Pre-clinical Investigation of the Wee1 Inhibitor, MK-1775, Using Pharmacodynamic and Mechanistic Markers in Diverse Cancer Models In Vivo

Abstract #4689

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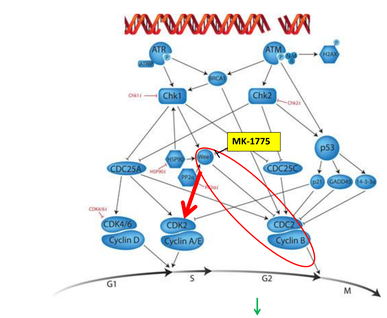
Introduction

Wee1 kinase is a critical regulator of the G2/M checkpoint by initiating inhibitory phosphorylation of the conserved tyrosine-15 residue of cdk1/cdc2. Wee1 also phosphorylates tyrosine 15 of cdk2 and plays a crucial role in maintaining genomic integrity during S-phase. AZD1775 (MK1775) (NSC 754352) is a small-molecule selective inhibitor of Wee1 kinase that is currently under clinical evaluation. We have developed a quantitative immunofluorescence assay to measure inhibition of Wee1 by detection of pY15 of cyclin-dependent kinases. A full analysis of the pharmacodynamic (PD) effects of Wee1 inhibition by MK1775, as well as the downstream mechanistic consequences of Wee1 inhibition on DNA repair, apoptosis, and premature mitotic entry markers, was performed using two xenograft models: A673 Ewing sarcoma and U87-MG glioblastoma. DNA repair markers (γH2AX, pNbs1^{S343}) were examined as well as the mitotic marker pHistone H3. Advanced quantitative image analysis was performed using Definiens software to measure changes in PD markers by total nuclear area measurements. Definiens software enables enhanced analysis of the markers by enumeration of the nuclei in the imaged xenograft tissues, over an entire data set with high-content capacity. Our data demonstrate greater than 80% inhibition of pY15-cdk in vivo at the clinically relevant dose of 60 mg/kg (180 mg/m^2) MK1775 in two xenograft models. In addition, γH2AX induction was observed after multiple doses of MK1775 as a single agent. A PD biomarker time course was determined for MK1775 and gemcitabine (NSC 750927) as single agents to develop a drug administration schedule for combination studies in a Ewing sarcoma model. The time point at which the DNA damage response peaked following administration of the maximum tolerated dose of gemcitabine in the mouse was determined. This study elucidates a broad profile of PD marker response, as well as the corresponding levels of MK1775 in the xenografts. Funded by NCI Contract No. HHSN261200800001E.

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CDK1  1 ----------ME---------DYTKIEKIGEGTYGVVYKGR—H-KTTGQVVAMKKIRLE    35
CDK2  1 ----------ME---------NFQKVEKIGEGTYGVVYKAR—N-KLTGEVVALKKIRLD    35
CDK3  1 ----------MD---------MFQKVEKIGEGTYGVVYKAK—N-RETGQLVALKKIRLD    35
CDK4  1 ----------M--------ATSRYEPVAEIGVGAYGTVYKAR—D-PHSGHFVALKSVRVP    40
CDK5  1 ----------MQ--------KYEKLEKIGEGTYGTVFKAK—N-RETHEIVALKRVRLD    35
CDK6  1 ----------MEKDGLCRADQQYECVAEIGEGAYGKVFKAR—DLKNGGRFVALKRVRVQ    45
CDK7  1 ----------MALDVKSRA-KRYEKLDFLGEGQFATVYKAR—D-KNTNQIVAIKKIKLG    46
CDK8  1 MDYDPKVKLSSERE-RVEDLFEYE-GCKVGRGTYGHVYKAKRKD-GKDDKDYALKQIE--    50
CDK9  1 ---MAKQYDSVECP-FCDEVSKYEKLAKIGQGTFGEVFKAR—H-RKTGQKVALKKVLME    60
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Methods

Immunofluorescence staining: Xenografts were formalin fixed and paraffin embedded, and were cut onto glass slides that were stained with antibodies for pY15-cdk2 (EPR2233Y, Abcam), Nbs1 pS343 (EP178, Abcam), γH2AX (JBW301, Millipore), Phospho-Histone H3 (Ser10) (D2C8), Biotinylated (Cell Signaling Tech), and Ki67 (Sp6, Abcam), using a modified version of the published protocol (Kinders et al., *Clin Cancer Res* 2010; 16(22): 5447–5457).

Image acquisition and analysis: Slides were scanned using the Aperio imaging system, and 20x images were extracted using ImageScope software. Images were analyzed for nuclear area positive using Definiens Tissue Studio IF software. Images are representative of the mean.

SOPs for the validated γH2AX immunofluorescence assay are posted on the DCTD web site: http://dctd.cancer.gov/ResearchResources/ResearchResources-biomarkers.htm

Data

PD Marker Analysis in U87-MG Xenograft Analysis

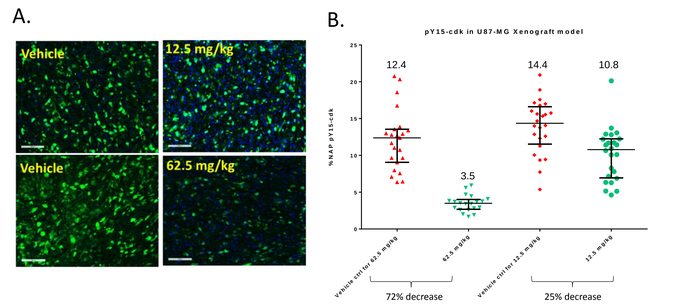


Figure 1: Quantitative analysis of pY15-cdk in response to MK1775 treatment in a U87-MG glioblastoma xenograft model. MK1775 was administered BID X 10 by IP route. Tumor quadrants were formalin fixed and paraffin embedded, and cut slides were stained for pY15-cdk by immunofluorescent assay. Quantitative analysis was performed using Definiens software. Each analyzed 20x image is represented as a point on the graph and contains over 1,000 cells. Median with interquartile range was plotted.

Quantitative analysis

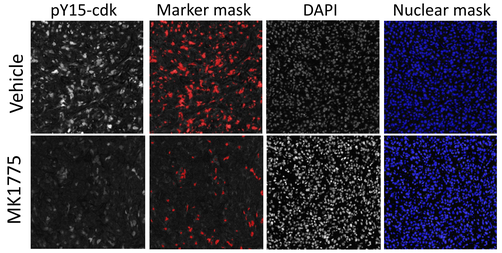


Figure 2: Definiens analysis of the pY15-cdk marker area after exposure to MK1775 (62.5 mg/kg QDx5, 4 h post-dose 5) in U87-MG xenograft tissues using pY15-cdk2 (EPR2233Y). Nuclei are classified using intensity criteria. The marker area mask was created by thresholding the marker intensity; nuclear area positive was calculated using the marker area and nuclear mask information.

Assay Development

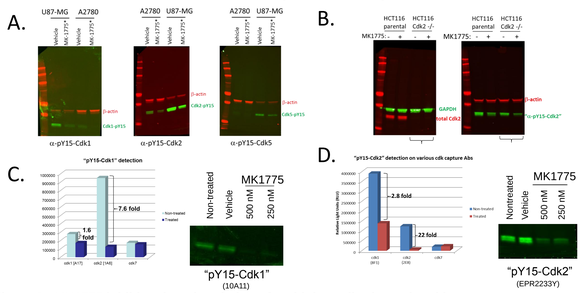
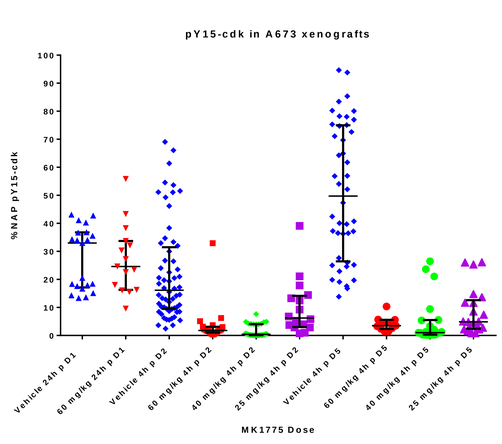


Figure 3: MK1775 inhibits phosphorylation of multiple cyclin-dependent kinases.
A. Cell lines treated with 250 nM of MK1775 for 4 h (A2780) or 8 h (U87). **B.** A Cdk2 -/- isogenic cell line was used to illustrate that pY15-Cdk2 Ab recognizes other Cdks. MK1775 drug modulates all cdk proteins recognized by pY15 Cdk2 antibody (EPR2233Y) (Abcam) **C and D.** ELISA demonstrates that cdk1 and -2 are modulated by MK1775 and that cdk2 capture is consistently more sensitive than Cdk1. Selection of reagent: pY15-cdk2 (EPR2233Y); Abcam to measure both cdk1 and cdk2 phosphorylation.

PD Marker and Mechanistic Marker Analysis in A673 Ewing Sarcoma Xenograft Model



Mouse Dose	Human Equivalent
60 mg/kg	~ 288 mg
40 mg/kg	~ 192 mg
25 mg/kg	~ 120 mg
12.5 mg/kg	~ 60 mg

Sample	Nuclei	# of Tumors analyzed	Mean % NAP pY15-cdk
Vehicle (24h p D1)	35274	4	27.50
60 mpk 24h p D1	24666	3	27.16
Vehicle (4h p D2)	73423	2	22.12
60 mpk	26842	2	3.76
40 mpk	38429	3	1.77
25 mpk	20679	2	10.02
Vehicle (4h p D5)	39219	2	51.19
60 mpk	20586	2	4.08
40 mpk	27670	2	5.0
25 mpk	28881	3	8.52

4h p D2 Mean Veh vs 60 & 40 mg/kg: p<0.05
4h p D5 Mean Veh vs 60, 40 & 25 mg/kg: p<0.05

Figure 4: Quantitative analysis of pY15-cdk in response to MK1775 treatment in A673 Ewing sarcoma xenograft model. MK1775 was administered QD for 5 doses and harvested 24 h post-dose 1 and 4 h post-dose 2 and dose 5. Tumor quadrants were formalin fixed and paraffin embedded, and cut slides were stained for pY15-cdk by immunofluorescent assay. Quantitative analysis was performed using Definiens software. Each analyzed 20x image is represented as a point and contains over 1,000 cells. Median with interquartile range was plotted.

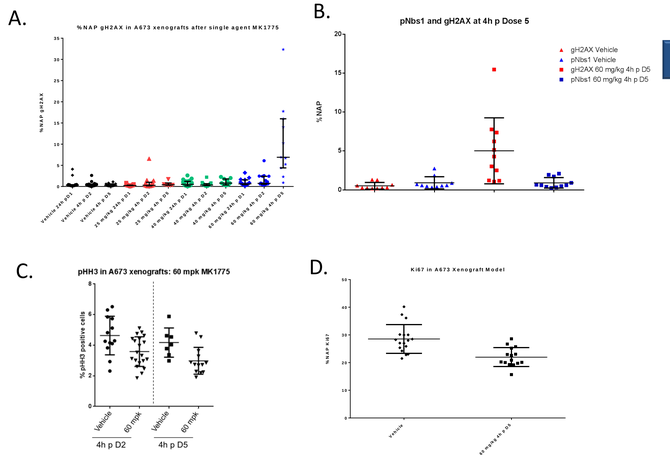


Figure 5: Quantitative analysis of markers of DNA repair, apoptosis, proliferation, and mitotic arrest in response to single-agent MK1775 treatment in A673 Ewing sarcoma xenograft model by immunofluorescence assay. A. gH2AX was induced at 4 h post-dose 5 at 60 mg/kg, but not at earlier time points. **B.** DNA repair marker pNbs1 was not induced at 4 h post-dose 5 at 60 mg/kg. **C.** pHistone H3, a marker of mitotic arrest, is not induced by MK1775. **D.** Ki67, a marker of proliferation is reduced at 4 h post-d5 at 60 mg/kg. Quantitative analysis was performed using Definiens software. Each analyzed 20x image is represented as a point and contains over 1,000 cells. Median with interquartile range was plotted.

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PD Marker Time Course of Gemcitabine Single-Agent In Vivo

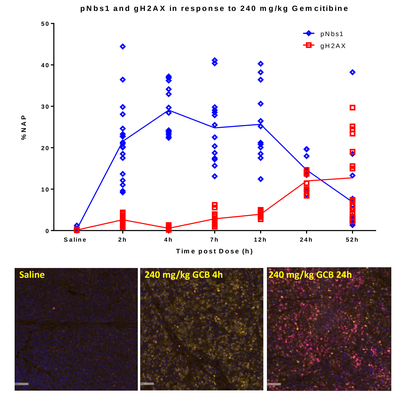

Figure 6: PD marker time course of gemcitabine single agent in vivo.
A. Analysis of percent NAP (nuclear area positive) pNbs1 and gH2AX after one dose of gemcitabine (240 mg/kg) in the A673 xenograft model. **B.** Example images of xenograft tissues following multiplex immunofluorescence assay for gH2AX (pink) and pNbs1 (yellow). DAPI (blue).

Summary and Conclusions

- pY15-cdk assay was developed in support of CTEP # 9350: A Phase I Study of Single-Agent MK-1775, a Wee1 Inhibitor, in Patients with Advanced Refractory Solid Tumors

MK1775 Single Agent:

- U87-MG glioblastoma model: There is a clear reduction (~70% decrease) in pY15-cdk PD marker at the 4 h post-dose 10 time point at 62.5 mg/kg MK1775, but not at 12.5 mg/kg.
- A673 model: There is a clear reduction of pY15-cdk PD Marker:
  - At 4 h post-dose 5: at all doses - 60, 40, and 25 mg/kg. (>80% decrease from average)
  - At 4 h post-dose 2: at two doses - 60 and 40 mg/kg doses (not 25 mg/kg).
  - There is no significant reduction of pY15-cdk after a single dose at the 24 h time point.
- Downstream indicators with MK1775 single agent:
  - pHistone H3 was not induced.
  - pNbs1 was not induced.
  - γH2AX is induced at the latest time point and highest dose (60 mg/kg, 4 h post-dose5). γH2AX induction is probably due to apoptosis.
  - Ki67 proliferation marker decreased at 4 h post-dose 5 (60 mg/kg) MK1775.

Gemcitabine Single-Agent Time Course:

- PD marker timecourse for single-agent gemcitabine shows a clear "wave" of expression: pNbs1 early (4–12 h) and γH2AX later (24 h).
- The DDR effects of gemcitabine are independent of MK1775 and can be clearly evaluated in future combination studies. MK1775 did not induce DDR effects.

All animals used in this research project were cared for and used humanely according to the following policies: the *U.S. Public Health Service Policy on Humane Care and Use of Animals* (2000); the *Guide for the Care and Use of Laboratory Animals* (1996); and the *U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* (1985). All Frederick National Laboratory animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.