

MK-1775, a Potent Wee1 Inhibitor, Synergizes with Gemcitabine to Achieve Tumor Regressions, Selectively in p53-Deficient Pancreatic Cancer Xenografts

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Abstract

Purpose: Investigate the efficacy and pharmacodynamic effects of MK-1775, a potent Wee1 inhibitor, in both monotherapy and in combination with gemcitabine (GEM) using a panel of p53-deficient and p53 wild-type human pancreatic cancer xenografts.

Experimental Design: Nine individual patient-derived pancreatic cancer xenografts (6 with p53-deficient and 3 with p53 wild-type status) from the PancXenoBank collection at Johns Hopkins were treated with MK-1775, GEM, or GEM followed 24 hour later by MK-1775, for 4 weeks. Tumor growth rate/regressions were calculated on day 28. Target modulation was assessed by Western blotting and immunohistochemistry.

Results: MK-1775 treatment led to the inhibition of Wee1 kinase and reduced inhibitory phosphorylation of its substrate Cdc2. MK-1775, when dosed with GEM, abrogated the checkpoint arrest to promote mitotic entry and facilitated tumor cell death as compared to control and GEM-treated tumors. MK-1775 monotherapy did not induce tumor regressions. However, the combination of GEM with MK-1775 produced robust antitumor activity and remarkably enhanced tumor regression response (4.01-fold) compared to GEM treatment in p53-deficient tumors. Tumor regrowth curves plotted after the drug treatment period suggest that the effect of the combination therapy is longer-lasting than that of GEM. None of the agents produced tumor regressions in p53 wild-type xenografts.

Conclusions: These results indicate that MK-1775 selectively synergizes with GEM to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts. *Clin Cancer Res*; 17(9); 2799–806. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDA) remains one of the least curable cancers, ranking fourth in cancer related

deaths in the United States (1). Worldwide, PDA contributes to more than 230,000 deaths annually (2). Gemcitabine (GEM), a pyrimidine antimetabolite, is the accepted standard treatment for patients with advanced and metastatic PDA, but the benefit is small (3). The addition of other chemotherapeutics or monoclonal antibodies or radiation to GEM has not resulted any meaningful improvement in the survival of PDA patients (4–6). As the current therapies offer very limited benefit, there is significant unmet medical need to identify novel molecular targets and develop novel therapeutic strategies to treat this devastating disease (7).

Treatment efficacy of DNA damaging agents is determined not only by the amount of therapy-induced DNA damage but also by the capacity of tumor cells to repair the damaged DNA. Many of the conventional anticancer treatments including antimetabolites, ionizing radiation, alkylating agents, platinum compounds, and DNA topoisomerase inhibitors exert their antitumor effects by damaging the DNA in tumor cells which leads to apoptosis (8). However, when cells are treated with DNA damaging agents, multiple checkpoints are activated, including G₁, intra-S, and G₂/M, leading to cell cycle arrest, thus providing time for the cell to repair the

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Translational Relevance

Treatment efficacy of DNA damaging agents is determined not only by the amount of therapy-induced DNA damage but also by the capacity of tumor cells to repair the damaged DNA. The G₂ DNA damage checkpoint ensures maintenance of cell viability by delaying progression into mitosis when cells have suffered DNA damage. Wee1 is a cellular protein kinase which inhibits Cdc2 activity, thereby preventing cells from proceeding through mitosis by maintaining G₂ arrest. MK-1775 is the first reported Wee1 inhibitor with high potency, selectivity, and oral bioavailability in preclinical animal models, and is currently being evaluated in several phase I clinical trials. Here we provide compelling evidence that MK-1775 synergizes with gemcitabine to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts. These results may help to frame clinical investigation of Wee1 inhibitors along with chemotherapy to benefit pancreatic cancer patients whose tumor cells are devoid of functional p53 gene.

damage and to evade apoptosis before resuming the cell cycle (9–11). Tumor cells can exploit these repair mechanisms in response to DNA damaging chemotherapeutics, rendering tumors refractory to current therapeutic interventions. Therefore, abrogation of checkpoints function may drive tumor cells toward apoptosis and enhance the efficacy of oncotherapy.

p53 is a key regulator of the G₁ checkpoint and is one of the most frequently mutated genes in cancer. Since G₁ checkpoint is frequently compromised due to loss-of-function mutation of p53 gene in 50% to 70% of all cancers, the G₂/M checkpoint plays a pivotal role in preventing the programmed cell death in p53-deficient tumors (12, 13). Hence, inhibitors of the G₂ checkpoint can selectively sensitize cancer cells with deficient p53 to killing by DNA-damaging anticancer agents while sparing normal tissue from toxicity.

The G₂ DNA damage checkpoint ensures maintenance of cell viability by delaying progression into mitosis when cells have suffered DNA damage. Wee1 is a cellular protein kinase which inhibits Cdc2 activity, thereby preventing cells from proceeding through mitosis by maintaining G₂ arrest (14). Wee1 reversibly arrests the cell cycle by inactivating Cdc2 through phosphorylation at Tyr-15 (15). Disruption of this phosphorylation site abrogates checkpoint-mediated regulation of Cdc2 (16). Wee1 knockdown with siRNA has been reported to abrogate the G₂ DNA damage checkpoint arrest and to sensitize cancer cells to DNA damaging agents (17). Wee1 kinase inhibition is expected to potentiate the antitumor effect of DNA damaging chemotherapeutics by overcoming the G₂ arrest and thereby promoting checkpoint bypass to facilitate the preferential killing of p53-deficient tumor cells via mitotic catastrophe.

MK-1775 is the first reported Wee1 inhibitor with high potency, selectivity, and oral bioavailability in preclinical animal models and is currently being evaluated in several phase I clinical trials (18–21). Given that p53 mutations are common in pancreatic cancer (22), we sought to investigate efficacy and pharmacodynamic effects of MK-1775 alone and in combination with GEM in pancreatic cancer xenografts with p53 wild-type or p53-deficient status. Our results provide compelling evidence that MK-1775 synergizes with GEM to achieve tumor regressions, selectively in p53-deficient pancreatic cancer xenografts. These results may help to frame clinical investigation of Wee1 inhibitors along with chemotherapy to benefit cancer patients whose tumor cells are devoid of functional p53 gene.

Materials and Methods

Animals and establishment of xenografts model

Animal experiments were conducted following approval and accordance with Animal Care and Use Committee guidelines of Johns Hopkins University. Fresh pancreatic cancer specimens resected from patients at the time of surgery, with informed written patient consent, were implanted subcutaneously into the flanks of 6-week-old female *nu/nu* athymic mice (Harlan). The patients had not undergone chemotherapy or radiation therapy before surgery. Grafted tumors were subsequently transplanted from mouse to mouse and maintained as a live PancXenoBank according to an Institutional Review Board (IRB) approved protocol (23). Tumor-specific mutations of protein-coding genes (exomic sequencing) in these xenografts have been recently reported (24). Most importantly, these xenografts were not placed in culture and appear to retain most of the genetic features of the original tumor, despite serial passing across several generations of mice (25, 26).

Drugs

The Wee1 inhibitor, MK-1775, was provided by Merck Research Laboratories. GEM (Eli Lilly) was purchased from pharmacy.

In vivo efficacy experiments

Nine pancreatic cancer xenografts (6 with p53-deficient and 3 with p53 wild-type status) from PancXenoBank were allowed to grow separately on both flanks of athymic mice. When tumors reached a volume of approximately 200 mm³, mice were individually identified and randomly assigned to treatment groups, with 5 to 6 mice (8–10 evaluable tumors) in each group: (1) control; (2) MK-1775 (30 mg/kg, p.o., once daily for 4 weeks); (3) GEM (100 mg/kg, i.p., twice weekly on days 1 and 4) for 4 weeks; (4) GEM followed 24 hour later by MK-1775 in the above mentioned dose. Tumor growth was evaluated twice per week by measurement of 2 perpendicular diameters of tumors with a digital caliper. Individual tumor volumes were calculated as $V = ab^2/2$, where a being the largest diameter, b the smallest. Relative tumor growth index (TGI)

on day 28 was calculated using the formula: (mean tumor volume of drug-treated group/mean tumor volume of control group) \times 100. Number of tumors that regressed more than 50% of its initial size in each xenograft was noted. Animals were sacrificed 1 hour after the last dose of GEM or MK-1775 and tumors were harvested for analysis except 3 mice each from GEM and combination treatment group, which were kept longer to check tumor regrowth after the treatment. Mice kept for the regrowth study were sacrificed when the tumors reached the size of control tumors in that xenograft.

Protein extraction and Western blotting

Protein extracts were prepared from tumors according to previously published method (27). Briefly, tumors (50 mg) were minced on ice in prechilled lysis buffer containing protease cocktail (Roche Molecular Biochemicals). The minced tissue was homogenized and centrifuged at $16,000 \times g$ at 4°C for 10 minutes. Protein content in the supernatants was measured with the Pierce Protein Assay kit using bovine serum albumin (BSA) as a standard. Protein lysates (30 μg) were boiled in Laemmli sample buffer (Bio-Rad Laboratories) resolved by electrophoresis on 4% to 12% Bis-Tris precast gels (Bio-Rad Laboratories), and transferred to Immobilon-P membranes (Millipore). Membranes were blocked at room temperature with 5% nonfat milk (Pierce) for 1 hour. Primary antibodies against Wee1, phospho-Wee1^{Ser642}, Cdc2 (p-Cdc2), phospho-Cdc2^{Tyr15} (p-Cdc2^{Tyr15}), phospho-histone H3^{Ser10} (p-HH3^{Ser10}), cleaved poly (ADP-ribose) polymerase^{Asp214} (C-PARP^{Asp214}), phospho-H2AX^{Ser139} (γ -H2AX), cellular inhibitor of apoptosis protein-2 (cIAP2) and cyclin B1 (Cell Signaling Technology) were diluted (1:1,000) in 5% BSA and incubated overnight at 4°C with mild rocking. Membranes were probed with secondary anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (GE Healthcare) at a final dilution of 1:2,000 for 2 hours. After washing 3 times with TBST (Tris-buffered saline and Tween 20), bound antibodies were detected by enhanced chemiluminescence (GE Healthcare) or Supersignal West Femto (Pierce). Equal loading was verified with β -actin antibody.

Immunohistochemistry

Sections were prepared from formalin-fixed, paraffin-embedded tumor samples. To ensure uniform handling of samples, all sections were processed simultaneously. Sections were deparaffinized by routine techniques and were quenched with 3% H_2O_2 for 10 minutes. The slides were steamed in citrate buffer (pH 6.0) for 20 minutes at $90^{\circ}\text{C}+$ and were blocked with 10% FBS solution (Invitrogen) for 30 minutes prior to incubation with primary antibodies. For the p-Cdc2 staining, p-Cdc2^{Tyr15} antibody (Cell Signaling Technology) was used at a dilution of 1:200 followed by 1 hour incubation. For p-HH3 detection, p-HH3^{Ser10} antibody (Cell Signaling Technology) was used at a 1:250 dilution and incubated overnight at 4°C . Sections were incubated with EnVision/HRP rabbit antibody for 30 minutes and diaminobenzidine (DAB+) chromogen

for 10 minutes. The sections were counterstained with hematoxylin, dehydrated, and mounted. Slides were evaluated by a pathologist under 20X objective and digitized using a color camera mounted to the microscope. p-Cdc2^{Tyr15} and p-HH3^{Ser10} stained tumor cells were counted over total number of tumor cells and two sided Fisher's exact test (GraphPad Prism 5.03 software) was used for significance calculation.

Statistical analysis

All error bars are represented as the standard error of the mean. Significance was analyzed using unpaired Student's *t*-test. The differences were considered significant when *P* value was less than 0.05.

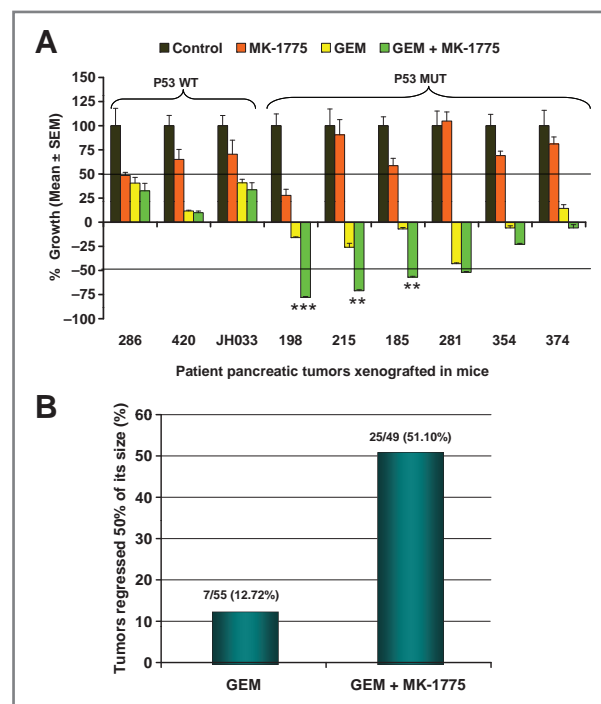


Figure 1. Combination of MK-1775 and gemcitabine potentiates the efficacy of gemcitabine in established human pancreatic cancer xenografts. Nine individual patient-derived low passage pancreatic cancer xenografts (3 with wild-type p53 (WT) and 6 with deficient p53 (MUT) were implanted in athymic mice. Animals with established tumors were dosed with MK-1775, GEM, or a combination of GEM with MK-1775 as mentioned in Materials and Methods. Tumor size was evaluated twice per week by caliper measurements. Relative tumor growth index (TGI) was calculated by relative tumor growth of treated mice (T) divided by relative tumor growth of control mice (C) \times 100. A, efficacy of MK-1775, GEM, and MK-1775, and GEM combination on the growth inhibition of pancreatic cancer xenografts. MK-1775 treatment produced greater than 50% inhibition of tumor growth in two xenografts (286, 198) compared to control tumors. Five of 9 xenografts treated with GEM and 6 of 9 xenografts treated with combination of GEM and MK-1775 produced complete tumor growth inhibition resulting in tumor shrinkage. Combination treatment caused greater than 50% regression in tumor size in 4 of 6 xenografts with p53-deficient tumors. Error bars represent SE. ***, *P* < 0.0001; **, *P* < 0.005, compared with GEM-treated tumors. B, combination therapy leads to tumor regression in p53-deficient tumors. Tumors that regressed greater than 50% of its size upon treatment on day 28 were calculated. Error bars, SE.

Results and Discussion

The principal aim of this study was to investigate the efficacy of MK-1775 as a single agent and in combination with GEM in PDA xenografts and to assess whether the status of the p53 gene had any role in dictating treatment efficacy. We used a xenograft model which is freshly generated from the tumors taken from pancreatic cancer patients and selected nine xenografts (6 xenografts with p53-deficient and 3 xenografts with p53 wild-type status) for this study. As shown in Figure 1A, tumors in vehicle-treated animals grew rapidly. Single agent MK-1775 treatment produced greater than 50% inhibition of tumor growth in 2 xenografts (PANC286 and PANC198). However, 5 of 9 xenografts treated with GEM and 6 of 9 xenografts treated with GEM plus MK-1775 produced complete tumor growth inhibition and in fact resulted tumor shrinkage compared to control and MK-1775 treated animals (Fig. 1A). These data suggest that single agent MK-1775 is unlikely to be effective in patients with PDA but that the combination of this agent with GEM has a substantial level of activity, and should be prioritized for clinical development.

Overall, none of the xenografts with p53-deficient status in the GEM treatment group produced 50% regression of initial tumor volume (Fig. 1A). However, combination of GEM and MK-1775 resulted in greater than 50% regression of initial tumor volume in 4 of 6 xenografts (66.66%) with p53-deficient status (Fig. 1A). The number of tumors that regressed more than 50% of its initial tumor size in each xenograft upon completion of treatment is provided in Table 1. Tumors with wild-type p53 status did not regress

with treatments (Table 1). Among the xenografts with p53-deficient status, GEM alone treatment-induced regression in 7 of 55 tumors (12.72%), while MK-1775 in combination with GEM induced regressions in 25 of 49 tumors (51.10%) in the 6 xenografts. Tumor growth regressions in GEM plus MK-1775 treated mice were found to be significant in PANC198 ($P < 0.0001$), PANC215 ($P < 0.005$), and PANC185 ($P < 0.005$) as compared to GEM-treated mice. There was an overall 4.01-fold increase in total number of tumors regressed in the combination treatment group compared to GEM alone treatment (Fig. 1B). *K-ras* and *SMAD4* status do not influence the tumor regression pattern in the xenografts (Table 1). One limitation of preclinical studies is that the threshold of activity that translates into positive clinical outcome is not known. Often, drugs are selected for clinical development based on tumor growth inhibition in preclinical models. As our experience with freshly generated PDA models increases and more comparison data is available, we are observing that indeed only agents that result in marked tumor regressions in this model have the potential to impact patient outcome. This is illustrated by our recent work on AZD0530 and nab-paclitaxel. AZD0530, a Src kinase inhibitor, induced only modest inhibition of tumor growth in PDA xenografts and, as expected, failed in a phase II clinical trial (27, 28). In contrast, nab-paclitaxel, in combination with GEM, resulted in marked tumor regression in this model, which successfully predicted a positive phase II study (29, 30).

The selective augmentation of antitumor effects in tumors with deficient-p53 was anticipated based on the mechanism of action of the agent. Mammalian cells

Table 1. Mutational status and number of tumors regressed more than 50% of initial size as on day 28

Xenograft	Genetic background			GEM	GEM + MK-1775
	P53	K-ras	SMAD4		
PANC286	WT	MUT	WT	0 (8)	0 (6)
PANC420	WT	WT	WT	0 (10)	0 (8)
JH033	WT	MUT	WT	0 (8)	0 (9)
PANC198	MUT	MUT	MUT	1 (8)	8 (8)
PANC215	MUT	MUT	WT	1 (9)	5 (7)
PANC185	MUT	MUT	MUT	1 (10)	3 (8)
PANC281	MUT	MUT	MUT	3 (10)	8 (10)
PANC354	MUT	WT	MUT	1 (10)	0 (8)
PANC374	MUT	MUT	WT	0 (8)	1 (8)
		Total with p53 MUT		7/55 (12.72%)	25/49 (51.10%)
		Total with p53 WT		0/26 (0%)	0/23 (0%)

NOTE: Animals were treated with vehicle, MK-1775, GEM, or GEM + MK-1775 for 4 weeks. Tumors that regressed greater than 50% of its size on day 28 in each xenograft were counted. Numbers in parenthesis denotes total number of tumors in that xenograft. Over all, 7 of 55 (12.72%) tumors with p53-deficient (MUT) status regressed in the GEM treatment group. However, 25 of 49 (51.10%) of tumors with p53 MUT status regressed in the GEM + MK-1775 group. None of the tumors with p53 wild-type (WT) regressed in GEM + MK-1775 (0 of 23) or GEM (0 of 26) treatment groups. *K-ras* and *SMAD4* status do not influence the tumor regression pattern in these xenografts.

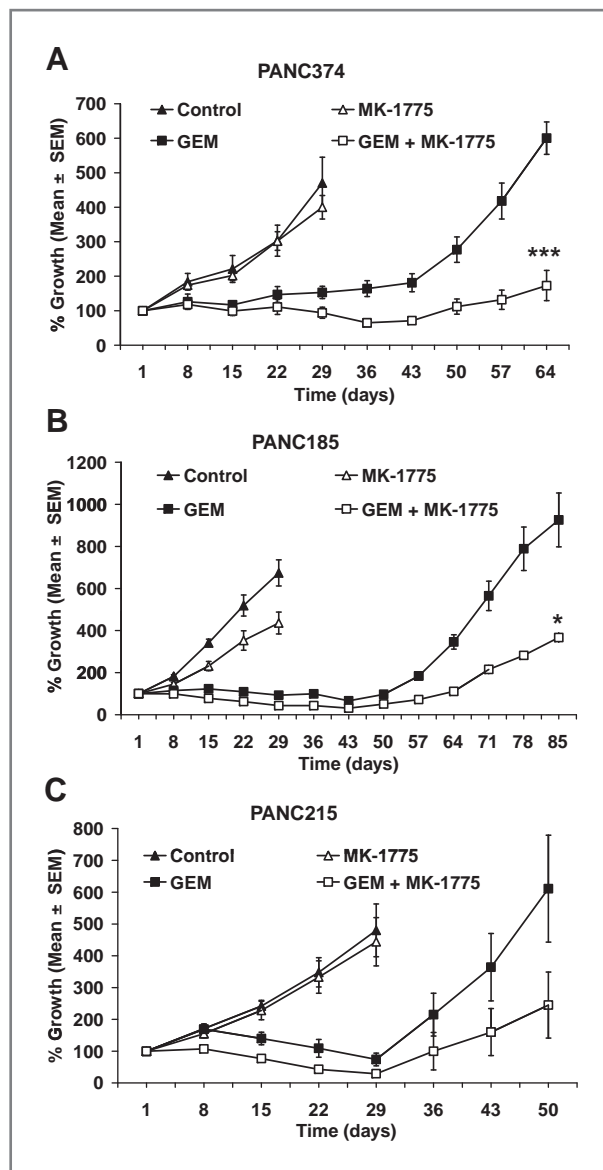


Figure 2. MK-1775 synergize with gemcitabine to inhibit tumor growth of human pancreatic cancer xenografts. Animals were dosed with MK-1775, GEM, or a combination of GEM with MK-1775 for 4 weeks as mentioned in Materials and Methods. Tumor growth curves of (A) PANC374, (B) PANC185, and (C) PANC215 suggest that the combination of MK-1775 and GEM lead to synergistic growth inhibition. Tumors in the vehicle and MK-1775 treated animals grew progressively and were sacrificed on day 28 due to tumor burden. Animals in the GEM and combination of GEM with MK-1775 were kept longer after the 4 week treatment. Combination of GEM with MK-1775 slowed the tumor growth progression compared to GEM alone treated animals. Points, mean ($n = 8$ to 10 tumors per group); bars, SE. Error bars, SE. ***, $P < 0.0001$; *, $P < 0.01$, compared with GEM-treated tumors.

undergo cell cycle arrest in response to DNA damage due to the existence of multiple checkpoint response mechanisms. In response to DNA damage, the cell cycle halts, preventing the propagation of cells with damaged DNA. DNA damage culminates in the enforcement of cell cycle

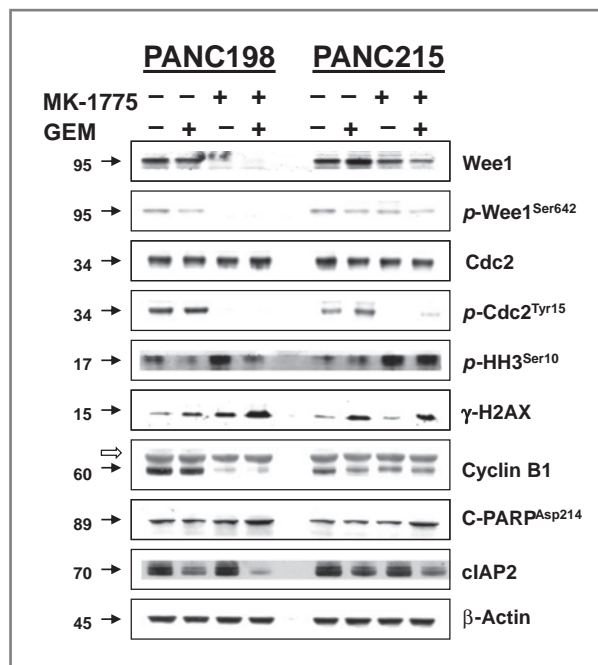


Figure 3. Combination of MK-1775 and gemcitabine inhibits Wee1 and attenuates Cdc2 phosphorylation to promote mitotic entry and apoptosis. Tumor lysates collected from vehicle, GEM, MK-1775, and combination of GEM with MK-1775 treated mice were resolved in SDS-PAGE and probed with specific antibodies against indicated proteins. MK-1775 as well as combination of MK-1775 and GEM treatment strongly inhibit Wee1 and p-Cdc2. Combination of MK-1775 and GEM treatment upregulate the expression of p-HH3, γ -H2AX, and cleaved PARP. Upon combination with GEM, MK-1775 downregulate the expression of cIAP2 as compared to GEM. MK-1775 as well as combination of MK-1775 and GEM treatment strongly inhibit the cyclin B1 expression as compared to control and GEM treatment of PANC198. Filled arrowheads indicate the molecular weight of corresponding proteins (KD). The empty arrowhead indicates the presence of a nonspecific band in cyclin B1. β -Actin was used as a loading control.

arrest, mainly at G_1 and G_2 phases. Checkpoint pathways operating at the G_1 phase are frequently lost in cancer cells due to mutation of the p53 tumor suppressor gene. Cells lacking functional p53 would not be anticipated to arrest at the G_1 checkpoint and would depend on the G_2 checkpoint to permit DNA repair prior to undergoing mitosis. Thus, G_2 checkpoint abrogation should preferentially kill p53-deficient cancer cells by removing the only checkpoint that protects these cells from premature entry into mitosis in response to DNA damage. Our data strongly suggest that the clinical development of MK-1775 with GEM should be restricted to patients with p53-deficient PDA.

Cdc2 initiates mitosis, which is the ultimate target of DNA replication and repair checkpoints. Chk1, Chk2, Wee1, and Myt1 are key regulators of G_2 checkpoint, which act directly or indirectly to inhibit Cdc2 activity (17, 31). Chk1 and Chk2 are downstream effectors of ataxia telangiectasia-mutated kinase (ATM) and ataxia telangiectasia and Rad3-related kinase (ATR), which

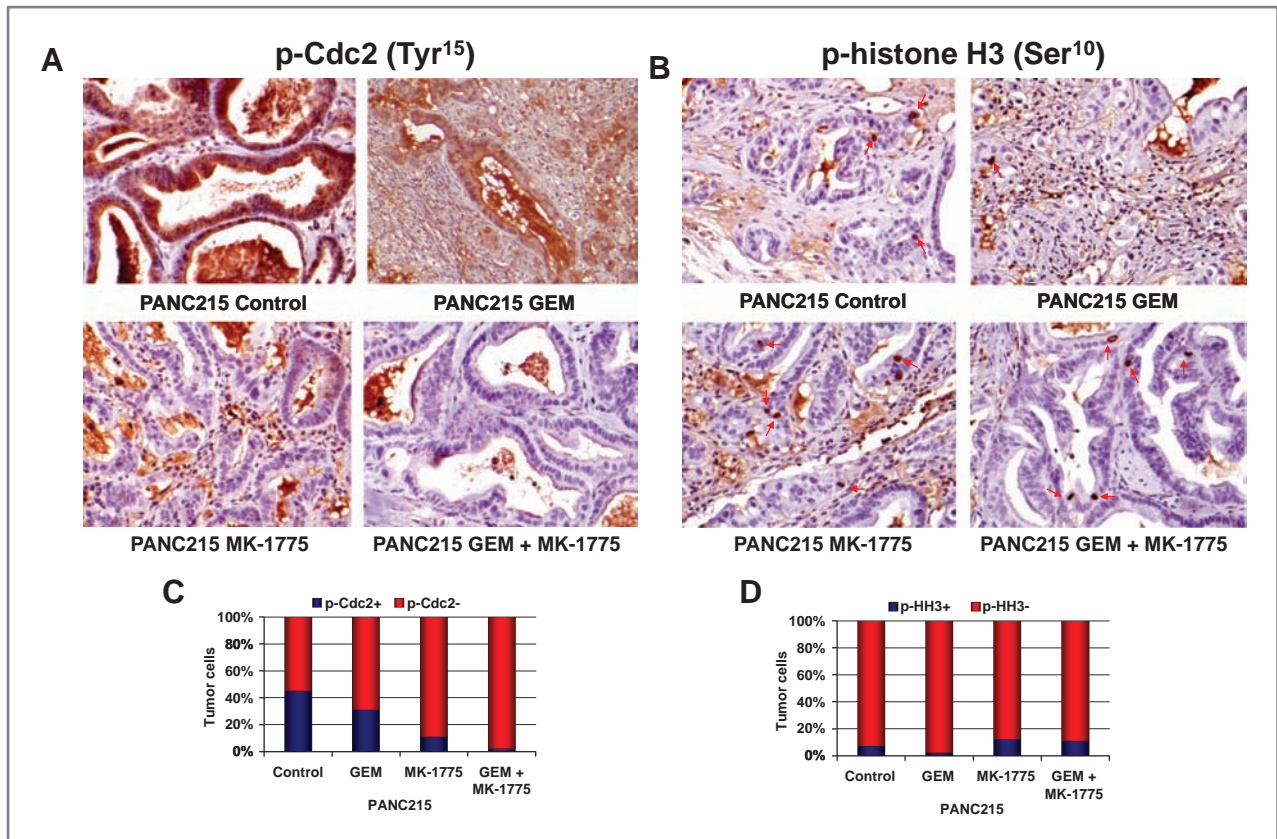


Figure 4. Immunohistochemical staining of p-Cdc2 and p-HH3. **A**, representative micrographs of p-Cdc2 from PANC215 showing high immunoreactivity for p-Cdc2 in the vehicle and GEM-treated tumors. MK-1775 and combination treatment greatly inhibited the p-Cdc2 compared to vehicle and GEM-treated tumors (left). There was a 45% and 31% of tumor cells were stained positive for p-Cdc2 in the control and GEM-treated tumors, respectively, while only 11% ($P = 0.0061$ compared to GEM-treated tumors) and 2% ($P < 0.0001$ compared to GEM-treated tumors) of tumor cells were stained positive for p-Cdc2 in the MK-1775 and combination of MK-1775 and GEM-treated tumors, respectively. **B**, representative micrographs of p-HH3, a marker of mitosis, from PANC215 were shown in the right panel. Number of p-HH3 stained tumor cells were elevated in the MK-1775 and combination of MK-1775 and GEM-treated tumors as compared to control and GEM-treated tumors. A total of 7% and 2% of tumor cells were stained positive for p-HH3 in the control and GEM-treated tumors, respectively, while 12% ($P = 0.0115$ compared to GEM-treated tumors) and 11% ($P = 0.0201$ compared to GEM-treated tumors) of tumor cells were stained positive for p-HH3 in the MK-1775 and combination of MK-1775 and GEM-treated tumors, respectively. **C**, histogram showing the number of positively stained p-Cdc2 tumor cells over total number of tumor cells. **D**, histogram showing the number of positively stained p-HH3 tumor cells over total number of tumor cells.

induce G₂/M cell cycle arrest by inactivating Cdc25 tyrosine phosphatases through phosphorylation (32). Both Chk1 and Chk2 are known to phosphorylate Cdc25 on Ser216 and this phosphorylation makes Cdc25 functionally inactive (33). Cdc25 is required for removal of inhibitory phosphotyrosines on Cdc2/cyclin B1 kinase complexes that mediate entry into mitosis. On the other hand, the inhibitory phosphorylations at Thr-14 and Tyr-15 sites of Cdc2 are mediated by Myt1 and Wee1 kinases (34, 35). Wee1 is the major kinase phosphorylating the Tyr-15 site and Wee1-dependent phosphorylation of Cdc2 maintains the Cdc2/cyclin B1 complex in an inert form. While Myt1 preferentially phosphorylates the Thr-14 site, it can also phosphorylate the Tyr-15 site. Thus either Cdc25 inactivation and/or Wee1/Myt1 activation could contribute to G₂ cell cycle arrest in response to DNA damage.

Chk1/2 inhibitors are in clinical development (36–38). A recent report indicated that Chk1 is required to maintain genome integrity and cell viability, and that p53 wild-type cells are no less sensitive than p53-deficient cells to Chk1 inhibition in the presence of DNA damage. Thus, combining Chk1 inhibition with DNA damaging agents does not lead to preferential killing of p53-deficient over p53 wild-type cells, and inhibiting Chk1 does not appear to be a promising approach for potentiation of cancer chemotherapy (39). Here we showed that Wee1 inhibition by MK-1775 could potentiate GEM sensitivity and tumor regressions, selectively in p53-deficient pancreatic cancer xenografts.

We were also interested in long-term tumor growth control and followed 3 xenografts after treatment for an extended period of time. Tumor regrowth data, as shown in Figure 2 suggest that not only does the combination of

GEM with MK-1775 lead to synergistic tumor growth inhibition, but the effect of the combination therapy is also longer-lasting than that seen with GEM alone (Fig. 2A, B, and C). It was noteworthy, however, that tumors eventually recur, albeit at a slower pace.

In order to determine the target modulation by MK-1775, we examined Wee1, Cdc2, and their phosphorylated forms in posttreatment tumor specimens. MK-1775 treatment strongly inhibited phosphorylation of Tyr-15 of Cdc2, the primary substrate of Wee1 (Figs. 3 and 4A and C), suggesting increased Cdc2 kinase activity. In addition, the Wee1 protein was consistently reduced by MK-1775 treatment as shown by Western blotting (Fig. 3), likely due to degradation of Wee1 as MK-1775 treatment activates Cdc2 which in turn phosphorylates Wee1, ultimately leading to its ubiquitin-proteasome-dependent destruction (40).

To determine whether combination therapy promotes mitotic entry, we measured the expression of p-HH3 by Western blotting as well as by immunohistochemistry. When administered in combination with GEM, MK-1775 promoted mitotic entry as measured by enhanced p-HH3^{Ser10} expression (Fig. 3, 4B and D). In addition, the combined treatment resulted in the upregulation of C-PARP as well as downregulation of cIAP2, suggesting that combination therapy facilitates apoptotic death of tumor cells (Fig. 3). GEM, as a chain terminator, requires an active cell cycle to be effective for inhibiting tumor growth, and might induce cell cycle halt and enforce cell cycle checkpoints, which may play an important role in escalating the resistance to therapy. Thus, there is a strong rationale in combining checkpoint inhibitors with GEM as a means to enhance tumor response (41, 42). Here we showed that GEM induces G₂ arrest, which correlates with an increased Cdc2 inhibitory phosphorylation at Tyr-15 and prevents mitotic entry as evidenced by decreased p-HH3^{Ser10} (Fig. 4B and D). However, the decreased Cdc2 inhibitory phosphorylation at Tyr-15 caused by MK-1775 treatment indicates that MK-1775 has the ability to abrogate the G₂ arrest induced by GEM and promote mitotic entry as demonstrated by enhanced p-HH3^{Ser10} (Fig. 4B and D). Cyclin B1 was examined as a marker of G₂ phase (43). Expression of

Cdc2 was not altered by treatments, while the expression of cyclin B1 was strongly inhibited by MK-1775 as well as combination of MK-1775 and GEM treatment compared to control and GEM-treated tumors of PANC198 (Fig. 3). Loss of cyclin B1 accumulation in the MK-1775 as well as combination of MK-1775 and GEM-treated tumors indicate the exit from G₂ phase arrest (Fig. 3). The levels of γ -H2AX were used as a surrogate for unrepaired DNA damage (44). γ -H2AX expression was clearly elevated in the combination of MK-1775 and GEM treatment group compared to GEM-treated tumors of PANC198, indicating the persistence of unrepaired DNA damage in the tumors (Fig. 3). Overall, in addition to providing mechanistic support to the observations made above, the data provides important clues for potential biomarkers for clinical development of this drug combination.

In conclusion, our results provide compelling evidence that MK-1775 treatment leads to the inhibition and subsequent loss of Wee1 and activation of its substrate, Cdc2. The MK-1775 and GEM combination promoted the mitotic entry of tumor cells and eventually led to apoptotic death, and delayed the tumor progression compared to the GEM treatment. These findings have important clinical implications and raise the hope for potential therapeutic benefit to many PDA patients whose cancer cells are deficient for p53 function.

Disclosure of Potential Conflicts of Interest

M. Hidalgo received funding from Merck Research Laboratories. J. Watters, D. Brooks, T. Demuth, S.D. Shumway, S. Mizuarai, and H. Hirai are employees of Merck. The other authors declare no potential conflicts of interest.

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