# Discovery of BH-30236: A Novel Macrocyclic CLK Inhibitor Targeting Alternative Splicing in Cancers

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## Abstract

Aberrant alternative splicing is a newly recognized hallmark of cancer, that has been shown to play a critical role in tumorigenesis, cancer progression, and therapeutic resistance *via* multiple mechanisms, including increased proliferation, decreased apoptosis, enhanced migration and metastatic potential, and induced evasion of immune surveillance. Serine and arginine-rich splicing factors (SRSFs) are RNA-binding proteins (RBPs) that regulate constitutive and alternative splicing. SRSFs are often mutated or overexpressed in cancers, resulting in widespread alterations in splicing patterns. The CDC-like kinase (CLK) family and dual-specificity tyrosine-regulated kinase (DYRK) phosphorylate SRSFs, influencing the assembly of spliceosome machinery, exon recognition, and splicing. Therefore, targeting CLK/DYRK kinases can modulate cancer specific splicing isoforms, opening avenues for new therapeutic interventions. BH-30236 was designed as a novel orally bioavailable, ATP-competitive, macrocyclic inhibitor of CLK with $IC_{50}$s of 0.134, 0.165, and 0.446 nM against CLK1, CLK2, and CLK4, respectively in enzymatic kinase assays. At clinically relevant concentrations, BH-30236 also inhibited DYRK1A/1B/2, proviral insertion site of Moloney murine leukemia virus kinase 3 (PIM3), and FMS-like tyrosine kinase 3 (FLT3) with $IC_{50}$s of 0.110, 0.148, 0.562, 0.115 and 0.248 nM, respectively. In cancer cells, BH-30236 impaired the phosphorylation of SRSFs, Tau and 4EBP1, the direct downstream substrates of CLK, DYRK, and PIM kinases with $IC_{50}$s of 40-60 , ~50, and ~80 nM, respectively. Furthermore, BH-30236 also potently inhibited the FLT3 phosphorylation with an $IC_{50}$ of 0.16 nM. Overall, BH-30236 regulated alternative splicing by primarily inducing skipped exons in favor of anti-tumor isoforms, leading to cancer cell death and growth suppression in a broad panel of cancer cell lines and *in vivo* efficacy studies. For example, BH-30236 potently inhibited cell proliferation with an $IC_{50}$ of 0.98 nM in *FLT3-ITD* positive MV-4-11 cells and achieved complete tumor regression in MV-4-11 tumor model, even after stopping dosing for 30 days. In MV-4-11 cells, BH-30236 increased pro-apoptosis isoform BCL-xS, downregulated RNA expression of BCL2, MCL1, and AML stem cell markers CD33 and CD123. In addition, BH-30236 has also demonstrated good human ADME and preclinical safety profiles. Collectively, the preclinical study results strongly support the clinical applications of this novel multikinase CLK inhibitor BH-30236 in hematological malignancies and solid tumors, as a single agent or in combination with other therapies.

## Introduction

Pre-mRNA splicing is a complex, highly regulated process involving the removal of introns and the ligation of exons to produce mature mRNAs for protein translation. More than 94% of human protein-coding genes are alternatively spliced in nearly all human organs, leading to proteome diversity and regulation of cell functions. Dysregulated pre-mRNA splicing has been identified in almost all tumor types. Cancer-associated splicing alterations arise from either recurrent mutation in splicing factors or altered expression of trans-acting factors governing splicing catalysis and regulation. This leads to cancer cell proliferation, migration, and metastasis, escaping from cell death, rewiring cell metabolism or cell signaling, promoting an abetting microenvironment, altering immune response or enabling drug resistance.[1] The expression of the key splicing regulator SRSFs is tightly regulated at the post-translational level. The localization and activity of SRSFs are modulated by a dynamic cycle of phosphorylation/dephosphorylation, mostly at serine residues within the RS-domain of SRSF proteins. The phosphorylation of SRSFs is controlled by three main families of splicing kinases: serine-rich protein kinases (SRPKs), CDC-like kinases (CLKs), and dual-specificity tyrosine-regulated kinases (DYRKs). The CLK family, which comprises CLK1-4, collaborates with SRPKs at nuclei to adjust the degree of phosphorylation of SR proteins. SRSFs are often upregulated in many cancers. Therefore, inhibition of CLK/DYRK kinases may be an effective approach to modulate aberrant alternative splicing for cancer therapy.

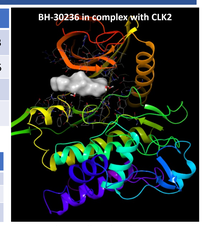
## Characterization of BH-30236

| Compd | Recombinant Enzymatic Assay[a] $IC_{50}$ (nM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CLK1 | CLK2 | CLK3 | CLK4 | DYRK1A | DYRK1B | FLT3 | PIM3 |
| BH-30236 | 0.134 | 0.165 | 5.87 | 0.446 | 0.11 | 0.148 | 0.248 | 0.115 |
| CTX-712[b] | 0.205 | 0.064 | 1.66 | 0.518 | 0.153 | NA | NA | NA |
| SM08502[b] | 0.097 | 0.262 | 5.86 | 0.407 | 0.078 | NA | 5.9 | 623 |

[a] CLK enzymatic activities were determined at Nanosyn, and DYRK, FLT3-ITD and PIMs were determined at Reaction Biology
[b] Proxy chemical compound purchased from a commercial source

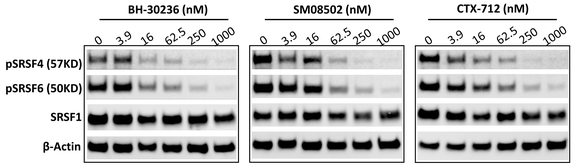
| Compound ID | NanoBRET™ $IC_{50}$ (nM)[c] | | |
|---|---|---|---|
| | CLK1 | CLK2 | CLK4 |
| BH-30236 | 0.111 | 1.387 | < 0.058 |
| SM08502 | < 0.058 | 1.954 | < 0.058 |

[c] CLK NanoBRET™ activities were determined at Reaction Biology

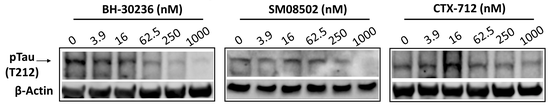



Macrocyclic small molecule: MW 431.49

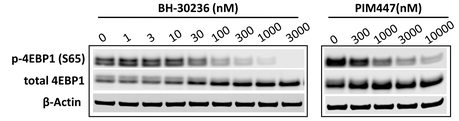
- **BH-30236 effectively inhibited the phosphorylation of CLK substrate SRSFs in Kasumi-1 Cells**




- **BH-30236 modulated the phosphorylation of DYRK downstream effector Tau in SH-SY5Y Cells**



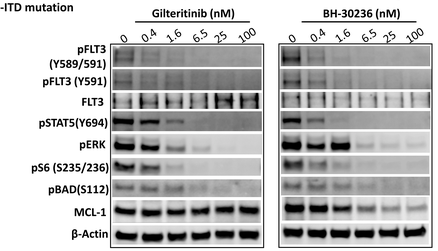

- **BH-30236 suppressed the phosphorylation of PIM downstream effector 4EBP1 in MM1S Cells**




Note: PIM447 is a proxy chemical compound purchased from a commercial source

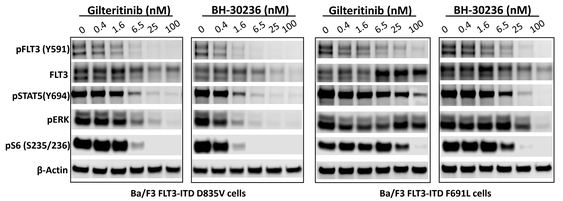
## BH-30236 Potently Inhibited FLT3-ITD and Resistant Mutations

- **BH-30236 potently inhibited auto-phosphorylation of FLT3 and downstream targets in MV-4-11 Cells with FLT3-ITD mutation**

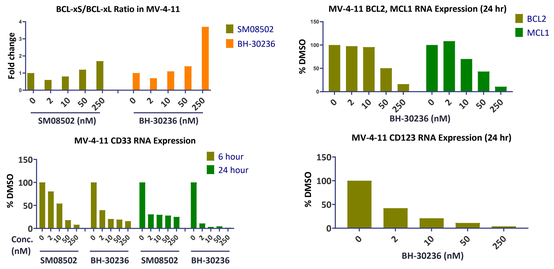



Note: Gilteritinib is a proxy chemical compound purchased from a commercial source

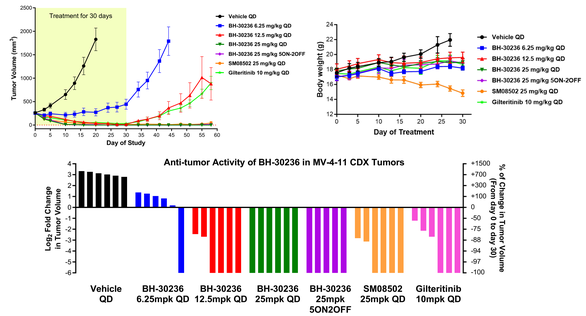
- **BH-30236 effectively modulated auto-phosphorylation of FLT3 and downstream signaling in engineered Ba/F3 FLT3-ITD D835V and F691L cells**




- **BH-30236 dose-dependently modulated alternative splicing to favor the apoptosis isoform BCL-xS and downregulated BCL2, MCL-1 and stem cell markers CD33 and CD123 in MV-4-11 cells**



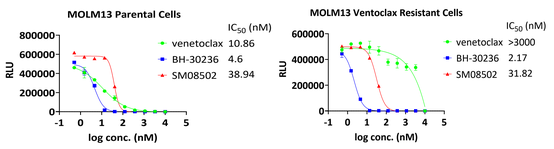

- **BH-30236 treatment achieved complete tumor regression in MV-4-11 cell-derived xenograft (CDX) tumor model without tumor regrowth after cessation of treatment**




- BH-30236, a dual CLK/FLT3 inhibitor, demonstrated dose-dependent inhibition of tumor growth and induced complete tumor regression at 25 mg/kg once a day (QD) in MV-4-11 cell xenograft model
- Gilteritinib, a FLT3 inhibitor achieved 79% tumor regression at 10 mg/kg QD in MV-4-11 CDX model
- SM08502, a CLK inhibitor, achieved 84% tumor regression but also caused significant body weight loss at 25 mg/kg QD dosing schedule in MV-4-11 CDX model
- After cessation of treatment for 4 weeks, tumor grew back quickly in FLT3 inhibitor gilteritinib-treated mice, however, minimum regrowth were observed in BH-30236 and SM08502 treated mice at 25 mg/kg QD
- The dual inhibition of CLK/FLT3 may provide deeper response and longer duration of remission in AML patients with *FLT3* mutation

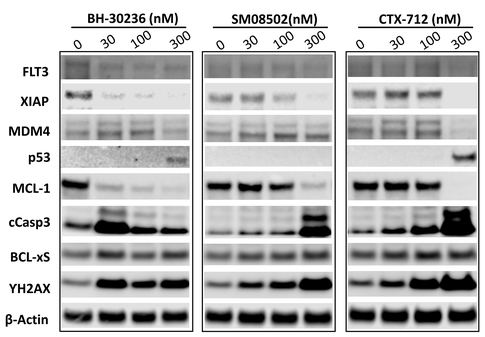
## BH-30236 Overcame Resistance to BCL2 Inhibitor Venetoclax and Demonstrated Strong Synergy with Venetoclax

- **BH-30236 demonstrated equal potency in MOLM-13 parental cells and venetoclax resistance cells**

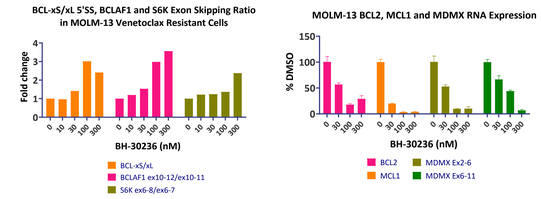



Note: Venetoclax is a proxy chemical compound purchased from a commercial source

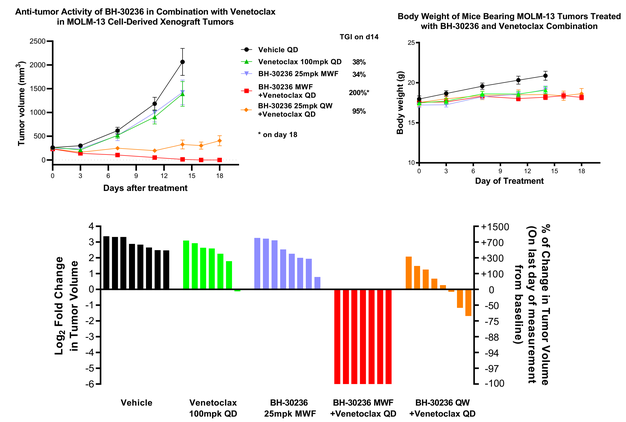
- **BH-30236 effectively modulated apoptosis and DNA damage response pathways in MOLM-13 cells**




- **BH-30236 dose-dependently modulated alternative splicing to favor anti-tumor isoforms and downregulated BCL2, MCL-1 and MDMX in MOLM-13 cells**

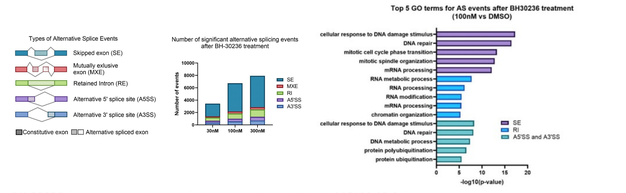



- **BH-30236 demonstrated strong synergy with selective BCL2 inhibitor venetoclax in MOLM-13 CDX tumor model in female SCID/Beige mice**

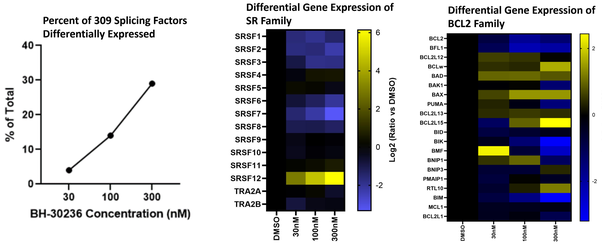



## BH-30236 Effectively Regulated Aberrant Alternative Splicing in Cancers

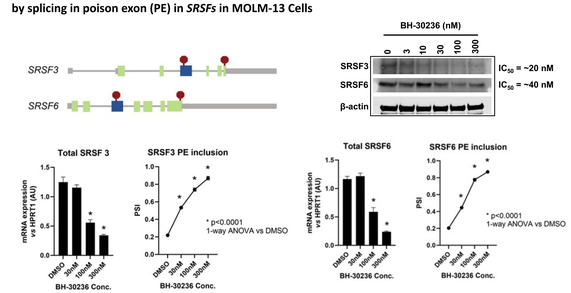
- **BH-30236 dose-dependently modulated alternative splicing with skipped exon as the dominated event**
- **Gene ontology enrichment analysis indicated that BH-30236 treatment was mainly associated with DNA repair, DNA damage response, mRNA splicing and processing, and mitotic cell cycle/spindle organization in MOLM-13 Cells**



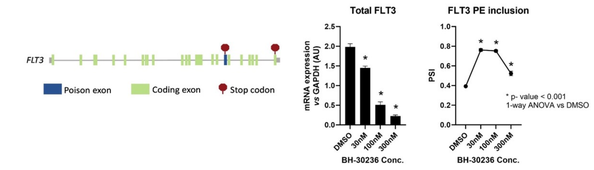

- **BH-30236 dose-dependently regulated alternative splicing in MOLM-13 Cells**




- **BH-30236 suppressed protein expression of splicing factors via Nonsense-Mediated mRNA Decay (NMD) by splicing in poison exon (PE) in *SRSFs* in MOLM-13 Cells**




- **BH-30236 down-regulated FLT3 expression via NMD by splicing in PE in MOLM-13 cells that will avoid the generation of treatment resistance**




## Conclusion

- BH-30236 is a potent CLK/PIM/FLT3 inhibitor
- BH-30236 potently inhibited FLT3-ITD and secondary FLT3-ITD resistant mutations commonly observed in AML patients
- Complete tumor regression was observed in MV-4-11 FLT3-ITD CDX tumor model without tumor recurrence 4 weeks after cessation of BH-30236 treatment
- BH-30236 can overcome venetoclax resistance in AML-derived MOLM-13 cells and achieved complete tumor regression when combined with venetoclax in the highly resistant MOLM-13 CDX tumor model
- Anti-tumor activity of BH-30236 can be attributed to its regulation of apoptosis, DNA damage response, and mRNA processing
- BH-30236 effectively modulated splicing of *BCL2L1*, *BCLAF1*, and *RPS6KB1* in favor of apoptosis and impaired cell growth
- SRSFs and FLT3 are commonly upregulated or mutated in primary AML tumors or as part of secondary resistance mechanism, and BH-30236 downregulated the expression of SRSFs and FLT3 via alternative splicing couple to NMD
- Preclinical pharmacology evaluation strongly supports further evaluation of BH-30236 in a planned Phase1/1b first-in-human study in relapsed or refractory acute myelogenous leukemia or higher risk myelodysplastic syndrome

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Reference
1. Bradley R, Anczuków O. RNA splicing dysregulation and the hallmarks of cancer. *Nat Rev Cancer*. 2023;23(3):135-155