Small Molecule Inhibition of the Ubiquitin-specific Protease USP2 Accelerates Cyclin D1 Degradation and Leads to Cell Cycle Arrest in Colorectal Cancer and Mantle Cell Lymphoma Models

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Running title: USP2 inhibition impacts Cyclin D1 and cell cycle

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ABSTRACT

Deubiquitinases are important components of the protein degradation regulatory network. We report the discovery of ML364, a small molecule inhibitor of the deubiquitinase USP2 and its use to interrogate the biology of USP2 and its putative substrate Cyclin D1. ML364 has an IC\textsubscript{50} of 1.1 µM in a biochemical assay using an internally quenched fluorescent di-ubiquitin substrate. Direct binding of ML364 to USP2 was demonstrated using microscale thermophoresis. ML364 induced an increase in cellular Cyclin D1 degradation and caused cell-cycle arrest as shown in western blots and flow cytometry assays utilizing both mino and HCT116 cancer cell lines. ML364, and not the inactive analog 2, was antiproliferative in cancer cell lines. Consistent with the role of Cyclin D1 in DNA damage response, ML364 also caused a decrease in homologous recombination (HR)-mediated DNA repair. These effects by a small molecule inhibitor support a key role for USP2 as a regulator of cell cycle, DNA repair and tumor cell growth.

The ubiquitination status of proteins can affect their degradation by the proteasome, as well as their conformation and subcellular localization, thereby modulating diverse processes including cell cycle and apoptosis. This status is tightly regulated at both the ubiquitination level by the E1, E2, E3 ubiquitin ligase system and the deubiquitination level by over 90 deubiquitinases (DUBs) (1). The largest of the five DUB families is the ubiquitin-specific proteases (USPs). USP2 is a DUB that removes ubiquitin thereby stabilizing a range of substrates including MDM2 (a regulator of p53) (2), fatty acid synthase (3), Cyclin D1 (4) and proteins important for the circadian rhythm, such
as Cry1 (5). Typically, ubiquitinated proteins with K48-linkages are directed to the proteasome for degradation, while K63-linked ubiquitinated proteins can regulate more diverse functions, such as innate immunity, localization and activation (6,7). USP2 has been shown to catalyze the hydrolysis of both linkage types (8). USP2 is upregulated in various cancers including prostate cancer and ovarian carcinoma, which would be expected to lead to an increase in the levels of deubiquitinated variants of its corresponding substrates (3), and has also been described as an oncogene due to its ability to transform cells and provide resistance to apoptosis (9). The USP2 substrate fatty acid synthase is upregulated in breast, prostate and ovarian cancers and is associated with a poor prognosis (10-12). The best-characterized substrate of USP2, Cyclin D1, is important for cell cycle progression from G1 to S in many cell types and is activated or overexpressed in several cancers including mantle cell lymphoma (13), a sub-type of non-Hodgkin’s lymphoma, in which the disease is often quite advanced by the time of diagnosis. The most common genetic alteration in this cancer is a t(11:14) chromosomal translocation that leads to overexpression of Cyclin D1 (14,15). In 2009, Shan et al. reported that the knockdown of USP2 arrests cancer cell growth by promoting the proteasome-mediated degradation of Cyclin D1, thus raising the possibility that small molecules specifically targeting this deubiquitinase could be effective chemotherapeutic agents for cancers addicted to Cyclin D1 expression (4). A crystal structure of USP2 and kinetic analysis of its interaction with ubiquitin have been reported (16,17); however only a few USP2 inhibitors have been described and several of these bind covalently and/or are nonselective (18-20). Herein we report the identification of a small molecule USP2 inhibitor, ML364, demonstrate its activity in USP2 biochemical assays, and profile its selectivity across a panel of proteases and kinases. We also characterize the binding of ML364 to USP2 and test its effects on cell viability and the levels of Cyclin D1. Our results suggest that ML364 acts on USP2 and can be used to interrogate the effect on USP2 substrates in a cellular context.

**RESULTS**

**ML364 Reversibly Inhibits USP2 in a Biochemical Assay and Its Selectivity is Assessed**- A high-throughput screen resulted in the identification of a sulfamido benzamide chemical series that inhibited USP2 biochemical activity. Further optimization through medicinal chemistry, led to development of the active compound ML364 and a structurally related inactive counterpart compound 2 (Fig. 1A). Using internally quenched fluorescent di-ubiquitin substrates (Di-Ub IQF; Fig. 1B), ML364 was determined to have an IC<sub>50</sub> of 1.1 µM for the K48-linked substrate and 1.7 µM for the K63-linked substrate, while 2 was inactive. The compounds did not have an effect on the detection system alone. The interaction of ML364 with USP2 was reversible as determined by measuring the recovery of enzyme activity following a large dilution of the USP2-ML364 complex (Fig. 1G). ML364 was also tested in other protease assays to assess selectivity (Table 1 and Fig. 1C): it was inactive against Caspase 6, Caspase 7, MMP1, MMP9, and USP15, but did inhibit USP8 with an IC<sub>50</sub> of 0.95 µM, in agreement with the similarity between the active sites of these two isozymes. In a panel of 102 kinases that include cell cycle regulators the cell cycle was not observed to any of the 102 enzymes tested using 10 µM of ML364. (Table 2 and Fig. 2).

**ADME Characterization of ML364**- Preliminary ADME properties were determined and while ML364 had low solubility in PBS 7.4 buffer (<2 µM), its solubility was more than 10-fold higher in the assay buffer (28.9 µM; 20 mM Tris pH 8, 2 mM β-mercaptoethanol, 0.05% CHAPS), where it was stable for more than 48 hours (Table 3a). The logD at pH 7.4 (a measure of lipophilicity) was 2.31 and the PAMPA permeability was 82.2 x 10<sup>-6</sup> cm/s, indicating that the compound has properties that should allow for cell membrane permeation. While the stability in rat microsomes was modest (t<sub>1/2</sub> = 15
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minutes), the stability in mouse (61% remaining at 30 minutes) and human (84% remaining at 30 minutes) microsomes and in mouse plasma (100% remaining at 30 minutes) were quite good (Table 3b).

Binding of ML364 to USP2 Demonstrated by Microscale Thermophoresis-
Using label-free microscale thermophoresis, a method to measure binding affinities by monitoring differential movement of particles in a microscopic temperature gradient (21), the interactions of ML364 and 2 with USP2 were examined. ML364 bound USP2 with a K_d of 5.2 µM while inactive analog 2 did not bind (Fig. 1, D-E); control experiments with USP2 alone confirmed that the thermophoresis signature was due to binding of active compound to the enzyme (Fig. 1F). USP2 is fluorescent due to the presence of several tryptophan residues, two of which line the ubiquitin binding pocket (PDB ID: 2NHE). Upon addition of ML364 there was a dose-dependent decrease in USP2 tryptophan fluorescence yield (Fig. 1D). To determine that the observed binding interaction was not due to quenching of tryptophan fluorescence but rather to thermophoresis that probes the size, charge, and solution entropy of molecules, dilutions of USP2 alone were tested and the dose response curve produced was in the opposite direction indicating that the curve observed for USP2 in the presence of dilutions of ML364 was not due to the tryptophan quenching.

Effect of USP2 Inhibition by ML364 in Cells Investigated by Western Blotting- To determine the effect of USP2 inhibition on Cyclin D1 stability, HCT116 colorectal cancer cells were treated with ML364 and Cyclin D1 protein levels were monitored by both western blotting (Fig. 3, A-C) and in-cell ELISA (Fig. 4A). Whereas Cyclin D1 protein level remained unchanged in cells treated with the inactive compound 2, it decreased in both a time- and dose-dependent manner following treatment with ML364. Quantification of the western blot results revealed an IC_{50} value of 0.97 µM, which is consistent with the results from the biochemical assays discussed above (Fig. 1 and Table 1). The similar potencies for ML364 in the cell-based and biochemical assays are in concert with the expected cell permeability of the inhibitor and support the notion that ML364 engages USP2 in the cell. ML364-mediated Cyclin D1 destabilization was not due to a reduction in USP2 protein levels (Fig. 3A), and was proteasome dependent, as the addition of the proteasome inhibitor MG132 restored Cyclin D1 protein levels to those observed in the DMSO control (Fig. 3C). To test whether ML364 promoted Cyclin D1 degradation in a cell line that relies on this protein for transformation, the above experiments were repeated using the mantle cell lymphoma cell line, Mino. Again, ML364, but not compound 2, reduced Cyclin D1 protein levels in a time-, dose-, and proteasome-dependent manner (Fig. 3, D-F).

To directly identify the changes in Cyclin D1 protein stability by ML364, we expressed a Cyclin D1-firefly fusion protein in cells as such fusion proteins are usually directly targeted by ubiquitin ligases (22,23). Such an approach is used to identify changes in protein stability in cells exposed to Lenalidomide (22). We used a plasmid encoding Cyclin D1 open reading frame (ORF) fused to firefly luciferase (FLuc) reporter and Renilla luciferase (RLuc) reporter for normalization purpose as described (22). When 293T cells were transfected with this plasmid, the cells expressed Cyclin D1-FLuc fusion protein as detected by western blotting with antibodies against FLuc and Cyclin D1 (Fig. 4, B-C). We next transfected HCT116 cells with this plasmid, treated them with inactive compound or ML364, and measured Fluc as well as RLuc enzyme activity. As expected, a decrease in relative FLuc/RLuc activity was observed in cells exposed to ML364 suggesting a downregulation of Cyclin D1 fusion protein (Fig. 4D). As a positive control, exposure of cells to BIIB021 (a HSP90 inhibitor) also resulted in decrease in relative FLuc/RLuc activity (Fig. 4E). Reduced FLuc/RLuc activity was also observed in 293T cells exposed to ML364 (Fig. 4F). Collectively, these results suggest that ML364 directly targets Cyclin D1 for degradation via USP2 inhibition.
**Effect of USP2 Inhibition by ML364 in Cells Investigated by Flow Cytometry**-

Since Cyclin D1 regulates the G1-to-S phase transition of the cell cycle, the cell cycle profiles of ML364-treated HCT116 and Mino cells were determined by both flow cytometry (Fig. 5, A-B) and high content imaging (IC50 = 2 µM; Fig. 5C): in contrast to cells treated with 2, which were able to progress to the G2/M phase of the cell cycle, both cell types remained largely in G1 phase when treated with ML364. It is important to note that for these assays, cells were co-dosed with nocodazole, which blocks cells in G2/M phase, to increase the ability to visualize the changes in the G1 levels. Consistent with ML364-induced cell cycle arrest, clonogenic assays revealed that the growth of HCT116 cells was severely inhibited following chronic treatment with ML364 (Fig. 5, D-E, IC50 = 3.6 µM), whereas cells treated with 2 proliferated at a rate comparable to the DMSO-treated control (Fig. 5, F-G). Assessment of cell viability in HCT116 cells and Mino cells using cellular ATP content as the readout also showed that ML364 decreased cell viability (Fig. 6A). In addition to HCT116 and Mino cells, LnCAP cells (prostate cancer cells) and MCF7 cells (breast cancer cells) also showed decrease in cell viability upon exposure to ML364 in a dose dependent manner (Fig. 6, B-C). The decrease in cell viability in these cell lines correlated with Cyclin D1 degradation (Fig. 6D). We next determined whether additional targets of USP2, namely, MDM2 and FAS, are affected by ML364. Interestingly, FAS but not MDM2 protein levels were moderately reduced after ML364 exposure in LnCAP prostate cancer cells (Fig. 6E,F).

**Effect of USP2 inhibition on homologous recombination (HR)-mediated DNA repair**—Recent studies suggest that Cyclin D1 regulates DNA damage response (24). Cyclin D1 interacts with RAD51, a critical component of homologous recombination (HR)-mediated DNA repair. Reduction of Cyclin D1 levels in cancer cells results in impaired HR-mediated DNA repair and increased DNA damage (24). We therefore tested whether inactivation of USP2 by ML364 alters DNA repair. We used a cell-based DR-GFP reporter assay to measure the activity of HR-mediated DNA repair. Interestingly, ML364 caused a decrease in HR-mediated DNA repair in DR-GFP U2OS cells (Fig. 7A). We next analyzed RAD51 foci, a surrogate marker for efficient HR-mediated DNA repair, after inhibition of USP2 by ML364. Consistent with the results from DR-GFP reporter assay, ML364 exposure in Hela cells caused a decrease in IR-induced RAD51 foci (Fig. 7, B-C). Together, these data indicate that inhibition of USP2 by ML364 causes a decrease in HR suggesting the role of USP2 in DNA repair.

**DISCUSSION**

Bortezomib and carfilzomib are clinically used to treat multiple myeloma (both) and mantle cell lymphoma (bortezomib only) (25,26). While these drugs inhibit the proteasome in both normal and cancer cells, herein we sought to impact one enzyme (USP2) that controls the fate and levels of several oncogenic substrates by specifically promoting their degradation. With the DUB activity of USP2 inhibited, these oncogenic proteins are expected to remain ubiquitinated and targeted for proteasomal degradation as shown herein for Cyclin D1, while leaving the proteasome functionally intact. Shan et al. had previously tested 76 DUBs and found that only USP2 was able to deubiquitinate Cyclin D1 in human cells (4). Importantly, knockdown of USP2 was shown previously to induce growth arrest in cells that depend on Cyclin D1 for cell growth but to have minimal effects on normal fibroblasts and no effect on cancer cell types that do not express Cyclin D1, such as SAOS2 (4).

Using both HCT116 cells, used in the previous siRNA work by Shan et al. (4), and the Mino mantle cell lymphoma line, noted for its very high Cyclin D1 levels (Broad-Novartis Cancer Cell Line Encyclopedia), ML364 was shown to impact the levels of Cyclin D1 through an increase in proteasome-mediated degradation. Interestingly, the IC50 observed in the mantle cell lymphoma model was right-shifted relative to that observed in HCT116, which could be a
consequence of the very high levels of Cyclin D1 in those cells (14,15). Decrease in Cyclin D1 stability by ML364 was also confirmed by expressing a Cyclin D1 luciferase fusion protein in a reporter assay. ML364 affected cell cycle, blocking the cells in G0/G1 phase and preventing cell cycle progression as observed by flow cytometry and high content imaging (Figure 8). As mitotic arrest is poorly tolerated by cancer cells, viability was decreased in multiple cancer cell lines upon treatment with ML364. Additionally, the size and number of colonies in a 10-day colony forming assay were reduced upon treatment with the inhibitor. While ML364 displays similar potency against USP8 and USP2, USP8 is not known to be involved in the cell cycle, but rather has a role in endosomal trafficking (27). Indeed, USP8 has been described as a novel target for overcoming gefitinib-resistance in lung cancer indicating a potential additional use for ML364 (28). Our data suggest that besides regulating cell cycle, USP2 also plays a role in HR-mediated DNA repair by stabilizing Cyclin D1. Recent studies suggest that Cyclin D1 regulates DNA damage repair (24,29). Cyclin D1 facilitates HR-mediated DNA repair through the recruitment of RAD51 to double stranded breaks and Cyclin D1 depletion causes a reduction in HR (24). Therefore, promoting the degradation of Cyclin D1 through the inhibition of USP2 using ML364 presents a new avenue for treating Cyclin D1 driven cancers. Indeed, using a DR-GFP reporter assay and by assessing DNA damage-induced RAD51 foci, we found that ML364 causes HR defect. These data suggest that USP2 may be a novel therapeutic target for inhibiting HR activity in Cyclin D1 driven cancers. Suppressing homologous recombination using this strategy would enhance the effect of genotoxins, such as PARP inhibitors, which specifically target HR deficient tumors.

Previously reported USP2 inhibitors include patented 2-cyano pyrimidines (potency range 100 nM - 50 µM, likely reversible covalent modifiers) (18), and chalcone compounds (double-digit micromolar potency but also shown to inhibit many other DUBs) (19). ML364 is an extensively characterized non-covalent USP2 inhibitor and as such is an important probe of USP2 biology. Of the six additional proteases tested, only the closely related USP8 was inhibited, indicating that ML364 is not a general protease inhibitor. Further, 102 kinases were tested, many of which are involved in the cell cycle, and ML364 did not bind to any, thereby supporting USP2 as the target leading to the observed cell cycle arrest phenotype observed. While the ADME properties of ML364 are suitable for the biochemical and cell-based assays used here, an improved molecule may be needed for future in vivo testing. While in this initial study we focused on Cyclin D1, there are additional putative USP2 substrates that can be interrogated in relevant cell lines using ML364. It is envisioned that ML364 can be used in proteomic studies to elucidate heretofore unknown substrates of USP2. Our provision of ML364 to the research community should spur studies in these and other directions.

EXPERIMENTAL PROCEDURES

General Methods for Chemistry- All air- or moisture-sensitive reactions were performed under positive pressure of nitrogen with oven-dried glassware. Anhydrous solvents such as dichloromethane, N,N-dimethylformamide (DMF), acetonitrile, methanol and triethylamine were purchased from Sigma-Aldrich. Preparative purification was performed on a Waters semi-preparative HPLC system. The column used was a Phenomenex Luna C18 (5 micron, 30 x 75 mm) at a flow rate of 45 mL/min. The mobile phase consisted of acetonitrile and water (each containing 0.1% trifluoroacetic acid). A gradient of 10% to 50% acetonitrile over 8 minutes was used during the purification. Fraction collection was triggered by UV detection (220 nM). Analytical analysis was performed on an Agilent LC/MS (Agilent Technologies, Santa Clara, CA). Purity analysis was determined using a 7 minute gradient of 4% to 100% acetonitrile (containing 0.1% trifluoroacetic acid) in water (containing 0.05%
trifluoroacetic acid) with an 8 minute run time at a flow rate of 1 mL/min. A Phenomenex Luna C18 column (3 micron, 3 x 75 mm) was used at a temperature of 50 °C using an Agilent Diode Array Detector. Mass determination was performed using an Agilent 6130 mass spectrometer with electrospray ionization in the positive mode. 1H NMR spectra were recorded on Varian 400 MHz spectrometers. Chemical shifts are reported in ppm with non-deuterated solvent (DMSO-d6) peak at 2.50 ppm as internal standard for DMSO-d6 solutions. All of the analogs tested in the biological assays have a purity greater than 95% based on LCMS analysis. High resolution mass spectrometry was recorded on Agilent 6210 Time-of-Flight LC/MS system. Confirmation of molecular formulae was accomplished using electrospray ionization in the positive mode with the Agilent Masshunter software (version B.02).

2-(4-methylphenylsulfonamido)-4-trifluoromethylbenzoic acid (SI-I) - A mixture of 2-amino-4-trifluoromethylbenzoic acid (1.08 g, 5.25 mmol), sodium carbonate (1.39 g, 13.11 mmol), and water (10.5 mL) was treated with tosyl chloride (1.0 g, 5.25 mmol) (Fig. 9A). This mixture was stirred at 80 °C for 2 hr. After cooling to 23 °C, 1N HCl was added dropwise (gas evolves from sodium carbonate neutralization) until the mixture reaches pH 2. The resulting precipitated solid was collected by vacuum filtration, washed with water, and dried by high vacuum overnight to afford intermediate sulfonamide SI-1 as a pure off-white solid (1.07 g, 57%).

2-(4-methylphenylsulfonamido)-N-(4-phenylthiazol-2-yl)-4-(trifluoromethyl)benzamide (ML364) - A mixture of sulfonamide SI-1 (0.4 g, 1.11 mmol), 4-phenylthiazol-2-amine (0.20 g, 1.11 mmol), DIPEA (0.78 mL, 4.45 mmol), and DMF (2.78 mL) was treated with HATU (0.51 g, 1.34 mmol). This mixture was stirred at 80 °C for 2 hr. The resulting solution was quenched with water, and the organic layer was extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed with saturated ammonium chloride (2 x 30 mL), saturated sodium bicarbonate (2 x 30 mL), and saturated brine (2 x 30 mL). The resulting organic solution was dried over MgSO4, and concentrated under reduced vacuum. The crude oil was then purified via reverse phase chromatography to furnish ML364 as an off-white solid (219 mg, 31%). 1H NMR (400 MHz, DMSO-d6) δ 12.83 (bs, 1H), 10.27 (bs, 1H), 8.05 – 7.88 (m, 3H), 7.76 (s, 1H), 7.67 – 7.55 (m, 3H), 7.45 – 7.42 (m, 3H), 7.36 (t, J = 7.3 Hz, 1H), 7.32 – 7.22 (m, 2H), 2.26 (s, 3H); 19F NMR (376 MHz, DMSO-d6) δ –62.1; LC-MS Retention Time: t1 = 7.107 min; HRMS (ESI) m/z (M+H)+ calcd. for C24H18F3N3O3S2, 518.0814; found 518.0810.

3-(4-methylphenylsulfonamido)isonicotinic acid (SI-2) - A mixture of 3-aminonicotinic acid (181 mg, 1.311 mmol), sodium carbonate (347 mg, 3.28 mmol), and water (3.28 mL) was treated with tosyl chloride (250 mg, 3.28 mmol) (Fig. 9B). This mixture was stirred at 80 °C for 2 hr. After cooling to 23 °C, 1N HCl was added dropwise (gas evolves from sodium carbonate neutralization) until the mixture reaches pH 2. The resulting precipitated solid was collected by vacuum filtration, washed with water, and dried by high vacuum overnight to afford intermediate sulfonamide SI-2 as a pure off-white solid (60 mg, 16%).

3-(4-methylphenylsulfonamido)-N-(pyridin-4-yl)isonicotinamide (2) - A mixture of sulfonamide SI-2 (40 mg, 0.137 mmol), pyridin-4-amine (12.9 mg, 0.137 mmol), DIPEA (96 µL, 0.547 mmol), and DMF (0.68 mL) was treated with HATU (78 mg, 0.205 mmol). This mixture was stirred at 80 °C for 12 hr. The resulting solution was purified directly via reverse phase chromatography to furnish the TFA salt of compound 2 as an off-white solid (41 mg, 62%).

1H NMR (400 MHz, DMSO-d6) δ 8.74 (d, J = 7.1 Hz, 2H), 8.54 (s, 1H), 8.28 (s, 1H), 8.03 (d, J = 7.1 Hz, 2H), 7.66 (d, J = 5.1 Hz, 1H), 7.56 (d, J = 8.2 Hz, 2H), 7.30 (d, J = 7.5 Hz, 2H), 2.31 (s, 3H); 19F NMR (376 MHz, DMSO-d6) δ –74.2; LC-MS Retention Time: t1 = 1.527 min; HRMS (ESI) m/z (M+H)+ calcd. for C18H16N4O3S, 369.1016; found 369.1005.
General Methods for Biology- The catalytic core of USP2a (EC 3.4.19.12 and UniProt # O75604) was obtained from Lifesensors and Progenra (Malvern, PA, USA) for the high-throughput screen. We produced USP2 for additional studies as had been previously reported in PDB ID 3NHE. Buffer components were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified. Experiments were run at room temperature unless otherwise specified. Medium binding black or white solid-bottom 1536-well plates (assay plates) and 1536-well polypropylene plates (compound plates) were purchased from Greiner Bio One (Monroe, NC, USA).

Compound plating and data analysis - Preparation of the compound library for quantitative high-throughput screening (qHTS) has been described previously (30). The library was formatted into columns 5-48 of 1536-well compound plates. A control plate was made in a compound plate in columns 1-4 using a Cybi-Well (CyBio, Jena, Germany) to transfer solutions from a 384 compound storage plate to the 1536 plate compound plate. Columns 1, 3, and 4 contained DMSO and column 2 contained 2 M N-ethylmaleimide in dimethyl sulfoxide (DMSO, certified ACS grade, Thermo-Fisher Scientific (Pittsburg, PA, USA)). Data were normalized to control columns representing maximum signal (no enzyme or no cells) and minimum signal (all components). Screening data were corrected and normalized, and concentration-response curves were derived using in-house algorithms (31).

Ub-Chop2 Screen- This assay technology was described previously (20), and details of the high-throughput screen and countscreen executed in 1536-well plate format from which the chemical series discussed herein was identified is publicly available at PubMed ID: 463254 and 493169.

K48-4 and K63-3 Assay- Using a Flying Reagent Dispenser (Beckman Coulter, Fullerton, CA, USA), 2 μL of 10 nM USP2 in 20 mM Tris pH 8, 2 mM β-mercaptoethanol and 0.05% CHAPS was dispensed in a black 1536-well plate in columns 1-3, 5-48 and a no enzyme control was dispensed into column 4. Using a 1536 pintool (Kalypsys Systems, San Diego, CA, USA), 23 nL of compounds were then added and the plate was incubated for 10 minutes. To initiate the reaction, 1 μL of 100 nM Di-Ub IQF substrate (Lifesensors, K48-4 or K63-3) in 20 mM Tris pH 8, 2 mM β-mercaptoethanol and 0.05% CHAPS was added and the fluorescence signal (excitation 525 nm and emission 598 nm, bodipy mirror and 0.5 sec exposure) was monitored in kinetic mode on a Viewlux high-throughput CCD imager (Perkin Elmer, Waltham, MA, USA); the apparent reaction rate was calculated using the signal change during the first 15 minutes, which was within the linear region of this assay. Additionally, to verify that the compounds did not interfere with the detection system, the effect of compound on the substrate alone was tested by omitting the enzyme from the above reaction.

Reversibility and Substrate Competition- Following the method described in Copeland (32), to test the reversibility of the ML364 binding to USP2, ML364 at a concentration ten times its IC₅₀ and 100x the concentration of USP2 used in the K48-4 assay described above were incubated for 1 hour at which point the sample was diluted 100-fold in assay buffer. The activity of this diluted sample was tested as described above for the miniaturized K48-4 and K63-3 enzyme assays.

Selectivity Assays- Using Kinomescan (DiscoveRx, Fremont, CA, USA), 102 kinases were tested for inhibition by 10 μM ML364. MMP1 and MMP9 data were obtained from Reaction Biology (Malvern, PA, USA) using their standard conditions (5 μM of the FRET peptide). Caspase 6 and caspase 7 assays were run using the Caspase 6 and Caspase 3/7 Glo kits from Promega (Madison, WI, USA). This kit couples the activity of the cysteine-aspartic acid protease, i.e., the caspase, to luciferase. First, 2.5 μL of Caspase 6 (0.5 U/mL; Enzo Life Sciences, Farmingdale, NY, USA) of Caspase 7 (0.5 U/mL; Enzo Life Sciences) in 10 mM Hepes pH 7.2, 2 mM DTT, 10% glycerol and 0.05% CHAPS was dispensed into a white 1536-well plate. The substrate is at 5 μM in this assay,
which is the K_m for the substrate per the manufacturer. Then, 23 nL of compounds were dispensed using the pintool and incubated for 30 minutes at room temperature. Lastly, 2.5 µL of Caspase Glo reagent (either Caspase Glo 6 or Caspase Glo 3/7) was added and the luminescence was monitored kinetically using a Viewlux for a total of 50 minutes (1 sec exposure).

Three µL of USP8core (140 nM, Lifesensors) or USP15 (125 nM, Lifesensors) were added to black 1536-well plates that had been prespotted with either 23 nL of DMSO or test compound using an acoustic dispenser (ATS-100 acoustic dispenser, EDC Biosystems, Freemont, CA, USA) to transfer the DMSO or DMSO/compound from a Greiner cyclic olefin copolymer source plate to the 1536-well assay plate. Following incubation for 30 minutes, 1 µL of Di-Ub IQF K48-02 (100 nM) was added and the reaction was monitored kinetically using a Viewlux as for the USP2 assay above.

Microscale Thermophoresis- Following the Manufacturer’s protocol, a 16-point titration series of ML364 in DMSO (40 µM final, 1:2 dilutions), were transferred to the protein (1 µM) in a buffer containing 20 mM Tris pH 8, 2 mM β-mercaptoethanol and 0.05% CHAPS, samples were loaded into Monolith NT hydrophilic-treated Label-free capillaries (NanoTemper Technologies, München, Germany). The buffer alone and compound in buffer were tested and found not to be fluorescent. A capillary scan was performed followed by the successive measurement of thermophoresis in each capillary on a NanoTemper Monolith NT.115 LabelFree instrument at room-temperature (UV-light emitting diode excitation and 350 nm emission). The IR laser power and the LED power conditions are described in the figure captions. A laser on-time of 30 seconds and a laser-off time of 5 seconds were used. The experiment was performed in duplicate. Curve fitting was performed using GraphPad Prism 5 (La Jolla, CA, USA). As a control, dilutions of USP2 were also tested per the manufacturer’s protocol to determine whether tryptophan quenching by the compound could be responsible for the observed IC_{50} curve.

Cell culture- HCT116 cells were cultured in Dulbecco’s Minimum Eagle Medium (DMEM) + GlutaMAX (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone Laboratories, Waltham, MA, USA) and 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen). Cells were maintained at 37 °C under a humidified atmosphere and 5% CO_2. Mino cells, a cell line established and characterized as described in (33) and LnCAP cells were cultured in RPMI-1640 (Gibco, Carlsbad, CA, USA) with 10% FBS and 50 U/mL penicillin and 50 µg/mL streptomycin. MCF7 cells were grown in DMEM with 10% FBS, 10 µg/ml insulin (Sigma) and 50 U/mL penicillin and 50 µg/mL streptomycin. U2OS-DRGFP cells and Hela cells were cultured in DMEM with 10% FBS, and 50 U/mL penicillin and 50 µg/mL streptomycin.

Western Blotting- Cells were grown in 6-well tissue-culture plates and treated as indicated. To obtain total lysate, the cells were resuspended in lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1X protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA, USA)] and lysed on ice for 30 min. Proteins were separated by SDS-PAGE using a Novex 4–12% Tris-glycine gel (Life Technologies, Grand Island, NY, USA) and transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Life Technologies). The membrane was blocked with 5% nonfat dry milk in PBS/0.1% Tween 20 for 1 h at room temperature, incubated with primary antibody in PBS/0.1% Tween 20 overnight at 4 °C, and then incubated with HRP-conjugated secondary antibody in PBS/0.1% Tween 20 for 1 h at room temperature. After each incubation, the membrane was washed with PBS/0.1% Tween 20. Proteins were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher, Waltham, MA, USA) and the Chemidoc XRS Imaging System (Bio-Rad, Hercules, CA, USA). The following antibodies were used: mouse anti-Cyclin D1
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(DCS6, #2926, Cell Signaling, 1:1,000 dilution), rabbit anti-Cyclin D1 (#2922, Cell Signaling, 1:1,000 dilution), fatty acid synthase (FAS) (SC-48537, Santa Cruz, 1:1,000 dilution), MDM2 (Ab-1, # OP46, Calbiochem, 1:500 dilution), rabbit anti-USP2 (AP2131c-ev, Abgent, 1:1,000 dilution), fatty acid synthase (FAS) (SC-48537, Santa Cruz, 1:1,000 dilution), MDM2 (Ab-1, # OP46, Calbiochem, 1:500 dilution), rabbit anti-USP2 (AP2131c-ev, Abgent, 1:1,000 dilution), rabbit anti-GAPDH (ab9485, Abcam, San Diego, CA, USA, 1:5,000 dilution), anti-tubulin (sc-32293, Santa Cruz, 1:1,000 dilution), HRP-conjugated anti-mouse IgG (#7076, Cell Signaling, 1:5,000 dilution), and HRP-conjugated anti-rabbit IgG (#JM-6401-05, MBL International Corporation, Woburn, MA, USA, 1:5,000 dilution. When necessary, the membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher). Band intensities were quantified using ImageJ. The ratio of Cyclin D1 or USP2 to GAPDH protein levels at each compound concentration, normalized against the start time or vehicle controls, was used to determine the IC50. Cyclin D1 and tubulin proteins in LnCAP cells and MCF7 cells were assessed by western blotting, as described (34).

Cell cycle analysis- Cells were treated with 10 µM compound and 100 nM Nocodazole for 16 hours, harvested, and fixed with 70% ethanol. The cells were then resuspended in 500 µl RNase/Propidium Iodide solution (Cell Signaling Technology) supplemented with a 1:50 dilution of 1 mg/mL Propidium Iodide (Life Technologies) and incubated at room temperature for 15 minutes. Cell cycle analysis was carried out by flow cytometry using the IntelliCyt HTFC screening system (Albuquerque, NM, USA).

Cell Cycle Imaging Assay- HCT116 cells were seeded at a density of 600 cells/well in a 96-well clear bottom black Costar plate and incubated overnight at 37 °C and 5% CO2. The media was removed and 50 µL of media (+/-100 nM nocadazole) with 196 nL of compound in DMSO was added to the cells. The media/compound mix was created in a separate 96-well plate using an acoustic dispenser for the compound followed by pipet addition of the media. Cells were washed PBS, fixed with formaldehyde (8%, Electron Microscopy Sciences, Hatfield, PA) and permeabilized with 0.2% Triton X in PBS. This was removed and 25 µL of propidium iodide/RNase (Cell Signaling Technology) was added and the plate was incubated for 1 hr at 37 °C. Plates were then read on an Acumen eX3® microplate cytometer (TTP LabTech Ltd., Melbourne, UK; excitation 488 nm, emission 575-640 band pass and 640 dichroic, PMT 500). The presence of active USP2 leads to higher fluorescence due to cell cycle progression from G0/G1 to S/M/G2.

Clonogenic Assay- 500 cells in 2 mL media were incubated in a 6-well dish at 37 °C for 2 days at which time 3 µL of compound (15, 5, 1.6, 0.56, 0.19 or 0 µM final concentration) in DMSO was added and the cells were allowed to grow for an additional 10 days. Then the media is removed, the cells are washed with PBS and crystal violet in 10% methanol was added to fix and stain the cells. After a 30 min incubation,
the crystal violet was removed and the plate was rinsed with water and allowed to air dry overnight. The cells are imaged using a Typhoon FLA 9500 (GE Healthcare Life Sciences, Pittsburg, PA, USA) in end-point mode using Cy5 detection (Ex 635 and Em long pass red filter) and the colony size and number was quantitated using the Typhoon analysis software. The total fluorescence per well was used to derive the IC50 values.

**Cell Viability Assay** - Cells were seeded at a density of 500 cells/5 µL well in a 1536-well white solid bottom plate and incubated for four hours at 37 °C and 5% CO2. Then, 23 nL of compound solution in DMSO was added and the plate was incubated for 72 hours. 3 µL Promega CellTiter Glo reagent (Madison, WI, USA) was then added to each well, and the plates were read for luminescence using a Viewlux to determine any compound effects on cell viability. For experiments with LnCAP and MCF7 cells, cells were seeded at a density of 2000 cells/100 µL well in a flat bottom 96-well plate for 24 hrs at 37 °C and 5% CO2. Then DMSO or **ML364** was added and the plates were incubated for 24 or 48 hrs. 40 µL of CellTiter Glo reagent was added to each well before reading the luminescence.

**Dual-Glo luciferase assay** - The ORFeome Luciferase fusion plasmid containing Cyclin D1 ORFeome in a pCMV-IRES-Renilla Luciferase-IRES-Gateway-Firefly Luciferase (pIRIGF) vector was kindly provided by Dr. Richard Middleton (Belfer Center for Applied Cancer Science, Boston, MA, USA). The vector contains a Cyclin D1 ORFeome fused with Firefly luciferase (22). Renilla luciferase (RLuc) encoded by the plasmid is used for normalization purpose. The expression of Cyclin D1-Firefly luciferase (FLuc) protein in 293T cells was confirmed by transfecting the cells with the plasmid followed by western blotting of the cell lysates with Cyclin D1 (Santa Cruz, # SC-753) and FLuc (Abcam, # ab16466) antibodies. To investigate the changes in Cyclin D1-Firefly luciferase protein after treatment with compounds, the luciferase assay was carried out in 96-well plate. Briefly, the day before transfection, HCT116 cells were seeded into 96-well plates (BD Biosciences) with 20,000 cells/100 µL per well. A transfection mixture (20 µL per well) containing lipofectamine and ORFeome Luciferase fusion plasmid DNA was added on the day of transfection. 24 hrs after transfection, cells were treated with DMSO or compounds (20 µL per well). The firefly and renilla luciferase signals were quantified using the Dual-glo assay kit (Promega, # E2920) according to manufacturer’s instructions at 24 hrs after the drug treatments.

**Homologous recombination (HR) analysis** - HR activity was analyzed by DR-GFP reporter assay as previously described (34,35). U2OS-DRGFP cells carrying a chromosomally integrated single copy of HR repair substrate were used. Double strand break (DSB)-induced HR in these cells results in restoration and expression of GFP (35). Cells were transfected with a plasmid encoding I-SceI endonuclease to induce DSBs. 24 hrs after transfections, cells were exposed to DMSO or **ML364** for 24 hrs. Cells were then subjected to fluorescence-activated cell sorting (FACS) analysis to quantify the percentage of viable GFP-positive cells. The RAD51 foci in Hela cells were detected by immunofluorescence as described (34) using anti-RAD51 (EMD Millipore) and Alexa Fluor 488-conjugated secondary antibodies. The quantification of cells with RAD51 foci was performed by counting the number of cells with at least ten RAD51 foci per cell.

**ADME Profile** - Stability studies were conducted by dissolving a 10 mM DMSO stock of compound into a solution of diubiquitin (DiUb) IQF assay buffer (2% DMSO, 15% MeCN, 83% DiUb assay buffer). The amount of remaining compound was monitored through 15 µL injections using LCMS analysis and determining the area under curve (AUC) of the corresponding peak (monitored at wavelength 254 nm) at time points of 0 and 48 hours. **ML364** solubility in aqueous solution and assay buffer, logD, PAMPA permeability and stability in rat microsomes and mouse plasma, and mouse plasma were tested following methods described previously (36,37). Data in mouse and human
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Microsomes were obtained from Pharmaron, Inc (Irvine, CA, USA).

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FOOTNOTES

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The abbreviations used are: USP2, ubiquitin-specific protease 2; USP8, ubiquitin-specific protease 8; IQF, internally quenched fluorescence; Di-Ub, di-ubiquitin; DUB, deubiquitinase; DMF, N,N-dimethylformamide; qHTS, quantitative high-throughput screening; DMEM, Dulbecco’s Minimal Eagle Medium; DMSO, dimethyl sulfoxide; AUC, area under curve

FIGURE LEGENDS
FIGURE 1. ML364 binds to USP2 and inhibits its activity, while a related chemical analogue 2 does not. A, Chemical structures of ML364 and 2. B, Plot of inhibition of USP2 biochemical activity of ML364 and 2, assessed using K48- and K63-linked IQF Di-Ub substrates. Colors indicate the compound/substrate combinations, as follows: Blue is ML364/K48-4, green is ML364/ K63-3, orange is 2/ K48-4 and red is 2 /K63-3. C, Inhibition of activity of caspase 6 (orange), caspase 7 (purple), MMP1 (red), MMP9 (yellow), USP8 (green) and USP15 (blue) by ML364. D-F, Demonstration of ML364 binding to USP2 through microscale thermophoresis. Microscale thermophoresis curves (Normalized fluorescence vs. time (s)) and resultant concentration responses plotted as log (concentration) in M versus normalized thermophoresis without temperature jump ((average hot)/(average cold)*1000 where hot is the average value between the pink vertical lines and cold is the average between the blue vertical lines) for (D) ML364 and (E) 2. LED was 20% and laser power was 17%. F, Microscale thermophoresis curves (Normalized fluorescence vs. time (s)) and resultant IC50 curves plotted as log (concentration, M) versus normalized thermophoresis without temperature jump ((average hot)/(average cold)*1000 where hot is the average value between the pink vertical lines and cold is the average between the blue vertical lines) for titration of USP2 alone. LED= 20% and MST power 60%. G, ML364 and 2 bind reversibly to USP2. The fluorescence over time is plotted for ML364 (blue), 2 (red), enzyme alone (green) and no enzyme (purple).

FIGURE 2. Kinomescan visualization for ML364 (10 µM) tested for binding to 102 kinases.

FIGURE 3. ML364 reduces Cyclin D1 protein levels in a time-, dose-, and proteasome-dependent manner in HCT116 cells and Mino cells. A, HCT116 cells were treated with 10 µM ML364 for the indicated amount of time (top panel) or with the indicated concentration of ML364 (bottom panel) for 4 hours. B, HCT116 cells were treated with 10 µM 2 for the indicated amount of time (top panel) or with the indicated concentration of 2 (bottom panel) for 4 hours. C, HCT116 cells were treated with 10 µM ML364 or 10 µM 2 for 4 hours in the absence or presence of 10 µM MG132. D, Mino cells were treated with 10 µM ML364 for the indicated amount of time (top panel) or with the indicated concentration of ML364 (bottom panel) for 4 hours. E, Mino cells were treated with 10 µM 2 for the indicated amount of time (top panel) or with the indicated concentration of 2 (bottom panel) for 4 hours. F, Mino cells were treated with 10 µM ML364 or 10 µM 2 for 4 hours in the absence or presence of 10 µM MG132. Cyclin D1 and USP2 protein levels were assessed by western blotting using a GAPDH control. Bands were quantitated by ImageJ.

FIGURE 4. ML364 directly targets Cyclin D1 for degradation. A, The effect of ML364 on Cyclin D1 protein levels is confirmed by in-cell ELISA. HCT116 cells were treated with the indicated concentration of ML364 or 2 for 24 hours, and Cyclin D1 protein levels were quantified by in-cell ELISA. B-C, Expression of Cyclin D1-FLuc fusion protein in 293-T cells. Cells were transfected with a plasmid vector encoding Cyclin D1-Fluc and RLuc. 48 hrs after transfection, Cyclin D1-FLuc fusion protein levels were detected by western blotting using a tubulin control. D, ML364 downregulates Cyclin D1 in HCT116 cells. Twenty four hrs after transfection with a plasmid vector encoding Cyclin D1-Fluc and RLuc (as indicated in panels b and c), the cells were exposed to ML364. 24 hrs after ML364 exposure, cellular FLuc and RLuc activity was measured. The fold change of FLuc/RLuc ratio normalized to corresponding to DMSO-treated cells is shown. E, HSP90 inhibitor BIIB021 reduces FLuc activity in HCT116 cells. The cells were treated and analyzed as described in (D). F, ML364 downregulates Cyclin D1 in 293T cells. Forty four hrs after transfection with a plasmid vector encoding Cyclin D1-Fluc and RLuc (as indicated in panels b and c), the cells were exposed to ML364. Four hrs after ML364 exposure, cellular FLuc and RLuc activity was measured as described in (D).
FIGURE 5. ML364 induces cell cycle arrest and inhibits cell growth in HCT116 and Mino cells. A, HCT116 and (B) Mino cells were treated with DMSO (purple), 10 µM ML364 + 100 nM nocodazole (green), or 10 µM 2 + 100 nM nocodazole (red) or 100 nM nocodazole (blue) for 16 hours. The percentage of cells in each phase of the cell cycle was determined by flow cytometry and is displayed in each plot below the population brackets, which are, from left to right, G1, S, and G2/M phases, respectively. C, The percentage of G1 phase (circles) and G2 phase (triangles) for ML364 as determined by cell image analysis of fixed and stained HCT116 cells treated with ML364. The concentration-response curves and images of the 6-well plate for the colony forming assays with HCT116 cells for ML364 (D) and (E) and 2 (F) and (G), respectively.

FIGURE 6. ML364 exposure decreases cell viability and promotes Cyclin D1 degradation in cancer cell lines. A, The effect of ML364 on cell viability of Mino (squares) and HCT116 (circles) cells as measured by ATP content using Cell titer Glo™. B-C, The effect of ML364 on cell viability of LnCAP (circles) and MCF7 (squares) cells as measured by ATP content using Cell titer Glo™. The cells were exposed to ML364 for 24 hrs (B) or 48 hrs (C). D, ML364 promotes degradation of Cyclin D1 in LnCAP cells and MCF7 cells. Cells were treated with ML364 for 24 hrs and Cyclin D1 protein levels were assessed by western blotting using a tubulin control. E-F, ML364 exposure results in a moderate decrease in FAS levels. LnCAP cells were treated with ML364 for 24 hrs and FAS, MDM2 and Cyclin D1 protein levels were assessed by western blotting using a vinculin control.

FIGURE 7. Inhibition of USP2 activity by ML364 causes a decrease in HR-mediated DNA repair. A, U2OS-DRGFP cells were transfected with a plasmid encoding I-SceI endonuclease and cultured for 24 hrs. followed by exposure to ML364 at the indicated concentration for 24 h. Cells were then subjected to flow cytometric analysis. The relative GFP positive cells normalized by solvent vehicle treated group are shown. B, C, Hela cells were treated with ML364 (5 µM) for 24 hrs before exposing them with IR (10 Gy). Eight hrs after IR, cells were analyzed for RAD51 foci by immunofluorescence. Representative images (B) and quantification (C) of RAD51 foci are shown. At least one hundred cells were scored for RAD51 foci for each replicate and three replicates were scored.

FIGURE 8. Schematic showing the impact of ML364 on USP2 which leads to accelerated Cyclin D1 degradation and subsequent G0/G1 cell cycle arrest.

FIGURE 9. A, Synthetic route to ML364. B, Synthetic route to compound 2.
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**TABLES**

**Table 1. Summary of Biochemical and Cell Assay Results**

|                              | ML364 | Compound 2<sup>a</sup> |
|------------------------------|-------|-------------------------|
| USP2 Di-Ub IQF K48-4 (IC<sub>50</sub>) | 1.1 µM | inactive                |
| USP2 Di-Ub IQF K63-3 (IC<sub>50</sub>) | 1.7 µM | inactive                |
| HCT116 Cell Viability (EC<sub>50</sub>) | 3 µM  | inactive                |
| Mino Cell Viability (EC<sub>50</sub>) | 4.7 µM | inactive                |
| HCT116 Clonogenic (EC<sub>50</sub>) | 3.6 µM | inactive                |
| HCT116 Acumen Cell Cycle (EC<sub>50</sub>) | 2 µM  | inactive                |
| HCT116 Flow, Cell cycle arrest in G1 | inactive | inactive                |
| Western HCT116, Cyclin D1 destabilization | 0.97 µM | inactive                |
| Western Mino, Cyclin D1 destabilization | 8.5 µM | inactive                |
| Caspase 6 (IC<sub>50</sub>) | inactive | inactive                |
| Caspase 7 (IC<sub>50</sub>) | inactive | inactive                |
| USP8 Di-Ub IQF K48-2 (IC<sub>50</sub>) | 0.95 µM | inactive                |
| USP20 Di-Ub IQF K48-2 (IC<sub>50</sub>) | inactive | inactive                |
| MMP1 (IC<sub>50</sub>) | inactive | ND                      |
| MMP9 (IC<sub>50</sub>) | inactive | ND                      |
| 102 Kinases Screened at 10 µM | inactive | ND                      |

<sup>a</sup> ND = Not determined
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Table 2. Kinase Competition Binding for ML364 from Kinomescan

| Entrez Gene Symbol | Percent Control | Entrez Gene Symbol | Percent Control | Entrez Gene Symbol | Percent Control |
|--------------------|-----------------|--------------------|-----------------|--------------------|-----------------|
| ABL1               | 100             | EPHA2              | 90              | PAK2               | 100             |
| ABL1               | 82              | ERBB2              | 100             | PAK4               | 98              |
| ABL1               | 90              | ERBB4              | 99              | CDK16              | 96              |
| ABL1               | 90              | MAPK3              | 100             | PDGFRA             | 91              |
| ACVR1B             | 94              | PTK2               | 100             | PDGFRB             | 95              |
| CABC1              | 84              | FGFR2              | 85              | PDK1               | 100             |
| AKT1               | 92              | FGFR3              | 88              | PIK3C2B            | 79              |
| AKT2               | 56              | FLT3               | 71              | PIK3CA             | 87              |
| ALK                | 68              | GSK3B              | 100             | PIK3CG             | 89              |
| AURKA              | 87              | IGF1R              | 88              | PIM1               | 94              |
| AURKB              | 87              | CHUK               | 83              | PIM2               | 100             |
| AXL                | 62              | IKBKB              | 79              | PIM3               | 90              |
| BMPR2              | 85              | INSR               | 81              | PRKACA             | 95              |
| BRAF               | 82              | JAK2               | 75              | PLK1               | 98              |
| BRAF               | 94              | JAK3               | 98              | PLK3               | 78              |
| BTK                | 84              | MAPK8              | 88              | PLK4               | 83              |
| CAMK2B             | 93              | MAPK9              | 84              | PRKCE              | 88              |
| CDK19              | 100             | MAPK10             | 84              | RAF1               | 93              |
| CDK2               | 100             | KIT                | 97              | RET                | 92              |
| CDK3               | 88              | KIT                | 85              | RIOK2              | 100             |
| CDK4               | 88              | KIT                | 96              | ROCK2              | 89              |
| CDK4               | 81              | STK11              | 100             | RPS6KA3            | 100             |
| CDK5               | 100             | MAP3K4             | 73              | NUAK2              | 72              |
| CDK7               | 76              | MAPKAPK2           | 81              | SRC                | 96              |
| CDK8               | 100             | MARK3              | 83              | SRPK3              | 96              |
| CDK9               | 93              | MAP2K1             | 89              | TGFB1              | 100             |
| CHEK1              | 84              | MAP2K2             | 84              | TEK                | 98              |
| CSF1R              | 100             | MET                | 90              | NTRK1              | 100             |
| CSNK1D             | 100             | MKNK1              | 69              | TSSK1B             | 44              |
| CSNK1G2            | 98              | MKNK2              | 100             | TYK2               | 83              |
| DCLK1              | 100             | MAP3K9             | 92              | ULK2               | 81              |
| DYRK1B             | 100             | MAPK14             | 92              | KDR                | 79              |
| EGFR               | 86              | MAPK11             | 95              |                    |                 |
| EGFR               | 95              | PAK1               | 99              |                    |                 |
Table 3. ADME Properties of ML364

|                  | Aq. Kinetic Solubility (pH 7.4, PBS Buffer) | Aq. Kinetic Solubility (pH 8, DiUb Assay Buffer without β-ME) | Aq. Stability (2% DMSO, 15% MeCN, 83% pH 8 DiUb assay buffer) | Log D (measured at pH 7.4, PBS Buffer) | PAMPA Permeability |
|------------------|-------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|--------------------------------------|-------------------|
| ML364            | <2 µM                                     | 28.9 µM                                                       | >48 hrs                                                       | 2.31                                 | 82.2 x 10^{-6} cm/s |

|                  | Rat Microsomal Stability, $t_{1/2}$ | Mouse Microsomal Stability, (remaining at 30 minutes) | Human Microsomal Stability, (remaining at 30 minutes) | Mouse Plasma Stability (remaining at 30 minutes) |
|------------------|-----------------------------------|-----------------------------------------------------|-----------------------------------------------------|-----------------------------------------------|
| ML364            | 15 minutes                        | 61 %                                                | 84 %                                                | 100 %                                         |

*a* Performed in-house.; *b* Contracted to Analiza, Inc.; *c* Contracted to Pharmaron, Inc.
FIGURES

Fig. 1
Fig. 2

USP2 inhibition impacts cyclin D1 and cell cycle
**USP2 inhibition impacts cyclin D1 and cell cycle**

Fig. 3

| A | 0 | 2 | 4 | 8 | 16 | 24 |
|---|---|---|---|---|----|----|
| Time (h) | 1.00 | 0.43 | 0.27 | 0.25 | 0.12 | 0.05 |
| Relative band intensity | Cyclin D1 | 1.00 | 1.10 | 1.07 | 0.90 | 0.87 |
| Relative band intensity | USP2 | 1.00 | 0.54 | 0.34 | 0.32 | 0.28 |
| Relative band intensity | GAPDH | 1.00 | 0.91 | 1.04 | 1.08 | 1.14 | 1.19 |

| B | 0 | 2 | 4 | 8 | 24 |
|---|---|---|---|---|----|
| Time (h) | 1.00 | 1.04 | 1.10 | 1.06 | 0.96 |
| Relative band intensity | Cyclin D1 | 1.00 | 0.86 | 0.78 | 0.76 | 0.83 | 0.79 |
| Relative band intensity | GAPDH | 1.00 | 1.19 | 0.18 | 0.66 | 0.90 | 1.14 |

| C | - | - | + | + | - |
|---|---|---|---|---|---|
| - | - | - | + | + | + |
| - | + | - | + | + |
| 1.00 | 1.04 | 1.10 | 1.06 |
| Relative band intensity | Cyclin D1 | 1.00 | 0.95 | 1.14 | 1.10 | 1.10 | 1.06 |

| D | 0 | 2 | 4 | 8 | 16 | 24 |
|---|---|---|---|---|----|----|
| Time (h) | 1.00 | 0.54 | 0.49 | 0.37 | 0.28 | 0.22 |
| Relative band intensity | Cyclin D1 | 1.00 | 0.92 | 0.79 | 0.80 | 0.78 | 0.82 |
| Relative band intensity | USP2 | 1.00 | 0.98 | 0.78 | 0.83 | 0.85 | 1.04 |
| Relative band intensity | GAPDH | 1.00 | 0.91 | 1.04 | 1.08 | 1.14 | 1.19 |

| E | 0 | 2 | 4 | 8 | 16 | 24 |
|---|---|---|---|---|----|----|
| Time (h) | 1.00 | 1.04 | 1.10 | 1.06 | 1.04 | 0.97 |
| Relative band intensity | Cyclin D1 | 1.00 | 0.95 | 1.14 | 1.10 | 1.10 | 1.06 |
| Relative band intensity | GAPDH | 1.00 | 0.98 | 0.78 | 0.83 | 0.85 | 1.04 |

| F | - | - | + | + | - |
|---|---|---|---|---|---|
| - | - | - | + | + | + |
| - | + | - | + | + |
| - | - | - | + | + | + |
| 1.00 | 1.04 | 1.10 | 1.06 | 0.97 | 0.95 | 1.14 | 1.10 | 1.10 | 1.06 | 1.04 | 0.97 |
| Relative band intensity | Cyclin D1 | 1.00 | 0.95 | 1.14 | 1.10 | 1.10 | 1.06 |
| Relative band intensity | GAPDH | 1.00 | 0.98 | 0.78 | 0.83 | 0.85 | 1.04 |
USP2 inhibition impacts cyclin D1 and cell cycle
USP2 inhibition impacts cyclin D1 and cell cycle

Fig. 5

A

B

C

D

E

F

G
USP2 inhibition impacts cyclin D1 and cell cycle

Fig. 6
USP2 inhibition impacts cyclin D1 and cell cycle
USP2 inhibition impacts cyclin D1 and cell cycle

Fig. 8

ML364 Inhibition of USP2

Cyclin D1 Degradation

G0 G1 G2 M
S

G1 Arrest
USP2 inhibition impacts cyclin D1 and cell cycle

Fig. 9

A

\[
\text{HO-CONH}_2 \xrightarrow{\text{ClSO}_2 \text{C}_6 \text{H}_4, \text{H}_2 \text{O}} \xrightarrow{\text{Na}_2 \text{CO}_3, \text{H}_2 \text{O}} \text{HNOSO}_3 \text{C}_6 \text{H}_4 \text{CF}_3 \xrightarrow{\text{HATU, DIPEA, DMF}} \text{ML364}
\]

\[
\text{SI-4} \xrightarrow{80^\circ \text{C}, \text{2h}, 57\%} \text{ML364}
\]

B

\[
\text{HO-CO-NH}_2 \xrightarrow{\text{ClSO}_2 \text{C}_6 \text{H}_4, \text{H}_2 \text{O}} \xrightarrow{\text{Na}_2 \text{CO}_3, \text{H}_2 \text{O}} \text{HNOSO}_3 \text{C}_6 \text{H}_4 \text{CF}_3 \xrightarrow{\text{HATU, DIPEA, DMF}} \text{2}
\]

\[
\text{SI-2} \xrightarrow{80^\circ \text{C}, \text{2h}, 16\%} \text{2}
\]

\[
\text{SI-2} \xrightarrow{80^\circ \text{C}, \text{12h}, 62\%} \text{2}
\]
Small Molecule Inhibition of the Ubiquitin-specific Protease USP2 Accelerates Cyclin D1 Degradation and Leads to Cell Cycle Arrest in Colorectal Cancer and Mantle Cell Lymphoma Models

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