

Brostallicin Context of Vulnerabilities Revealed Through HT-RNAi Based Cellular Pharmacogenomics

Holly Yin¹ Jeffrey A. Kiefer¹, Chris Sereduk¹, Pinar Tuzman¹, Kandavel Shanmugam², Shannon Shulby², Steven D. Weitman², Daniel D. Von Hoff¹, Spyro Mousses¹, Elizabeth M. Bruckheimer².

¹Translational Genomics Research Institute, Scottsdale, AZ, ²Systems Medicine LLC, Scottsdale, AZ

ABSTRACT

Brostallicin is a dastamycin-like, second-generation, minor groove DNA binding agent (MGB), currently in clinical development given its improved pharmacological and clinical safety profile and unique chemistry that requires glutathione for its activation. In order to identify molecular determinants of Brostallicin response that could further inform the clinical development and rational drug combination strategies, we undertook an integrated cellular pharmacogenomics study. In brief, a High Throughput (HT) RNAi screen utilizing the drugable genome, encompassing 7000 gene targets, and a supplemental set of approximately 100 genes obtained through knowledge mining of NC160 Brostallicin response data was performed to identify genetic determinants of Brostallicin response. This HT RNAi screen identified 380 unique sensitizing hits which resulted in increased Brostallicin sensitivity in the A2780 cisplatin resistant cell line model. Upon further evaluation, 59 out of the 380 hits were validated in the A2780 cisplatin resistant and A2780 parental cell lines. Additional knowledge mining was applied which resulted in the identification of two predominant cellular concepts for Brostallicin response, DNA repair and histone modification. Additional studies focused on these two biological pathways included confirmation of the hits across additional cell lines, characterization of the siRNA response, and identification and testing of rational drug combinations. A consolidated list of 18 genes were utilized to confirm the siRNA response in two additional cell lines models, the MDA-MB-231 and BT474 breast cancer cell lines. Western blot analysis confirmed protein knock down of key genes and demonstrated a strong correlation between siRNA concordance and cell viability. Given that DNA repair mechanisms and associated processes emerged as key contexts of vulnerability, compounds known to inhibit DNA repair and histone modification pathways were selected for *in vitro* drug combinations with Brostallicin. The combined results from this study will assist in the identification of potential patient populations as well as putative drug combinations which will support the further clinical development of Brostallicin.

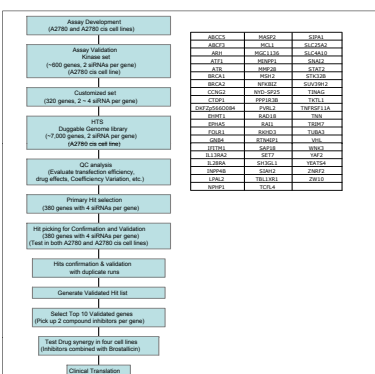


Table 1: List of validated potential sensitizers from HTS in A2780 and A2780cis Cells. A high throughput screen was performed first using A2780cis cells against Glagien's Human Druggable G2.0 siRNA library to arrive at total of 400 genes as potential Brostallicin sensitizers. For these genes, four siRNAs (sequences A through D) were hi-picked and printed onto assay plates followed by confirmation assays in both A2780 and A2780cis cell lines. Two experimental replicates were performed using the same techniques from the HTS. From the analysis of the confirmation experiments, only genes with a minimum of two (2) sequences that demonstrated Brostallicin sensitization in BOTH experimental replicates were considered a confirmed HTS hit. A total of 148 confirmed hits (siRNA sequences) targeting 59 genes were identified in the A2780cis cell line during the confirmation step (table on the right). These hits were also tested in the A2780wt cells, although less than five of these hits were validated in this cell line.

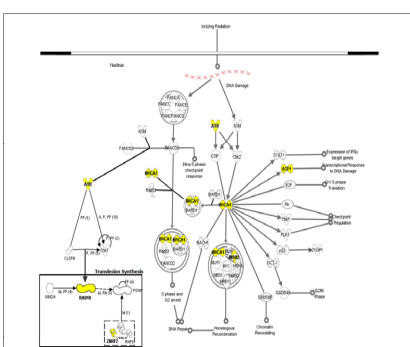


Figure 2: Schematic of the DNA repair associated hits in the Brostallicin siRNA screen. Hits in the screen are colored in yellow. That ATR/RAD18 hits are intimately associated with a DNA repair process called translesion synthesis (TLS). TLS is a mechanism during DNA replication in which the standard DNA polymerase is temporarily exchanged for a specialized polymerase that can synthesize DNA across base damage on the template strand. TLS activity has been associated with repairing lesions from monofunctional alkylators (nitrosourea compds, temozolomide) and bifunctional alkylators (mitomycin C, CDDP). Targeting of CHEK1 could be strategy to inhibit TLS to increase sensitivity to brostallicin. Inhibiting CHEK1 would reduce stabilization of claspin which will inhibit RAD18 complex binding to chromatin. RAD18 ubiquitinates PCNA creating a permissive environment for polymerase exchange to allow synthesis through sites of damage.

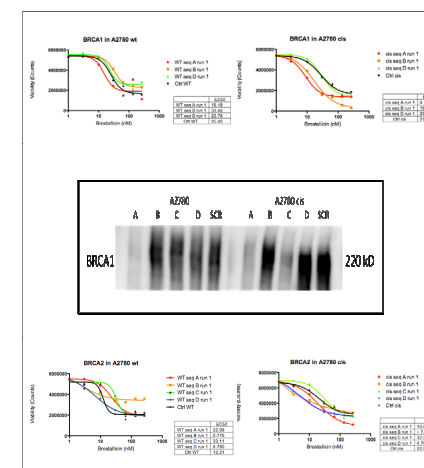


Figure 4: BRCA1/2 – brostallicin sensitizers in cisplatin resistant ovarian cancer cells. In both the A2780 parental and cisplatin resistant cell lines, siRNA A resulted in almost a complete loss of BRCA1 protein. Additionally, in the A2780 cisplatin resistant cell line, siRNA C also decreased BRCA1 protein levels. Interestingly, only the siRNA A knockdown correlated with decreased proliferation. In the case of BRCA2 protein, it proved to be quite difficult to identify correlate the knockdown effects observed in proliferation experiments by western blotting due to the fact it is extremely sensitive and prone to degradation.

INTRODUCTION

Cancer has been shown to be a disease of genetics. That is, changes in the way genes are controlled lead to the formation of cancer. This insight has set the stage for modern cancer drug development paradigm, wherein, identification of genetic contexts that are vulnerable to specific therapies would be critical to tailor therapies to specific Contexts of Vulnerabilities (COV).

Brostallicin, a synthetic second generation DNA minor groove binder, was selected for clinical development by Systems Medicine LLC. In view of its outstanding antitumor activity in a number of preclinical tests, its proapoptotic effect and its activity in cancer cells resistant to alkylating agents and topoisomerase I inhibitors. Emerging work has identified certain defects in DNA repair genes, and cancer associated translocations as potentially important in determining cellular sensitivity to this class of drugs, but further investigation is needed to clarify the mechanisms underlying these observations. A systematic pharmacogenomics approach helped achieve this goal through the discovery of important genetic factors in breast and ovarian cancer cells that determine and regulate Brostallicin response. The work presented here has identified certain defects in DNA repair genes, namely ATR and BRCA1/2 and genes associated with chromatin remodeling and epigenetic methylation defects as potentially important in determining cellular sensitivity to this drug. Further investigation to confirm the HT-RNAi results using a complementary approach was carried out in ovarian and breast cancer cell lines by combining Brostallicin with either rapamycin, wortmannin, 5-Aza-deoxycytidine, Valcade, SAHA, CGK 733, SNDX 275 or gemtacinib, which impinge specific effects either directly or indirectly on gene(s) products and their respective genetic pathways, identified in the HT-RNAi screen. These studies have provided us with significant information with regards to contexts in cancer cells that render them vulnerable to Brostallicin therapy, which would provide rational starting points for the development of patient and indication selection, as well as new additional combinations that will synergize with Brostallicin to provide a greater therapeutic benefit.



Figure 1: Biological Concept Enrichment of Validated Brostallicin Sensitizer Genes. Enrichment analysis was performed to identify common biological themes represented in the listing of the 59 sensitizers. GeneGo's Metacore database was used for enrichment analysis. Three different different categories were used for the enrichment analysis: GeneGo canonical maps, GeneGo process networks and Gene Ontology processes. Statistically significant categories passes FDR filtering using a significance of 0.05 are marked in bold. Note the convergence on DNA repair, apoptosis and chromosome associated concepts.

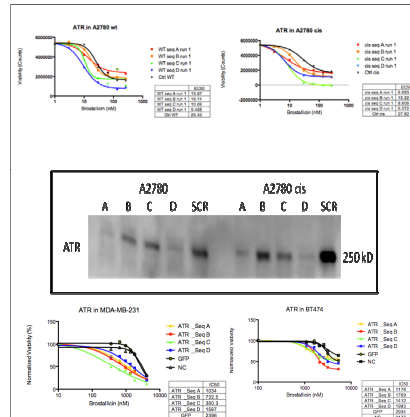


Figure 3: ATR – a Brostallicin sensitizer in Breast and ovarian cancer cells. All 4 siRNAs tested resulted in decreased protein levels in both cell lines tested versus the scrambled control siRNA. While all showed a decrease in protein expression versus control, the amount of decrease was variable. siRNA A and D were much more efficient at reducing protein levels in both cell lines than siRNA B and C. This pattern was observed in both the A2780 parental and cisplatin resistant cell lines. Interestingly, the decreased protein expression correlated with the anti-proliferation results, where siRNA A and D demonstrated marked responses in the A2780 parental and cisplatin resistant cell lines.

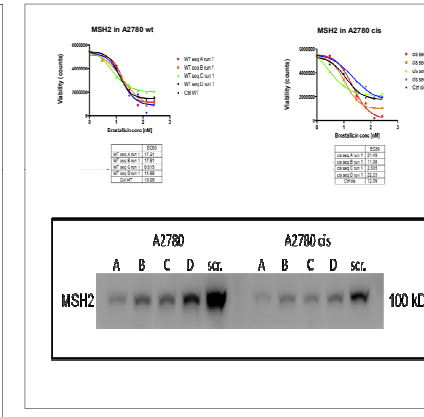


Figure 5: MSH2 – a Brostallicin sensitizer in ovarian cancer cells. All four siRNAs seem to knock down the protein relative to the scrambled control in both the parental and cisplatin resistant A2780 cell lines. The knockdown seems to be more variable between siRNAs in the A2780 parental line, while the knockdown in the cisplatin resistant line is more uniform among the 4 siRNAs. Interestingly, in the anti-proliferation studies, MSH2 siRNA B and C demonstrated a greater effect in the cisplatin resistant cell line.

Compound Name	Pathway/Target	Source	Product Number
wortmannin	ATR inhibitor	Sigma	W1628-SMG
5-aza-cytidine	DNA Methylation	Sigma	A1287
5-aza-deoxycytidine	DNA Methylation	Sigma	A3656-10MG
SNDX-275	HDACs	Bayer	2112316
SAHA	HDACs	Cayman Chemical	1000929
rapamycin	MCL modulator	Sigma	80395-1MG
Valcade	proteasome	LC Laboratories	B-1498
Methotrexate	Replication inhibitor	Alexis Biochemicals	ALX-440-045-2050
S-Flourouracil	Replication inhibitor	LKT cat# LKT-F4480	F4480
gemtacinib	Replication inhibitor	Toxis	2359
CGK733	ATR inhibitor	Toxis	2639

Table 2: List of compounds selected for synergy test.

Drug/Compound	Pathway /Gene Target	A2780 wild type	A2780 cis	MDA-MB-231	BT474
Wortmannin	ATR inhibitor	Synergy	Synergy	Synergy at high concentrations	Synergy at high concentrations
Rapamycin	MCL modulator	Synergy	Synergy	-	-
5-aza-deoxycytidine	DNA Methylation	-	-	Synergy	Mild synergy at high concentrations
Gemtacinib	Replication inhibitor	-	-	No synergy	Mild synergy at high concentrations
SAHA	HDACs	Synergy	Synergy	Synergy	Mild synergy
Valcade	Proteasome	Mild synergy	Synergy	No synergy	Synergy at high concentrations
CGK 733	ATR inhibitor	Mild synergy	Mild synergy	No synergy	Mild synergy
SNDX 275	HDACs	Mild synergy	Synergy	Synergy	Synergy

Table 3: Summary of Observed Drug Synergy of Brostallicin

CONCLUSIONS

- Drug/compound synergistic effects with Brostallicin, in the four cell lines tested, are summarized in tables 2 and 3.
- ATR inhibitors (Wortmannin and CGK733) and HDAC inhibitors (SNDX 275 and SAHA) showed synergy in at least three out of four cell lines.
- Very high correlation was observed for the targets showing synergy between siRNA and compound experiments.
- Our study demonstrated that both approaches (siRNA knock down and drug synergy) are functional/comparable and can help with the clinical development of Brostallicin.

Acknowledgement

•We thank Syndax Pharmaceuticals Inc for providing the SNDX-275 for the drug synergy test.

Syndax Pharmaceuticals, Inc.
11260 El Camino Real
Suite 220
San Diego, CA 92130