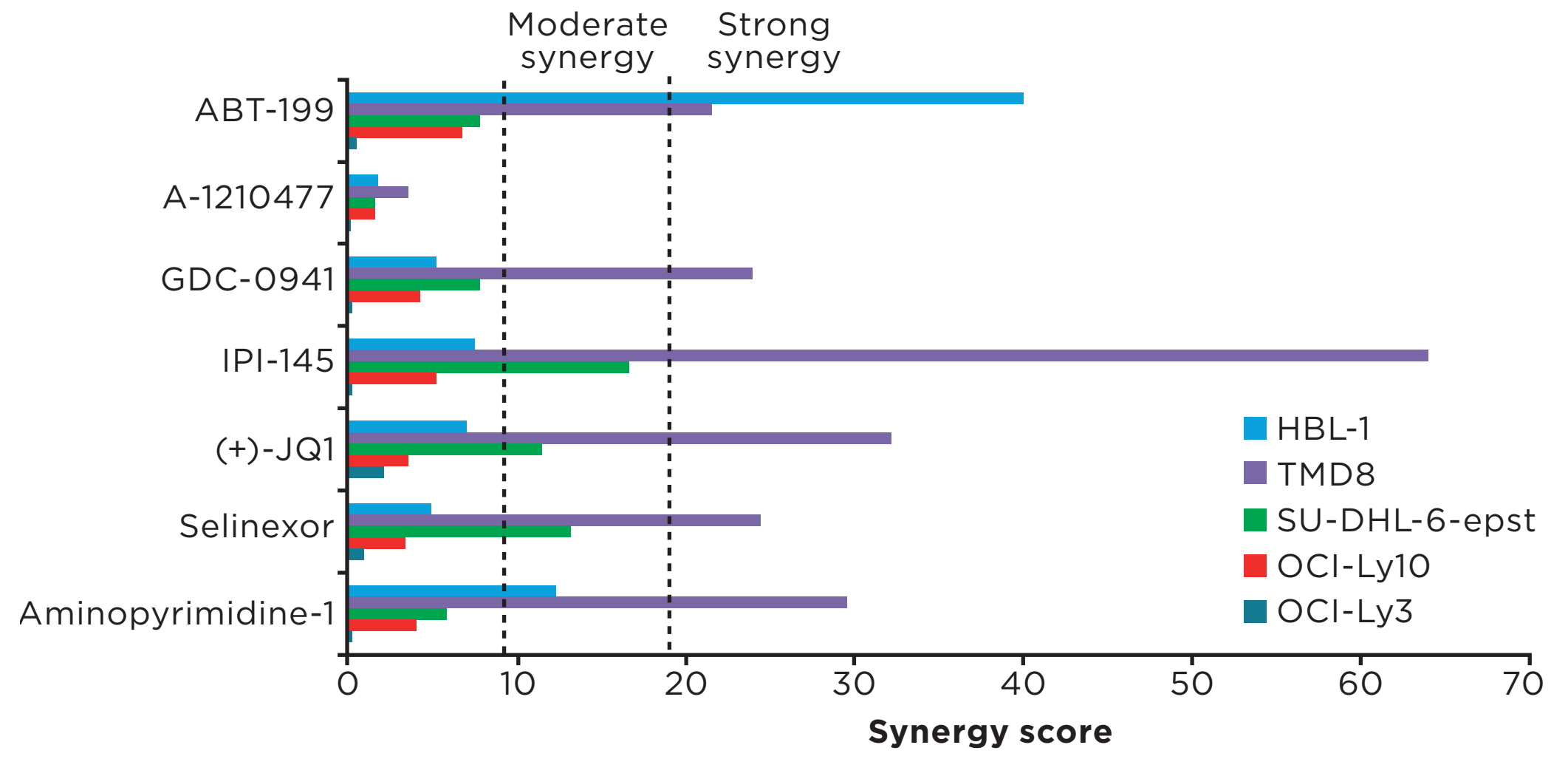
		DLBCL					FL				MCL					AML					BL	
	Target	HBL-1	OCI-Ly3	OCI-Ly10	SU-DHL-6-epst	TMD8	DOHH-2	RL	SC-1	WSU-NHL	GRANTA-519	MAVER-1	Mino	REC-1	Z-138	F-36P-j	KG-1	HL-60	KASUMI-1	MOLM-13	Raji	Ramos (RA #1)
ABT-199	BCL-2	40	2.56	6.83	7.88	21.6	46.9	13	10.8	7.96	11.8	6.32	6.21	28.2	1.17	0.152	18	1.02	17.4	15.4	0.066	0.215
A-1210477	MCL-1	1.68	0.0146	1.44	1.52	3.59	10.4	0.718	0.146	1.54	1.02	1.74	2.55	2.67	0.154	0.0263	4.49	0.0439	5.67	8.78	6.35	0.371
GDC-0941	PI3Ka/d	5.33	0.659	4.18	7.92	23.9	22.9	4.38	0.324	10.4	4.22	6.91	7.92	5.48	0.999	0.338	7.22	0.305	8.37	17.1	0.014	0.668
IPI-145	PI3Kd/g	7.37	0.48	5.25	16.5	63.9	27.8	3.88	0.0992	26.9	7.88	2.56	11.4	9.63	1.12	0.364	6.39	0.442	8.71	12.4	0.069	0.249
(+)-JQ1	BET bromodomain	6.92	4.74	3.56	11.4	32	12.5	7.02	1.46	7.82	6.82	13.8	5.43	19	5.65	0.196	7.55	0.487	6.02	5.77	1.97	10.9
Selinexor	XPO1	4.79	2.43	3.34	13.1	24.3	3.53	0.54	1.84	12.2	3.81	14	9.74	23	2.5	1.03	8.57	1.63	8.51	10.6	0.883	12.9
Aminopyrimidine-1	IRAK4	12.1	0.752	3.86	5.64	29.4	17.4	3.3	2.95	18.5	9.86	6.11	11	14.7	6.54	1.01	7.82	6.18	5.57	4.89	1.78	2.17

Synergy score values: white, < 10.6; pink, 10.6-19; red, > 19. Synergy scores shown with no concentration gating.

BCL-2 inhibitors

- Combination activity was observed with ibrutinib and the BCL-2 inhibitor ABT-199 below clinically achievable concentrations[12] in select DLBCL cell lines (**Figures 3** and **4**).
- Strong synergy scores of 40.0 and 21.6 were calculated for the ibrutinib/ABT-199 combination in DLBCL cell lines HBL-1 and TMD8, respectively.
- Strong synergy was also observed in FL line DOHH2 and MCL line REC-1, and medium synergy was observed in several cell lines, including AML lines KG-1, KASUMI-1, and MOLM-13, and the BL line Ramos (RA #1), indicating broad synergy across several histologies (**Table 1**).

Figure 3. Synergy Scores for DLBCL Cell Lines by Ibrutinib Drug Combination



Ibrutinib concentration gating at 200 nM. Scores in the range 9.59-19.2 indicate moderate synergy, and scores > 19.2 indicate strong synergy.

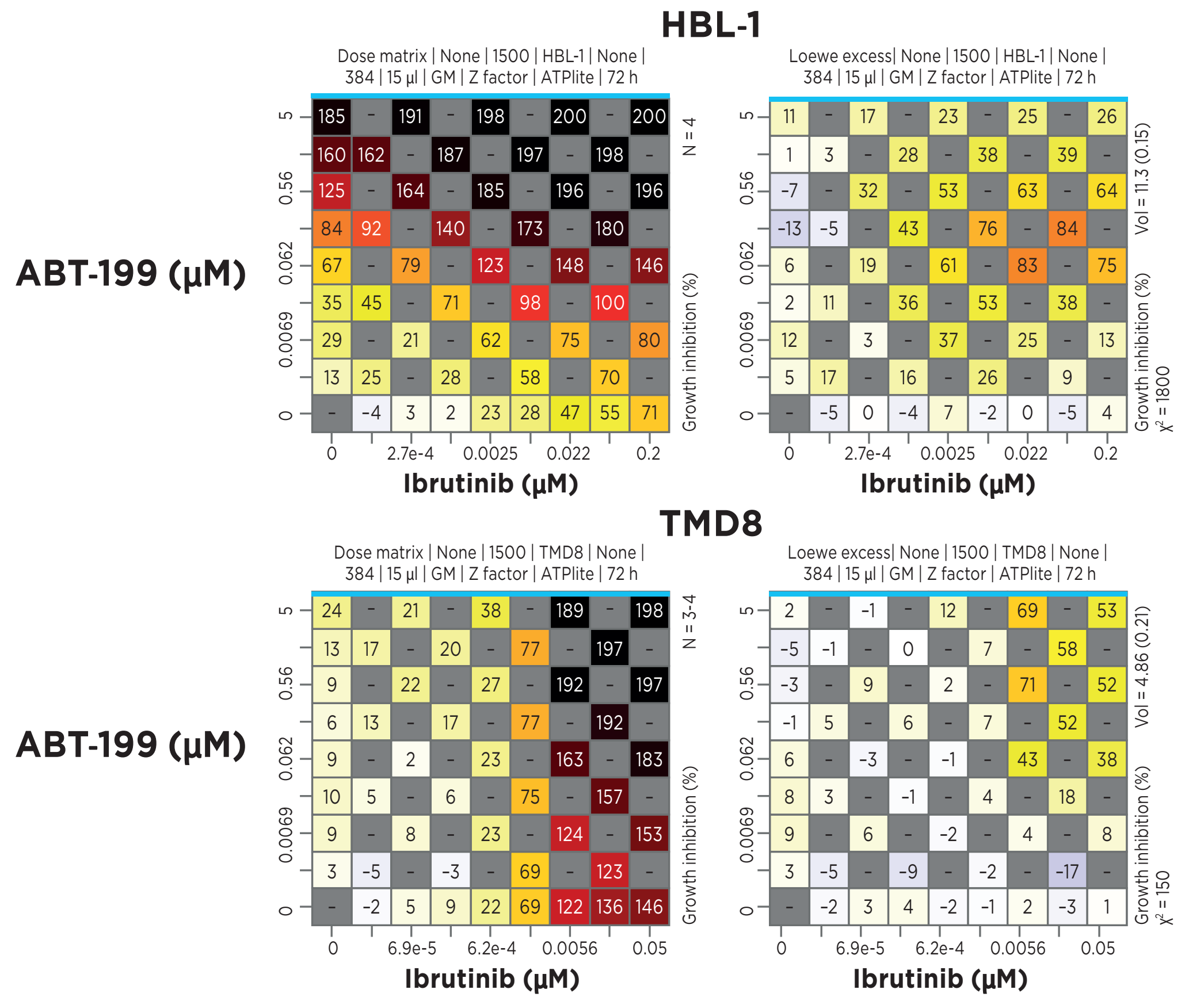
PI3K pathway inhibitors

- Synergistic combination activity was observed with ibrutinib and PI3K inhibitors (of varying isoform specificity) in select DLBCL cell lines at concentrations below levels that have been reported to be achievable in vivo (GDC-0941 [PI3Ka/d] ~ 0.3 μM; IPI-145 [PI3Kd/g] ~ 2 μM)[13,14] (**Figures 3** and **5**).
- Ibrutinib combination with the PI3K inhibitors IPI-145 and GDC-0941 resulted in strong synergy scores of 63.9 and 23.9, respectively, for the DLBCL cell line TMD8.

Other molecular synergies

- Strong/moderate synergistic activity was observed in select DLBCL cell lines with ibrutinib in combination with the potent BET bromodomain inhibitor (+)-JQ1 and the XPO1 inhibitor selinexor (selective inhibitor of nuclear export) at concentrations below those known to be achievable in vivo,[15,16] and with the IRAK4 inhibitor aminopyrimidine-1 at ≤ 1 μM (0.042 μM in TMD8 cell line) (**Figure 3**).
- There was no observed antagonism with any of the ibrutinib combinations screened.

Figure 4. Dose Matrices (9 × 9) for the Ibrutinib/BCL-2 Inhibitor (ABT-199) Combination in DLBCL Cell Lines HBL-1 and TMD8

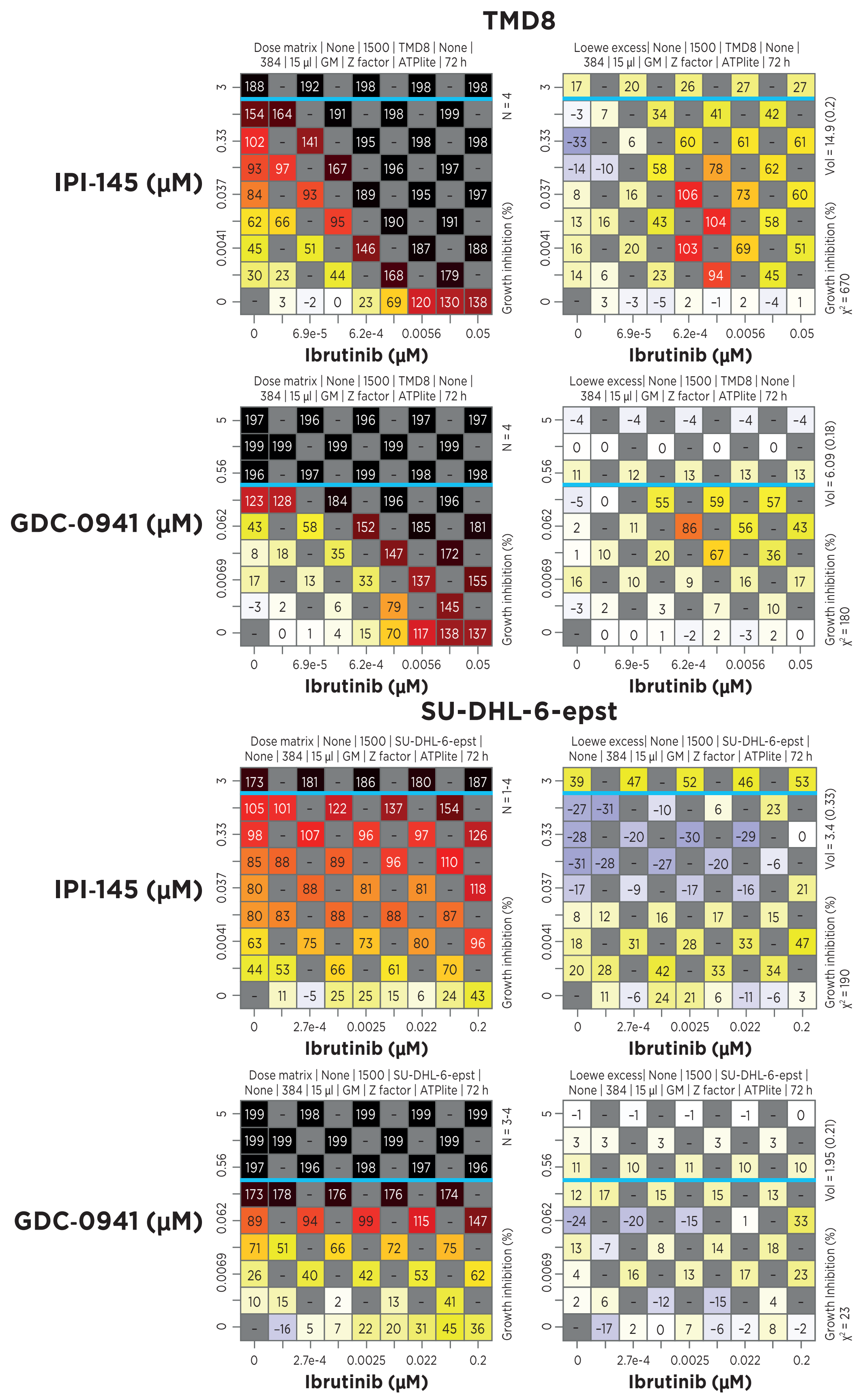


Dose-matrix format allows identification of combination activity at multiple ratios across a wide concentration range. Each point from observed combination data (left matrix) is compared with single-agent responses (Loewe dose-additivity model) to calculate activity in excess of the dose-additivity model (right matrix). Blue line denotes drug levels below clinically achievable concentrations.

CONCLUSIONS

- Ibrutinib shows cell-line–specific combination activity with several distinct classes of compound that target the BCR signaling pathway at clinically achievable concentrations, providing good rationale for future clinical testing.
  - In specific DLBCL cell lines, maximum activity was shown when ibrutinib was combined with BCL-2 and PI3K inhibitors.
  - Strong synergy was also observed with inhibitors of XPO1, BET bromodomain, and IRAK4 in DLBCL.
  - Ibrutinib was also synergistic with other agents, with no observed antagonism, suggesting that further study in specific histologies is warranted.
- Broad synergy across several histologies (DLBCL, FL, MCL, AML) was observed with ibrutinib and the BCL-2 inhibitor ABT-199.

Figure 5. Dose Matrices (9 × 9) for Ibrutinib/PI3K Inhibitor Combinations (IPI-145 and GDC-0941) in the DLBCL Cell Lines TMD8 and SU-DHL-6-epst



Blue line denotes concentrations below levels that have been reported to be achievable in vivo.

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ACKNOWLEDGMENTS

This study was funded by Janssen Research & Development, LLC. Writing assistance was provided by Michelle Olsher, PhD, of PAREXEL, and was funded by Janssen Global Services, LLC.

Poster presented at the American Association for Cancer Research (AACR) Annual Meeting, April 16-20, 2016; New Orleans, LA, USA.

Sponsored by Janssen Research & Development, LLC.