Tuspetinib Oral Myeloid Kinase Inhibitor Creates Synthetic Lethal Vulnerability to Venetoclax

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Background

Tuspetinib (HM43239; TUS), a once daily oral kinase inhibitor, suppresses key oncogenic signaling pathways operative in acute myeloid leukemia (AML) by inhibiting SYK, FLT3, RSK2, JAK1/2, mutant KIT, and TAK1-TAB1 kinases. In AML animal models, tuspetinib exhibited greater potency than gilteritinib and entospletinib when given as single agents and combined favorably with venetoclax (VEN) and azacytidine (AZA). Tuspetinib is being evaluated as monotherapy (TUS) and in combination (TUS/VEN) in a global Phase 1/2 trial of patients with R/R AML (NCT03850574). In AML, drug combination treatment overcomes drug-induced resistance. In this study, Tuspetinib resistant AML cells (TUS-Res) were created to investigate the resistance mechanism and sensitivity to other drugs.

Dissociation and inhibition constants for TUS against key kinases operative in AML

Assay Methodology	Kinase	Mutation Status	Activity
Binding Affinity (K_D, nM)	FLT3	WT	0.58
		ITD	0.37
		D835Y	0.29
		D835H	0.4
		ITD/D835V	0.48
		ITD/F691L	1.3
Inhibition of Kinase Enzyme Activity (IC_{50}, nM)	FLT3	WT	1.1
		ITD	1.8
		D835Y	1.0
	SYK	WT	2.9
	JAK	JAK-1	2.8
		JAK-2	6.3
		JAK-2 (V617F)	9.9
	c-KIT	WT	> 500
		D816H	3.6
		D816V	3.5
	RSK	RSK2	9.7
	TAK1-TAB1	TAK1-TAB1	7.0

Objectives

- Generate Tuspetinib-resistant (**TUS-Res**) AML cell lines
- Investigate mechanistic changes in TUS-Res cell lines relative to parental
- Investigate the vulnerabilities to other inhibitors of AML on TUS-Res cells

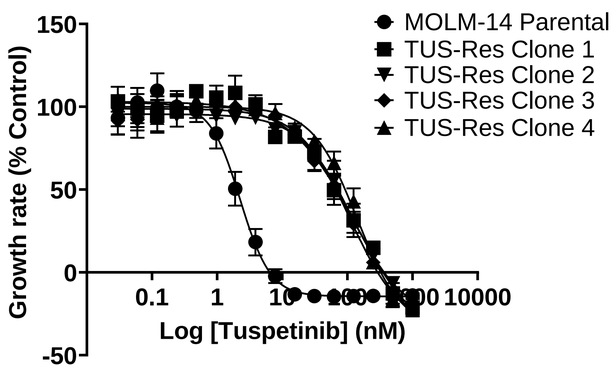
Methods

- **Generation of TUS-Res cells:** MOLM-14 cells were grown in progressively higher concentrations of tuspetinib (TUS) over a period of 4 months. TUS-Res clones were then maintained in the presence of 75nM Tuspetinib and sub-cultured at 2-3 days.
- **Cytotoxicity assay:** Tuspetinib was washed from TUS-Res cells to test the sensitivity of multiple inhibitors, including Gilteritinib, Quizartinib, Ruxolitinib, Fostamatinib, Venetoclax, Brequinar, Luxeptinib, IMP1088, 5-Azacytidine and Zelavespib. Cytotoxicity assays using CCK8 reagent were performed over a 72-h period of drug exposure, and inhibition of growth rate was determined.
- **Western Blot:** Cell signaling targets and pro-and anti-apoptotic targets were analyzed by Western Blot. TUS-Res MOLM-14 cells (maintained in 75nM TUS) were analyzed for stability of phenotype by culturing cells in Tuspetinib-free media for 60 days (Sampling performed at 15, 30, and 60 days).
- **Statistical Analysis:** Data reflect the mean ± SEM from 3 independent experiments and Student's t-test analysis. $IC_{50}$was calculated using a curve fitting function in Prism.

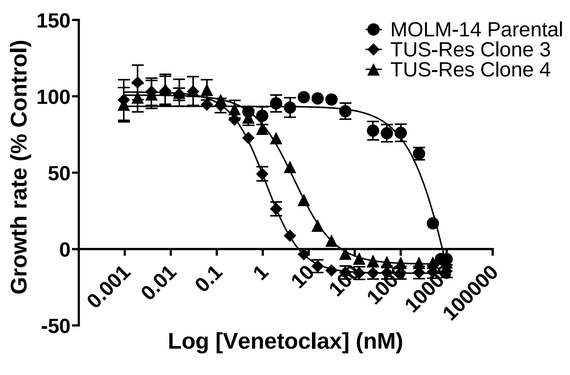
Sensitivity of Tuspetinib-Resistant Cell Clones

- Four clones of TUS-Res cells selected by continuous treatment of MOLM-14 cells in progressively higher concentrations of Tuspetinib for a period of 4 month
- TUS-Res clones (maintained in 75nM Tuspetinib) were 139 ± 17-fold resistant to Tuspetinib
- **TUS-Res clones showed 2645-fold increased sensitivity to venetoclax**
- TUS-Res clones showed 156 ±22-fold resistance to gilteritinib
- TUS-Res clones showed slightly increased sensitivity to quizartinib and luxeptinib (not shown)

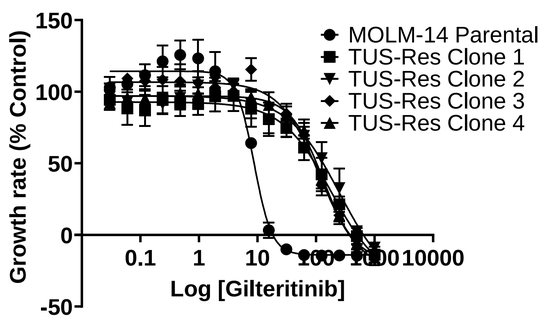
Effect of Tuspetinib on MOLM-14 parental and TUS-resistant clones



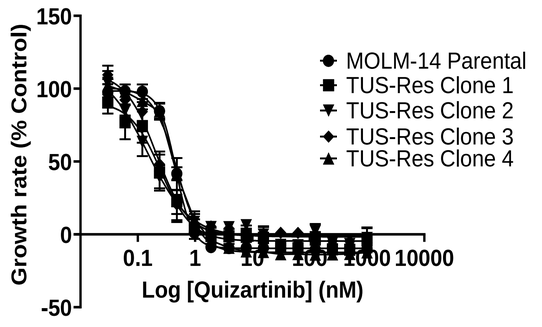
Effect of Venetoclax on MOLM-14 parental and TUS-resistant clones



Effect of Gilteritinib on MOLM-14 parental and TUS-resistant clones



Effect of Quizartinib on MOLM-14 parental and TUS-resistant clones



IC_{50} (nM, Mean ± SD) of Various Drugs for Tuspetinib-Resistant Clones

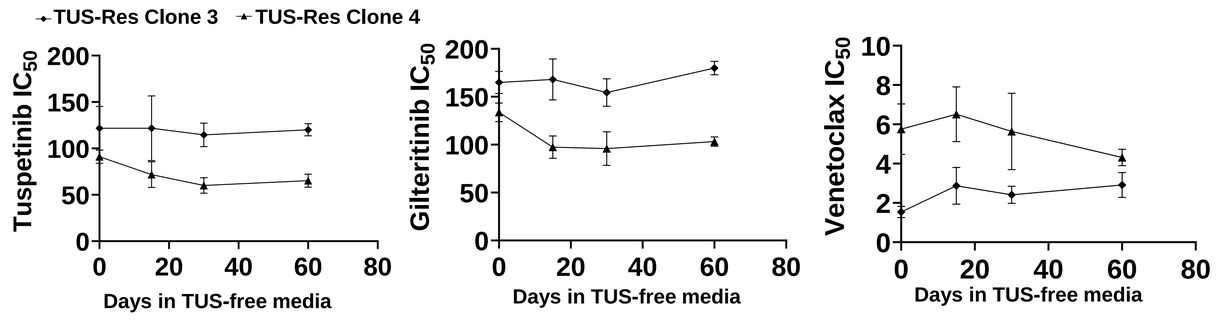
	MOLM-14 parental	TUS-Res. Clone 1	TUS-Res. Clone 2	TUS-Res. Clone 3	TUS-Res. Clone 4
Tuspetinib	2.33 ± 0.28	183.18 ± 32.76	119.92 ± 13.88	107.96 ± 11.32	144.40 ± 14.86
Venetoclax	3016.37 ± 172.91	ND	ND	1.14 ± 0.09	4.95 ± 0.38
Gilteritinib	8.58 ± 0.08	ND	ND	118.43 ± 3.99	128.07 ± 4.20
Quizartinib	0.46 ± 0.01	ND	ND	0.21 ± 0.03	0.50 ± 0.04
Brequinar	116.20 ± 1.82	ND	ND	73.67 ± 3.26	66.28 ± 2.07
Luxeptinib	0.19 ± 0.01	ND	ND	0.06 ± 0.01	0.08 ± 0.003
IMP-1088	161.61 ± 17.53	ND	ND	66.30 ± 7.08	53.03 ± 6.53
5-azacytidine	2137.67 ± 109.00	ND	ND	1615.33 ± 138.85	1580.67 ± 68.47
Zelavespib	74.98 ± 3.86	ND	ND	86.23 ± 2.30	114.07 ± 2.36
Fostamatinib	99.55. ± 10.25	ND	ND	175.65 ± 10.95	459.25 ± 28.72
Ruxolitinib	≈10000	ND	ND	≈10000	≈10000

ND- Not Determined

Stability of Tuspetinib-Resistant Phenotype

- Stability of resistance to Tuspetinib was tested by removal Tuspetinib from TUS-Res cells and then monitoring for sensitivity to Tuspetinib for 15-, 30-, and 60-days. TUS-Res clones maintained similar IC_{50} values for Tuspetinib, Gilteritinib and Venetoclax even after removal of 75nM Tuspetinib for 60 days.

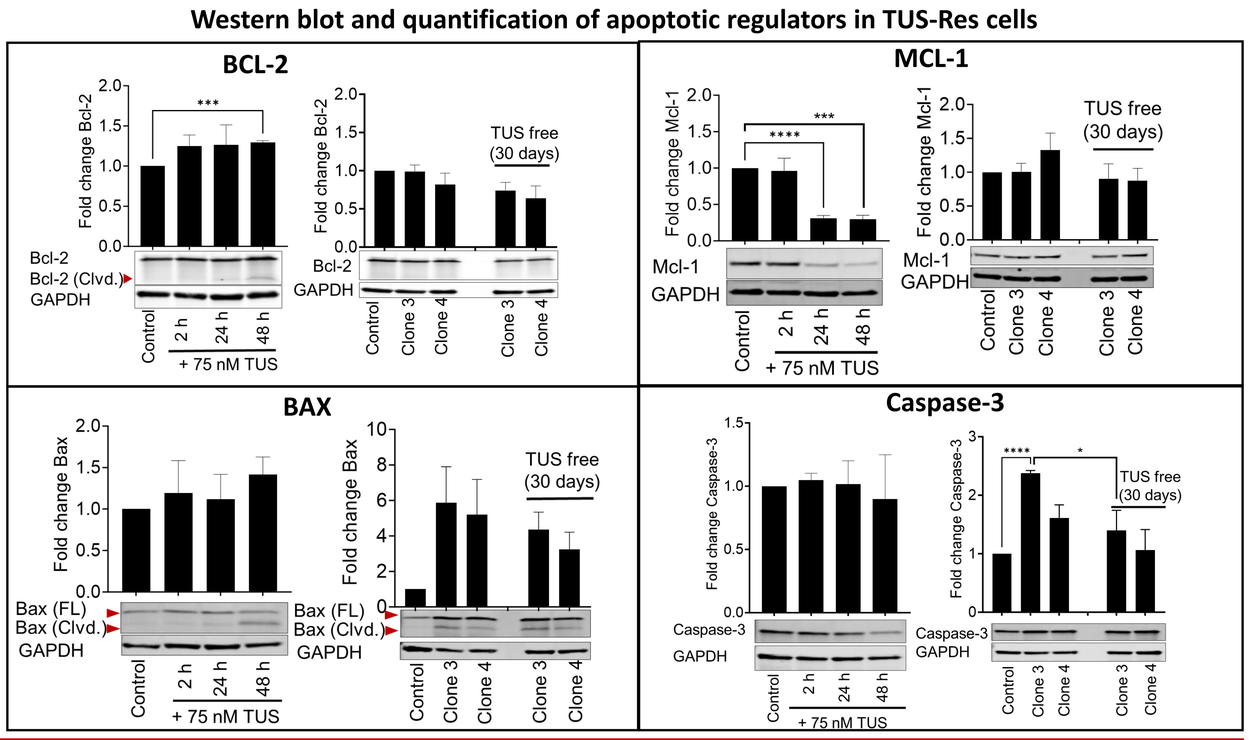
IC_{50} over 60 days following removal of Tuspetinib from growth medium of TUS-Res clones



Apoptotic Machinery in Tuspetinib-Resistant Clones

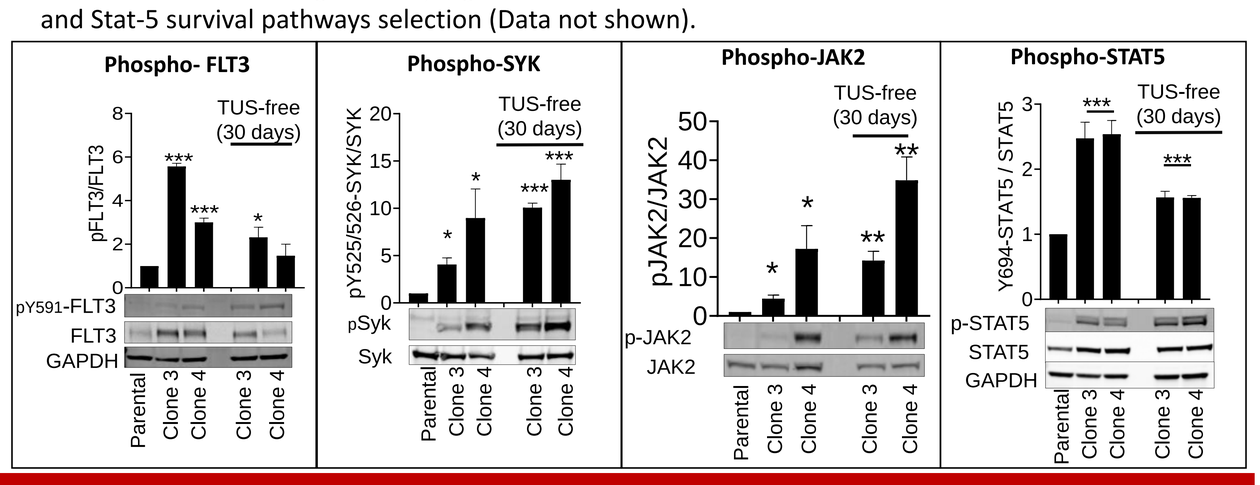
- In TUS-Res cells, the anti-apoptotic protein, Bcl-2 was marginally reduced and remained stable over 30 days in the absence of Tuspetinib, whereas Mcl-1 expression was unchanged. In contrast, parental cells showed higher Bcl-2 expression and reduction in Mcl-1 expression after 48 hr treatment of high dose Tuspetinib.
- The pro-apoptotic protein BAX was highly expressed in TUS-Res cells and retained stable expression for 30 days in the absence of Tuspetinib.

Western blot and quantification of apoptotic regulators in TUS-Res cells

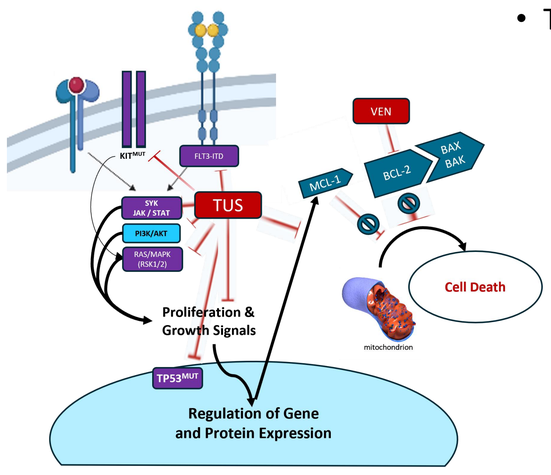


SYK-JAK-STAT5 signaling pathway is activated in Tuspetinib-Resistant Clones

- Relative to parental cells, TUS-Res cells showed an increase in phosphorylation of FLT3, JAK2, STAT5 and SYK, which persisted over 30 days after removal of Tuspetinib.
- TUS-Res cells showed significantly higher levels of total FLT3 and STAT5 which indicates FLT3-mutant and Stat-5 survival pathways selection (Data not shown).



Tuspetinib Targets Venetoclax-Resistance Mechanisms



- TUS inhibits kinase-driven abnormal signaling
  - TUS reduces MCL-1 protein expression
  - VEN continues to inhibit BCL-2 block on cell death
  - TUS/VEN combine to kill AML cells
  - TUS can deliver responses in $TP53^{MUT}$ patients

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Conclusions

- **Stable MOLM-14 Tuspetinib-resistant clones (TUS-Res) were generated**
- **TUS-Res clones showed 139 ±17-fold resistance to tuspetinib**
- **TUS-Res clones remarkably are >2000-fold more sensitive to venetoclax**
- **TUS-Res clones retain sensitivity to luxeptinib, quizartinib, IMP-1088, 5-azacytidine, zelavespib, fostamatinib, and ruxolitinib similar to that of the parental MOLM-14 clone**
- **TUS-Res clones have impaired apoptotic regulators**
  - Bcl-2 reduced | Caspase 3, BAX, and BIM upregulated | No change in Mcl-1 or PARP
- **TUS-Res clones expressed higher levels of phospho-FLT3, -STAT5, -SYK and -JAK2**
- **TUS-Res cells are hypersensitive to Venetoclax, suggesting the TUS/VEN combination may prevent the development of resistance to Tuspetinib**
- **Tuspetinib targets the resistance mechanisms of Venetoclax and the TUS/VEN combination may re-sensitize VEN-resistant AML to Venetoclax – See the ESH2023 Abstract# 6046059**

Disclosures: Current study is sponsored by Aptose Biosciences. The following authors are employees of Aptose Biosciences: R Sinha, W Rice, and R Bejar. **Contact: showell@health.ucsd.edu; rbejar@aptose.com**

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