# TUSPETINIB RETAINS NANOMOLAR POTENCY AGAINST AML CELLS ENGINEERED TO EXPRESS THE NRAS G12D MUTATION OR SELECTED FOR RESISTANCE TO VENETOCLAX

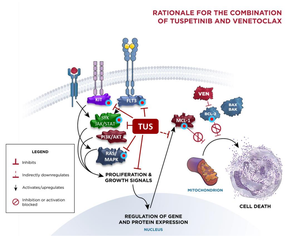
Himangshu Sonowal[1], Seung Hyun Jung[2], Joo-Yun Byun[2], Marina Konopleva[3], William Rice[4], RafaelBejar[4], Ranjeet Sinha[4], Stephen Howell MD*[1]

[1]UCSD Moores Cancer Center, San Diego, United States of America, [2]Hanmi R&D Center, Gyeonggi-do, Korea, Rep. of Korea, [3]Albert Einstein College of Medicine and Montefiore Medical Center, Bronx, United States of America, [4]Aptose Biosciences, San Diego, United States of America

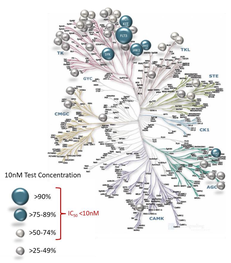
EHA EUROPEAN HEMATOLOGY ASSOCIATION
EHA2024 JUNE 13 - 16 | MADRID

## INTRODUCTION

**Tuspetinib** (HM43239; TUS) with VEN and AZA **(TUS+VEN+AZA) is being evaluated in 1st line newly diagnosed AML patients in a global Phase 1/2 trial**. TUS is a potent, once daily, oral myeloid kinase inhibitor targeting SYK, FLT3, RSK2, JAK1/2, mutant KIT, and TAK1-TAB1 kinases, involved in dysregulated cellular proliferation in AML. In preclinical AML models, TUS has exhibited superior potency compared to gilteritinib and entospletinib, both as a single agent and in combination with venetoclax (VEN) or azacitidine (AZA). In an ongoing clinical trial (NCT03850574), TUS alone and TUS combined with VEN showed excellent safety and tolerability and produced complete remissions in relapsed/refractory AML harboring a diverse array of adverse resistance mutations, including NRAS G12D. This clinical activity led us to conduct studies of how the occurrence of an NRAS G12D mutation, or the development of *in vitro* VEN resistance, affected the potency of TUS against AML cell lines.




Kinome Tree of Tuspetinib Kinase Inhibition Profile




## AIM

Investigate mechanistic changes and vulnerabilities to other inhibitors in tuspetinib-resistant (TUS-Res) cell lines relative to parental.

Investigate the potency of TUS on AML cell lines with engineered NRAS G12D mutations or with acquired venetoclax resistance.

## METHOD

**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 75 nM tuspetinib and sub-cultured at 2-3 days.

**NRAS-G12D-Res cells:** Two groups of AML cells were used in these studies: 1) Three clones of MV-4-11 cells engineered to express FLAG-tagged NRAS G12D (clones A, B, and C), 2) MV-4-11, MOLM-13 and OCI-AML2 cell selected to grow in 1 µM VEN (doi: 10.1038/s41392-021-00870-3).

**Cytotoxicity assay:** Tuspetinib was washed from TUS-Res cells to test the sensitivity of multiple inhibitors, including gilteritinib, quizartinib, ruxolitinib, fostamatinib, venetoclax, brequinar, luxeptinib, IMP-1088, 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 75 nM 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).

**Preclinical Mice model:** FLT3-ITD/ITD-positive MV-4-11 or NRAS G12D expressing clone B MV-4-11 cells were used in subcutaneous xenograft mouse models.

**Statistical Analysis:** Data reflect the mean ± SEM from 3 independent experiments and Student's t-test analysis. IC50 was calculated using a curve fitting function in Prism.

ABSTRACT : P-1756

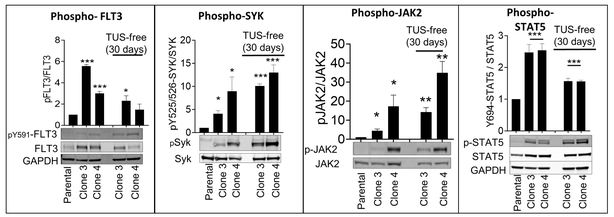
## RESULTS

### $IC_{50}$ (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** | **2.3 ± 0.28** | **183 ± 33** | **120 ± 14** | **108 ± 11** | **144 ± 15** |
| **Venetoclax** | **3016 ± 464** | ND | ND | **1.1 ± 0.09** | **5 ± 0.38** |
| **Gilteritinib** | 8.6 ± 0.08 | ND | ND | 118 ± 4 | 128 ± 4 |
| **Quizartinib** | 0.46 ± 0.01 | ND | ND | 0.21 ± 0.03 | 0.50 ± 0.04 |
| **Brequinar** | 116 ± 2 | ND | ND | 74 ± 3 | 66 ± 2 |
| **Luxeptinib** | 0.19 ± 0.01 | ND | ND | 0.06 ± 0.01 | 0.08 ± 0.003 |
| **IMP-1088** | 162 ± 18 | ND | ND | 66 ± 7 | 53 ± 7 |
| **5-azacytidine** | 2138 ± 109 | ND | ND | 1615 ± 139 | 1581 ± 68 |
| **Zelavespib** | 75 ± 4 | ND | ND | 86 ± 2 | 114 ± 2 |
| **Fostamatinib** | 100 ± 10 | ND | ND | 176 ± 11 | 459 ± 29 |
| **Ruxolitinib** | ≈10000 | ND | ND | ≈10000 | ≈10000 |

- Four clones of TUS-Res cells selected by continuous treatment of MOLM-14 cells in progressively higher concentrations of tuspetinib over 4 months.
- TUS-Res clones (maintained in 75 nM tuspetinib) were 60-fold resistant to tuspetinib on average.
- **TUS-Res clones showed 1900-fold increased sensitivity to venetoclax**
- TUS-Res clones showed 14-fold resistance to gilteritinib.
- TUS-Res clones showed slightly increased sensitivity to quizartinib and luxeptinib (not shown).

### 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 selection for FLT3 and STAT5-5 signaling pathways (Data not shown).

### Distinct levels of RAS protein were expressed in NRAS G12D clones

- NRAS G12D mutation is a strong negative prognostic indicator in R/R AML patients. NRAS G12D overexpressing MV-4-11 cell lines were engineered.
- Three clones showed differential expression; Clone 4 has the greatest expression of NRAS G12D than clones 1 and 15 which have more modest levels of NRAS G12D.

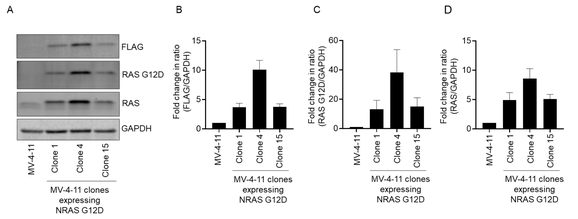



**Figure .** Western blot analysis of total cell lysates from parental MV-4-11 and clones engineered to express $NRAS^{G12D}$ containing a FLAG tag probed with anti-FLAG, anti-RAS G12D, anti-RAS and anti-GAPDH. A representative blot is shown in figure A. Quantification of band intensities from 3 blots for the indicted proteins is shown in B-D. Data is mean ± SEM.

### TUS and VEN Interaction Landscapes for Synergy

- TUS combined with VEN has an additive or synergistic effect in MV-4-11 AML cells, even those with the NRAS G12D mutation.
- TUS+VEN could be an effective treatment for AML patients with RAS mutations.

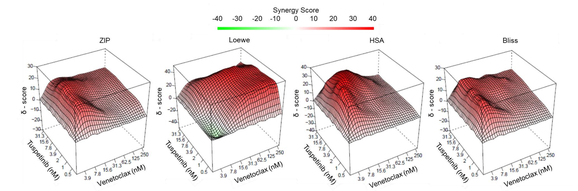



**Figure** . 3D drug interaction landscapes depicting the interaction between TUS and venetoclax in MV-4-11 cells using data averaged from 2 experiments. Table presents mean ± SEM synergy scores in different models. Note: Synergy scores <-10 indicate antagonism, scores between -10 and 10 denote additive effects, and >10 predict synergy.

| Drug combination (TUS + VEN) | Synergy Scores | | | |
|---|---|---|---|---|
| | ZIP | Loewe | HSA | Bliss |
| MV-4-11 | 12.6 ± 1.2 | 29.1 ± 1.6 | 16.1 ± 1.3 | 12.5 ± 1.3 |
| MV-4-11-NRAS G12D | 11.9 ± 0.9 | 19.9 ± 1.6 | 17.2 ± 1.3 | 11.7 ± 1.1 |

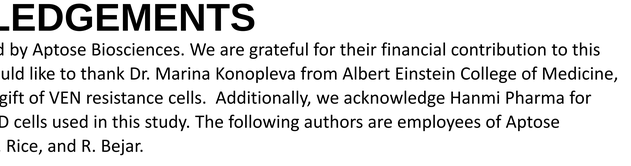
### $IC_{50}$ (nM, Mean ± SD) of Various Drugs for NRAS G12D Clones

- MV-4-11 NRAS G12D Clones A and C did not alter sensitivity to TUS or to VEN. In contrast, clone B was 7.6-fold resistant to TUS and 214-fold resistant to VEN.
- MV-4-11 NRAS G12D cells showed RAS-RAF pathway dependency and sensitive towards RAF-inhibitor.
- See table in next column.

| Drug | $IC_{50}$ (nM) | | | |
|---|---|---|---|---|
| | Parental (MV-4-11) | Clone A | Clone B | Clone C |
| **Tuspetinib** | 9.1 ± 2.3 | 8.7 ± 3.0 | 69.9 ± 7.4** | 7.7 ± 3.1 |
| Venetoclax | 10.5 ± 1.7 | 5.5 ± 1.4 | 2256.0 ± 156.2*** | 4.9 ± 1.3 |
| Belvarafenib | 750.0 ± 92.8 | 186.7 ± 38.6** | 209.3 ± 33.9** | 104.1 ± 11.1** |
| Trametinib | 6.7 ± 1.6 | 1.6 ± 0.4 | 2.5 ± 0.7 | 2.3 ± 0.8 |
| S63845 | 4.6 ± 0.4 | 1.1 ± 0.3** | 7.3 ± 1.5 | 1.5 ± 0.1** |
| AZD5991 | 32.5 ± 5.4 | 10.1 ± 3.2# | 44.3 ± 8.3 | 7.2 ± 0.4 |
| Gilteritinib | 2.4 ± 0.7 | 3.3 ± 0.7 | 51.0 ± 12.2* | 5.6 ± 1.1 |
| Quizartinib | 0.5 ± 0.1 | 1.1 ± 0.2ns | 1.9 ± 0.5ns | 1.3 ± 0.4ns |

*Data is mean ± SEM. *p<0.01, **p<0.001, #p = 0.02, ns = not significant vs parental MV-4-11 cells.

### TUS+VEN or TUS+Belvarafenib is Effective in FLT3 ITD and / or NRAS G12D Mutant Tumor Elimination in Mice




- TUS+VEN showed synergistic anti-leukemic activity in FLT3-ITD/ITD-positive MV-4-11 subcutaneous xenograft mouse models.
- TUS plus the RAF-inhibitor belvarafenib showed significant antitumor effect in a FLT3-ITD/NRAS G12D mutant MV-4-11 subcutaneous xenograft mouse model suggesting TUS could sensitize cells to RAS pathway inhibition in FLT3-ITD and NRAS G12D-mutant AML patients.

### $IC_{50}$ (nM, Mean ± SD) of TUS and Various Drugs on VEN-Resistant AML Cells

- VEN-resistant AML cell lines showed greater resistance in FLT3-ITD mutant cells compared to AML cells lines with wild type FLT3.
- VEN resistant cells carrying either wild type or mutant FLT3-ITD are sensitive to TUS and other tyrosine kinase inhibitors.
- VEN+TUS combination may help prevent the development of VEN resistance.

| Cell type | $IC_{50}$ (nM) (mean ± SEM) | | | | |
|---|---|---|---|---|---|
| | Venetoclax[1] | Tuspetinib | Gilteritinib | Midostaurin | Quizartinib |
| **MOLM-13** | 37 ± 0.019 | 3.4 ± 0.9 | 15.7 ± 1.2 | 14.5 ± 2.5 | 3.5 ± 1.5 |
| **MOLM-13 VEN/R** | 3263 ± 1.730 | 5.0 ± 0.6 | 23.6 ± 1.3* | 31.4 ± 6.2 | 5.4 ± 2.3 |
| **MV-4-11** | 138 ± 0.043 | 2.7 ± 0.3 | 2.5 ± 0.3 | 8.9 ± 1.0 | 2.5 ± 1.1 |
| **MV-4-11 VEN/R** | 13753 ± 4.422 | 2.3 ± 0.4 | 3.1 ± 0.3 | 6.9 ± 0.8 | 2.0 ± 0.9 |
| **OCI-AML-2** | 150 ± 0.053 | 751 ± 39.6 | 429 ± 52.5 | 170 ± 15.4 | 692.0 ± 32.4 |
| **OCI-AML-2 VEN/R** | 3095 ±0.779 | 824 ± 120.0 | 1061 ± 142.8* | 288 ± 20.7* | 976 ± 545.3 |

1- $IC_{50}$ reported in Zhang et al 2022; *Data is mean ± SEM. *p<0.01,

## CONCLUSIONS

1. The level of NRAS G12D mutant protein expression level is the key determinant of resistance to both TUS and VEN in the MV-4-11 FLT3-ITD model.
2. TUS exhibits additivity in cytotoxic assays in both the wild type MV-4-11 and the NRAS G12D clones, hence combination of TUS+VEN can overcome resistance due to NRAS G12D mutation.
3. TUS resistant cells showed hypersensitivity to VEN such that treatment with both drugs could interfere with the emergence of TUS resistance.
4. This study establishes a strong rationale for combining TUS with VEN to treat NRAS G12D AML in the clinical trial (NCT03850574. Abs# P4557).
5. VEN resistant cells remain highly sensitive to TUS and VEN+TUS which may help avoid the development of VEN resistance.

## ACKNOWLEDGEMENTS

This work was supported by Aptose Biosciences. We are grateful for their financial contribution to this research project. We would like to thank Dr. Marina Konopleva from Albert Einstein College of Medicine, Bronx, for her generous gift of VEN resistance cells. Additionally, we acknowledge Hanmi Pharma for providing the NRAS G12D cells used in this study. The following authors are employees of Aptose Biosciences: R. Sinha, W. Rice, and R. Bejar.

## REFERENCES

Zhang, Q., Riley-Gillis, B., Han, L., Jia, Y., Lodi, A., Zhang, H., Ganesan, S., Pan, R., Konoplev, S. N., Sweeney, S. R., Ryan, J. A., Jitkova, Y., Dunner, K., Jr, Grosskurth, S. E., Vijay, P., Ghosh, S., Lu, C., Ma, W., Kurtz, S., Ruvolo, V. R., … Konopleva, M. (2022). Activation of RAS/MAPK pathway confers MCL-1 mediated acquired resistance to BCL-2 inhibitor venetoclax in acute myeloid leukemia. Signal transduction and targeted therapy, 7(1), 51.

## CONTACT INFORMATION

Aptose Biosciences , CA, USA | Email: showell@health.ucsd.edu; rbejar@aptose.com.