BTM-3655 co-opts mitochondrial quality control pathways to induce apoptosis and tumor regression in DLBCL cell lines, xenografts and PDX models

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is a highly heterogeneous tumor, for which novel therapies are needed. Herein we describe a family of orally available small molecule compounds, exemplified by BTM-3528 and BTM-3566, which induce apoptosis in DLBCL cell lines, including double- and triple-hit subtypes.

- **Robust *in vivo* Activity:** BTM-3566 induces complete and durable regression in a model of human DLBCL. Importantly, no regrowth is seen through 21 days post therapy. In an expanded panel of high-risk human DLBCL PDX models BTM-3566 demonstrated a 100% response rate, and complete tumor regression in 6 of 9 PDX models.
- **Novel mechanism of action:** High content cellular imaging and gene expression analysis revealed that BTM-3566 fragments the mitochondrial network in an OMA1 dependent manner and activates the ATF4 ISR.
- Activation of the mitochondrial i-AAA protease OMA1 is coupled to activation of eIF2α kinase HRI, phosphorylation of eIF2α and induction of the ATF4 Integrated StressResponse (ISR). CRISPR-Cas9 depletion of HRI and OMA1 eliminates BTM-3566's ability to induce ATF4 protein and significantly attenuates apoptosis.
- Substrates of OMA1 include the dynamin, OPA1 and DELE1. Cleavage of both are sensors for mitochondrial dysfunction leading to fragmentation of the mitochondrial network or activation of HRI kinase and ATF4. Transfection of BJAB cells with a cleavage resistant OPA1 mutant has no effect on BTM-3566 induced apoptosis. In contrast, KO of DELE1 suppresses BTM-3566 mediated apoptosis.
- **Dependence on mitochondrial protein expression.** BTM-3566 induces OMA1 activity through a novel mechanism regulated by the mitochondrial protein FAM210B. FAM210B expression is negatively correlated with response to BTM-3566, and overexpression of FAM210B blocks causes complete resistance to BTM-3566 induced apoptosis. FAM210B expression is a strong predictor of sensitivity to BTM-3566 and reveals a novel mechanism of regulation of OMA1 activation.

Objectives

BTM-3566 is member of a family of compounds with a novel pyrazolo-thiazole core structure. Our studies are intended to evaluate the activity of BTM-3566 in tumor lines *in vitro* and *in vivo* as well as to elucidate the mechanism of action.

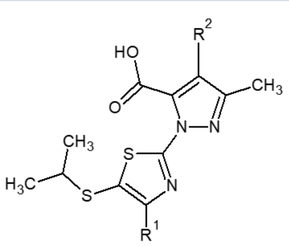

Figure 1: Core pyrazolo-thiazole structure.

Results

1. BTM-3528 and BTM-3566 inhibits the growth of both solid and hematopoietic tumor cell lines

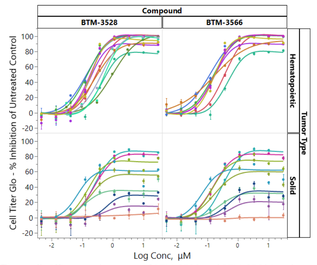

Figure 2 Efficacy and potency of BTM-3528 and BTM-3566 in select tumor cell lines. Cell lines were seeded into 96-well plates and incubated for 72 hours with either compound or vehicle. At the end of the incubation period cell number were determined using Cell-Titre Glo (Promega). All data are plotted as the average percent of cell numbers as compared to vehicles. Each data point is the mean of duplicate plates; error bars indicate the range. Left panel: BTM-3528, Right panel BTM-3566; Upper Panels Hematopoietic tumors, Lower Panels Solid Tumors

2. BTM-3566 treatment leads to sustained tumor regressions xenograft model of DLBCL

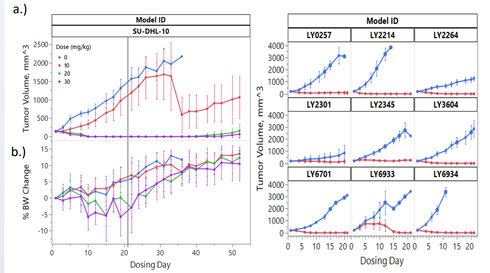

Figure 3: **Efficacy of BTM-3566 in human xenograft models.** Su-DHL-10 cells were implanted subcutaneously in SCID beige mice and randomized into four groups when tumor volume reached 200 mm^3. Mice were dosed po, qd with vehicle or a solution of 10, 20 or 30 mg/kg BTM-3566. a) Tumor volume and b) body weights were determined every 3 days. The vertical line at day 21 represents the end of drug dosing. All data are the mean +/- SD (n=10 animals). c) Nine patient derived human DLBCL tumors were implanted into scid mice (n=6 per tumor group) and grown to 200 mm^3. At staging each tumor group was split into treatment or vehicle arms (n=3 animals per arm). Treatment arms received BTM-3566, 20 mg/kg, po, qd. All groups received drug or vehicle for 21 days. Tumor volume was measured and recorded every other day. All data represented as mean ± SD. •=Vehicle; •= BTM-3566

3. BTM-3528 and BTM-3566 inhibit the growth of all subtypes of DLBCL *in vitro* and *in vivo*

Cell Line	COO Subtype	CCC Subtype	Potency, µM BTM-3528	Potency, µM BTM-3566
BJAB	GCB		0.26	0.44
Su-DHL-2	ABC		0.288	
SU-DHL 4	GCB	BCR	0.177	0.179
SU-DHL 6	GCB	BCR	0.136	0.119
SU-DHL 8	ABC		0.9	
SU-DHL 10	GCB		0.136	0.116
DOHH-2	GCB		0.288	0.36
Farage	GCB			0.89
HT	GCB			0.72
OCI-Ly1	GCB	BCR	0.179	0.166
OCI-Ly3	ABC	BCR	0.68	
OCI-Ly7	Type 3		0.146	0.166
Pfeiffer	GCB	OxPhos	0.187	0.173
Toledo	GCB		1.45	0.68
WSU-DLCL-2	GCB		0.83	

PDX Model ID	Genotype	Response, Day 21 % TGI	CR	PR	SD	PD
LY0257	ABC, MYD88, L265P	97	1	1	1	
LY2214	GCB	100	3			
LY2264	ABC, MYD88, L265P	100	3			
LY2301	ABC	89				
LY2345	ABC/GCB	100		1	1	1
LY3604	ABC/GCB	100	3			
LY6701	GCB	100	3			
LY6933	ABC/GCB	100	3			
LY6934	ABC/GCB	100	3			

4. BTM-3528 induces the ATF4 ISR and eIF2α phosphorylation

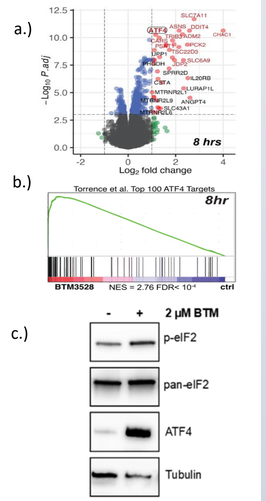

Figure 4 a) RNA Seq Analysis of BTM-3528 regulated gene expression in HCT-116 cells treated with 3 µM BTM-3528 for 8 hours. Treatment data are 3 replicates compared to equivalent vehicle control; b) GSEA analysis reveals that ATF4 regulated genes are highly enriched for increased expression in BTM-3528 treated cells; c) eIF2α phosphorylation and ATF4 protein are increased following treatment with BTM-3528

5. BTM compounds induce fragmentation of the mitochondrial network in an OMA1 dependent manner

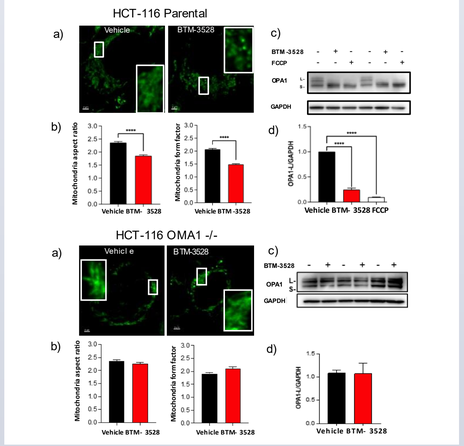

Figure 5. BTM compound treatment fragments the mitochondria and induces OPA1 cleavage in an OMA1 dependent manner. a-d) HCT116 WT and OMA1 -/- treated with 3 µM BTM-3566 for 3 hours. a) Representative images from HCT116 cells stained with mitotracker green and live imaged using confocal laser microscopy. b) Quantification of the average mitochondrial aspect ratio and form factor per cell. Values of form factor and aspect ratio close to 1 represent isolated and spherical mitochondria. Bar graphs show the average of n=4 independent experiments, with the values of Aspect Ratio and Form Factor per experiment being the average of n= 5 cells, with mitochondria detected and properly segmented in one cell analyzed. C) Representative Western blot analysis of OPA-1 long forms (L) cleavage to short isoforms (S) in total lysates, with GAPDH being used as a loading control. FCCP is a compound known to induce OPA1 cleavage by depolarizing the mitochondria, being a positive control. D) Quantification of OPA1 L isoforms determined by Western blot as in panel C. Bar graph show the average of n=3-4 independent experiments with error bars being SEM. ****p<0.0001.

6. CRISPR/Cas9 KO of ISR pathway genes eliminates activity of BTM 3566

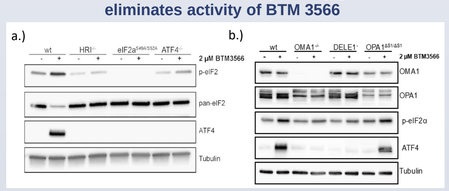

Figure 6 CRIPSR KO of ISR pathway components but not OPA1 block action of BTM-3566 in BJAB cells. CRISPR/Cas9 was used to create gene KO's for HRI, DELE1, OMA1 and ATF4. Homology directed repair was used to create a non-phosphorylatable mutant of eIF2α and and OMA1 non-cleavable mutant of OPA1. a.) BTM-3566 induced eIF2α phosphorylation and ATF4 protein expression are eliminated by deletion of HRI and ATF4 and mutation of eIF2α; b.) BTM-3566 induced eIF2α phosphorylation and ATF4 protein expression are eliminated by deletion of OMA1 and DELE1. Mutation of OPA1 has no effect on BTM-3566 activity.

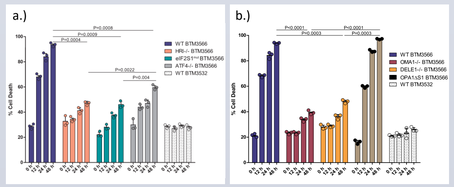

Figure 7 CRIPSR KO of ISR pathway components but not OPA1 blocks action of BTM-3566. Annexin-PI apoptosis assays investigating the cell death kinetics of a) BJAB wt, BJAB HRI$^{-/-}$, BJAB eIF2a$^{S48A/S51A}$, and BJAB ATF4$^{-/-}$ cells treated with 2 µM BTM3566 for the indicated time. BTM3532 served as inactive control. b) Annexin-PI Cell death kinetic of BJAB wt, BJAB OMA1$^{-/-}$, BJAB DELE1$^{-/-}$, and BJAB OPA1$^{\Delta S1/\Delta S1}$ cells treated with 2 µM BTM3566 for the indicated time. Depicted data represents n=3 independent experiments. P-Values were calculated with a two-sided Welsh's T-test.

Results

7. FAM210B expression is inversely correlated with BTM-3566 activity

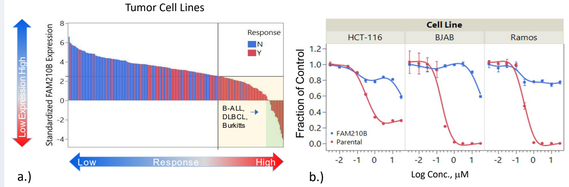

Figure7 a) **Waterfall plot of FAM210B mRNA expression data in 303 cell lines**. Data are plotted by descending FAM210B expression level. Values in red represent cell lines found responsive to compound. A positive response to compound was defined as an AUC of < 3.1. Values in blue are non-responsive cell lines with an AUC > 3.4. Responsive to drug is correlated with decreasing FAM210B expression. Cells of the B-cell lineage have the lowest levels of FAM210B and are most responsive to drug; b.) **Ectopic overexpression of FAM210B rescues cells from the effects of BTM-3566.** All responsive cell lines were transfected with FAM210B cDNA and selected by puromycin selection. Clones with the highest level of FAM210B expression were tested for sensitivity to BTM-3566. All cell lines proved resistant to the action of compound.

8. Summary and Conclusions

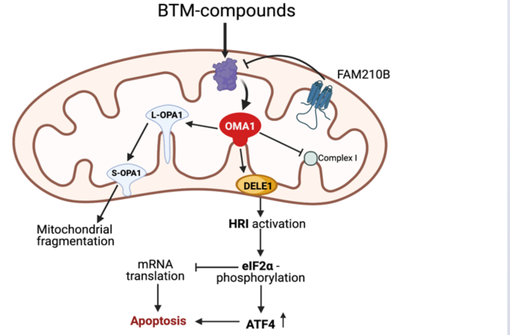

Figure 8 Model for the mechanism of action of BTM compounds. BTM compounds induce the activation of OMA1 leading to the cleavage of DELE1 and OPA1. OPA1 activation leads to fragmentation of the mitochondrial network. DELE1 cleavage leads to activation of HRI and the downstream effector pathways leading to cell death in DLBCL cell lines.

- BTM-3566 shows significant therapeutic activity and a favorable toxicity profile in *in vitro* and *in vivo* models of DLBCL.
- BTM compounds are novel orally bioavailable agents with a mechanism of action dependent on the OMA1 dependent activation of HRI and the eIF2α ISR.
- We have identified novel protein that regulates BTM compound activity.
- An Investigational New Drug application for BTM3566 in B-cell malignancies will be submitted by early Q2 2022 with initiation of first in human clinical trials Q3 2022.