Molecular Cancer Therapeutics

Identification of Preferred Chemotherapeutics for Combining with a *CHK1* Inhibitor

Yang Xiao[1], Judi Ramiscal[1], Kaska Kowanetz[2], Christopher Del Nagro[1], Shiva Malek[3], Marie Evangelista[2], Elizabeth Blackwood[1], Peter K. Jackson[4], and Thomas O'Brien[1]

Abstract

Here we report that GNE-783, a novel checkpoint kinase-1 (*CHK1*) inhibitor, enhances the activity of gemcitabine by disabling the S- and G_2 cell-cycle checkpoints following DNA damage. Using a focused library of 51 DNA-damaging agents, we undertook a systematic screen using three different cell lines to determine which chemotherapeutics have their activity enhanced when combined with GNE-783. We found that GNE-783 was most effective at enhancing activity of antimetabolite-based DNA-damaging agents; however, there was a surprisingly wide range of activity within each class of agents. We, next, selected six different therapeutic agents and screened these in combination with GNE-783 across a panel of cell lines. This revealed a preference for enhanced chemopotentiation of select agents within tumor types, as, for instance, GNE-783 preferentially enhanced the activity of temozolomide only in melanoma cell lines. Additionally, although p53 mutant status was important for the overall response to combinations with some agents; our data indicate that this alone was insufficient to predict synergy. We finally compared the ability of a structurally related *CHK1* inhibitor, GNE-900, to enhance the *in vivo* activity of gemcitabine, CPT-11, and temozolomide in xenograft models. GNE-900 significantly enhanced activity of only gemcitabine *in vivo*, suggesting that strong chemopotentiation *in vitro* can translate into chemopotentiation *in vivo*. In conclusion, our results show that selection of an appropriate agent to combine with a *CHK1* inhibitor needs to be carefully evaluated in the context of the genetic background and tumor type in which it will be used. *Mol Cancer Ther; 12(11); 2285–95. ©2013 AACR.*

Introduction

The checkpoint kinase 1 (*CHK1*) activates the S and G_2 checkpoints in response to DNA damage, which prevents cells from entering mitosis with damaged DNA (1). Inactivation of *CHK1* following DNA damage is a very attractive therapeutic strategy as it promotes cells to prematurely enter mitosis and undergo mitotic catastrophe (1). On the basis of this strategy, a select number of *CHK1* inhibitors have progressed into clinical trials and are being evaluated for their ability to enhance the activity of DNA-damaging agents (2).

In response to stretches of single-strand DNA, cells activate Ataxia telengiectasia and Rad3 related (ATR) protein kinase, which phosphorylates and activates *CHK1* (3, 4). A critical function of *CHK1* is to phosphorylate a number of downstream targets (including *CDC25A*) to prevent cell-cycle progression into mitosis (1). Numerous *CHK1* small-molecule inhibitors have been identified (5), and used to verify the essential role of *CHK1* in maintaining the G_2 checkpoint response following DNA damage (6–9).

The tumor suppressor p53 is primarily responsible for activation of the G_1 and, to a lesser extent, the G_2 checkpoint response in cells experiencing DNA damage (10). Following DNA damage, phosphorylation of p53 results in its enhanced stabilization and a corresponding increase in expression of its downstream transcriptional targets (11). One of these targets, p21, is required for repressing the activity of the cyclin–cdk kinase complex (12) and, thus, cells with wild-type p53 can activate the G_1- and G_2-checkpoints in response to stress. However, cells lacking p53 accumulate at the G_2–M boundary due to activation of *CHK1* (10). Thus, inhibition of *CHK1* in p53-deficient cells exposed to a DNA-damaging agent promotes rapid bypass of both the G_1 and G_2 checkpoints, and cells proceed into mitosis with unrepaired DNA damage and rapidly undergo cell death.

Many of the studies describing *CHK1* inhibitors have used a limited number of chemotherapeutics including gemcitabine (6, 13–17), SN-38 (6, 13, 16–18), camptothecin (13, 18, 19), cytarabine (16), cisplatin/carboplatin (16), hydroxyurea (14, 16), topotecan (6), or ionizing radiation (8, 19–21). As these agents span different classes of DNA-damaging agents, it suggests that a *CHK1* inhibitor

Authors' Affiliations: Departments of [1]Translational Oncology, [2]Research Diagnostics, [3]Biochemical Pharmacology, and [4]Research Oncology, Genentech, South San Francisco, California

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Corresponding Author: Thomas O'Brien, Department of Translational Oncology, Genentech, 1 DNA Way, South San Francisco, CA 94080. Phone: 650-467-7463; Fax: 650-467-5470; E-mail: obrien.tom@gene.com

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may have broad utility. However, there was no systematic analysis of the ability of a single *CHK1* inhibitor to effectively combine with a wide range of chemotherapeutics.

Given the role that both p53 and *CHK1* have in establishing the G_1- and G_2-checkpoints in response to DNA damage, it was proposed that inhibition or knockdown of *CHK1* in cells that are p53 deficient would sensitize these cells to chemotherapeutic agents. Although this has been shown using genetically matched p53 wild-type or small numbers of mutant cell lines (6, 8, 19, 20, 22, 23), there was no systematic evaluation of the importance of p53 status to chemopotentiation of a wide range of agents.

Here, we evaluate the ability of a *CHK1* inhibitor, GNE-783, to potentiate the activity of 51 different chemotherapeutics across a range of cell lines. We find that GNE-783 preferentially potentiates the activity of antimetabolite-based DNA-damaging agents, and potentiation is greater in cells lacking wild-type p53 activity. Moreover, a detailed analysis of a select number of agents reveals that p53 mutation alone is not sufficient to predict synergy. Thus, our results reveal that predicting cellular response to *CHK1* inhibitor–chemotherapeutic combinations depends upon multiple factors, with p53 status being only one of these.

Materials and Methods

Cell lines

Cell lines were obtained from the American Type Culture Collection and stored at early passage in a central cell bank; lines were authenticated by short tandem repeat (STR) and genotyped upon reexpansion. HCT116 p53 genetically matched cell lines (10) were obtained from Horizon Discovery Inc.

Cell-based assays

Cells were exposed to a dose titration of each agent and with a fixed dose (1 μmol/L) or dose titration (0.04–10 μmol/L) of GNE-783 for 72 hours, and viability was measured by Celltiter Glo (Promega). AZD7762 was screened at 100 nmol/L ($\sim EC_{10}$; data shown in Fig. 2B). For assays with temozolomide and GNE-783 or GNE-900, cells were treated with a dose titration of temozolomide (from 0.1 μmol/L to 1 mmol/L) and a dose titration (0.04–10 μmol/L) of GNE-783 or GNE-900 for 72 hours. Viability was measured by Celltiter Glo (Promega). Bliss scores were calculated as described previously (24).

Cells were fixed in 1% paraformaldehyde and stained with BD Perm/wash buffer; intracellular flow staining was performed with anti-phospho-histone H3-AF488 (#3465; Cell Signaling Technology). Flow cytometry collection was performed on an LSRII flow cytometer (BD Biosciences), and data was analyzed using FlowJo software (TreeStar Inc.).

Antibodies and Western blot analysis

Western blots were performed with a Li-COR Odyssey system (IRDye secondary antibody and blocking buffer). Antibodies used: *CHK1* (#2360; Cell Signaling Technology), *CHK1*-S345 (#2341; Cell Signaling Technology), γ-H2AX (#05-636; Millipore), *PARP1*-p25 (#1051-1; Epitomics), and *TUBULIN* (#32293; Santa Cruz Biotechnology).

In vivo efficacy studies

All *in vivo* studies were approved by Genentech's Institutional Animal Care and Use Committee (IACUC) and adhere to the NIH Guidelines for the Care and Use of Laboratory Animals. Xenograft models were established by subcutaneous injection of tissue culture tumor cells into NCR nude mice (Taconic Laboratories). Animals were divided into groups ($n = 10$/group) when tumors reached a mean volume of 150 to 300 mm^3. GNE-900 (20–40 mg/kg) was administered by oral gavage [*per os*, formulated in 25% hydroxypropyl-β−cyclodextrin (HP-β−CD)/50 mmol/L sodium citrate pH 3.3 or 0.5% w/v methylcellulose/0.2% v/v Tween 80 in reverse osmosis water (MCT)] 24 hours after intraperitoneal (i.p.) administration of gemcitabine (60 mg/kg, saline vehicle; Q3D×4). CPT-11 was delivered by i.p. injection (50 mg/kg; saline vehicle, Q4D×4), with GNE-900 doses administered 24 hours later. Temozolomide was administered by oral gavage (*per os*, MCT vehicle, QD×5) at 68 mg/kg; GNE-900 was administered 4 hours following each temozolomide dose.

Tumor volumes were determined using digital calipers (Fred V. Fowler Company, Inc.) by using the formula $(L \times W \times W)/2$. Curve fitting was applied to Log_2 transformed individual tumor volume data using a linear mixed-effects (LME) model with the R package nlme, version 3.1–97 in R v2.13.0 (R Development Core Team 2008; R Foundation for Statistical Computing, Vienna, Austria). Tumor growth inhibition (%TGI) was calculated as the percentage of the area under the fitted curve (AUC) for each drug-treated group per day relative to vehicle control animals, such that: $\%TGI = 100 \times (1 - (AUC_{treatment}/day) / (AUC_{vehicle}/day)$. One Hundred percent TGI is characterized as tumor stasis, whereas a TGI > 100% indicates tumor regression; 95% confidence intervals (CI) were defined by the lower and upper range of %TGI values for each drug treatment group. Tumor size and body weight were recorded twice weekly over the course of the study. Mice with tumor volumes of 2,000 mm^3 or more or recorded body weight loss of 20% or more from their weight at the start of treatment were promptly euthanized.

Results

GNE-783 is a selective *CHK1* inhibitor

Through a combination of high-throughput screening followed by structure-based design, GNE-783 was identified as a novel small-molecule *CHK1* kinase inhibitor (Fig 1A) (25) with an IC_{50} of 0.001 μmol/L and with approximately 444-fold selectivity against *CHK2* (IC_{50} of 0.44 μmol/L; Fig 1B). To examine the broader selectivity of GNE-783, it was screened at 100 nmol/L against

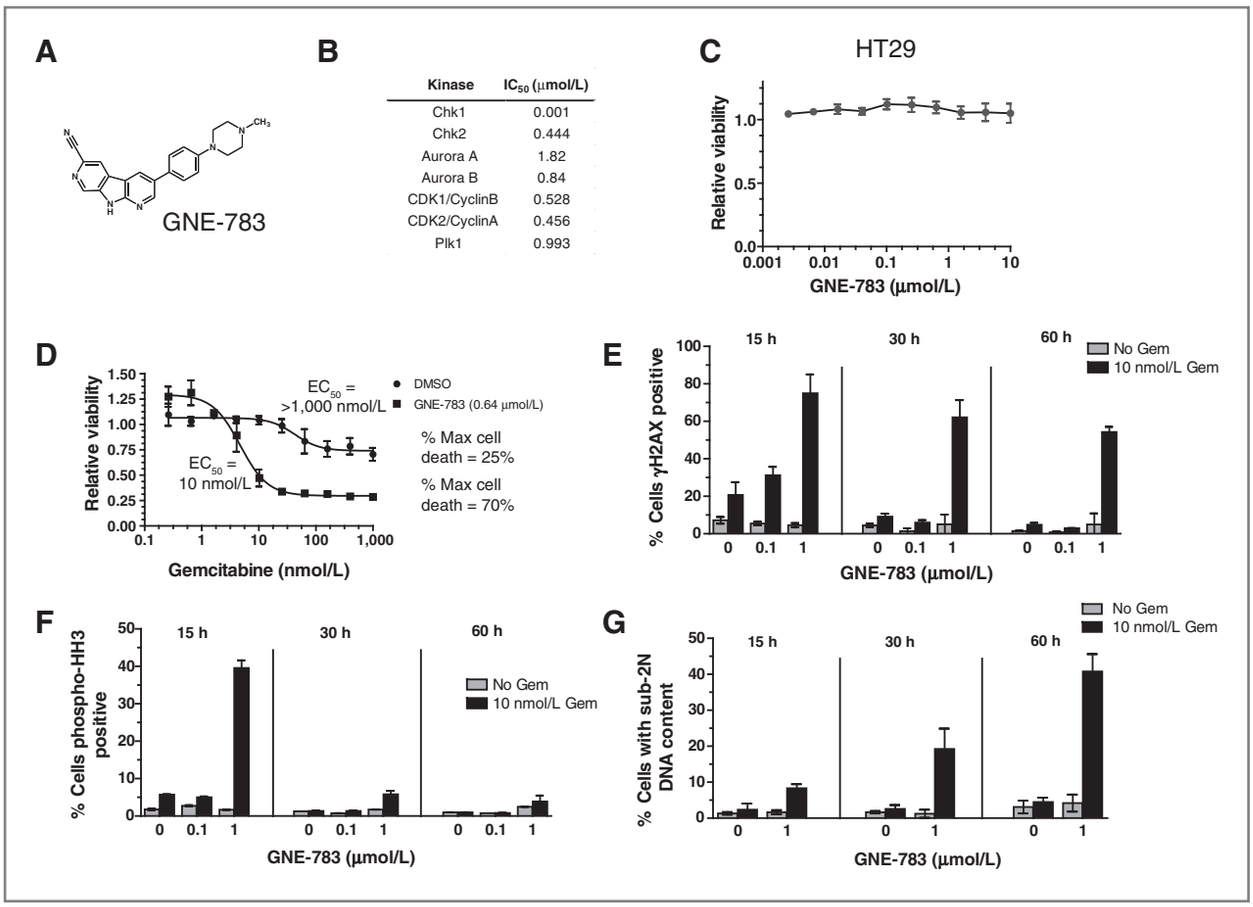

Figure 1. The *CHK1* inhibitor GNE-783 promotes enhanced DNA damage and premature mitotic entry in HT-29 cells when combined with gemcitabine. A, structure of GNE-783. B, the IC_{50} values (μmol/L) for GNE-783 against *CHK1* and other kinases involved in cell-cycle control. C, a dose titration of GNE-783 in a 72-hour proliferation assay (mean ± SEM, $n = 6$). D, the graph illustrates a dose titration of gemcitabine in the presence or absence of 0.64 μmol/L GNE-783 (mean ± SD, $n = 2$). E–G, the addition of GNE-783 (0.1 or 1 μmol/L) to 0.01 μmol/L gemcitabine increases the percentage of cells staining positive for γH2AX (E), histone H3 phospho-Serine10 (F), or the percentage of cells with a sub-2N DNA content in a time- and concentration-dependent manner. Quantitation was conducted by fluorescence activated cell sorting cell-cycle analysis (mean ± SD, $n = 2$).

a panel of 71 kinases, and found to only inhibit two kinases by >80%, one of which was *CHK1* (Supplementary Table S1). Moreover, GNE-783 displayed >400-fold selectivity against key relevant kinases involved in cell-cycle progression (Fig. 1B). Consistent with the selectivity of this compound, GNE-783 had minimal single-agent activity in HT-29 cells at concentrations as high as 10 μmol/L in a 72-hour proliferation assay (Fig. 1C). Exposing HT-29 cells to gemcitabine and GNE-783 continuously for 72 hours enhanced gemcitabine activity (Fig. 1D).

DNA damage and cell-cycle distribution was examined at various times by flow cytometry following exposure of cells to gemcitabine and either dimethyl sulfoxide (DMSO), 0.1 μmol/L or 1 μmol/L GNE-783. Compared with DMSO or GNE-783 alone, gemcitabine treatment modestly increased the percentage of γ-*H2AX*–positive cells (to ∼20%) within 15 hours (Fig. 1E). In contrast, cells exposed to gemcitabine/GNE-783 contained high levels of DNA damage (∼75% of cells within 15 hours were γ-*H2AX* positive; Fig. 1E). In addition, cells exposed to gemcitabine/GNE-783 showed a significant increase in the percentage of cells staining positive for phosphorylated ser-10 on histone H3 (p-HH3) within 15 hours (Fig. 1F), consistent with cells entering mitosis. Moreover, an increase in the sub-2N population was observed within 15 to 30 hours (Fig. 1G), indicating enhanced cell death. Thus, our data indicates that GNE-783 enhances gemcitabine-induced DNA damage, promotes premature entry into mitosis, and increases cell death.

GNE-783 preferentially enhances the activity of antimetabolite agents in HT-29 cells

A variety of reports have shown that *CHK1* small-molecule inhibitors can potentiate the activity of DNA-damaging agents such as gemcitabine, 5-fluorouracil (5-FU), and SN-38 (6, 13–17, 26); however, there is no reported systematic analysis that compared the ability of a single *CHK1* inhibitor to potentiate the activity of a wide range of DNA-damaging agents.

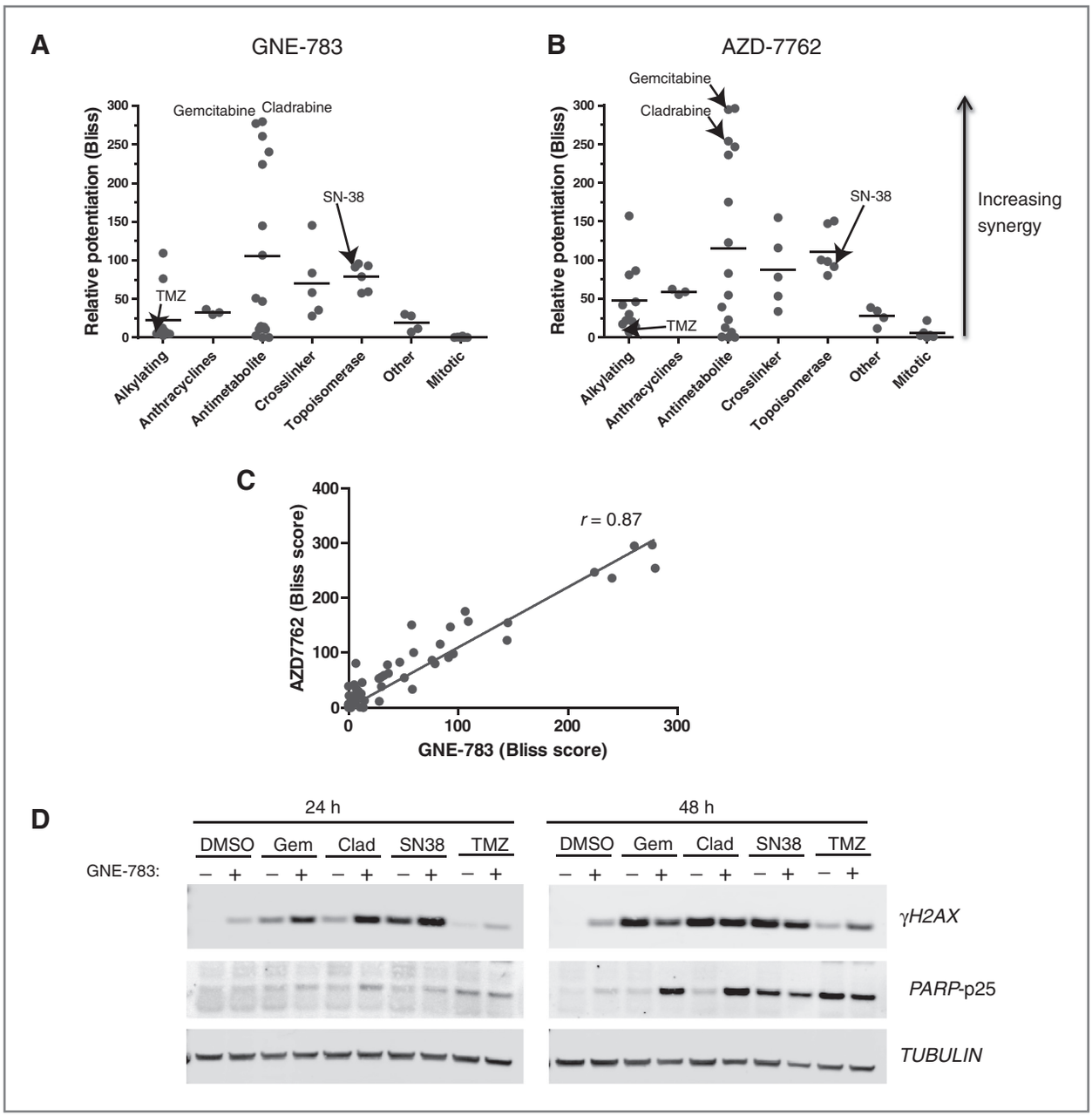

Figure 2. *CHK1* inhibition preferentially enhances the activity of antimetabolite-based DNA-damaging agents in HT-29 cells. The ability of GNE-783 (A) or AZD-7762 (B) to potentiate the activity of each agent was assessed by Bliss analysis, and the calculated Bliss score for each combination is shown. C, scatter plot analysis of the bliss scores obtained for GNE-783 and AZD-7762 in HT29 cells (Spearman $r = 0.87$, $P < 0.001$, $n = 51$). D, Western blot analysis of γ-H2AX, cleaved *PARP* (PARP-p25), and *TUBULIN* in response to DMSO or GNE-783 (1 μmol/L) in combination with gemcitabine (1 μmol/L), cladribine (10 μmol/L), SN-38 (0.1 μmol/L), and temozolomide (TMZ; 500 μmol/L) at 24 and 48 hours.

Here, we compiled a library of 51 different agents spanning alkylating, anthracycline, antimetabolite, cross-linking, topoisomerase, and antimitotic agents. Each agent was titrated either in the presence or absence of 1 μmol/L GNE-783, and assayed to determine whether GNE-783 would potentiate its cytotoxic activity. Synergy was quantified using a Bliss analysis (24), which compares the observed activity of the two agents when combined to their predicted effect if they are only additive. Our data revealed that *CHK1* signaling plays a critical role in the cellular response to some DNA-damaging agents, as, for example, GNE-783 predominantly enhanced activity of antimetabolite-based agents, in particular gemcitabine and cladribine (Fig 2A; Supplementary Table S2). Interestingly, even within a single class of DNA-damaging agents such as antimetabolites, there is a wide range in the ability of

GNE-783 to potentiate their activity, indicating that *CHK1* has a differential role in the response to each agent. This library was also screened using AZD-7762, a structurally diverse *CHK1* small-molecule inhibitor (6). The profile of chemopotentiation of AZD-7762 strongly correlated with the profile obtained with GNE-783 (Spearman $r = 0.87$, $P < 0.001$, $n = 51$; Fig 2B and C; Supplementary Table S2), supporting the conclusion that synergy of these chemotherapeutics with GNE-783 is due to *CHK1* inhibition.

To verify these findings, we combined an 8-point dose titration of selected agents with a 9-point dose titration of GNE-783 in HT-29 cells (Supplementary Fig. S1) and confirmed that GNE-783 strongly enhanced the activity of gemcitabine and cladribine (Bliss scores of 3698 ± 55 and 1919 ± 24, respectively). Our primary screen (Fig. 2A) showed an intermediate bliss score for SN-38 (topoisomerase inhibitor) and a low score for temozolomide, and our follow-up experiment confirmed these observations with modestly enhanced activity of SN-38 (1056 ± 57) compared with low or no enhanced activity of temozolomide (29 ± 18; Supplementary Fig. S1A).

To verify that enhanced activity of various chemotherapeutics is consistent with *CHK1* inhibition, we examined levels of DNA damage and cleavage of *PARP* in HT-29 cells. Within 24 hours, GNE-783 enhanced levels of γ-*H2AX* levels (a marker of double-strand breaks) when combined with gemcitabine, cladribine, SN-38, and, to a lesser extent temozolomide (Fig. 2D). Combinations that resulted in high Bliss scores tended to have the largest differential between *PARP* cleavage in the absence or presence of GNE-783 at 48 hours. In addition, at 48 hours levels of γ-*H2AX* have decreased in the samples combining GNE-783 with gemcitabine, cladribine, and SN-38, which is likely due to the fact that cells with high levels of DNA damage may have already undergone cell death (as observed for gem/GNE-783 within 30 hours; Fig. 1G). Thus, our pathway analysis confirms that combinations that result in high Bliss scores correlate with enhanced DNA damage and *PARP* cleavage.

Synergy between gemcitabine and GNE-783 is greater in p53-deficient cells

As our initial screen used HT-29 cells that are p53 mutant (R273H; ref. 27), we expanded this screen to other cell lines to evaluate the importance of p53 status to chemopotentiation. We found that GNE-783 enhanced the activity of gemcitabine more efficiently in p53-deficient HCT116 cells compared with genetically matched p53 wild-type cells (Supplementary Fig. S1B). In addition, gemcitabine activity is better enhanced in p53-deficient HCT116 cells when *CHK1* protein is depleted by siRNA (Supplementary Fig. S1C). When we screened our focused library, we found that HCT116 cells (wild-type p53) tend to have lower Bliss scores, in general, than genetically matched p53-null cells (Fig. 3A; Supplementary Table S3), and, as observed in p53-mutant HT29 cells, antimetabolite agents showed significantly greater Bliss scores in p53-null cells. In addition, synergy between GNE-783 and anthracyclines, topoisomerase inhibitors, and mitotic inhibitors is greater in p53-null cells compared with p53 wild-type cells (Supplementary Table S3).

To broaden this analysis, we screened a panel of 24 cell lines of various tumor types to determine whether potentiation of gemcitabine activity by GNE-783 correlated with p53 mutation status. Cell lines harboring mutations in p53 generally showed stronger synergy between gemcitabine and GNE-783 than cell lines with functional p53 ($P = 0.03$; Fig. 3B and C, Supplementary Table S4). Although there is a trend that p53-mutant cell lines are more resistant to gemcitabine (shown as an EC_{25} because an EC_{50} could not be determined for many cell lines; Fig. 3D), the difference is not significant ($P = 0.41$). Thus, lack of synergy does not correlate with the ability of these cells to respond to gemcitabine. Moreover, GNE-783 has minimal single-agent activity in these cell lines; thus, sensitivity to GNE-783 does not correlate with p53 status (Fig. 3E).

GNE-783 can be broadly combined with key standard of care chemotherapeutics across cell lines

We then evaluated whether the correlation between p53 status and chemopotentiation would apply to other chemotherapeutics when assessed across a broader panel of tumor cell lines. For this analysis, we used a set of standard chemotherapeutics that included gemcitabine and 5-FU (both antimetabolites), carboplatin (a cross-linking agent), SN-38 (a topoisomerase inhibitor), temozolomide (an alkylating agent), and taxol (a mitotic inhibitor). Each of these agents displayed a wide range of potentiation with GNE-783 across these cell lines (Fig. 4A; Supplementary Table S4).

Potentiation of only gemcitabine (Fig. 3C) and SN-38 (Fig. 4B) activity with GNE-783 was greater ($P < 0.05$) in p53-deficient cell lines than in wild-type cell lines. Although combining GNE-783 with temozolomide, carboplatin, or 5-FU showed better synergy in p53-deficient cell lines, this was not statistically significant in our panel of cell lines; there is minimal potentiation of taxol activity with GNE-783. In general, p53 status did not correlate with single-agent activity of each DNA-damaging agent ($P \geq 0.05$), with the single exception of carboplatin ($P = 0.04$), where p53-deficient cell lines were more resistant to carboplatin (Fig. 4C). It is not clear whether this correlation would hold up across a broader panel of cell lines.

GNE-783 potentiation of temozolomide is enhanced in p53-mutant melanoma cells

Although temozolomide did not show significant synergy in combination with GNE-783 in HT-29 cells (Fig 2A, Supplementary Fig. S1), it did show potentiation with GNE-783 in a small number of p53-mutant cell lines (Fig. 4B). On closer examination, we found that melanoma cell lines tended to respond better to a combination of GNE-783/temozolomide than all other cell lines ($P = 0.041$; Fig. 5A). Given that temozolomide

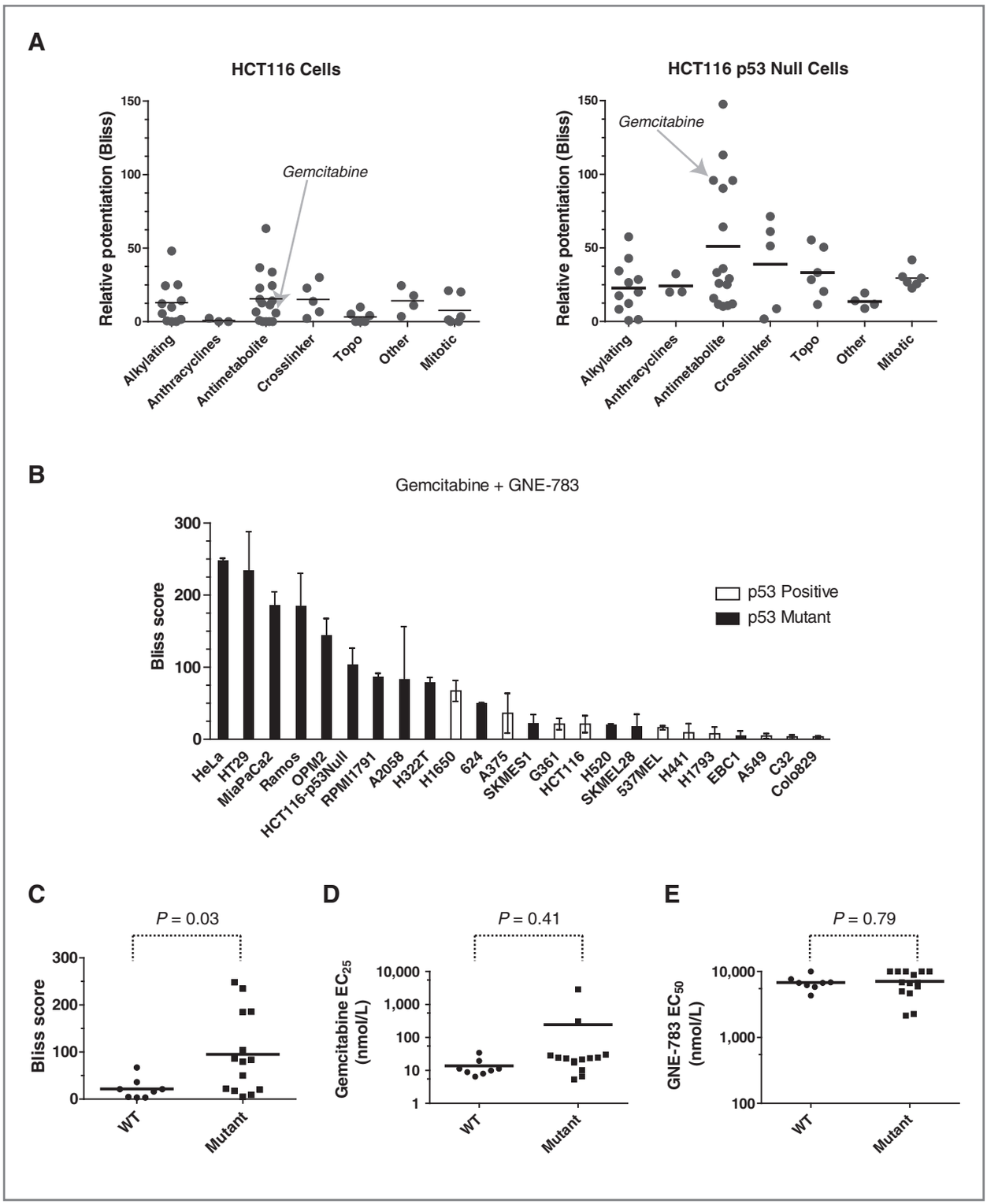

Figure 3. Synergy between gemcitabine and GNE-783 is p53 dependent. A, a library of 51 agents was screened with DMSO or 2 μmol/L GNE-783 in genetically matched HCT116 cell lines. The ability of GNE-783 to potentiate the activity of each chemotherapeutic agent was assessed by Bliss analysis, and the Bliss score for each combination is shown. B, a panel of 24 cell lines was screened using a dose titration of gemcitabine and GNE-783. The average and SD associated with each bliss score is shown ($n = 2$–9 for each cell line). C, Bliss scores (B) were grouped according to the p53 status of each cell line. D–E, the calculated EC_{25} for gemcitabine (D) or the EC_{50} for GNE-783 (E) for each cell line was grouped according to p53 status.

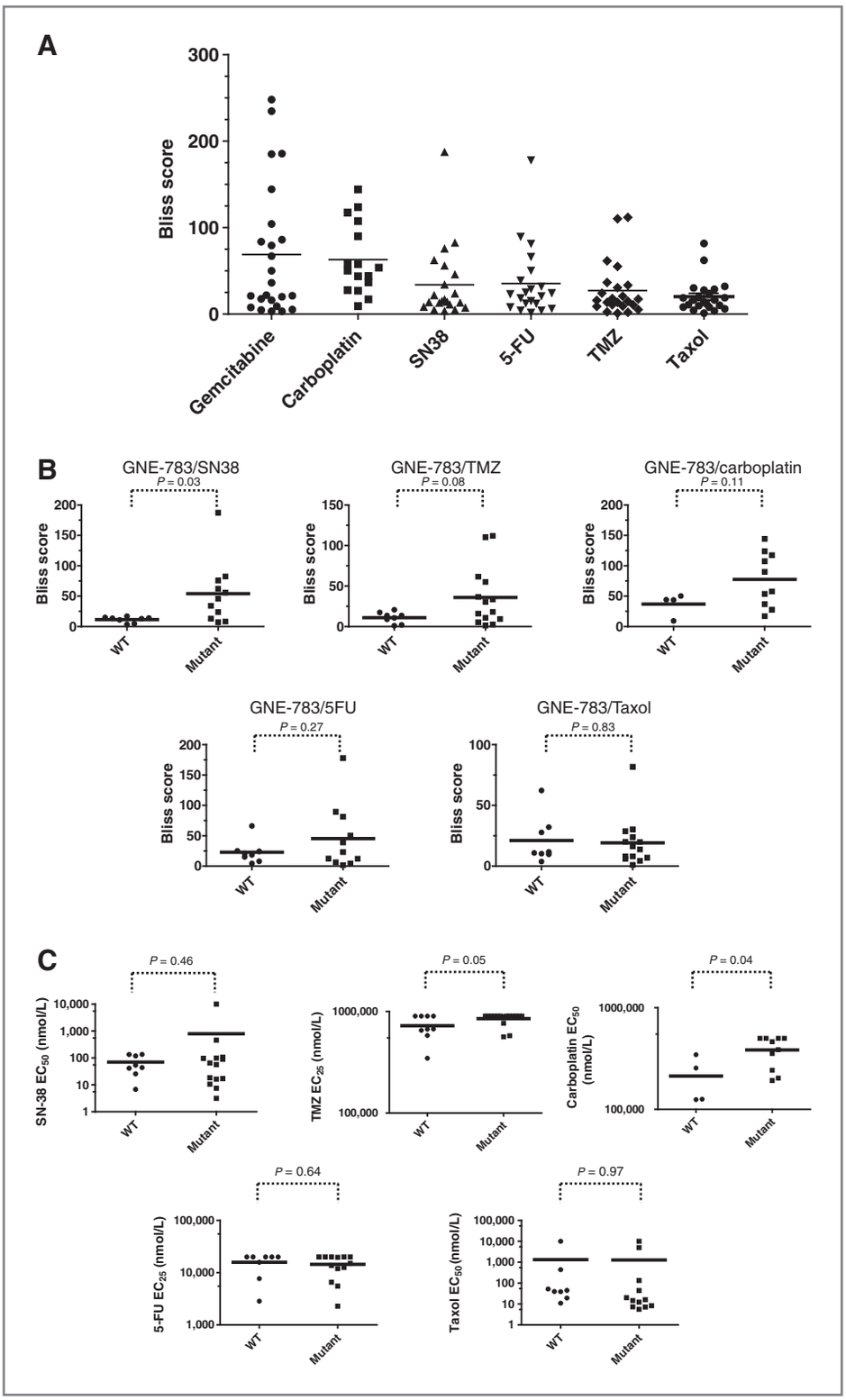

Figure 4. The ability of GNE-783 to potentiate a select set of chemotherapeutics was assessed across a panel of 24 cell lines. A, GNE-783 was tested for its ability to potentiate the activity of gemcitabine, carboplatin, SN-38, 5-FU, temozolomide, and taxol across a panel of cell lines. The associated Bliss score for each combination in each cell line is shown ($n = 2$–6 for each combination). B, the ability of GNE-783 to potentiate the activity of each agent was compared based on the p53 status of each cell line. C, correlation of single-agent activity with p53 status for each cell line.

is used for the treatment of metastatic melanoma (28), we explored this combination across a panel of 15 melanoma cell lines using a full combination dose matrix (9 × 9) of each compound (Fig. 5B) and found that p53-mutant cell lines were more sensitive to the combination than wild-type cells (Fig. 5C).

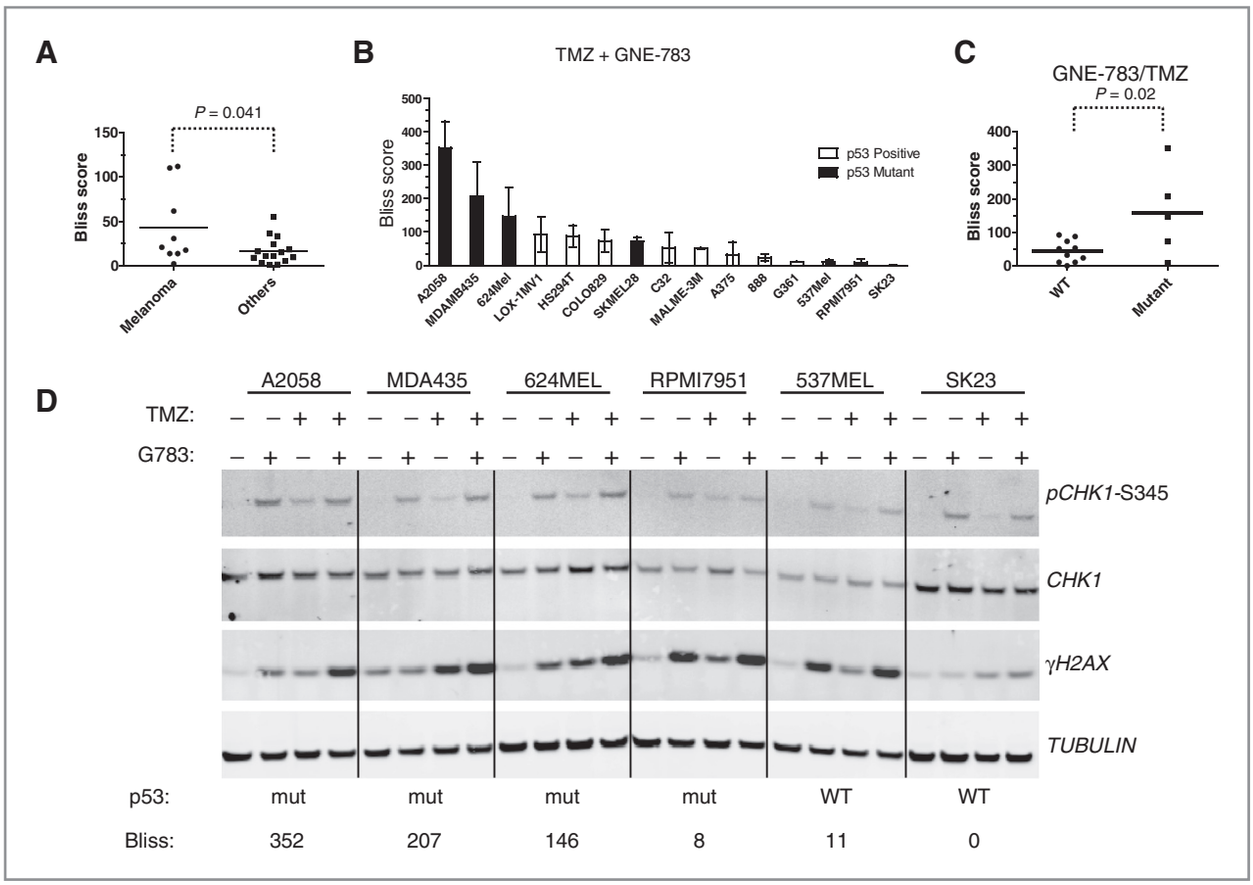

Figure 5. Potentiation of temozolomide with GNE-783 is enhanced in melanoma cell lines in a p53-dependent manner. A, melanoma cell lines support enhanced potentiation of temozolomide activity by GNE-783 compared with other cell lines. B, 15 melanoma cell lines were screened with temozolomide and/or GNE-783, and Bliss scores are shown (average ± SD, $n = 3$). C, Bliss scores for each cell line (B) were grouped according to the p53 status of each cell line. D, pathway response as assessed by Western blot analysis for *CHK1*, p*CHK1*-S345, and γ-H2AX in the indicated melanoma cell lines following treatment with temozolomide (500 μmol/L), GNE-783 (1 μmol/L), or both for 24 hours. Bliss scores and p53 status for each cell line are indicated.

Activation of *CHK1* to varying levels, as evident by an increase in levels of phosphorylation of *CHK1* serine-345, was observed in all melanoma cell lines in response to temozolomide or GNE-783 (Fig. 5D). In cell lines with high levels of potentiation (A2058, MDA-MB-435, 624MEL), we also observed an increase in γ-*H2AX* levels in the presence of both agents compared with either agent alone (Fig. 5D). In cell lines with low levels of potentiation, γ-*H2AX* levels did not increase between single agent and combination treatment (Fig. 5D).

Robust chemopotentiation *in vitro* translates into enhanced efficacy *in vivo*

Further optimization of our GNE-783 chemical series lead to the discovery of GNE-900 (Supplementary Fig. S2A), which has a similar *in vitro* profile but has improved pharmacokinetic properties and supports efficient chemopotentiation *in vivo* (29). GNE-900 can be effectively combined with gemcitabine or SN38 in HT29 cells and with temozolomide in A2058 cells (Supplementary Fig. S2B). In addition, GNE-900 can effectively combine with temozolomide across the same panel of melanoma cell lines as tested in Fig. 5B, and, similar to GNE-783, displays better synergy in p53-deficient cell lines (Supplementary Fig. S2C).

To understand how our *in vitro* findings relate to chemopotentiation *in vivo*, we compared the ability of GNE-900 to enhance the *in vivo* response of three different chemotherapeutics with varying levels of synergy with *CHK1* Inhibition *in vitro*. In an HT-29 xenograft model, GNE-900 in combination with gemcitabine produced a more robust *in vivo* response than gemcitabine alone (Fig. 6A, Supplementary Fig. S3A); combining GNE-900 and gemcitabine resulted in tumor growth inhibition (TGI) of 116% (lower:upper CI of 102:136), which was statistically different to the TGI obtained with gemcitabine alone (61%; lower:upper CI of 29:83). In contrast, combining GNE-900 with CPT-11 (the prodrug formulation of SN-38, which showed modest chemopotentiation *in vitro*) resulted in a TGI of 93% (lower:upper CI of 63–112), which was only modestly better, but not statistically significant, than that obtained with

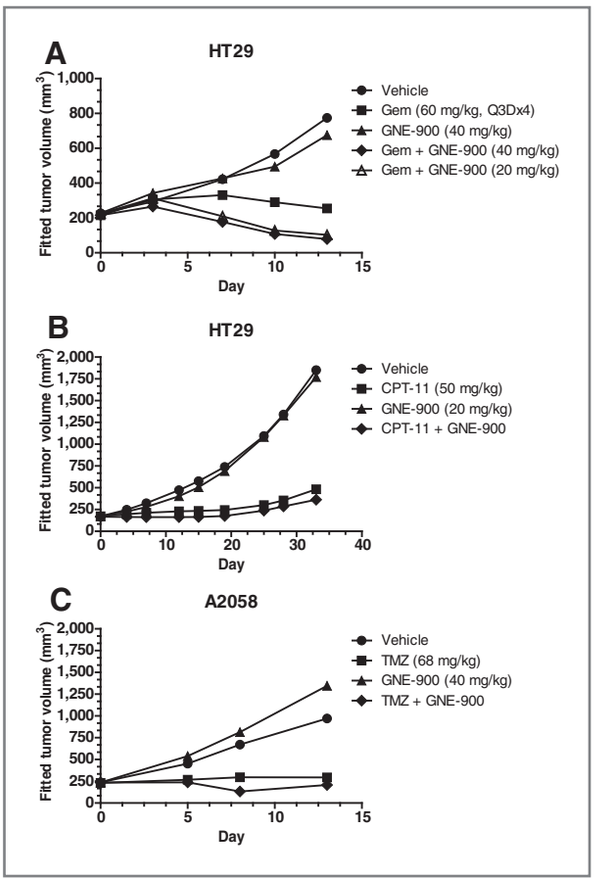

Figure 6. *In vivo* evaluation of the ability of GNE-900 to enhance the activity of gemcitabine, CPT-11, and temozolomide in tumor xenograft models. A, GNE-900 enhances the activity of gemcitabine in an HT-29 xenograft model. Gemcitabine (60 mg/kg) was administered i.p. once every 3 days (Q3D) for a total of 4 doses. GNE-900 was administered orally at 20 or 40 mg/kg 24 hours after gemcitabine ($n = 10$ animals per group). B, combination of GNE-900 and CPT-11 in an HT-29 xenograft model. CPT-11 was administered at 50 mg/kg i.p. every 4 days (Q4D) for a total of 4 doses. GNE-900 was administered orally at 20 mg/kg 24 hours following CPT-11 ($n = 10$ animals per group). C, combination of GNE-900 and temozolomide in an A2058 xenograft model. Temozolomide was administered at 68 mg/kg orally daily for 5 days (QD×5) for 1 cycle. GNE-900 was administered orally at 40 mg/kg 4 hours after temozolomide ($n = 10$ animals per group).

CPT-11 alone (82%TGI: lower:upper CI of 34:100; Fig. 6B, Supplementary Fig. S3B).

Finally, we evaluated the ability of GNE-900 to enhance the activity of temozolomide in the A2058 xenograft tumor model (Fig. 6C, Supplementary Fig. S3C). Temozolomide alone is effective in this model, and resulted in an 87% TGI (lower:upper CI of 53:105) when administered orally for 5 days. In comparison, the combination of temozolomide and GNE-900 resulted in TGI of 111% (lower:upper CI of 97:128), which is not statistically different. In all *in vivo* models, there was minimal body weight loss during treatment (Supplementary Fig. S3D–F).

Our results suggest that robust chemopotentiation *in vitro* may be required to observe enhanced efficacy *in vivo*. Although animals exposed to GNE-900/CPT-11 or GNE-900/temozolomide did not result in a statistical improvement in TGI versus animals exposed to either agent alone, there was an increase in the number of animals displaying a partial response in the combination treated animals (Supplementary Fig. S3A–C), suggesting that these combinations may be modestly more effective than either CPT-11 or temozolomide alone.

Discussion

CHK1 is implicated in a wide range of activities including DNA damage (1), initiation of DNA replication (30, 31), DNA replication fork progression (32), mitotic spindle checkpoint (33), and regulating activity of the spleen tyrosine kinase (long form) in hepatocellular carcinoma (34). There is a great deal of interest in *CHK1* as a therapeutic target, given its ability to enhance the activity of some DNA-damaging agents, and numerous *CHK1* inhibitors are undergoing clinical evaluation (2). However, there was neither a systematic study comparing the ability of a single *CHK1* inhibitor to potentiate the activity of different types of DNA-damaging agents, nor was there a good understanding of the importance of p53 for chemopotentiation.

CHK1 inhibitors have been shown to enhance the activity of gemcitabine both *in vitro* and *in vivo* (6, 13–17), and CHK1 inhibitors are being clinically evaluated to determine if they can increase the response to gemcitabine (2). Gemcitabine and cytarabine (both antimetabolite-based agents) induce replication fork stalling, which was shown to activate the ATR/CHK1 signaling pathway (35), and we find that both agents can be effectively combined with GNE-783 (Supplementary Table S2). Moreover, CHK1 is induced in AML samples from patients infused with cytarabine (36), and a combination of cytarabine and the CHK1 inhibitor (SCH900776) is undergoing evaluation in the clinic (37). Consistent with CHK1 being important in the cellular response to these agents, we found that the activity of antimetabolite-based DNA-damaging agents was preferentially enhanced in p53-deficient cells compared to all other classes of DNA-damaging agents (Figs. 2, 3). Interestingly, the ability of GNE-783 to enhance the activity of DNA-damaging agents was more dependent on the specific agent and the mechanism by which cells respond to the type of DNA damage, rather than on the class of agent used. Furthermore, it can be difficult to predict which combinations may be effective, as we found that some combinations are only effective in certain tumor types.

We screened five other agents across a broad panel of cell lines, and found that combining GNE-783 with gemcitabine generated an overall better response than with any of the other agents (Fig. 4A); moreover, GNE-783 preferentially enhanced the activity of gemcitabine and SN-38 in p53 mutant cell lines. In addition, GNE-783 enhanced the activity of temozolomide, but this predominantly occurred in p53-deficient melanoma cell lines (Fig. 5A–C). Our data is consistent with previous reports

showing that p53 deficiency improves response to chemopotentiation with a *CHK1* inhibitor (6, 8, 19, 20, 22, 23), although it has also been shown that *BRCA*, *XRCC3*, *DNA-PK* (38), and *CYCLIN B1* (39) levels may influence the effectiveness of combining a *CHK1* inhibitor with a DNA-damaging agent. Thus, while p53-mutant cell lines tend to have a better response to some combinations with GNE-783, it also indicates that p53 deficiency alone may not be sufficient to ensure a robust response.

We evaluated the ability of the Chk1 inhibitor, GNE-900 (Supplementary Fig. S2; ref. 29), to enhance the activity of three different standard-of-care therapeutics in tumor xenograft models. For gemcitabine, CPT-11, and temozolomide, we chose a dose that was within approximately 2-fold of their maximum-tolerated dose in these xenograft models. Thus, we designed our *in vivo* studies to closely approximate how these chemotherapeutics may be used in the clinic, that is, at or close to their maximum-tolerated dose. Combining GNE-900 with gemcitabine significantly reduced tumor growth in an HT-29 model compared with gemcitabine alone (Fig. 6A). In contrast, there was no statistically significant difference in TGI when GNE-900 was combined with CPT-11 or temozolomide versus either agent alone (Fig. 6B–C); however, there was an increase in the number of animals that displayed a partial response in the combination groups compared with animals treated with CPT-11 or temozolomide alone (Supplementary Fig. S3A–C). Although it is possible that using lower doses of each chemotherapeutic would potentially favor a positive effect with combination treatment, we believe the way we designed our experiments would be more informative in understanding the potential for a positive benefit in the clinic. In addition, it is possible that the dosing regimen used in these studies may not be optimal, either for the chemotherapeutic or for the timing of administration of GNE-900. In addition, these combinations may result in enhanced efficacy in other tumor xenograft models not tested here. Nevertheless, our data suggests that robust chemopotentiation *in vitro* may be required to translate into enhanced efficacy *in vivo*.

In conclusion, our results indicate that p53 status can clearly impact the ability of a *CHK1* inhibitor to potentiate the activity of some chemotherapeutics, most noticeably gemcitabine and SN-38. However, although p53 status may increase the likelihood of a robust response, it is not sufficient, as many p53-deficient cell lines do not display synergy with these same combinations. In addition, our results have implications for the selection of preferred combinations to pursue *in vivo*, because without rigorous cell line profiling in a relevant panel of cell lines, it is difficult to predict how effective a specific combination will be.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Malek, E. Blackwood, P.K. Jackson, T. O'Brien
Development of methodology: Y. Xiao, C. DelNagro, E. Blackwood, T. O'Brien
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Xiao, J. Ramiscal, K. Kowanetz, C. DelNagro, S. Malek, M. Evangelista, E. Blackwood
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Xiao, C. DelNagro, S. Malek, M. Evangelista, E. Blackwood, P.K. Jackson, T. O'Brien
Writing, review, and/or revision of the manuscript: Y. Xiao, J. Ramiscal, M. Evangelista, E. Blackwood, P.K. Jackson, T. O'Brien
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Ramiscal, E. Blackwood
Study supervision: S. Malek, E. Blackwood, P.K. Jackson, T. O'Brien

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