

**Abstract #412**

**The PI3K inhibitor ON 01910.Na Inhibits Critical Survival Pathways in the Tumor Microenvironment and Induces Apoptosis in CLL cells Through Induction of NOXA and BIM**

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Chronic Lymphocytic leukemia (CLL) is the most common leukemia in Western countries and clinically is a heterogeneous disease characterized by the accumulation of mature B lymphocytes. Progression is driven by a concomitant defect in both cell survival and proliferation signals coming from the tumor microenvironment through different receptors including B-cell receptors, TNF family receptors, chemokine and cytokine receptors, and cell-cell interaction. ON 01910.Na (Onconova Therapeutics) belongs to the family of styryl benzyl sulfones, a novel family of non-ATP competitive compounds that are currently under development as potential anticancer treatments. These compounds have shown activity in a variety of xenograft mouse models. Interestingly, it has recently been described that ON 01910.Na exerts potent antitumor activity against Mantle Cell Lymphoma (MCL) cells by inhibition of PI-3K/Akt/mTOR pathway and downregulation of Cyclin D1 translation.

Here, we evaluated the cytotoxic effect of ON 01910.Na after 48 hours of *in vitro* exposure on PBMCs from CLL and normal donors by flow cytometry using AnnexinV-PE. We co-stained with CD3-APC and CD19-FITC to assess the effect on B and T cells separately. ON 01910.Na induced apoptosis of the leukemic cells in all CLL samples tested (n=24), without affecting T-cell viability. Noteworthy, ON 01910.Na did not affect B and T cells from normal donors at concentrations that killed CLL cells. Thirteen CLL samples were highly sensitive towards ON 01910.Na (mean B-cell IC<sub>50</sub> 0.71 μM) in the concentration range achieved in phase I clinical trials, eight samples were sensitive (mean B-cell IC<sub>50</sub> 1.38 μM) and three showed moderate sensitivity (mean B-cell IC<sub>50</sub> 4.26 μM). In contrast, the IC<sub>50</sub> of T-cells was >8 μM in all samples. No significant differences were found in ON 01910.Na cytotoxicity against CLL expressing mutated (n=9, mean B-cell IC<sub>50</sub> 1.45 μM) or unmutated (n=15, mean B-cell IC<sub>50</sub> 1.34 μM) IgVH sequences. ON 01910.Na was also effective against cases carrying 17p deletions (n=4, mean B-cell IC<sub>50</sub> = 1.45 μM) or 11q deletions (n=3, mean B-cell IC<sub>50</sub> = 0.96 μM). ON 01910.Na activated the mitochondrial apoptosis pathway, as shown by flow cytometry using conformation specific antibodies to detect activation of Bax and Bak, leading to mitochondrial depolarization (measured by mitotracker) and caspase-3 activation. ON 01910.Na also induced the upregulation of the proapoptotic BH3-only proteins Noxa and Bim and decreased expression of Mcl-1 at 4 hours, well before the onset of apoptosis. Inhibition of Noxa expression by retroviral transfection reduced the sensitivity to drug by 60%. ON 01910.Na induced reactive oxygen species (ROS) that may contribute to Noxa induction and ROS blockade using N-acetyl-cysteine also reduced ON 01910.Na cytotoxic activity.

To further delineate the biological processes underlying ON 01910.Na induced apoptosis, we performed Gene Expression Profiling (GEP) in CLL cells treated *in vitro* for 4 and 10h with ON 01910.Na. GEP revealed induction of Noxa, ATF3 and an AP-1 gene signature that was validated by the nuclear accumulation of c-jun at 4 hours. Activation of FOXO gene signatures, which correlate with the upregulation of Bim, was also apparent. Strikingly, ON 01910.Na downregulated B-cell receptor, PI3K and NF-κB gene signatures and decreased TCL-1 mRNA expression by 50%, which was confirmed by TaqMan analysis. Consistent with inhibition of the BCR/PI3K/AKT axis, ON 01910.Na also inhibited AKT phosphorylation by 65% after *in vitro* BCR activation.

Our results identify ON 01910.Na as a promising compound for the treatment of CLL with interesting combined mechanisms of action; one being the activation of apoptotic stress signals leading to Noxa and BIM up regulation, and another being the inhibition of the BCR/PI3K/AKT pathway that blocks microenvironment-induced survival signals. These data support the development of ON 01910.Na in CLL and a clinical trial has been initiated at our institution.

**Abstract #3815**

**Initial Evaluation of a 48-h Continuous Intravenous Infusion Weekly Regimen of ON 01910.Na in Advanced Myelodysplastic Syndrome (MDS)**

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Following initial promising clinical results reported at ASH 2008 (Shenoy et al) in RAEB MDS and AML patients treated with the new investigational agent ON 01910.Na, we have initiated a phase 1/2 single arm escalating dose study in advanced MDS patients with a shorter schedule of administration of ON 01910.Na. The first 13 patients enrolled in our ongoing trial (6 high risk, 1 Intermediate-2 and 5 Intermediate-1 according to IPSS classification; 7 females/6 males, age range: 47-83ys), most of them having failed prior MDS therapies were administered ON 01910.Na as a 48 h continuous intravenous infusion weekly for 3 weeks of a 4-week cycle. Ten patients were treated at the 800 mg/m<sup>2</sup>/day dose level and 3 at the 1500 mg/m<sup>2</sup>/day dose level for 4 to 27+ weeks (1 to 6 four-week cycles). Most patients had grade 3 or 4 cytopenias at baseline. Overall the therapy was well tolerated and a few patients reported improvements of well being and pain relief. The most frequently reported adverse events were thrombocytopenia, neutropenia, anemia, fatigue and nausea. Serious adverse events were typical of this patient population and none of them was related to ON 01910.Na except for one case of neutropenia. Bone marrow blastic response was evaluated by bone marrow morphology. Five patients had a pretreatment blast count lower than 5% and no worsening of blast count was observed in the 3 patients who had follow-up bone marrow examination. Another six patients had more than 5% pretreatment bone marrow blasts. Among these patients, two had significant decreases in blast counts compared to pre-treatment values, while two showed stabilization and two progressed. Two patients had hematological improvements (neutrophil and erythroid responses). These results are encouraging and the trial is continuing.

| Patient | Bone Marrow Blasts |   | Hematological Improvement |
|---------|--------------------|---|---------------------------|
|         | Pre-Treatment      | 4-8 week Follow-up                                  |                           |
| 1       | <2%                | 2%  |                           |
| 2       | 10-13%             | Death end 1 <sup>st</sup> cycle                     |                           |
| 3       | 1-2%               | Not Done (Platelet =4)                              |                           |
| 4       | 20-30%             | 30%   | N wk 10-18                |
| 5       | <5%                | 3%  |                           |
| 6       | 3%                 | Death end 2 <sup>nd</sup> cycle                     |                           |
| 7       | <5%                | 1-2%  |                           |
| 8       | 25%                | 11%   |                           |
| 9       | 10-13%             | 15%*  | E wk 1-13                 |
| 10      | 35%                | Discontinued mid 2 <sup>nd</sup> cycle (transplant) |                           |
| 11      | 9%                 | 5%  |                           |
| 12      | 10%                | 2%  |                           |
| 13      | 15%                | 20%   |                           |

\* 16 week Follow-up: 25% blasts

Abstract #120

**ON 01910.Na Suppresses Cyclin D1 Accumulation in Trisomy 8 Myelodysplastic Syndromes Patients While Decreasing Bone Marrow CD34+ Blast Counts and Aneuploid Clone Size**

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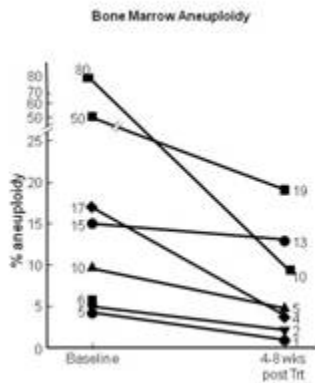
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Patients with high risk MDS can be successfully treated with 5-azacytidine, or with lenalidomide but non-responding patients have few treatment options. Chemotherapy produces significant morbidity and very short remissions and most patients are too old for bone marrow transplantation. We previously demonstrated up-regulation of c-myc, survivin, and cyclin D1 in CD34+ cells in patients with trisomy 8 (and selected patients with monosomy 7). siRNA-mediated knockdown of survivin or c-myc decreased trisomy 8 cell growth in vitro (Sloand et al, Blood 2007, 110: 822). We postulated that increased cyclin D1 causes upregulation of survivin, resulting in resistance of these cells to apoptosis. The styryl sulfone, ON 01910.Na, decreases cyclin D1 accumulation in cultured bone marrow from patients with high risk trisomy 8 MDS and in some monosomy 7 patients (who also show upregulation of cyclin D1), while selectively decreasing blasts and aneuploidy with this cytogenetic abnormality (ASH Abstracts Nov 2008; **112**: 1651). Here we examine the clinical response to ON 01910.Na in an ongoing phase I/II clinical trial in which 13 evaluable patients with intermediate-1(int-1) to high risk MDS and treatment-refractory trisomy 8 AML were enrolled. Patients were treated with escalating doses of ON 01910.Na at 800 mg/m<sup>2</sup> x 2 days every 3/4 weeks, 800 mg/m<sup>2</sup> x 3 days every 2 weeks, 800 mg/m<sup>2</sup> x 5 days every 2 weeks, and 1500 mg/m<sup>2</sup> x 2 days every 3/4 weeks at two institutions. No significant toxicity could be ascribed to the drug. Patients with trisomy 8 and monosomy 7 demonstrated significant declines in aneuploidy measured by fluorescence in situ hybridization (FISH) (mean aneuploidy; 50% before and 24% after 1 cycle of treatment; p=0.02; Fig below). Rather than becoming cytopenic, many patients showed substantial improvements of blood counts and one patient (01-02; graphic shown below) became red cell transfusion-independent and maintains his remission 14 months after stopping therapy. Cyclin D1 measurement by flow cytometry showed decreases of this protein in both CD34 and CD33 cells during infusion of ON 1910 infusion (example shown in Fig below). Results from individual evaluable patients are shown in **Table 1**. These results indicate that modulation of cell cycle control by cyclin D1 may represent a novel targeted approach for trisomy 8 and monosomy 7 MDS.

| Table 1<br>Protocol | PID    | ON 01910.Na<br>Dosage<br>mg/m <sup>2</sup> /24h | IPSS<br>Pre-Trt | Weeks<br>on<br>study | Cytogenetics | Prior<br>5-Aza | % Blasts BM |                   | HI  | Survival<br>weeks |
|---------------------|--------|---|-----------------|----------------------|--------------|----------------|-------------|-------------------|-----|-------------------|
|                     |        |   |                 |                      |              |                | Pre-<br>Rx  | Week 4-<br>8 F-Up |     |                   |
| 07-H-0225           | 01-01  | 800/72h Q2W                                     | High            | 10                   | +8, -7, 5q-  | Yes            | 17          | 4                 | N,P | 19                |
|                     | 01-02  | 800/72h Q2W                                     | High            | 16                   | -7           | No             | 5           | 2                 | E   | 56+               |
|                     | 01-03  | 800/72h Q2W                                     | High            | 8                    | -7, +8       | No             | 10          | 5                 | N   | 52+               |
|                     | 02-01  | 800/120h Q2W                                    | High            | 6                    | +8           | No             | 50          | 19                | P,E | 26                |
|                     | 02-02* | 800/120h Q2W                                    | High            | 5                    | +8/Complex   | Yes            | 15          | 13                |     | 33                |
|                     | 02-03  | 800/120h Q1W                                    | AML             | 2                    | +8           | No             | 80          | 10                |     | 34                |
|                     | 02-04  | 800/120h Q2W                                    | High            | 14                   | t (3,21)     | No             | 5           | 1                 | N   | 21+               |
|                     | 02-05  | 800/120h Q2W                                    | AML             | 2                    | +8 complex   | No             | 30          | ND                |     | 2                 |
|                     | 02-06  | 800/120h Q2W                                    | High            | 4+                   | +11          | No             | 6           | 62                |     | 4+                |
| 04-15               | 01-04  | 800/48h 3/4 wks                                 | High            | 19+                  | +14          | Yes            | 25          | 30                | N   | 19+               |
|                     | 01-08  | 800/48h 3/4 wks                                 | High            | 17+                  | Complex      | No             | 25          | 11                |     | 17+               |
|                     | 01-09  | 800/48h 3/4 wks                                 | Int-1           | 17+                  | Normal       | No             | 12          | 15                | E   | 17+               |
|                     | 01-11  | 1500/48h 3/4 wks                                | Int-2           | 15+                  | 5q-/7 abn    | No             | 9           | 5                 |     | 15+               |
|                     | 01-12  | 1500/48h 3/4 wks                                | Int-1           | 15+                  | ND           | No             | 10          | 2                 |     | 15+               |
|                     | 01-13  | 1500/48h 3/4 wks                                | Int-2           | 10                   | +8/+13       | Yes            | 15          | 20                |     | 13                |

BM=Bone Marrow; h=hours; wks=weeks; Q2W=every other week; SD=Stable disease; PD=Progressive disease; HI=Hematological Improvement (P=Platelet; E=Erythroid; N=Neutrophil); ND not done

\*Patient did not complete two infusions because of access problems



**Abstract #3827**

**Single Cell Network Profiling (SCNP) to Evaluate the Mechanism of Action of ON 01910.Na, a Novel Clinical Trial Stage Compound**

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**Background:** ON 01910.Na, a small molecule multikinase inhibitor, promotes G2/M arrest and apoptosis. Key targets for this inhibitor include Plk1 (polo-like kinase, a cell cycle regulator), Cdk1, (cyclin dependent kinase, a mitotic regulator) and the PI-3 kinase pathway (Ramana Reddy et al. J. Med. Chem. 2008, Park et al, Oncogene, 2007, Gumireddy et al., Cancer Cell, 2005). The drug has been shown to have anti-tumor activity in in vitro and in vivo models. Phase I studies in >100 advanced cancer patients revealed that the drug is well tolerated. Further, in several ongoing Phase 1 clinical trials in patients with myelodysplastic syndromes (MDS), positive effects on hematological indicators have been noted (Sloand et al, ASH 2008). Based on these data, a Phase 2 single-arm study is in progress to assess the efficacy and safety of the drug in IPSS Intermediate-2 and High risk MDS patients. Single Cell Network Profiling (SCNP) using flow cytometry is a platform that measures multiple fluorescent parameters (up to 10) in each cell, including both surface markers and intracellular signaling proteins in response to extracellular network inputs. By simultaneously measuring the effects of drug exposure on several pathways within each cell type in a heterogeneous patient tissue sample, valuable data can be gained about drug interactions with specific cellular pathways and cell type selectivity. This information has potential implications for dose/schedule optimization and development of patient stratification biomarkers.

**Objectives:** Studies were designed to evaluate the in vitro effects of ON 01910.Na, at clinically relevant concentrations, on intracellular pathways in the human GM-CSF-dependent erythroblastic TF-1 cell line using SCNP in order to monitor transitional changes in the cell cycle, with a focus on the G2-M phase and to perform dose-dependent titrations of drug using these cell cycle readouts.

**Methods:** The reagents chosen to measure cell cycle readouts were fluorochrome-conjugated antibodies that recognize cyclin B1, p-histone H3(S28) and p-Cdk1(Y15) and 4'6'-diamino-2-phenylindole (DAPI), a fluorescent dye that binds strongly to DNA. The phosphorylation status of p-histone H3(S28) and p-Cdk1(Y15), and the level of cyclin B1 expression are all determinants of the G2-M and/or M phase of the cell cycle. Dose dependent titrations of ON 01910.Na and its inactive analog ON 01911 were performed over a dose range starting at 10<sup>-5</sup> M and decreasing to 10<sup>-10</sup> M (dose range which includes pharmacologically achievable concentrations in humans) with 3-fold serial dilutions for eleven points after an exposure to the drug for either 24 or 48 hrs. Cells were processed for multiparameter flow cytometry by fixation, permeabilization and incubation with fluorochrome-conjugated antibodies.

**Results:** The data showed that at 24 hours after ON 01910.Na exposure there was a simultaneous increase in phosphorylation of histone H3(S28), a decrease in phosphorylation of Cdk-1(Y15), and accumulation of cyclin B1. These data suggest that ON 01910 exposure disrupted the G2/M cell cycle transition leading to mitotic arrest with subsequent apoptosis. TF-1 cell DNA content measured by DAPI verified this to be the case as increases in G2/M and sub-G1 (a measure of apoptotic cell death) were simultaneously observed. No significant effects on G2/M targets were observed when TF-1 cells were exposed to ON 01911, indicating the effects of ON 01910.Na on the cell cycle were specific to the drug. Maximal effects of ON-01910.Na on cell cycle signaling molecules were observed at a drug concentration of 0.37 mM and no further changes were seen at higher concentrations. These effects were also observed at 48 hours, although with more cell death.

**Conclusions:** These data indicate that intracellular phosphorylation changes of histone H3(S28) and Cdk-1(Y15), in addition to accumulation of cyclin B1 with subsequent apoptosis, reflect possible mechanisms of action of ON 01910.Na. The assay will be used in ongoing clinical trials to measure the pharmacodynamic activity of the drug in MDS patient samples pre- and post-treatment.