AVL-292: A Targeted Therapy For Bruton's Tyrosine Kinase in B Cell Malignancies

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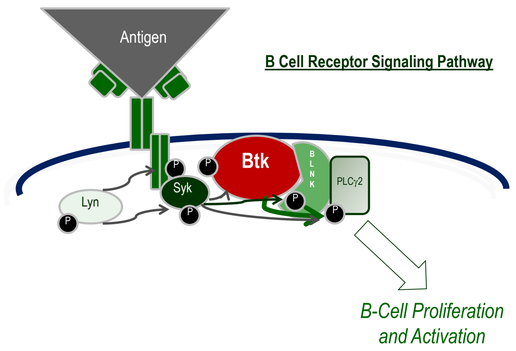
Background

Targeted therapies that suppress B cell receptor (BCR) signaling have emerged as promising agents in the treatment of B cell malignancies. Bruton's tyrosine kinase (Btk) plays a crucial role in promoting B cell proliferation and survival through participation in the BCR signaling pathway and represents a promising new drug target.



Aims

The aims of the study are to:

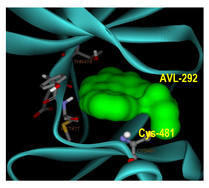
1) Demonstrate the utility of a potent and selective inhibitor of Btk in B cell malignancies
2) Investigate effects of clinical candidate, AVL-292, on the survival and BCR activation of primary CLL cells cultured with α-IgM or Nurse-like cells (NLC)
3) Determine, in a clinical setting, the minimum AVL-292 dose necessary for maximal Btk target site occupancy utilizing a novel translational medicine approach.

Methods

Patient Derived CLL Cells: After informed consent, blood samples were obtained from patients with CLL (Chronic Lymphocytic Leukemia). PBMCs ($1x10^7$ cells/ml complete RPMI media) were pre-incubated with or without inhibitors, followed by incubation with 10 μg/mL anti-IgM (polyclonal goat $F(ab')_2$ fragments to human IgM) or in co-cultures with nurse-like cells (NLC) at 37°C in 5% CO_2. To assess CLL cell viability, cells were collected after 24, 48 and 72 hours and assayed by flow cytometry by staining with $DiOC_6$ (to measure mitochondrial membrane potential) and PI (to measure cell membrane permeability). Concentrations of the chemokines CCL3 and CCL4 in CLL cell supernatants after 24 hours were analyzed by ELISA. CCL3 and CCL4 are secreted by CLL cells in response to BCR triggering and in NLC co-cultures and function as surrogate markers for BCR responsiveness (Blood 114:1029-37, 2009). Chemotaxis of CLL cells towards CXCL12 and CXCL13 was performed across polycarbonate transwell inserts. After 30 min incubation with the inhibitors and 30 min anti-IgM incubation, cells were allowed to migrate across the mesh for three hours. Then, the number of migrated cells in the lower chamber was measured by flow cytometry.
All other methods outlined in appropriate figure legends.

Why Covalent Inhibition of Btk?

- Achieve exceptional potency and selectivity
  - Difficult with traditional approaches
- Prolonged duration of action
  - Potential for lower systemic exposure
  - favorable dose frequency
- Translational medicine empowers development

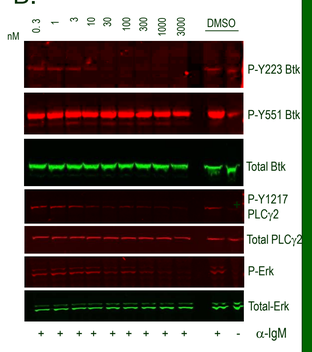


AVL-292 is a Potent, Selective Inhibitor of Btk

A.

Kinase	AVL-292 IC_{50} (nM)
Btk	<0.5
Brk	2430
c-Src	1729
Csk	>10000
Fyn	7146
Hck	14460
Lck	9079
Syk	1134
Lyn	4401
Yes	723

B.



C.

Kinase	Cell Line	AVL-292 Cellular EC_{50}
Btk	Ramos	1-10nM
EGFR	A431	>4000nM
Jak3	CTLL-2	500-1000nM
Itk	Jurkat	~1000nM

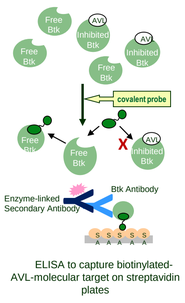
Figure 1. AVL-292 is a potent, selective inhibitor of Btk in both biochemical and cellular settings. A. AVL-292 was tested against full length recombinant Btk protein using OMNIA assay to determine the biochemical IC_{50app} value. AVL-292 was also profiled biochemically against a number of Src family kinases and B cell signaling components to demonstrate the specificity of AVL-292 for Btk in the B cell receptor signaling pathway. B. AVL-292 demonstrates dose dependent inhibition of Btk and downstream BCR signaling components in Ramos cells. Ramos cells were treated with AVL-292 for 1 hour followed by stimulation of the BCR with 5 μg/mL α-IgM for 10 minutes on ice. Cell lysates were immunoblotted for Btk autophosphorylation (Y223), PLCγ2 phosphorylation as well as activation of downstream Erk signaling. AVL-292 inhibited Btk kinase activity in a cellular setting with EC_{50} between 1-10 nM. C. AVL-292 was tested for inhibition of substrate phosphorylation in a cellular setting against several kinases with similar structural features to Btk including cysteine placement within the ATP binding pocket.

Development of a Btk Pharmacodynamic Assay: Dependence on Covalent Drug-Target Interaction

Avila Covalent Probe Assay

- **Directly measures extent and duration of target occupancy**
- **Correlates dose & response at molecular level**
- **Rationally determines optimal dose level & schedule**
- **Applicable to biological samples from a wide range of sources (cell culture, animal tissues, clinical samples)**

Biological sample exposed to AVL-292



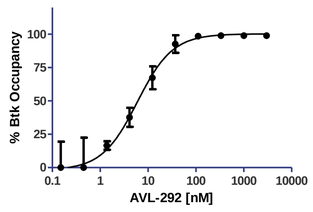
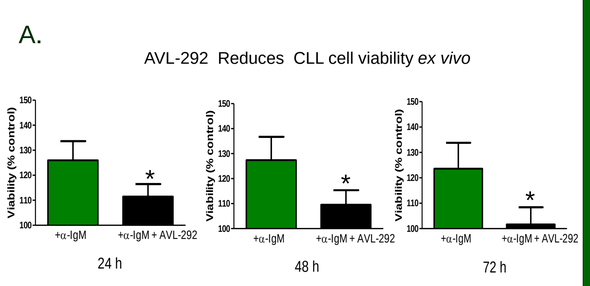


Figure 2. Covalent Probe Allows Direct Assessment of Btk Target Occupancy. The covalent mechanism of action of AVL-292 allows development of a covalent probe to detect free, uninhibited Btk in lysates derived from tissue culture, animal tissues or clinical samples. Lysates from samples previously treated with AVL-292 are incubated with the biotinylated covalent probe. Uninhibited Btk is captured by the covalent probe and quantitated by ELISA. Normalization to untreated control sample allows determination of the % Btk occupancy. B. Ramos cells were treated with increasing concentrations of AVL-292 for 1 hour (0.3 nM-3 μM as in Figure 1), lysed and percent Btk occupancy at each dose level was determined by ELISA. The concentration of AVL-292 required for 50% occupancy was 5.9 nM and correlated directly with the concentration needed for inhibition of Btk signaling in Ramos cells (EC_{50} 1-10 nM) seen in Figure 1.

AVL-292 Reduces Viability of CLL Cells Stimulated with α-IgM



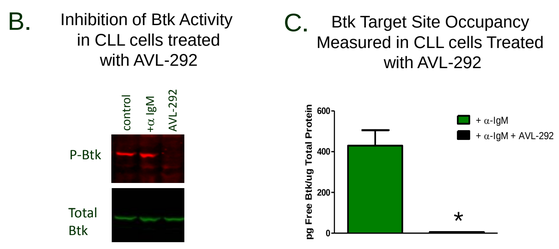
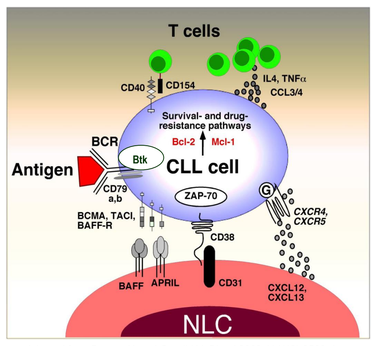


Figure 3. Abrogation of α-IgM-mediated BCR triggering and induction of apoptosis. A. Bar diagrams represent the mean relative viabilities after 24 h, 48 h, and 72 h incubation with 1 μM AVL-292 and 10 μg/mL α-IgM. Viabilities were normalized to the relative viability of control samples with medium only at the respective time points (100%), to account for differences in spontaneous apoptosis in samples from different patients. Shown are the means (+SEM) from 22 different patient samples. * indicates $p < 0.05$ (paired Student's t test). B. Immunoblot of representative CLL sample treated *ex vivo* with 1 μM AVL-292 demonstrating inhibition of Btk autophosphorylation at Y-223. C. Btk Target Site Occupancy analysis of CLL cell lysates isolated from 5 different patients demonstrate complete Btk target occupancy in CLL cell lysates treated ex vivo with 1 μM AVL-292. Plotted as the mean of 5 samples + SEM. * indicates $p<0.05$

AVL-292 Reduces Viability of CLL Cells Co-Cultured with Nurse-Like Cells (NLC)

A.



Adapted from Burger JA and Gandhi V, *Blood 2009;114(12):2560-1*

B.

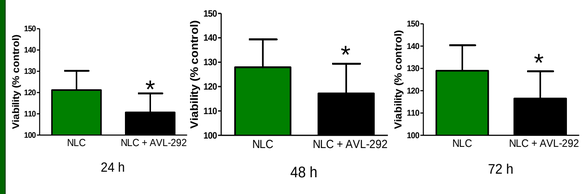


Figure 4. Abrogation of NLC-mediated protection of CLL cells and induction of apoptosis. A. Schematic adapted from Burger JA and Gandhi V, *Blood 2009;114(12):2560-1* demonstrating the interaction and support provided by nurse-like cells to foster survival of CLL cells *ex vivo* and mimic the lymph node microenvironment *in vivo*. B. Depicted are mean relative viabilities after 24 h, 48 h, and 72 h incubation with 1 μM AVL-292. Viabilities were normalized to the relative viability of control samples with medium only at the respective time points (100%), to account for differences in spontaneous apoptosis in samples from different patients. Shown are the means (+SEM) from 12 different patient samples. * indicates $p \leq 0.05$.

AVL-292 Inhibits CLL Cell Production of CCL3 and CCL4 in the Presence of Nurse-like cells (NLC)

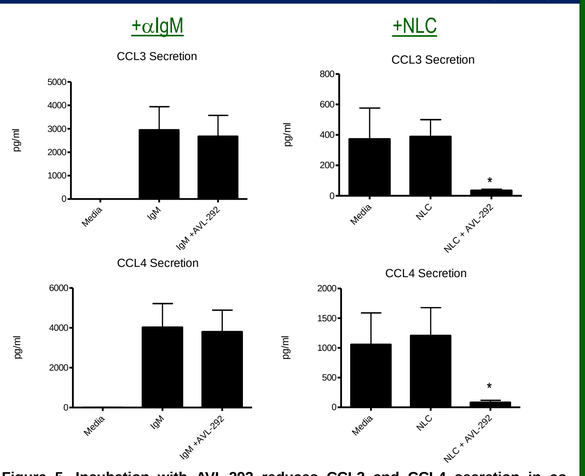


Figure 5. Incubation with AVL-292 reduces CCL3 and CCL4 secretion in co-cultures with NLC but not with α-IgM stimulation. CLL cells were incubated in media supplemented with 10 μg/mL α-IgM or co-cultured with NLC in the presence of 1 μM AVL-292. After 24 h, cell media supernatants were collected and the concentrations of secreted chemokines were determined by ELISA. When incubated with 10 μg/mL α-IgM, neither CCL3 nor CCL4 secretion were affected by the presence of AVL-292. However, when CLL cells were co-cultured with NLC, the secretion of both chemokines was significantly abrogated by 1 μM AVL-292 (CCL3: 35.4 pg/ml +/- 11.1 pg/ml in AVL-292, compared to 471.5 pg/ml +/- 160.56 pg/ml in the control; CCL4: 116.1 pg/ml +/- 52.7 pg/ml in AVL-292 compared to 1154.2 pg/ml +/- 286.8 pg/ml in the control). Displayed are mean ± SEM of 6 patient samples. * indicates $p \leq 0.05$ to the control.

AVL-292 Inhibits CXCL12- and CXCL13-induced Migration of CLL Cells

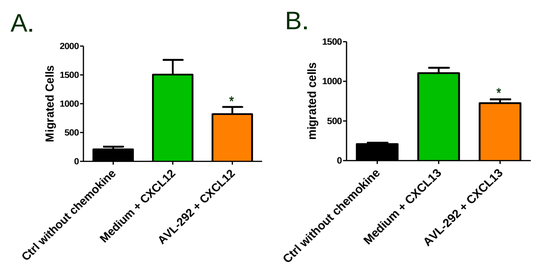


Figure 6. Incubation with AVL-292 reduces chemotaxis of CLL cells towards CXCL12 and CXCL13. (A) CLL cells were assayed in Transwell inserts for chemotaxis of CLL cells towards CXCL12 and CXCL13 after incubation for 30 min without (control) or with 1 μM AVL-292. All cells underwent BCR stimulation by α-IgM for 30 min. Incubation with AVL-292 reduced chemotaxis of CLL cells towards CXCL12 59.4% compared to control and towards CXCL13 54.0%. Displayed are mean ± SEM of 10 patient samples.* indicates $p \leq 0.05$ to the control.

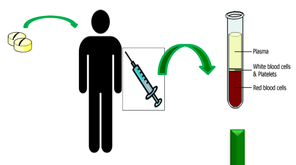
Phase 1a Clinical Trial in Healthy Normal Volunteers with AVL-292

Overview of AVL-292-001 Study Design

- **Healthy normal volunteers, 8 subjects per dose cohort (6 active, 2 placebo)**
- **Single oral dose, 5 dose cohorts: 0.5 mg/kg to 7 mg/kg**
- **Subjects monitored & assessed regularly following dose administration**
- **Data for all subjects at a given dose level reviewed for safety prior to escalation to next dose level**

Objective	Results
Safety and tolerability	• Generally safe and well tolerated • No SAEs and no apparent dose-related trends in AEs
Pharmaco-kinetics	• AVL-292 rapidly absorbed (T_{max} ~20-60 min) • C_{max} and AUC demonstrate dose-proportionality • $t_{1/2}$ ~ 2-3hr
Pharmaco-dynamics	• Maximal target occupancy goal achieved ➢ Occupancy sustained through 24hr ➢ Recovery towards pre-dose values between 48-96hr

Pharmacokinetic and Pharmacodynamic Insights Established in AVL-292 Phase 1a Clinical Trial



Analysis of subject samples for pharmacokinetics and Btk target occupancy

Provides mechanistic POC in 1st dose of First in Human study

Allows informed selection of starting dose in subsequent clinical trials to maximize patient benefit

AVL-292 Pharmacokinetics is Dose Proportional For All Cohorts

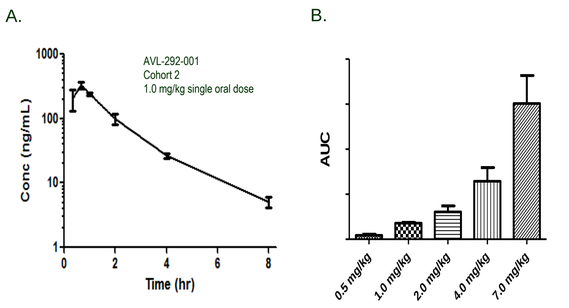


Figure 7. Human Pharmacokinetic Profile of AVL-292. A. The exposure of AVL-292 in cohort 2 (1.0 mg/kg dose, average value of 6 subjects) demonstrates the molecule is highly orally available and absorbed rapidly with Cmax occurring 20-60 minutes after dosing. As shown above, there is minimal subject-to-subject variability in AVL-292 exposure in plasma collected 15 min., 30 min., 1,2,4, or 8 hours after dosing and the $T_{1/2}$ of AVL-292 is between 2-3 hours. **B.** Comparison of the averaged AUC values across all cohorts in the Phase 1a study demonstrates linear and dose proportional exposure of AVL-292 throughout the 5 cohorts evaluated.

Complete Btk Target Occupancy Achieved With ≥ 2 mg/kg AVL-292

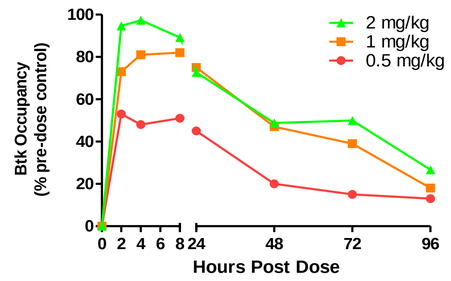


Figure 8. Maximum Btk Target Occupancy Detected with AVL-292 Doses of 2 mg/kg or Above in Human Subjects. B cells were isolated with RosetteSep B cell enrichment reagent from 22 mLs whole blood collected before compound administration and 2, 4, 8, 24, 48, 72 and 96 hours after dosing. Lysates made from the enriched B cell fraction were assayed for Btk target occupancy with the covalent probe ELISA and compared to pre-dose Btk values to determine % Btk occupancy. Btk target occupancy was detected at all dose levels and increased with dose escalation. Dose levels higher than 2 mg/kg resulted in complete Btk target occupancy at the 2-4 hour timepoints in all subjects. At 2 mg/kg, the cohort averaged >97% occupancy at 4 hours and recovery toward 50% pre-dose Btk values at 48 hours.

Uncoupling PK and PD With Covalent Btk Inhibitor AVL-292 : Confirmation in First In Human Trial

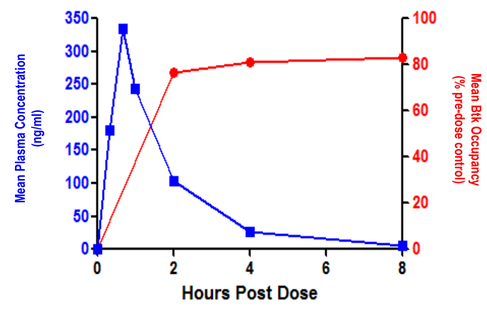


Figure 9. Pharmacokinetics and Pharmacodynamics are Uncoupled with AVL-292 in Human Subjects. The pharmacokinetic profile obtained from the 1.0 mg/kg dose (cohort 2) of AVL-292 is shown in blue and plotted with the mean Btk occupancy derived from the same subjects in red. Both pharmacokinetic and pharmacodynamic parameters are plotted against time. AVL-292 is rapidly absorbed with Tmax occuring 20 to 60 minutes after dosing. AVL-292 plasma levels decrease rapidly between 2 and 4 hours and are LLOQ at 24-48 hours. In contrast, PD measurements of Btk occupancy reach maximal levels 2-4 hours after dosing and are sustained at that level through 8 hours, despite decreasing drug levels in the plasma. In contrast to traditional reversible small molecule inhibition, the return of enzyme activity after covalent inhibition results from the re-synthesis of new Btk protein as opposed to decreasing circulating drug levels.

Conclusions

- **AVL-292 is a potent, selective, covalent inhibitor of Btk.**
- **Btk target occupancy can be used as a biomarker and assessed using a companion covalent probe with in vitro or ex vivo samples.**
- **AVL-292 reduces viability as well as markers of BCR activation, such as CCL3 and CCL4 chemokine production, in primary CLL cells cultured with Nurse-like Cells (NLC).**
- **AVL-292 reduces migration of CLL cells towards CXCL12 and CXCL13**
- **AVL-292 was safe and well tolerated in a Phase 1a Healthy Normal Volunteer trial with dose proportional exposure. Btk target occupancy was detected at all dose levels and complete occupation was demonstrated at doses ≥ 2 mg/kg.**
- **PK and PD results from Phase 1a trial establish starting dose level in subsequent clinical trials in B cell lymphomas/ CLL to maximize patient benefit.**
- **Phase 1b clinical trial with AVL-292 in B cell lymphoma /CLL will initiate June 2011.**

Acknowledgements

LEUKEMIA & LYMPHOMA SOCIETY® fighting blood cancers

AVL-292 is being developed for the treatment of B-cell leukemias and lymphomas with support from The Leukemia and Lymphoma Society