BTM Compounds Induce the Integrated Stress Response and Effectively Inhibit Growth of Diffuse Large B-cell Lymphoma

Matthew Kostura[1], Jedd Levine[1], Alan Cooper[1] and Michael Luther[1]

[1] Bantam Pharmaceutical, 505 Park Ave, New York, NY 10022

ABSTRACT

In response to diverse metabolic stress events, neoplastic cells commonly activate components of adaptive pathways denoted the Integrated Stress Response (ISR). Although the ISR is typically an adaptive cell survival mechanism designed to restore cellular homeostasis, under conditions of chronic and/or pharmacologically sustained cellular stress, ISR signaling can transition from an adaptive mode to induction of apoptosis in irreversibly stressed cells.

Bantam Pharmaceutical has developed novel, first-in-class small molecules that activate the ISR. In diffuse large B-cell lymphoma (DLBCL), Bantam (BTM) compounds lead to ISR activation, apoptosis, and potent anticancer activity *in vitro* and *in vivo*. Key features of ISR activation are phosphorylation of the translation initiation factor eIF2α and upregulation of the transcription factor ATF4. BTM compounds promote the phosphorylation of eIF2α, which results in general translational repression. ATF4-dependent gene transcripts are induced in DLBCL cells exposed to BTM compounds, but this is not observed in unresponsive normal or cancer cells. Alternate stress-related transcription factors/pathways (e.g., NRF2, ATF6, XBP1) are not notably activated *in vitro.*

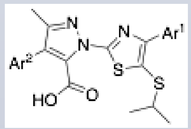
To further characterize BTM compound activity against DLBCL, 14 DLBCL cell lines were evaluated. BTM compounds were active against all tested cells lines with only 2/14 cell lines not demonstrating 100% growth inhibition after 72 hours of compound exposure. Compound potency ranged from IC_{50} 0.11 μM to 2.2 μM with 12/14 DLBCL cell lines demonstrating submicromolar IC_{50}. There were no evident differences in sensitivity to BTM compounds based on ABC or GCB status. Three double-hit cell lines and 1 triple-hit cell line were tested, demonstrating a mean IC_{50} of 0.3 μM. Apoptotic cell death was associated with caspase 3/7 activity. BTM compound significantly decreased expression of MCL-1 as early as 2 hours following compound exposure.

BTM-3601 was tested in vivo in a SCID mouse xenograft model using the double-hit SU-DHL 10 lymphoma. Tumors were established to a volume of ~150 mm^3. BTM-3601 administered once daily orally as a single agent for 21 days at 30 mg/kg led to rapid tumor regression in all animals (n=10) with complete tumor regressions achieved in all animals by treatment day 7. Treatment was continued for a total of 21 days with no adverse effects noted except for modest reversible weight loss. Following the 21-day treatment period, animals were observed off treatment for an additional 21 days with only one palpable tumor recurrence in animals treated at the 30 mg/kg dose.

BTM-3601 is a novel orally bioavailable compound with a unique mechanism of action based on modulation of cancer cell metabolism and induction of the ISR. It shows substantial activity and a favorable toxicity profile in *in vitro* and in *in vivo* models of DLBCL, including ABC, GCB, and double-hit subtypes.

BTM Compound Core Structure

Figure 1. Bantam Pharmaceutical compound core structure. The compounds were synthesized, prepared and evaluated for inhibition of growth and survival of the DLBCL BJAB cell line as described in Reference 1.



Objectives

A class of substituted 1-thiazol-2-yl-N—3-methyl-1H-pyrozole-5-carboxylic acid derivatives was found to have potent anti-proliferative activity (1). The activity of BTM-3528 was evaluated in a large panel of cell lines *in vitro* to judge potential therapeutic activity and suitability for progression into preclinical safety studies. Our preliminary data established that BTM compounds work broadly against hematopoietic cancer cell lines with excellent activity against DLBCL.

The molecular target for this class of compounds is as of yet undetermined. To provide a more meaningful understanding of the therapeutic potential and mechanism of action, our goal is to determine what pathway(s) are affected, identify relevant biomarkers associated with functional response to compound, and relate those markers to activity in sensitive DLBCL lines both *in vitro* and *in vivo.* We have previously determined that BTM compound class induces cell cycle arrest in G0/G1 with subsequent activation of caspase 3/7 and induction of apoptosis in BJAB cells. The latter is associated with a reduction in the expression of the anti-apoptotic protein MCL-1 (2). We have extended these observations and have determined that a central component of compound action is the activation of a genetic program termed the Integrated Stress Response.

Results

Human DLBCL tumor lines were tested for sensitivity to BTM compounds. Sensitivity of each cell line was determined by treating with compound for 72 hours and total cell numbers determined using Cell-Titer Glo. IC_{50} was determined relative to a point where maximal cell response (reduction in cell number) was achieved. The data summarized in Table I indicate that BTM-3601 is a potent inhibitor of DLBCL growth and survival. All COO subtypes are inhibited by the compound with double hit lymphoma cell lines being especially sensitive.

Table I. BTM-3601 is a potent inhibitor of DLBCL cell line growth and survival

Subtype	Cell Line	IC_{50} (μM)	Max Inhibition (%)
Double Hit (GCB)	SU-DHL 10	0.14	100
Double Hit (GCB)	SU-DHL 6	0.29	100
Double Hit (GCB)	SU-DHL 4	0.51	100
Triple Hit (GCB)	DOHH-2	0.18	100
GCB	Pfeiffer	0.18	100
GCB	BJAB	0.31	100
GCB	WSU-DLCL-2	0.75	100
GCB	Toledo	1.45	66
GCB	OCI-Ly1	2.2	100
ABC	SU-DHL 2	0.11	100
ABC	U2932	0.46	83
ABC	OCI-Ly3	0.68	100
ABC	SU-DHL 8	0.9	100
"Type 3"	OCI-Ly7	0.15	100

ATF4 gene expression is correlated with response to compound in hematopoietic and solid tumors

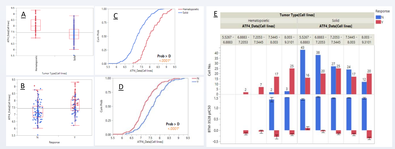


Figure 2. Gene Set Enrichment Analysis

406 tumor cell lines (73 hematopoietic tumor lines and 333 solid tumor lines) were screened to establish a relationship between gene expression and response to compound (Responsive is defined as IC_{50} < 1.0 μM and > 50% total inhibition of growth or survival). ATF4 gene expression is significantly higher in hematopoietic tumor lines as opposed to solid tumors (p<.001; Figures 2 A and C). ATF4 gene expression is higher in responsive as opposed to non-responsive tumor lines (p<.001; Figures 2 B, D and E). Overall 82% of hematopoietic tumor lines are responsive to compound and represent 36% of all responsive cell lines (Figure E).

The higher level of expression of ATF4 in hematopoietic tumors coupled with the association of ATF4 gene expression to compound response prompted us to determine whether the ATF4 pathway is activated by compound in DLBCL cell lines. To this end we used QPCR to measure the expression of a panel of genes regulated by distinct transcription factors (TF) (Table II).

Table II. Gene Targets

TF Pathway[Target Name]	Target Name
ATF4	ASNS
	DDIT3
	DDIT4
	PPP1R15A
	SARS
ATF6	DNAJC3
	HSP90B1
	HSPA5
	HSPA6
ERN1/XBP1	DNAJC7
	XBP1
Cell Cycle	CDKN1A
	PCNA
Nrf2	HMOX1

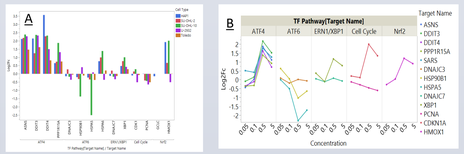


Figure 3. Induction of the ATF4 ISR pathway in hematopoietic tumor lines. ATF4 genes are preferentially regulated in a dose dependent manner

A panel of DLBCL lines and a responsive CML line were treated with 5 μM BTM-3601 for 8 hours, RNA harvested and gene expression changes determined by QPCR. All comparisons are to vehicle control. ATF4 regulated genes were preferentially regulated by compound treatment (Figure 3A). The induction of mRNA by compound was found to be dose dependent (as measured in SU-DHL-10 cells) with an IC_{50} consistent with inhibition of growth and survival. (Figure 3B). The observation that BTM-3601 induces ATF4 and not ATF6 or IRE-1a/XBP-1 regulated genes suggests that the classical UPR response is not activated *in vitro*.

BTM-3601 induces rapid and sustained tumor regression *in vivo*

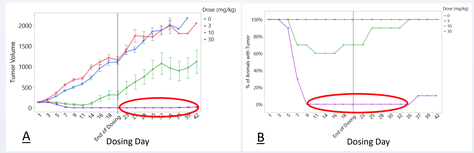


Figure 4. *In vivo* efficacy of BTM-3601 in a mouse xenograft model: SU-DHL-10 lymphoma. Female SCID beige mice (n=15 per group) were injected subcutaneously with $2x10^6$ SU-DHL-10 cells and allowed to establish solid tumors. Animals with tumors ≥ 150 mm^3 were treated with BTM-3601 at 3, 10 and 30 mg/kg once daily, orally for 21 days followed by a recovery period of 21 days. BTM-3601 was well tolerated with minimal weight loss and induced clear evidence of tumor regression in this model that was sustained following removal of compound on day 21 of the study. All data are plotted as the mean of tumor volume +/- CI ((Figure 4A) . (n=10 mice per group). Tumor regression is plotted as the % of animals bearing a tumor of greater than palpable size. (Figure 4B).

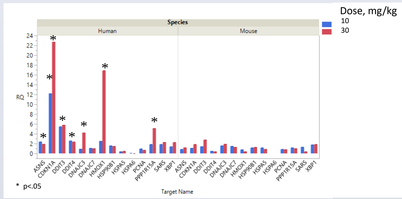
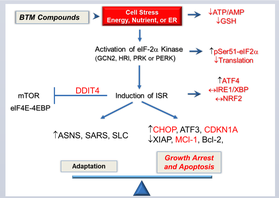


Figure 5. BTM-3601 induces ATF4 gene expression in tumor samples from a mouse xenograft model: SU-DHL-10 lymphoma. Tissue from animals treated with BTM-3601 in Figure 4 were tested for induction of ATF4 mediated gene expression using both human and murine probes. BTM-3601 induced clear signs of human, but not murine, ATF4 gene expression as seen *in vitro*. In contrast to *in vitro* conditions, there was also a significant elevation of CDKN1A and HMOX1 mRNA *in vivo.* All data are plotted as the mean fold induction, n=5 mice per group. (Dunnett's test comparing to control; * p<.05).

Figure 6. Working model for BTM compound Mechanism of Action



Conclusions

- BTM compounds exhibit potent activity against DLBCL *in vitro* and *in vivo*.
- The compounds are active against ABC and GCB subtypes with notable activity against Double Hit/Triple Hit lymphomas.
- BTM-3601 induces rapid, complete, and durable tumor regressions in a DLBCL/Double Hit xenograft model with once daily administration.
- Profiling across a panel of 406 hematopoietic and solid tumor lines identified correlation of the expression of ATF4 transcripts with sensitivity to BTM compound.
- BTM-3601 induces ATF4 ISR related transcripts in human DLBCL xenografts including DDIT3, DDIT4, and PPP1R15A. In addition, CDKN1A, HMOX1, and DNAJC3 are also detected in human DLBCL xenografts following treatment with BTM-3601. In contrast, murine homologs were not regulated in the same manner suggesting that compound activity is restricted to tumor cells.
- BTM compounds have been demonstrated to induce ATF4 mediated gene expression in DLBCL cell lines. We are currently determining whether an underlying causal role for ATF4 regulated gene induction is associated with the observed pharmacology of BTM compounds in DLBCL.
- The activity and mechanism of action of BTM-3601 support its potential development as a novel treatment for relapsed and refractory DLBCL, including Double Hit lymphomas.

References

1. Cooper AB, et al. (2017) 1-Thiazol-2-yl-N-3-methyl-1H-pyrozole-5-carboxylic acid derivatives as antitumor agents. *Bioorg Med Chem Lett* 27(18):4471–4477.
2. Kostura, M, Levine, J, et al. (2017) BTM-3528 potently induces G1/G0 cell cycle arrest and is efficacious in preclinical models of diffuse large B cell lymphoma. AACR Conference on Hematologic Malignancies 2017: Translating Discoveries to Novel Therapies, May 6-9, 2017, Boston, MA. Abstract 45, AACR.org/Heme17

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Please send correspondence to:
Dr. Michael Luther
Chief Executive Officer
Bantam Pharmaceutical
505 Park Ave, New York, NY 10022
mluther@bantampharma.com