

Richard J Rickles¹, Junji Matsui³, Ping Zhu¹, Yasuhiro Funahashi^{2,3}, Jill M Grenier¹, Janine Steiger¹, Nanding Zhao², Bruce A Littlefield², Kenichi Nomoto^{2,3} and Toshimitsu Uenaka^{2*}

¹Horizon Discovery Inc., MA, Japan

²Eisai Inc., MA, Japan

³Eisai Co., Ltd., Japan

Dates: Received: 17 October, 2015; Accepted: 03 November, 2015; Published: 04 November, 2015

*Corresponding author: Toshimitsu Uenaka, Ph.D., Executive Director, Production Creation Headquarter, Oncology & Antibody Drug Strategy, CINO (Chief Innovation Officer), E-mail: t-uenaka@hhc.eisai.co.jp

www.peertechz.com

Research Article

Identification of Combinatorial Drugs that Synergistically Kill both Eribulin-Sensitive and Eribulin-Insensitive Tumor Cells

Abstract

Eribulin sensitivity was examined in a panel of twenty-five human cancer cell lines representing a variety of tumor types, with a preponderance of breast and lung cancer cell lines. As expected, the cell lines vary in sensitivity to eribulin at clinically relevant concentrations. To identify combination drugs capable of increasing anticancer effects in patients already responsive to eribulin, as well as inducing *de novo* anticancer effects in non-responders, we performed a combinatorial high throughput screen to identify drugs that combine with eribulin to selectively kill tumor cells. Among other observations, we found that inhibitors of ErbB1/ErbB2 (lapatinib, BIBW-2992, erlotinib), MEK (E6201, trametinib), PI3K (BKM-120), mTOR (AZD 8055, everolimus), PI3K/mTOR (BEZ 235), and a BCL2 family antagonist (ABT-263) show combinatorial activity with eribulin. In addition, antagonistic pairings with other agents, such as a topoisomerase I inhibitor (topotecan hydrochloride), an HSP-90 inhibitor (17-DMAG), and gemcitabine and cytarabine, were identified. In summary, the preclinical studies described here have identified several combination drugs that have the potential to either augment or antagonize eribulin's anticancer activity. Further elucidation of the mechanisms responsible for such interactions may be important for identifying valuable therapeutic partners for eribulin.

Introduction

Eribulin mesylate, a microtubule dynamics inhibitor with a mechanism distinct from most other anti-tubulin therapeutics, is approved in the United States and many other countries for treatment of certain patients with advanced or metastatic breast cancer [1,2]. Eribulin works by binding to exposed beta-tubulin subunits at the plus ends of microtubules, where it acts through end-poisoning mechanisms to inhibit microtubule growth and sequester tubulin into non-functional aggregates. In mitosis, this results in G2/M cell cycle arrest, irreversible mitotic blockade, and ultimately cell death by apoptosis [3].

Eribulin has broad anticancer activity in a wide variety of preclinical cancer models; as a result, numerous clinical trials of its effectiveness under monotherapy conditions are ongoing in other non-breast tumor types [4-9]. Although eribulin failed to show meaningful activities in clinical trials of head and neck, pancreatic and advanced non-small cell lung cancer [10,11], improvements in overall survival by eribulin were reported in a Phase 3 trial in advanced soft tissue sarcoma compared with dacarbazine [12], pointing to additional clinical uses for eribulin. Clinical trials are ongoing to evaluate eribulin effectiveness for treatment of osteosarcoma, ovarian, cervical, urothelium, brain metastasis, metastatic salivary gland and pediatric cancers with the promise that additional cancer indications will be identified.

In certain ways, the clinical experience with eribulin has been similar to that of other chemotherapies: monotherapy benefits tend to be limited and it is often difficult to surpass the benefits of standard

of care. Chemotherapeutic drugs are often most effective when given in combination, in particular when synergistic killing is achieved without additive toxicity. To date, potential combination therapies for eribulin have been explored with only a limited number of anti-cancer agents. We therefore have performed an *in vitro* study of eribulin combined with 34 anticancer agents in 25 different tumor cell lines of various types, through use of a combination high throughput screening (cHTS) platform. Our goals were to identify drugs that might be paired with eribulin to increase clinical efficacy in metastatic breast cancer, to identify drugs that convert eribulin non-responders to responders, and to identify new therapeutic indications for eribulin utilization. Several compelling synergy effects with approved and emerging drugs were observed. The preclinical data provides insights about future clinical development strategies.

Materials and Methods

Materials

Cell lines were purchased from European Collection of Cell Cultures of Public Health England (A2780), Japanese Collection of Research Bioresources Cell Bank of the National Institute of Biomedical Innovation (SNG-M), German Collection of Microorganisms and Cell Cultures (KYSE-410), and American Type Culture Collection (all other cell lines). All cell lines are included in the Cancer Cell Line Encyclopedia [13]. Cell culture media, fetal bovine serum, and cell culture supplements were purchased from Gibco Life Technologies (Thermo Scientific). Chemical matter was purchased from Enzo Life Sciences, Inc., Micro Source Discovery Systems, Sigma-Aldrich Co. LLC, Selleck Chemicals, Sequoia Research Products Limited, and

Toronto Research Chemicals. Cell proliferation was measured by ATP Lite 1step Luminescence Assay System (Perkin Elmer).

Methods

Cells were thawed and grown in culture media according to vendor's recommendations. Detailed methods on the combination assay were reported in [14]. Combinations were analyzed using Synergy Score and Loewe Volume Score, as described in [15,16] using Horizon's proprietary Chalice™ Analyzer software.

Combination High-Throughput Screening

Cells were seeded in 384-well and 1536-well tissue culture treated assay plates at cell densities ranging from 100 to 500 cells per well. Twenty-four hours after cell seeding, compounds were added to assay plates with multiple replicates. Compounds were added to cells using a 6x6 dose matrix, formed by six treatment points (including DMSO control) of Eribulin and each combination partner as single agents and twenty-five combination points. Please see reference 12 for additional detail. Concentration sampling ranges were selected after review of single agent activity of each molecule across the cell line panel. Generally, concentrations between the EC10 and EC90 were sampled.

At the time of treatment, a set of assay plates (which do not receive treatment) were collected and ATP levels were measured by adding ATPLite. These Vehicle-zero (V_0) plates were measured using an EnVision Multilabel Reader (Perkin Elmer). Treated assay plates were incubated with compound for seventy-two hours. After seventy-two hours, treated assay plates were developed for endpoint analysis using ATPLite. All data points were collected via automated processes; quality controlled; and analyzed using Horizon's proprietary software.

Horizon utilizes Growth Inhibition (GI) as a measure of cell viability. The cell viability of vehicle is measured at the time of dosing (V_0) and after seventy-two hours (V). A GI reading of 0% represents no growth inhibition - cells treated with compound (T) and V vehicle signals are matched. A GI 100% represents complete growth inhibition - cells treated by compound and V_0 vehicle signals are matched. Cell numbers have not increased during the treatment period in wells with GI 100% and may suggest a cytostatic effect for compounds reaching a plateau at this effect level. A GI 200% represents complete death of all cells in the culture well. Compounds reaching an activity plateau of GI 200% are considered cytotoxic. Horizon calculates GI by applying the following test and equation:

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

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

Where T is the signal measure for a test article, V is the vehicle-treated control measure after seventy-two hours, and V_0 is the vehicle control measure at time zero.

Analysis of combination screening results

To measure combination effects in excess of Loewe additivity, Horizon has devised a scalar measure to characterize the strength of synergistic interaction termed the Synergy Score [13,14]. The Synergy

Score equation integrates the experimentally-observed activity volume at each point in the matrix in excess of a model surface numerically derived from the activity of the component agents using the Loewe model for additivity. Additional terms in the Synergy Score equation are used to normalize for various dilution factors used for individual agents and to allow for comparison of synergy scores across an entire experiment.

Loewe Volume is an additional combination model score used to assess the overall magnitude of the combination interaction in excess of the Loewe additivity model. Loewe Volume is particularly useful when distinguishing synergistic increases in a phenotypic activity (positive Loewe Volume) versus synergistic antagonisms (negative Loewe Volume). Please see reference 13 for more detail.

Self-cross based combination screening analysis

In order to objectively establish hit criteria for the combination screen analysis, twelve compounds were selected to be self-crossed across the twenty-five cell line panel as a means to empirically determine a baseline additive, non-synergistic response. The identity of the twelve self-cross compounds was determined by selecting compounds with a variety of maximum response values and single agent dose response steepness. Those drug combinations which yielded effect levels that statistically superseded those baseline additivity values were considered synergistic. The Synergy Score measure was used for the self-cross analysis. Synergy Scores of self-crosses are expected to be additive by definition and, therefore, maintain a synergy score of zero. However, while some self-cross Synergy Scores are near zero, many are greater suggesting that experimental noise or non-optimal curve fitting of the single agent dose responses are contributing to the slight perturbations in the score. Given the potential differences in cell line sensitivity to the eribulin combination activities, we chose to use a cell-line centric strategy for the self-cross based combination screen analysis, focusing on self-cross behavior in individual cell lines versus global review of the cell line panel activity. Combinations where the Synergy Score is greater than the mean self-cross plus two standard deviations (2σ 's) or three standard deviations (3σ 's) can be considered candidate synergies at the 95% and 99% confidence levels, respectively.

Results

Evaluation of eribulin potency using a cell-based assay

Eribulin's antiproliferative activity was assessed in a panel of twenty-five human cancer cells lines representing a variety of tumor types using a three-fold, ten-point dose titration of drug. Cells were incubated with drug for 72 hours. Cellular ATP levels were quantified as a measurement of effects on proliferation. A measurement was performed at the time of drug addition (time zero, T0) in order to calculate a Growth Inhibition percentage, providing information about cytostatic and cytotoxic activities. Cell lines were designated as sensitive to eribulin if the GI50 values were <1 nM, a concentration deemed achievable in a clinical setting [17]. Based on the 1 nM cutoff for drug sensitivity, 28% of the cell lines were (7/25) were deemed to be eribulin insensitive (Figure 1). Both sensitive and insensitive breast and lung cancer cell lines were identified. All cell lines shown

in **Figure 1** were included in the subsequent combination screen, with the following rationale: inclusion of eribulin sensitive cell lines would provide models to identify drugs with the potential to increase eribulin efficacy, while inclusion of insensitive lines would facilitate identification of drugs capable of converting eribulin insensitive cells to eribulin sensitive cells.

Receptor tyrosine kinase target-based cluster analysis

The thirty-five compound enhancer library (**Supplemental Table 1**) was screened in combination with eribulin in the twenty-five cell lines described above in **Figure 1**. In order to objectively establish hit criteria for the combination screen analysis, twelve compounds were selected to be self-crossed across the twenty-five cell line panel as a means to empirically determine a baseline additive, non-synergistic response. The identity of the twelve self-cross compounds was determined by selecting compounds with a variety of maximum response values and single agent dose response steepness. Those drug combinations which yielded effect levels that statistically superseded those baseline additivity values were considered synergistic.

A summary matrix view heat map of combination drug Synergy Scores which exceed the cell line specific self-cross thresholds at 2σ 's above the mean is shown in **Supplemental Figure 1**. Using this strategy, 19.7% of the combinations exhibited some synergistic activity at the 95% confidence level. Using a more stringent criteria of 3σ cutoff (99% confidence), synergy was observed for 12.5% of the combinations (**Supplemental Figure 2**).

The 35 compound enhancer library utilized contains some redundancy in terms of targets and pathway inhibitors. Whenever possible, target information was used to cluster similarly annotated enhancers across the cell line panel, and the Loewe Excess values for Growth Inhibition matrices with high-to-moderate synergy scores were individually reviewed to evaluate combination activities.

The strategy of using target or pathway cluster profiles with visual inspection of matrices provides additional evidence linking a particular target with combination activity, and helps to highlight subtle synergies that might otherwise be overlooked or insufficiently revealed due to steep single agent dose response curves. A Synergy Score heat map for select target/pathway clusters is displayed in **Figure 2**, with cell lines clustered first according to eribulin sensitivity/insensitivity then further demarcated based on tumor type.

As an example of enhancer library mechanistic redundancy, the combination screen contained three drugs known to inhibit ErbB1/ErbB2 (EGFR/HER2) in cell-based assays: lapatinib, BIBW-2992 and erlotinib [18-21]. BIBW-2992 exhibited the best breadth of combination activity. The mechanism of action of BIBW-2992 (irreversible inhibition) and higher potency for ErbB1 and ErbB2 may explain the greater breath-of-activity. Alternatively, synergies observed with BIBW-2992 but not lapatinib or erlotinib may be the result of unique polypharmacy with BIBW-2992 inhibiting secondary targets not affected by the other ErbB1/ErbB2 inhibitors. Representative Growth Inhibition and Loewe Excess dose matrices for some of the lapatinib combinations are shown in **Figure 3** with examples of erlotinib and BIBW-2992 combination activities shown in **Supplemental Figures 3, 4**. Low micromolar concentrations of lapatinib and erlotinib have been observed in pharmacokinetic studies [18,19] suggesting the potential for reproducing the observed preclinical synergy here in a clinical setting.

PI3K pathway inhibitors

Dysregulation of the PI3K pathway can transform cells by virtue of constitutive activation and ultimately, stimulation of cellular proliferation and suppression of pro-apoptotic signaling. Four PI3K pathway inhibitors were included in the screen. AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mTOR kinase inhibitor (TORC1 and TORC2); everolimus, an allosteric mTOR (TORC1) inhibitor; BEZ235, a dual pan-PI3K/mTOR inhibitor; and

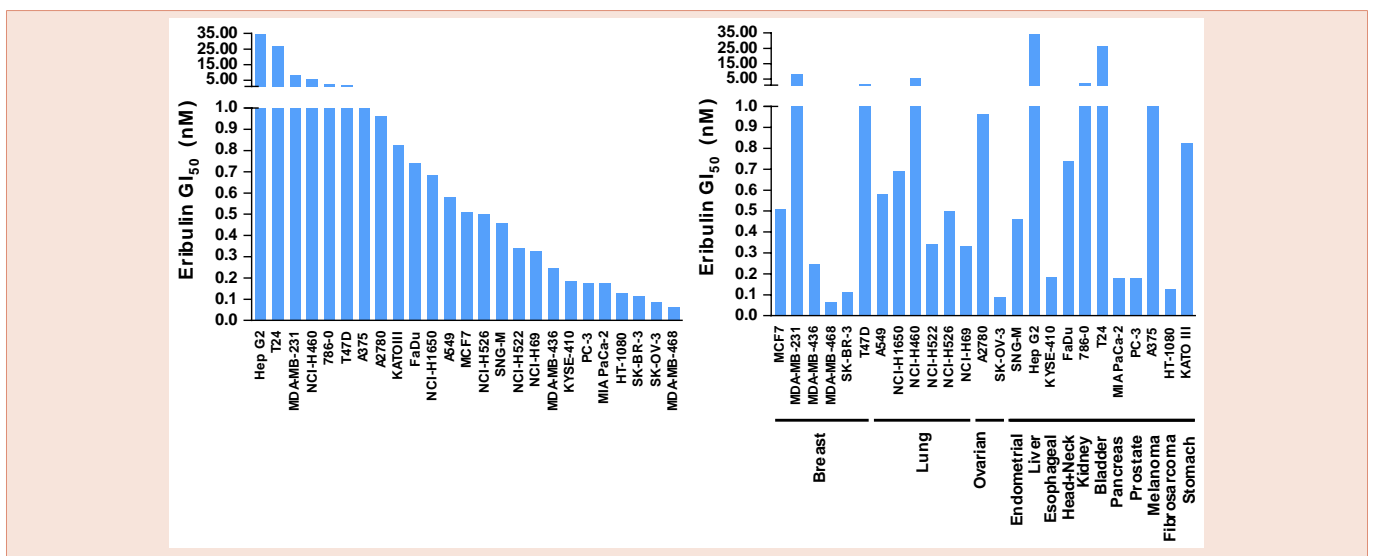


Figure 1: Assessment of eribulin activity in twenty-five cell lines. Bar graph display of GI50 values based on relative sensitivity (Waterfall plot, left) or after clustering cell lines based on tumor type (right). Cells were exposed to eribulin for 72 hours. The assay endpoint was measurement of ATP (as a surrogate for effects on proliferation). The median GI50 for the twenty-five cell line panel is 0.51 nM.



Target	Eribulin +	Eribulin-Sensitive Cell Lines															Eribulin-Insensitive Cell Lines												
		MCF7	MDA-MB-435	MDA-MB-468	SK-BR-3	LS459	NCI-H460	NCI-H222	NCI-H526	NCI-H469	A2780	SNG-M	KYSE-410	FaDu	Mia PaCa-2	PC-3	HepG2	ATCIII	MDA-MB-231	HepG2	786-0	T24	A375						
ErbB1/ErbB2	BIBW-2992	1.31	1.80	9.00	6.21	6.78	6.74	6.28	3.02	5.01	18.61	7.59	9.83	16.05	7.77	4.38	7.25	23.98	1.49	2.10	1.18	4.36	0.96	2.99	7.77	5.48	7.34	6.49	6.74
	Erlotinib	3.14	3.24	12.15	1.26	2.96	3.25	4.90	2.49	3.91	5.50	3.94	4.69	8.69	9.15	26.61	0.63	0.47	1.36	3.25	0.76	3.11	9.85	7.68	17.46	14.14	11.77		
	Lapatinib	7.86	1.77	9.33	2.35	1.65	2.20	0.43	1.89	4.55	6.87	4.80	8.69	9.15	26.61	0.63	0.47	1.36	3.25	0.76	3.11	9.85	7.68	17.46	14.14	11.77			
PI3K Pathway	AZD 8055	11.84	5.04	8.60	1.13	7.71	6.53	1.39	2.96	8.19	17.12	7.62	5.44	9.68	9.38	1.53	8.79	10.47	6.44	4.14	7.65	10.15	1.70	2.85	4.48	5.37			
	BEZ235	8.03	5.32	16.92	1.32	6.78	5.92	2.86	2.34	7.90	10.80	4.43	12.44	9.16	18.70	2.36	8.18	9.60	4.34	7.28	13.32	12.30	10.10	4.16	6.75	8.36			
	BKM-120	6.12	2.87	6.05	1.13	5.38	2.82	1.20	3.71	5.47	10.76	3.27	7.03	4.86	7.72	0.75	3.02	8.11	2.79	2.10	7.17	5.99	3.22	2.49	2.34	1.45			
MEK	Trametinib	1.32	3.98	10.18	0.50	5.63	3.66	0.26	0.87	2.09	14.55	4.80	0.71	4.10	4.76	3.87	0.88	0.72	1.69	14.92	1.58	9.31	10.41	3.65	2.90	1.10			
	E6201	0.71	1.72	2.19	0.00	7.06	4.05	1.02	1.61	2.42	19.08	0.36	1.19	3.32	7.22	12.64	0.23	1.26	1.93	11.75	2.35	6.26	18.64	2.78	2.01	1.22			

Figure 2: Synergy Score heat map of selected compounds screened in combination with eribulin and clustered based on target or pathway specificity. Twenty five cell lines were screened, shown as vertical columns labeled across the top and grouped first based on eribulin sensitivity/insensitivity then further clustered based on tumor type (breast cancer, blue; lung cancer, orange; ovarian cancer, green; single representative cancers, not highlighted; see Figure 1 for information on other cancer types). Each row lists one compound screened in combination with eribulin, with compounds clustered based on target or pathway specificity. Synergy Scores are shown, with higher scores highlighted in increasingly darker shades of red. For this analysis, a Synergy Score cut-off of 4.36 was chosen based on visual inspection of dose matrices and Loewe Excess matrices above and below this cut-off score.

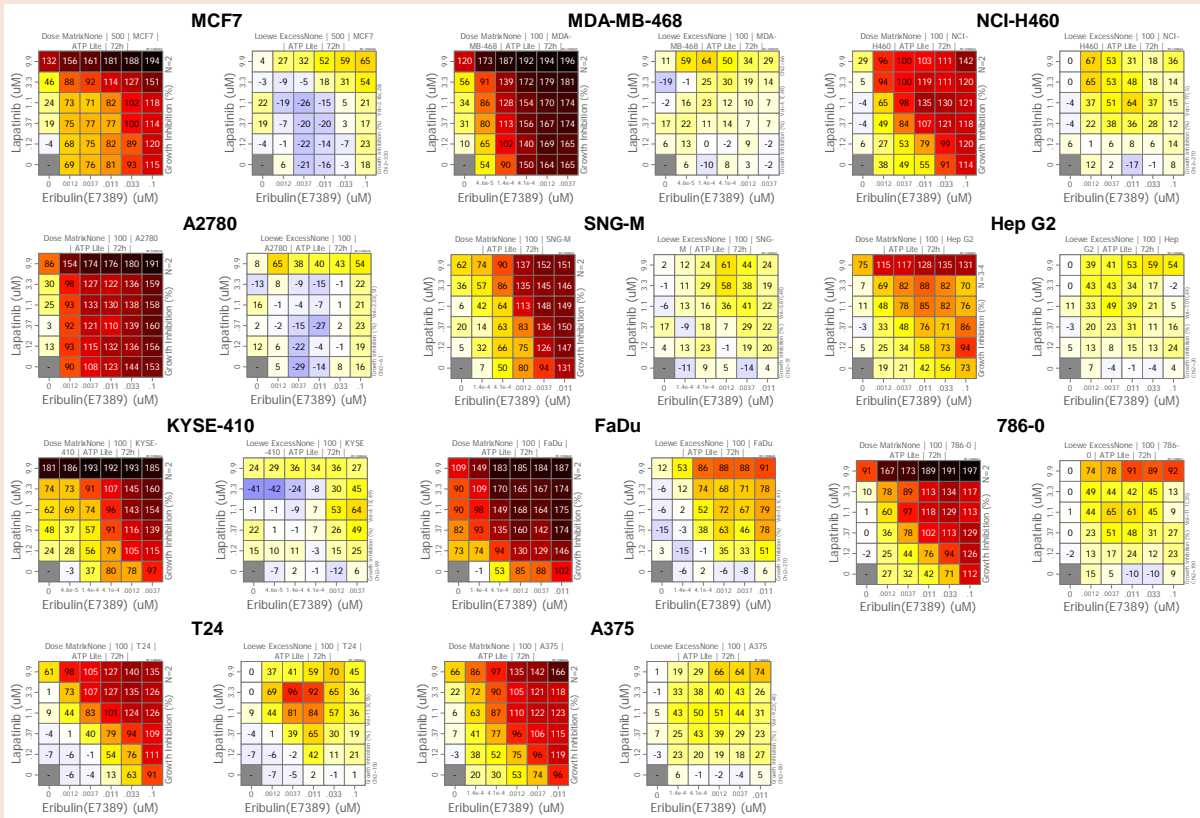


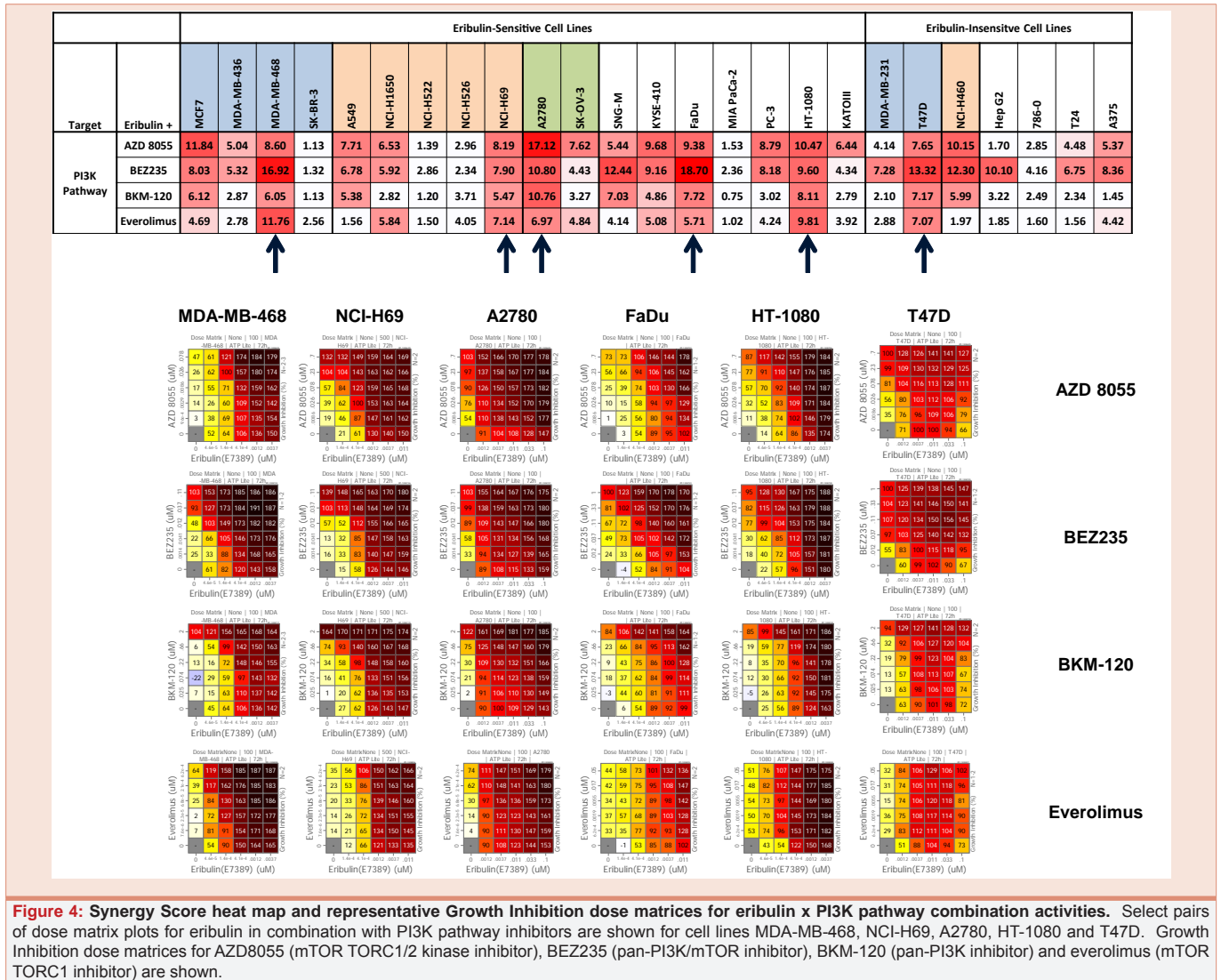
Figure 3: Representative dose matrices for eribulin x lapatinib combination activity. Selected dose matrix pairs for eribulin in combination with lapatinib are shown in cell lines MCF7, MDA-MB-468, NCI-H460, A2780, SNG-M, Hep G2, KYSE-410, FaDu, 786-0, T24 and A375. For each cell line, Growth Inhibition values (left matrix) and Loewe Excess values (right matrix) are shown. The Growth Inhibition dose matrix values are determined by measurement of ATP levels with a T0 measurement (at time of drug addition) performed at the whole well level in order to distinguish cytostasis from cytotoxicity. Values highlighted in medium red are those approximating GI100% and are indicative of compounds/combinations being cytostatic; values with highlighting closest to black are those approximating GI200% and indicate complete killing (cytotoxic effect).

BKM-120, a pan-PI3K inhibitor. As shown in Figures 2, 4, one or more of these inhibitors are synergistic in combination with eribulin in the majority of cell lines in the panel. For a subset of cell lines, synergy is observed with all four PI3K pathway inhibitors. The breadth of activity observed in the cell line panel and for the various PI3K pathway inhibitors highlight the importance of PI3K signaling for tumor cell proliferation and/or survival upon eribulin exposure

(Figure 4). Combination activity is selective (for example, little or no combination activity in SK-BR-3, NCI-H552, NCI-H526, Mia PaCa-2, and 786-0) pointing to specific genetic determinants in responsive cell lines as being important for combination activity.

MEK inhibitors

Two MEK inhibitors (E6201 and trametinib) were screened in



combination with eribulin (Figure 2); selected Growth Inhibition and Loewe Excess dose matrices are shown in Figure 5. There is good concordance for both inhibitors; when synergy is observed with one of the MEK inhibitors, it is most often observed with the other. MEK combination activity was strongest in MDA-MB-231, A2780 and Hep G2. For some cell lines, synergy is observed when eribulin is combined with either PI3K pathway inhibitors or MEK inhibitors (for example, FaDu, NCI-H460 and A2780). For other cell lines, (MCF7, NCI-H69, KYSE-410, HT-1080 and T47D), synergies are PI3K pathway-specific, with no synergies seen for MEK inhibitors.

BCL-2 Inhibition

ABT-263 (Navitoclax) is a potent Bcl-xL, Bcl-2, and Bcl-w inhibitor currently in multiple clinical trials as a monotherapy or in combination with approved drugs. Strong synergies with eribulin and good breadth of activity was observed with ABT-263, in both eribulin-sensitive and insensitive cell lines (Figure 6). Strong synergy was observed in most but not all of the cell lines, suggesting that an

eribulin x ABT-263 combination is not non-selectively synergistically toxic.

Antagonistic drug pairings

As described in Materials and Methods, Loewe Volume values can be used to gauge both synergies and antagonisms. A Loewe Volume heat map is shown in Figure 7 for 17-DMAG, cytarabine, topotecan, and gemcitabine, with greater negative values (antagonism) highlighted in increasingly darker shades of blue and increasing positive values (synergies) highlighted in increasingly darker shades of red. Also shown are representative combination dose matrices for selected antagonistic activities observed. The 17-DMAG Loewe Volume combination activity pattern is complex: for 6 cell lines (red shaded), combinations with eribulin are synergistic, while for 4 cell lines, antagonism was observed.

Discussion

Eribulin has broad anticancer activity in a wide variety of

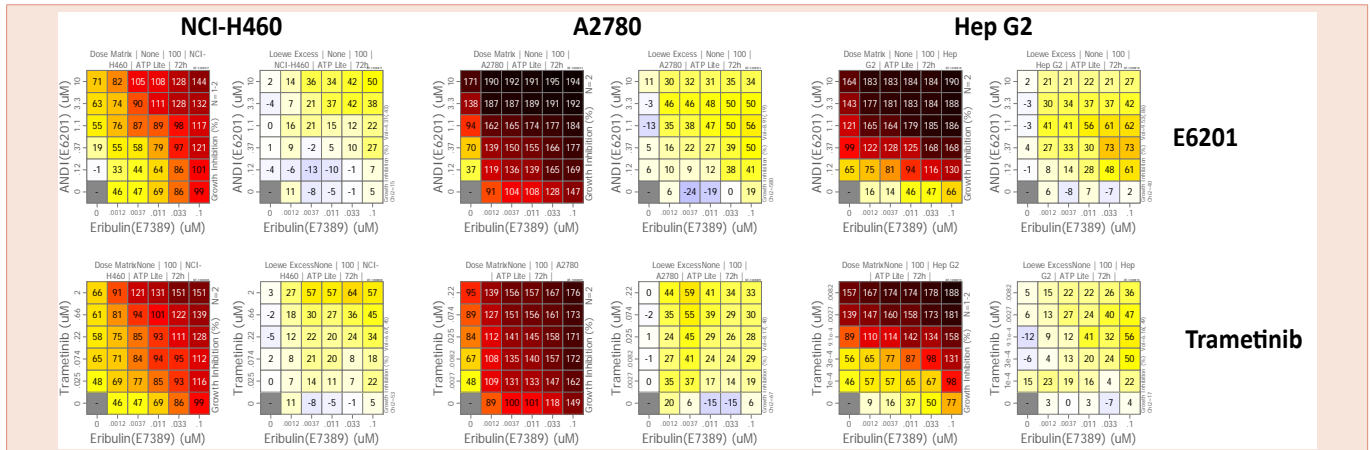


Figure 5: Eribulin x MEK inhibitor combination activities. Select dose matrices (Growth Inhibition and Loewe Excess) for eribulin in combination with the MEK inhibitors E6201 and trametinib are shown for cell lines NCI-H460, A2780 and Hep G2. Breadth of combination activity is shown in Figure 2 (Synergy Score heat map).

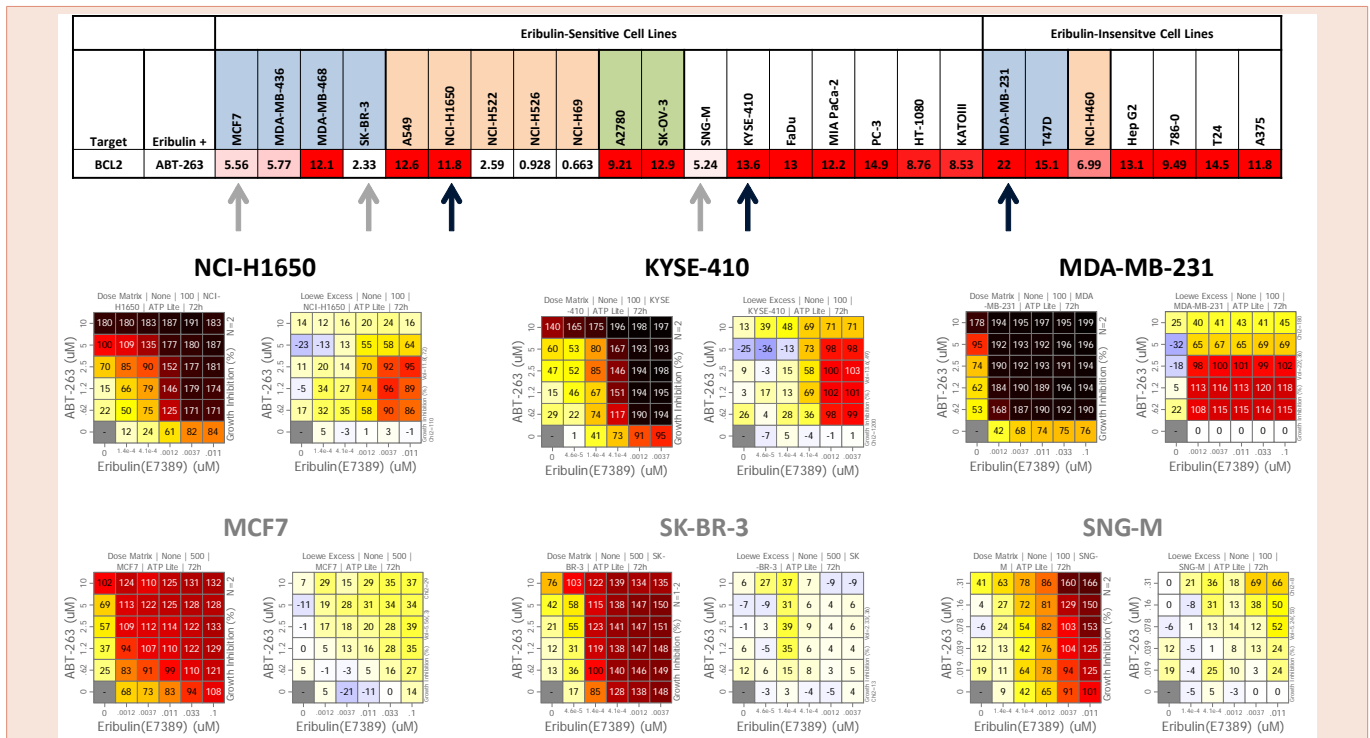
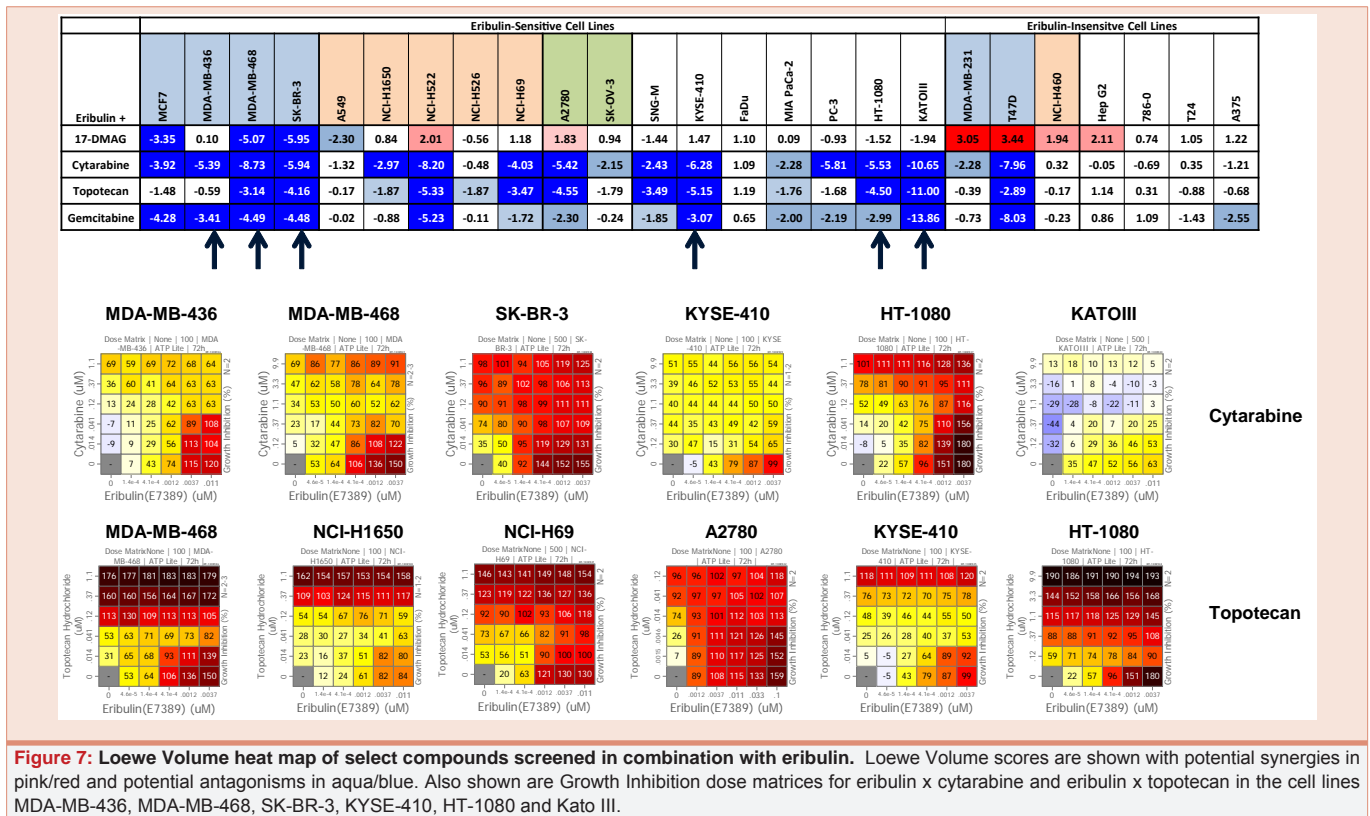


Figure 6: Synergy Score heat map and representative Growth Inhibition dose matrices for eribulin x ABT-263. Select pairs of dose matrix plots for eribulin in combination with the BCL-2 family inhibitor ABT-263 are shown for cell lines NCI-H1650, KYSE-410, MDA-MB-231, MCF7, SK-BR-3 and SNG-M. Three strong synergies are highlighted (black arrows, top row of matrices) as well as cell lines with weak combination effects (grey arrows, bottom row of matrices).

preclinical cancer models [4,7] and numerous monotherapy clinical trials are ongoing to investigate efficacy in non-breast tumor types (see www.clinicaltrials.gov). Consistent with utility for additional potential indications, a variety of tumor cell lines including lung, ovarian, endometrial, head and neck, prostate and melanoma are shown here to be sensitive to eribulin at clinically relevant concentrations. Since tumor cells have a robust capacity to lessen the therapeutic effects of cancer drugs through adaptive and acquired resistance mechanisms, the identification of drugs that can

paired with eribulin to trigger selective and synergistic tumor cell death represents a critical path toward optimizing patient benefit. Specifically, drug combinations need to be identified for situations in which the effects of eribulin monotherapy are suboptimal, or in which intrinsic or acquired drug resistance might be overcome with strategically selected drug combinations.

Not only eribulin, but also all anticancer drugs need to be looked for appropriate combination partners. A molecular targeted agent,



vemurafenib (BRAF inhibitor), demonstrated 48% anti-tumor response and extended PFS to 5.3 months in V600E melanoma patients in the BRIM3 study [21], in a first-line BRAF mutated melanoma therapy. However, the treatment of vemurafenib caused resistance in some patients through its 2-18 months post treatment [22]. Recently, four articles reported five resistance mechanisms for vemurafenib [23-27]. Those authors attributed their resistance mechanisms to the overexpression of PDGFR-β tyrosine kinase [23], and/or Q61K NRAS [24], COT kinase [25], IGF-1R [26], C121S MEK [27], in melanoma cells. Selective inhibitors against those molecules may be appropriate combination partners to prohibit these resistant mechanisms in V600E melanoma patients. Comprehensive combination analysis by using comprehensive compounds on several cancer cells harboring different molecular mutation are very useful to find out appropriate combination partners.

In this study, we describe the identification of approved and emerging drugs that synergize when combined with eribulin, with good breadth of combination activity observed in the twenty five cell line panel used for screening. Synergy is observed in both eribulin-sensitive and insensitive cell lines, suggesting potential clinical benefit for both responders and non-responders of eribulin monotherapy. In addition to approved drugs that target EGFR/HER2 (erlotinib, lapatinib, BIBW2992/afatinib), mTOR (everolimus), and MEK (trametinib), we show that emerging drugs such as BEZ235 (pan-PI3K/mTOR), BKM-120 (PI3Kα), and ABT-263 (BCL-2 family inhibitor) strongly synergize with eribulin in certain cell lines. Furthermore, the use of both target-specific (EGFR/HER2, MEK) and pathway-specific (PI3K), mechanistically redundant compounds

in our studies validates the identified target specificities as being important for synergy. By evaluating a wide concentration range for these molecules, we can interrogate combination activities and evaluate target selectivity; in addition, where pharmacokinetic data are available, we can obtain initial insights as to whether such effects could occur at clinically relevant concentrations. Looking at the eribulin's concentrations which showed synergy effects combined with these approval drugs, we found that they seem to be relatively wide-range. Not only at the highest concentration but also at the lower concentrations, eribulin could synergize with several drugs.

One challenge with the evaluation of *in vitro* drug activities is that patterns of drug exposure may not match what can be achieved *in vivo*. Limited drug exposure (pulse dosing) could be used to compare *in vitro* results with exposure in a clinical setting. In addition, tumor bearing animals could be treated with both drugs to validate combination activities to examine combination drug toxicities. To this end, *in vivo* animal studies are currently in progress; to date, eribulin combination activities with erlotinib, everolimus, and BKM-120 have been reproduced in human tumor xenograft models in mice, with these combinations showing acceptable toxicity profiles (manuscript in preparation).

An important goal for any monotherapy or drug combination is the identification of predictors of response so that clinicians can select the patient population most likely to benefit from treatment. The screen described here represents the discovery phase of such a project and additional work is required to identify predictors of response with a certainty that can drive patient selection. For instance, the kinetics

of the appearance of combination activity should be explored, and the relationships, if any, of synergy/non-synergy to intrinsic or acquired resistance should be further defined. To power such analyses, additional cell lines could be screened to generate a larger data set for more detailed genetic characterization. Additional analyses using larger data sets with sufficient breadth of known genetic profiles will be required to generate a fully vetted understanding of response prediction.

In addition to identifying drug synergies, we have also identified antagonistic combinations. For example, low concentrations of cytarabine or topotecan have little effect on proliferation as single agents, but can dramatically antagonize eribulin activity under combination conditions. Indeed, this is not without precedent: antagonism has been observed for a variety of cancer drug combinations in preclinical studies, prompting follow up analyses to investigate both the mechanisms of the synergies as well as the effects of drug combination sequencing [28-35]. For instance, in one extensively studied example, the anthracycline doxorubicin antagonized activity of the vinca alkaloid vincristine in 83% (15/18) of hematopoietic cell lines tested. This antagonism was reproduced *in vivo* in a xenograft model and was also observed in 34% (12/35) of childhood leukemia cells simultaneously treated with both drugs *ex vivo* [35]. Moreover, combination activity was shown to be sequence dependent: if cells are exposed to the anthracycline first, antagonism is observed, but if exposure to vinca alkaloid precedes anthracycline, the combination result is beneficial. A p53-dependent mechanism for this antagonism was proposed, where anthracycline effects stabilize anti-apoptotic BCL2 family members, thus reducing cytotoxic effects of the vinca alkaloid. With respect to our current study, the eribulin antagonisms observed with 17-DMAG, cytarabine, topotecan and gemcitabine do not necessarily require that p53 be functional as was the case in the preceding example, but further study will be required to determine this. Indeed, the mechanisms by which these compounds antagonize eribulin may overlap or be entirely unrelated.

In conclusion, we have identified promising preclinical *in vitro* synergy combination partners with eribulin, with compound mechanistic redundancy helping to validate particular targets and pathways as important for selective, synergistic tumor cell killing. In order to utilize the current results with the goal of clinical implementation, further studies, including sequencing of drugs and both *in vivo* efficacy and toxicology studies will be needed. Our results suggest that further investigation is merited to assess whether additional patient benefit with eribulin, in some patient populations, may be achieved when eribulin is administered in the clinic as part of a combination drug regimen in patients in the context of controlled clinical trials.

References

- Donoghue M, Lemery SJ, Yuan W, He K, Sridhara R, et al. (2012) Eribulin Mesylate for the Treatment of Patients with Refractory Metastatic Breast Cancer: Use of a "Physician's Choice" Control Arm in a Randomized Approval Trial. *Clin Cancer Res* 18: 1496-505.
- Shetty N, Gupta S (2014) Eribulin drug review. *South Asian J Cancer* 3: 57-59.
- Smith JA, Wilson L, Azarenko O, Zhu X, Lewis BM, et al. (2010) Eribulin binds at microtubule ends to a single site on tubulin to suppress dynamic instability. *Biochemistry* 49:1331-1337.
- Towle MJ, Salvato KA, Budrow J, Wels BF, Kuznetsov G, et al. (2001) *In vitro* and *in vivo* anticancer activities of synthetic macrocyclic ketone analogs of Halichondrin B. *Cancer Res* 61: 1013-1021.
- Schöffski P, Ray-Coquard IL, Cioffi A, Bui NB, Bauer S, et al. (2011) Activity of eribulin mesylate in patients with soft-tissue sarcoma: a phase 2 study in four independent histological subtypes. *Lancet Oncol* 12: 1045-1052.
- Swami U, Chaudhary I, Ghalib MH, Goel S. (2012) Eribulin--A review of preclinical and clinical studies. *Crit Rev Oncol Hemat* 81:163-184.
- Towle MJ, Nomoto K, Asano M, Kishi Y, Yu MJ, et al. (2012) Broad Spectrum Preclinical Antitumor Activity of Eribulin (Halaven®): Optimal Effectiveness under Intermittent Dosing Conditions. *Anticancer Research* 32: 1611-1620.
- Funahashi Y, Okamoto K, Nomoto K, ODA Y, et al. (2014) Eribulin mesylate reduces tumor microenvironment abnormality by vascular remodeling in preclinical human breast cancer models. *Cancer Sci* 105:1334-1342.
- Polastro L, Aftimos PG, Awada A (2014) Eribulin mesylate in the management of metastatic breast cancer and other solid cancers: a drug review. *Expert Rev Anticancer Ther* 14: 649-665.
- Scarpace SL (2012) Eribulin mesylate (E7389): review of efficacy and tolerability in breast, pancreatic, head and neck, and non-small cell lung cancer. *Clin Ther* 34: 1467-1473.
- Waller CF, Vynnychenko I, Bondarenko I, Shparyk Y, Hodge JP, et al. (2015) An Open-Label, Multicenter, Randomized Phase Ib/II Study of Eribulin Mesylate Administered in Combination With Pemetrexed Versus Pemetrexed Alone as Second-Line Therapy in Patients With Advanced Nonsquamous Non-Small-Cell Lung Cancer. *Clin Lung Cancer* 16: 92-99.
- Schöffski P, Maki RG, Italiano A, Gelderblom H, Grignani G, et al. (2015) Randomized, open-label, multicenter, phase III study of eribulin versus dacarbazine in patients (pts) with leiomyosarcoma (LMS) and adipocytic sarcoma (ADI). *J Clin Oncol* 33: LBA10502.
- Barrentina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, et al. (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* 483: 603-607.
- Saiki AY, Caenepeel S, Yu D, Lofgren JA, Osgood T, et al. (2014) MDM2 antagonists synergize broadly and robustly with compounds targeting fundamental oncogenic signaling pathways. *Oncotarget* 5: 2030-2043.
- Lehar J, Zimmermann GR, Krueger AS, Molnar RA, Ledell JY, et al. (2007) Chemical combination effects predict connectivity in biological systems. *Mol Syst Biol* 3:80
- Lehar J, Krueger AS, Avery W, Heilbut AM, Johansen LM, et al. (2009) Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nat Biotechnol* 27: 659-666.
- Jimeno A (2009) Eribulin: Rediscovering tubulin as an anticancer agent. *Clin Cancer Res* 15: 3903-3905.
- Sharma SV, Bell DW, Settleman J, Haber DA (2007) Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* 7: 169-181.
- Lapatinib product insert:
- Schaefer G, Shao L, Totpal K, Akita RW (2007) Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. *Cancer Res* 67: 1228-1238.
- Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, et al. (2008) BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models *Oncogene* 27: 4702-4711.
- Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, et al. (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364: 2507-2516.



23. Smalley KS, Sondak VK. (2010) Melanoma--an unlikely poster child for personalized cancer therapy. *N Engl J Med* 363: 876-878.
24. Nazarian R, Shi H, Wang Q, Kong X, Koya RC, et al. (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 468: 973-977.
25. Johannessen CM, Boehm JS, Kim SY, Thomas SR, Wardwell L, et al. (2010) COT drives resistance to RAF inhibition through MAP kinase pathway reactivation. *Nature* 468: 968-972.
26. Villanueva J, Vultur A, Lee JT, Somasundaram R, Fukunaga-Kalabis M, et al. (2010) Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K. *Cancer Cell* 18: 683-695.
27. Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, et al. (2011) Dissecting Therapeutic Resistance to RAF Inhibition in Melanoma by Tumor Genomic Profiling. *J Clin Oncol* 29: 3085-3096.
28. Faivre S, Raymond E, Woynarowski JM, Cvitkovic E (1999) Supraadditive effect of 2',2'-difluorodeoxycytidine (gemcitabine) in combination with oxaliplatin in human cancer cell lines. *Cancer Chemotherapy and Pharmacology* 44: 117-123.
29. Distefano M, Ferlini C, De Vincenzo R, Gaggini C, Mancuso S, et al. (2000) Antagonistic effect of the combination gemcitabine/topotecan in ovarian cancer cells. *Oncol Res* 12: 355-359.
30. Akutsu M, Furukawa Y, Tsunoda S, Izumi T, Ohmine K, et al. (2002) Schedule-dependent synergism and antagonism between methotrexate and cytarabine against leukemia cell lines in vitro. *Leukemia* 16: 1808-1817.
31. Budman DR, Calabro A, Kreis W (2002) synergistic and antagonistic combinations of drugs in human prostate cancer cell lines in vitro. *Anticancer Drugs* 13: 1011-1016.
32. Li T, Ling YH, Goldman ID, Perez-Soler R (2007) Schedule-dependent cytotoxic synergism of pemetrexed and erlotinib in human non-small cell lung cancer cells. *Clin Cancer Res* 13: 3413-3422.
33. Barone C, Landriscina M, Quirino M, Basso M, Pozzo C, et al. (2007) Schedule-dependent activity of 5-fluorouracil and irinotecan combination in the treatment of human colorectal cancer: in vitro evidence and a phase I dose-escalating clinical trial. *Br J Cancer* 96: 21-28.
34. Ikeda H, Taira N, Nogami T, Shien K, Okada M, et al. (2011) Combination treatment with fulvestrant and various cytotoxic agents (doxorubicin, paclitaxel, docetaxel, vinorelbine, and 5-fluorouracil) has a synergistic effect in estrogen receptor-positive breast cancer. *Cancer Science* 102: 2038-2042.
35. Ehrhardt H, Schrems D, Moritz C, Wachter F, Haldar S, et al. (2011) Optimized anti-tumor effects of anthracyclines plus Vinca alkaloids using a novel, mechanism-based application schedule. *Blood* 118: 6123-6131.

Copyright: © 2015 Rickles RJ, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Rickles RJ, Matsui J, Zhu P, Funahashi Y, Grenier JM, et al. (2015) Identification of Combinatorial Drugs that Synergistically Kill both Eribulin-Sensitive and Eribulin-Insensitive Tumor Cells. *Glob J Cancer Ther* 1(1): 009-017.