MLN0905, A Small Molecule PLK1 Inhibitor, Induces Anti-Tumor Responses in Human Models of Diffuse Large B-cell Lymphoma*

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Abbreviations: QD = dosing once/day; AUC = area under the curve; DAPI = 4',6-diamidino-2-phenylindole; PLK = polo-like kinase; MTD = maximum tolerated dose; s.c. = subcutaneous; LD50 = lethal dose 50%; IC50 = inhibitory concentration 50%; EC50 = effective concentration 50%.

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is the most common of the non-Hodgkin lymphomas, accounting for up to 30 percent of all newly diagnosed lymphoma cases. Current treatment options for this disease are effective, but not always curative; therefore experimental therapies continue to be investigated. We have discovered an experimental, potent and selective small molecule inhibitor of PLK1, MLN0905, which inhibits cell proliferation in a broad range of human tumor cells including DLBCL cell lines. In our report, we explored the pharmacokinetic, pharmacodynamic, and anti-tumor properties of MLN0905 in DLBCL xenograft models grown in mice. These studies indicate MLN0905 modulates the pharmacodynamic biomarker phospho-Histone H3 (pHisH3) in tumor tissue. The anti-tumor activity of MLN0905 was evaluated in three human subcutaneous DLBCL xenograft models, OCI LY-10, OCI LY-19, and PHTX-22L (primary lymphoma). In each model, MLN0905 yielded significant anti-tumor activity on both a continuous (daily) and intermittent dosing schedule, underscoring dosing flexibility. The anti-tumor activity of MLN0905 was also evaluated in a disseminated xenograft (OCI LY-19) model to better mimic human DLBCL disease. In the disseminated model, MLN0905 induced a highly significant survival advantage. Finally, MLN0905 was combined with a standard of care agent, Rituximab, in the disseminated OCI LY-19 xenograft model. Combining Rituximab and MLN0905 provided both a synergistic anti-tumor effect and a synergistic survival advantage. Our findings indicate that PLK1 inhibition leads to pharmacodynamic pHisH3 modulation and significant anti-tumor activity in multiple DLBCL models. These data strongly suggest evaluating PLK1 inhibitors as DLBCL anti-cancer agents in the clinic.
Introduction

DLBCL is the most common type of non-Hodgkins lymphoma (1), accounting for up to 30 percent of all newly diagnosed non-Hodgkins cases (2). DLBCL is an aggressive fast-growing cancer which arises in either lymph nodes or outside the lymphatic system, in gastrointestinal tract, testes, thyroid, skin, breast, bone or brain (2). This disease is more common in men (2) and the median age of diagnosis in both men and women is mid-60’s (3). Unfortunately, 40-50% of the patients diagnosed with DLBCL have extra-nodal disease at the time of diagnosis (4). Current treatment options for DLBCL include CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) +/- Rituxan, radiation, and bone marrow or stem cell transplantation (5). These treatment options can be effective but are not always curative (6); therefore, targeted investigational treatment options are being evaluated to improve clinical outcome.

Polo-like Kinase 1 (PLK1) is a serine/threonine protein kinase that plays a key role in cell cycle control (7). This mitotic kinase controls entry into and progression through mitosis at multiple stages by regulation of centrosome maturation, activation of initiating factors, degradation of inhibitory components, chromosome condensation, and exit from mitosis (reviewed in (8)). PLK1 is reported to be over-expressed in numerous cancers including: melanoma, prostate, ovarian, colorectal, pancreatic, non-small cell lung, esophageal, endometrial, glioma, squamous cell carcinoma of the head and neck, and non-Hodgkins lymphoma (reviewed in (8)). Moreover, over-expression of PLK1 in DLBCL is linked to poor patient prognosis (9).
We have discovered a highly potent small molecule inhibitor of PLK1 (MLN0905) which inhibits PLK1 enzyme activity in vitro using a final concentration of 5 nM (IC50 value) (10). In a cell based assay measuring phosphorylation of a direct PLK1 substrate, CDc25C-T96, MLN0905 was shown to be a highly potent PLK1 inhibitor (EC50=29 nM) (10). In addition, tissue culture experiments demonstrate cells treated with MLN0905 undergo a strong mitotic arrest followed by subsequent apoptosis (10), phenotypes consistently demonstrated to be associated with PLK1 inhibition (using both small molecule and genetic knockdown approaches) (11-14). Tissue culture cell viability experiments demonstrate MLN0905 yields LD50 values in the double digit nanomolar range for solid tumor cell lines (10). In vivo, MLN0905 demonstrates significant anti-tumor activity in an HT-29 solid tumor model of human cancer (10). In the current report, we evaluated the pharmacokinetic, pharmacodynamic, and anti-tumor properties of MLN0905 in mouse models harboring human DLBCL disease. Our findings indicate MLN0905 displays drug like pharmacokinetic properties, modulates an in vivo pharmacodynamic marker, and yields significant anti-tumor activity in multiple models of DLBCL. To our knowledge, this is the first time a small molecule PLK1 inhibitor has been evaluated in pre-clinical models of DLBCL.

**Materials and Methods**

**Cell culture and reagents.** HT-29, Daudi, Pfeiffer, and Ramos cells were obtained from ATCC. All cell lines used were passaged for less than 6 months after receipt or resuscitation (no further authentication was performed). HT29 cells were cultured in McCoy’s 5A media supplemented with 10% FBS. Daudi, Pfeiffer, and Ramos cells were cultured RPMI 1640 supplemented with 5% FBS. WSU cells were obtained from Wayne
State and cultured in RPMI1640 with 10% FBS and 1% L-glutamine. The OCI LY-19 and OCI LY-10 cells were a kind gift from Lou Staudt (National Cancer Institute, Bethesda, MD). OCI LY-10 cells were cultured in IMDM medium supplemented with 10% FBS, 1% L-glutamine, and 55 µM Betamercaptoethanol. Generation of the OCI LY-19-Luc cells was previously described (15); and cells were cultured in the presence of 1 mg/ml G418.

**RNAi experiments.** Cells (4 x 10⁴) were transfected as previously described (16). The PLK1 sequence used was as follows: sense, 5’CCGAGUUAUUCAUCGAGAC3’.

**Immunofluorescent staining in tissue culture.** Methodology was as previously described (16). Antibodies used were anti-pH3 (Cell Signaling), anti-Tubulin (Sigma), and secondary antibodies included the AlexaFluor series (Invitrogen). Cells were counter-stained with Hoechst # 33342 (Invitrogen).

**Western blotting.** Floating and adherent cells were harvested as previously described (17). Antibodies used included: anti-beta-actin (Abcam Inc), anti-Plk1 (Invitrogen), and goat anti-mouse (IgG)-horseradish peroxidase (HRP) (Santa Cruz).

**ATPlite assay.** As previously described (15).

**In vivo efficacy studies.** The OCI-LY10 and OCI-LY19 Luc models were previously described (15). The PHTX-22L primary lymphoma tumor was obtained from the Cooperative Human Tissue Network, University of Pennsylvania Medical Center, Philadelphia, PA. PHTX-22L tumor fragments were implanted into the dorsal flank of female NOD SCID mice (Taconic Farms) (15).

**In Vivo Efficacy Statistical Analysis with MLN0905 used as a single agent.**
Statistical methodology used to compare MLN0905 treatment to control has been previously described (10). Efficacy study p-values shown refer to dAUC comparisons.

**In Vivo Efficacy Statistical Analysis when combining MLN0905 with Rituximab.**

*Analysis during the treatment phase:* Photon flux area under the curve (AUC) from each animal was calculated from days 0-22. Let AUC$_i$ be the transformed photon flux level for the $i$th mouse, $i=1,2,3,…, 40$. Then the mean of AUC$_i$ is modeled as

$$AUC_i = \beta_0 + \beta_1 D_1 + \beta_2 D_2 + \beta_3 D_1 D_2$$

and the AUC$_i$ is assumed to be normally distributed and independent across mice.

The following hypothesis was tested $H_0: \beta_3 \geq 0$ versus $H_1: \beta_3 < 0$. Rejecting the null hypothesis $H_0$ indicates that the effects of Rituximab and MLN0905 are synergistic.

*Survival analysis:* The survival rates of the animals in each treatment group were plotted using Kaplan-Meier curves and a log rank test was used to compare the survival rates among treatment group to the vehicle control group. For the synergy analysis the median (50%) survival time = MST was used as benchmark. The following equation was used to determine synergy: $\beta = \text{MST (combination of drugs A and B)} - \text{MST (vehicle)} - (\text{MST (drug A)} - \text{MST (vehicle)}) + (\text{MST (drug B)} - \text{MST (vehicle)}$. If $\beta$ is significantly greater than 0, the effects of drug A and B are synergistic. The p-value (for one-sided null hypothesis) is calculated based on bootstrap re-sampling. It is correct only if we assume that the distribution for the estimator of $\beta$ under alternative hypothesis is a location shift of the distribution for the estimator of $\beta$ under null hypothesis. 95% confidence lower bounds for $\beta$ are calculated based on $B=10000$ bootstrap re-sampling of time to event data within each group.
In vivo immunohistochemistry. OCI LY-19-Luc subcutaneous tumors were processed, stained, and quantified as previously described (17). One tissue section/animal (n=3 animals/group) was used for the analysis.

MLN0905 pharmacokinetic analysis. MLN0905 was formulated and analyzed as previously described (10).

Results

In cells, MLN0905 phenocopies the affects of PLK1 knock-down using RNAi.

Previous studies indicate that PLK1 inhibition, using both small molecule and genetic RNAi knock-down approaches, lead to mitotic arrest, monopolor spindle formation, and subsequent cell death (11) (18). Here we treated HT-29 cells with a small molecule PLK1 inhibitor, MLN0905 (structure shown in Figure 1A), or PLK1 RNAi, and evaluated cells for mitotic arrest, i.e. pHisH3, and monopolar spindle formation. Targeting PLK1 with RNAi efficiently reduced PLK1 protein levels in HT29 cells (Figure 1B). Both RNAi and MLN0905 treatment yielded strong mitotic arrest characterized by robust pHisH3 staining (Figure 1C). This data is consistent with a previous report showing a robust dose-dependent pHisH3 increase in HT29 cells treated with MLN0905 (10). In addition, both RNAi and MLN0905 treatments yielded monopolar spindle formation. Monopolar spindle formation and mitotic arrest are phenotypes consistently shown to be associated with PLK1 inhibition (11) (18).
Evaluating cell viability in DLBCL cells treated with MLN0905. MLN0905 has been shown to reduce cell viability in solid tumor cells with LD50 values in the double digit nanomolar range (10). In the current report we treated a panel of human lymphoma cells with MLN0905 and evaluated cell viability at 72 hours using the ATPlite assay. Supplemental Table S1 indicates that lymphoma cell lines are highly sensitive to MLN0905 treatment with IC50 values between 3-24 nM.

Evaluating plasma and tumor pharmacokinetics following oral administration of MLN0905 into CB17-SCID mice bearing OCI LY-19 Luc xenograft tumors. To determine the pharmacokinetic paramaters of MLN0905 in vivo, CB17-SCID mice bearing OCI LY-19 Luc xenograft tumors were treated orally with increasing doses of MLN0905; and both plasma and tumor tissues were harvested and analyzed for MLN0905 exposure. After a single 1.6, 3.12, and 6.25 mg/kg dose, MLN0905 had plasma and tumor exposures (Supplemental Figure S1 and Table S2) which were greater than dose proportional, especially at the 6.25 mg/kg dose level. MLN0905 was rapidly distributed to tumor with a tumor-to-plasma AUC0-24hr of 2.1 to 2.9 across all dose levels. The compound showed reasonable retention in tumor with tumor half-life ranging from 4.5 to 6.9 hours.

Pharmacodynamic response of phosphorylated histone H3 (pHisH3) in OCI LY-19-Luc xenograft tumors following oral administration of MLN0905. Phosphorylated histone H3 (pHisH3 [ser 10]) is a biomarker routinely used to identify cells arrested in mitosis. Here pHisH3 was used as a downstream pathway biomarker to identify the percentage of cells arrested in mitosis following PLK1 inhibition. First we treated OCI
LY-19-Luc (and OCI LY-10) cells with MLN0905 in tissue culture and then evaluated pHisH3 levels using western blotting. Supplemental Figure S2 shows that MLN0905 leads to an increase of pHisH3 protein levels in the tissue culture setting. Next we wanted to evaluate the pHisH3 levels in vivo using animals bearing subcutaneous (s.c.) OCI LY-19 Luc xenograft tumors. Animals were given a single oral dose of MLN0905 using 1.6, 3.12, and 6.25 mg/kg, and tumors were harvested for pHisH3 analysis at 0, 1, 2, 4, 8, 24, 48, and 72 hours. Immunofluorescent staining was used to determine the percent tumor area staining positive for pHisH3 (Figure 2). A significant increase in pHisH3 staining (over control) was observed in the 3.12 and 6.25 mg/kg doses, which peaked at 8 hours with a 2.3-fold increase (p<0.05) using the 3.12 mg/kg dose and a 3.7-fold increase (p<0.05) using the 6.25 mg/kg dose.

**Evaluating the anti-tumor activity of MLN0905 in a subcutaneous OCI LY-19-Luc lymphoma xenograft model.** To determine the anti-tumor activity of MLN0905 in a model of human lymphoma, OCI LY19-Luc cells were grown as a s.c. xenograft tumor in SCID mice. Tolerability experiments in SCID mice determined the maximum tolerated MLN0905 dose (MTD) on the QD (daily) schedule to be 6.25 mg/kg and on the QDx3/week (3-days on/4-days off) schedule to be 14.5 mg/kg. Female SCID mice bearing s.c. OCI LY19-Luc tumors were treated orally with MLN0905 on a QDx3/week schedule using 10 and 14.5 mg/kg or on a QD schedule using 3.12 and 6.25 mg/kg. Animals were dosed for 20 days and the anti-tumor effect was calculated using the following two analyses: 1) a T/C analysis- which calculates the mean tumor volume of the treatment group divided by the mean tumor volume of the control group, and 2) an area under the tumor growth curve analysis (dAUC)- which accounts for tumor volume
over all treatment days. Using these two analyses, a significant anti-tumor effect was observed (Supplemental Figure S3) in all MLN0905 treated groups as follows: 3.12 mg/kg QD (T/C=0.57, dAUC p<0.001), 6.25 mg/kg QD (T/C=0.15, dAUC p<0.001), 10 mg/kg QDx3/week (T/C=0.34, dAUC p<0.001), and 14.5 mg/kg QDx3/week (T/C=0.26, dAUC p<0.001). An anti-tumor dose response was observed for the QD schedule but not for the QDx3/week schedule, likely due to efficacy saturation in the latter. All doses and schedules were well-tolerated with mean body weight loss less than 5% (data not shown).

Within this efficacy study the anti-tumor effect associated with dosing schedule (intermittent vs continuous) was compared. Comparing the anti-tumor effect of daily (QD) dosing to the 3-on/4-off (QDx3/week) schedule was possible because the same amount of drug was delivered (44 mg/kg) per week using the 6.25 mg/kg QD and 14.5 mg/kg QDx3/week groups. No significant anti-tumor difference was observed when comparing these two groups, using the dAUC analysis, suggesting that the anti-tumor effect was independent of schedule and driven by the total dose administered.

**Evaluating the anti-tumor activity of MLN0905 in a disseminated OCI LY-19-Luc lymphoma xenograft model.** To better mimic lymphoma disease in humans, a disseminated lymphoma model was used to evaluate the anti-tumor effect associated with MLN0905 treatment. To generate this model, OCI LY-19-Luc cells were inoculated into the tail vein of female SCID mice. The OCI LY-19-Luc cells constitutively express a luciferase reporter, allowing photon flux detection (following luciferin injection) with Xenogen imaging. Throughout the study animals were imaged once/week to determine tumor burden. Cancer hotspots were detected in femur, spine, and thymus (see vehicle treated Figure 3B), thereby mimicking lymphoma disease in humans. Once tumor burden
reached an average photon flux of 1.8x10^6 units (10 days post-inoculation), animals were treated daily (QD) for 21 days with MLN0905 (1.6, 3.12, and 6.25 mg/kg). Anti-tumor activity was assessed by calculating T/C values on day 20 and by calculating tumor growth curve AUC values during treatment. Significant (p<0.001, a Log-rank Mantel-Cox test) anti-tumor activity was observed using the medium and high dose levels of MLN0905 (Figure 3A; 3.12 mg/kg, T/C=0.31, dAUC=32.9; 6.25 mg/kg, T/C=0.02, dAUC=65.9). In the 6.25 mg/kg group, photon flux was almost undetectable on day 20 (Figure 3B). When comparing the anti-tumor effect of MLN0905 in the subcutaneous (Supplemental Figure S3) vs disseminated LY19-Luc model, MLN0905 yielded a better anti-tumor activity in the disseminated model. All treatments in this study were well-tolerated with no body weight loss.

To determine if MLN0905 treatment provided a survival advantage, animals were monitored for advanced disease (paralysis), a pre-defined humane endpoint, for 110 days after treatment initiation. A Kaplan-Meier graph (Figure 3C) summarizes mouse survival throughout the study. All vehicle treated mice reached paralysis and were euthanized between days 26 and 37 (median=26.5). Similarly, mice receiving 1.6 mg/kg of MLN0905 reached the pre-defined endpoint between days 26 and 35 days (median=33 days). Medium survival for animals treated with either 3.12 mg/kg (42 days) or 6.25 mg/kg (60.5 days) were significantly longer (p<0.0001) than that in the vehicle group (26.5 days). The MLN0905 survival advantage correlated nicely with PD response (Figure 2), suggesting the emergence of PD/Efficacy relationship.

**Evaluating the anti-tumor activity of MLN0905 in a subcutaneous OCI LY-10 lymphoma xenograft model.** To evaluate the anti-tumor effect of MLN0905 in a second
model of human lymphoma the OCI LY-10 subcutaneous lymphoma model was used. Female CB-17 SCID mice bearing OCI LY-10 tumors were treated orally with MLN0905 on a QD schedule using 1.6, 3.12, and 6.25 mg/kg, and on a QDx3/week schedule using 10 mg/kg for three weeks. The anti-tumor effect was evaluated on and up to day 20 (dosing was initiated on day 0, see Supplemental Figure S4A). In this model, a significant (p<0.001) anti-tumor effect was observed using all doses and schedules (1.6 mg/kg, T/C=0.73, dAUC=26.2; 3.12 mg/kg T/C=0.39, dAUC=80; 6.25 mg/kg T/C=0.01, dAUC=391.1; and 10 mg/kg T/C=0.06, dAUC=292.9). On the QD schedule, anti-tumor effect was dose dependent and well-tolerated (Supplemental Figure S4B). In the 6.25 mg/kg QD group, 10 of 10 tumors were undetectable (caliper measurement) on day 24. Eight of these tumors reformed between days 27 and 63, and the other two tumors in the group did not return. The study was terminated on day 125. Using the QDx3/week schedule (10 mg/kg), 4 of 10 tumors were undetectable on day 24, however all 4 tumors reformed between days 27 and 52. The QDx3/week schedule was well-tolerated.

In a separate study, dose scheduling was evaluated to determine the optimal schedule for MLN0905-induced tumor growth inhibition. This was accomplished by delivering equivalent units (mg/kg) of MLN0905 over a 3 week period using either a daily (QD) schedule or an intermittent schedule [3-on/4-off (QDx3/week) and 4-on/3-off (QDx4/week)]. An area under the tumor growth curve (dAUC) analysis was used to compare the anti-tumor effects observed between different schedules. Two different groups were used in the study, one group received 22 mg/kg/week and the other group received 30 mg/kg/week. As a positive control, animals were dosed QD using the MTD (6.25 mg/kg). Within each dosing group (22 and 30 mg/kg/week), no significant
(p≤0.037) anti-tumor differences were observed when comparing the QD to the intermittent dosing schedules (Figure 4). These data suggest the anti-tumor effect is driven by the total dose given, highlighting dosing flexibility with MLN0905. In the positive control (6.25 mg/kg dosed QD) no measurable tumor was detected in 10 of 10 mice on day 22. All doses and schedules were well-tolerated in this study with no weight loss (data not shown).

**Evaluating the anti-tumor activity of MLN0905 in a primary (PHTX-22L) human lymphoma xenograft model.** This primary lymphoma model was recently harvested from the lymph node of a 71 year old white male diagnosed with Diffuse Large B-Cell Lymphoma (DLBCL) (15). The model was generated by implanting fragments of the patient-derived tumor into the flanks of female NOD SCID mice. Efficacy experiments were performed with early passage tumors (less than 5 passages). In this study MLN0905 was administered orally to SCID NOD female mice bearing s.c. PHTX-22L primary xenograft tumors on a 3-on/4-off schedule using 6 mg/kg, 8mg/kg, 10mg/kg, 12.5mg/kg, and 14.5 mg/kg; and on a QD schedule using 6.25 mg/kg for three weeks. Tolerability studies in NOD SCID mice (data not shown) indicated 14.5 mg/kg was the MTD for the 3-on/4-off schedule and 6.25 mg/kg was the MTD for the QD schedule. The anti-tumor effect was evaluated on day 22 using T/C values and an area under the tumor growth curve (dosing was initiated on day 0, see Figure 5A). In this model, significant anti-tumor effect was observed using the following doses and schedules: 10 mg/kg dosed 3-on/4-off (T/C=0.59, dAUC=33.7, p=0.004), 12.5 mg/kg dosed 3-on/4-off (T/C=0.55, dAUC=67.6, p<0.001), 14.5 mg/kg dosed 3-on/4-off (T/C=0.41, dAUC=84.9, p<0.001), and 6.25
mg/kg dosed QD (T/C=0.34, dAUC=70.9, p<0.001). An anti-tumor dose response was observed using the 3-on/4-off schedule.

We also evaluated scheduling in this model by comparing the QD schedule (6.25 mg/kg) to the 3-on/4-off schedule (14.5 mg/kg), where both arms received 44 mg/kg of drug/week. Using the AUC tumor growth curves as a comparison, the two schedules yielded similar anti-tumor activities. However, mice dosed on 3-on/4-off schedule tolerated MLN0905 better than did those animals dosed on the QD schedule. One mouse in the 6.25 mg/kg QD group lost > 20% of its pre-treatment body weight by day 8 and was euthanized. The mean maximum body weight loss for the other 9 animals in this group was 9% (Figure 5B). In contrast, the mean maximum body weight loss for the 14.5 mg/kg 3-on/4-off group was 5%. All other doses and schedules were well-tolerated in this study.

**MLN0905 synergizes with Rituximab in a Disseminated OCI LY-19-Luc Lymphoma Model.**

Rituximab is a therapeutic monoclonal antibody directed against the B cell lymphoma phosphoprotein CD20 (19). Rituximab is routinely used in the clinic as a standard of care agent in combination with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). Using flow cytometry, the OCI LY-19-Luc cell line was confirmed to express cell surface CD20 (data not shown). The main goal of this OCI LY-19-Luc efficacy study was to determine if Rituximab would synergize with the PLK1 inhibitor MLN0905 in a disseminated model of lymphoma. Prior to the efficacy study, it was determined that Rituximab did not alter the pharmacokinetic profile of MLN0905 in either mouse plasma or OCI LY-19-Luc tumor tissue (data not shown). For the efficacy study, animals with established OCI LY-19-Luc disease were treated for 3 weeks with
either Rituximab (10 mg/kg dosed IV 1x/week), MLN0905 (3.64 and 7.28 mg/kg, QDx3/week), or simultaneous combination. Figure 6A shows the average tumor Xenogen signal plotted as a function of time during the treatment period. The anti-tumor effect was evaluated using T/C values on day 22 and using an area under the tumor growth curve analysis during the treatment period (days 0-22). Using a chi-squared evaluation, significant anti-tumor activity was observed with single agent Rituximab alone (T/C=0.04, dAUC=51.5, p-value<0.001) and using the high dose (7.28 mg/kg) MLN0905 (T/C=0.22, dAUC=17.0, p-value<0.001). Combining Rituximab with MLN0905 significantly enhanced the anti-tumor activity, and more importantly, yielded a synergistic anti-tumor response (Figure 6A). All doses in this study were well-tolerated with no observed weight loss (data not shown).

To determine if MLN0905 +/- Rituximab treatment provided a survival advantage, animals were monitored for paralysis out to day 130 after treatment initiation. All vehicle treated mice reached the pre-defined endpoint of paralysis, and were euthanized between days 16 and 35 (median = 29 days, Figure 6B). Similarly, mice receiving single agent MLN0905 (3.64 mg/kg) reached the pre-defined endpoint between days 24 and 36 (median = 28), neither significantly different from the vehicle group. In contrast, the medium survival time for animals treated with the high dose (7.28 mg/kg) MLN0905 (36 days, p<0.0134) or with 10 mg/kg Rituximab alone (57 days, p<0.0001) was statistically longer compared to that in the vehicle group (29 days).

To determine if combining Rituximab with MLN0905 yielded synergistic survival advantage, the 50% survival mark was used as a benchmark (see materials and methods). Low dose MLN0905 combined with Rituximab did not yield a synergistic response,
likely due to a sub-optimal MLN0905 dosing. In contrast, combining Rituximab with the high dose (7.28 mg/kg) MLN0905 yielded a synergistic enhancement of lifespan (p=0.0008).
Discussion

Small molecule PLK1 inhibitors have entered phase I/II clinical trials both in solid and hematological settings (20). Despite this advancement, little clinical data is available for evaluating PLK1 inhibition in patients diagnosed with DLBCL, and to our knowledge no pre-clinical data exists evaluating a PLK1 inhibitor in xenograft models of lymphoma. In 2008, phase I clinical trial data was presented evaluating PLK1 inhibition (BI-2536) in 41 non-Hodgkins Lymphoma patients (21). The number of DLBCL patients treated in this trial was not disclosed, however the authors stated that one DLBCL patient had a complete disease response. The observation that PLK1 inhibition led to a complete response in this patient is particularly encouraging.

In our current report, our efforts were focused on evaluating PLK1 as an oncology drug target in pre-clinical models of DLBCL. We have discovered a potent small molecule inhibitor of PLK1, MLN0905 (10). MLN0905 was screened against a panel of 359 known kinases (Ambit) (10) and found to have partial inhibitory activity against Anaplastic Lymphoma Kinase (ALK). ALK translocations are rarely found in DLBCL’s (22, 23) and all of the cell lines used in our studies were negative for ALK phosphorylation of Y1604 (data not shown), a marker indicative of ALK translocation activity (24). The absence of this marker provides evidence that the ALK translocation is unlikely relevant in the model systems used in our study. Moreover, the observed cellular phenotype (G2/M mitotic arrest) is consistent with that of PLK1 inhibition not an ALK inhibitor.

In our current report, our efforts were focused on evaluation of PLK1 as an oncology drug target in pre-clinical models of DLBCL. Using MLN0905 as a small
molecule PLK1 inhibitor, we demonstrate pHisH3 can be used as a biomarker to track PLK1 pathway modulation in *in vivo* models of DLBCL. Moreover, the anti-tumor activity associated with PLK1 inhibition was evaluated in three human xenograft DLBCL models. In the OCI LY-10 model, MLN0905 treatment induced significant anti-tumor activity when administered using a variety of different doses and schedules. The highlight of this model was that 20% of the animals treated with the daily maximum tolerated dose responded with complete responses. In the OCI LY-19 model, the anti-tumor activity of MLN0905 was evaluated in animals harboring both subcutaneous and disseminated lymphoma disease. Significant anti-tumor activity was observed in both settings, and most importantly, MLN0905 treatment yielded a significant survival advantage (over control) in mice harboring the disseminated disease, the most fatal setting in humans. Also in the OCI LY-19 model, combining MLN0905 with the standard of care agent Rituximab, yielded both synergistic anti-tumor and synergistic survival activity. These synergistic activities suggest, in the clinical setting, a PLK1 inhibitor could be successfully combined with a standard of care agent to realize enhanced anti-tumor responses. The anti-tumor activity of MLN0905 was also evaluated in a primary lymphoma model which was recently harvested from a DLBCL patient and represents, in many ways, the closest experimental system for mimicking dosing MLN0905 in a human patient. MLN0905 yielded significant anti-tumor activity in this primary model. When taken together, the above pre-clinical data indicate PLK1 is an exciting oncology drug target. Combining our pre-clinical data package with the encouraging clinical data from the BI-2536 phase I trial suggests PLK1 inhibitors should be evaluated in the clinical DLBCL setting.
MLN0905 was also used to compare the effect scheduling imparts on anti-tumor activity when dosing a PLK1 inhibitor on either a continuous or intermittent dosing schedule. In three different models (OCI LY-19, OCI LY-10, and PHTX-22L) the anti-tumor effect was similar when comparing the continuous to the intermittent dosing schedules. These data suggest, when comparing these two schedules, the primary driver of efficacy is the total amount of drug given, highlighting dosing flexibility.

In conclusion, PLK1 inhibition leads to robust anti-tumor activity in multiple DLBCL xenograft models using both a subcutaneous and disseminated setting. These preclinical data validate PLK1 as an oncology drug target in the indication of DLBCL. With dosing flexibility as an option, PLK1 inhibitors should be further evaluated in patients suffering from DLBCL.
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References


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Figure Legends

Figure 1. MLN0905, a small molecule inhibitor of PLK1, phenocopies the affects of PLK1 knock-down using RNAi in HT29 cells. A) Chemical structure of MLN0905. B) HT29 cells were transiently transfected with RNAi directed against PLK1 or a scrambled RNAi control. Using western blotting, PLK1 RNAi was found to efficiently reduce PLK1 protein levels. C) HT29 cells have been treated with MLN0905 (125 nM) or PLK1 RNAi for 24 hours and then labeled with DAPI or fluorescent antibodies directed against pHisH3 and tubulin. Both MLN0905 and PLK1 RNAi treatments led to robust increases in pHisH3 staining (pink staining). A scrambled RNAi control was used as the control (similar effects were observed using the vehicle of MLN0905- data not shown). PLK1 inhibition has previously been shown to result in monopolar spindle formation. Representative examples of monopolar spindle formation are shown in the bottom of panel C following both MLN0905 and PLK1 RNAi treatments (green- tubulin and blue- DAPI).

Figure 2. Phosphorylated histone H3 (pHisH3) was used as a biomarker to determine the level of mitotic arrest in OCI LY-19-Luc xenograft tumors. Tumor bearing mice were treated with a single oral dose of MLN0905 (vehicle was used as a control=c). At the indicated times tumor tissue was harvested and assayed for pHisH3 using immunohistochemistry. A two-tailed t-test for unequal variances was used to determine statistical significance (*p<0.05, n=3/time point).
**Figure 3.** MLN0905 induces a significant anti-tumor response in mice bearing disseminated (human) OCI LY-19-Luc lymphoma disease. CB-17 SCID mice were inoculated in the tail vein with OCI LY-19-Luc cells. After 10 days, mice (n=10/group) were treated once a day (QD) with an oral dose of MLN0905 using 1.6, 3.12, and 6.25 mg/kg for 21 days (treatment initiated on day 0). A) Tumor cell photon flux was measured using photon flux imaging and the tumor growth curves are shown using a linear scale. B) Photon flux signal from 5 representative animals are shown for both the vehicle and the 6.25 mg/kg MLN0905 groups on day 20. Heat map key: bright red=highest photon emission, cool blue=lowest. C) Kaplan-meier survival curves show animal survival during and after the MLN0905 treatment. *The 3.12 and 6.25 mg/kg curves are significantly different (p<0.0001) from the vehicle.

**Figure 4.** Evaluating the effect of dosing schedule vs anti-tumor effect with MLN0905 in the OCI LY-10 subcutaneous tumor model. Intermittent (3-on/4-off and 4-on/3-off) vs continuous (QD) dosing was initiated when tumors reached 200 mm³. Tumor growth curves (n=10 animals/group) are shown during the 21 days of treatment (dosing was initiated on day 0). No significant differences were observed when comparing the different dosing schedule as indicated by the following comparisons: 3.12 mg/kg vs 7.28 mg/kg dAUC=106.2, p<0.043; 3.12 mg/kg vs 5.46 mg/kg dAUC=22.9, p<0.584; 5.46 mg/kg vs 7.28 mg/kg dAUC=-354.4, p<0.037; 10 mg/kg vs 7.5 mg/kg dAUC=-36.8, p<0.125; 4.3 mg/kg vs 10 mg/kg dAUC=55.6, p<0.147; 4.3 mg/kg vs 7.5 mg/kg dAUC=-2.4, p<0.921. As a positive control, 6.25 mg/kg of MLN0905 was dosed daily (QD).
**Figure 5.** MLN0905 induces a significant anti-tumor response in a primary human lymphoma model (PHTX-22L). NOD SCID mice bearing subcutaneous PHTX-22L xenografts were treated orally for 21 days with MLN0905 using both a 3-on/4-off and QD schedule. A) Median tumor growth curves ± SEM are shown (n=10 animals/group). B) Median body weight change during treatment.

**Figure 6.** MLN0905 and Rituximab yield a synergistic anti-tumor response in a disseminated xenograft model of human lymphoma (OCI LY-19-Luc). Treatment was initiated in animals with established disease, i.e. 10 days after tail vein injection of OCI LY-19-Luc cells. Animals (n=10/group) were treated with either Rituximab (IV, 1x/week), MLN0905 (orally, 3-days on/4-days off), or the combination of agents. Xenogen imaging was used to measure photon flux (disease) once per week. A) Tumor growth curves ± SEM are plotted over time. During the treatment phase of the experiment, synergy was observed when combining Rituximab with both the low and high MLN0905 doses (p-value for the low dose combo = 0.0343, p-value for the high dose combo = 0.0067). B) Kaplan-meier survival curves illustrating animal survival during and after the treatment (day 0 is the first day of dosing). *Combining Rituximab with 7.28 mg/kg MLN0905 yielded a synergistic prolongation of lifespan (p-value=0.0008, β=51).
Figure 1

A

B

CTL RNAi:

Plk1 RNAi:

Plk1

Actin

24h  48h

Ctl RNAi: +  -  +  -

Plk1 RNAi: -  +  -  +

C

Control  PLK RNAi  MLN0905

pH3 S10

DAPI

Tubulin

DAPI
Figure 2

![Graph showing % pH3-positive area over time for different doses of drug.](image-url)
Figure 3

A

![Graph showing photon flux (p/s) over days for different treatments.](image)

B

![Dorsal and ventral views of mice with different treatments.](image)

C

![Graph showing percent survival over days for different treatments.](image)
Figure 4

The figure shows the average tumor volume (mm$^3$) over days for different treatment groups. The x-axis represents the number of days, ranging from 0 to 25. The y-axis represents the average tumor volume, ranging from 0 to 1000 mm$^3$. There are two treatment regimens: 22 mg/kg/wk and 30 mg/kg/wk. The treatments include:

- Vehicle
- 3.12 mg/kg QD
- 5.46 mg/kg 4 on/3 off
- 7.28 mg/kg 3 on/4 off
- 4.3 mg/kg QD
- 7.5 mg/kg 4 on/3 off
- 10 mg/kg 3 on/4 off
- 6.25 mg/kg QD

Each treatment group is represented by different symbols and colors. The error bars indicate the variability in the average tumor volume measurements. The data points show an increase in tumor volume over time for most treatment groups, with some showing a decrease or stabilization at higher doses.
Figure 5

A

![Graph A](image)

- Vehicle
- 6 mg/kg 3-on/4-off
- 8 mg/kg 3-on/4-off
- 10 mg/kg 3-on/4-off
- 12.5 mg/kg 3-on/4-off
- 14.5 mg/kg 3-on/4-off
- 6.25 mg/kg QD

Average Tumor Volume (mm$^3$)

Days

B

![Graph B](image)

- Vehicle
- 6 mg/kg 3-on/4-off
- 8 mg/kg 3-on/4-off
- 10 mg/kg 3-on/4-off
- 12.5 mg/kg 3-on/4-off
- 14.5 mg/kg 3-on/4-off
- 6.25 mg/kg QD

Average Percent Body Weight Change

Days
Figure 6

A

Photon flux (p/s)

100E+10

100E+9

100E+8

100E+7

100E+6

0 5 10 15 20 25

Treatment day

Vehicle

Rituximab 10mg/kg 1x/week

MLN0905 3.64mg/kg QDx3/week

MLN0905 7.28mg/kg QDx3/week

Rituximab +MLN0905 3.64mg/kg

Rituximab +MLN0905 7.28mg/kg

B

Percent survival

100

80

60

40

20

0

0 10 20 30 40 50 60 70 80 90 100 110 120 130

days

Vehicle

Rituximab 10mpk Q7D

MLN0905 3.64mpk QDx3/week

MLN0905 7.28mpk QDx3/week

MLN0905 3.64mpk & Rituximab

MLN0905 7.28mpk & Rituximab

*
MLN0905, A Small Molecule PLK1 Inhibitor, Induces Anti-Tumor Responses in Human Models of Diffuse Large B-cell Lymphoma

Judy Shi, Kerri Lasky, Vaishali Shinde, et al.

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