CK2 Inhibitor CX-4945 Suppresses DNA Repair Response Triggered by DNA Targeted Anticancer Drugs and Augments Efficacy: Mechanistic Rationale for Drug Combination Therapy

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Abbreviations list: DRR: DNA Repair Response; PARP: Poly (ADP-ribose) polymerase; SSB: strand break; DSB: double strand break; BER: base excision repair; NER: nucleotide excision repair; PNK: polynucleotide kinase; WT: wild type; TTE: time to endpoint; TGI: tumor growth inhibition; NHEJ: non-homologous end joining; HR: homologous repair

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ABSTRACT

Drug combination therapies are commonly used for the treatment of cancers to increase therapeutic efficacy, reduce toxicity and decrease the incidence of drug resistance. While drug combination therapies were originally devised primarily by empirical methods, the increased understanding of drug mechanisms and the pathways they modulate provides a unique opportunity to design combinations that are based on mechanistic rationale. We have identified protein kinase CK2 as a promising therapeutic target for combination therapy because CK2 regulates not just one, but many oncogenic pathways and processes that play important roles in drug resistance, including DNA repair, EGFR signaling, PI3K/AKT/mTOR signaling, Hsp90 machinery activity, hypoxia and IL-6 expression. In this report we demonstrate that CX-4945, a clinical stage selective small molecule inhibitor of CK2, blocks the DNA repair response induced by gemcitabine and cisplatin and synergizes with these agents in models of ovarian cancer. Mechanistic studies show that the enhanced activity is a result of inactivation of XRCC1 and MDC1, two mediator/adaptor proteins that are essential for DNA repair and that require phosphorylation by CK2 for their function. These data position CK2 as a valid pharmacologic target for intelligent drug combinations and support the evaluation of CX-4945 in combination with gemcitabine and platinum-based chemotherapeutics in the clinical setting.
INTRODUCTION

DNA targeted chemotherapeutics represent fundamental components of modern cancer therapy and are currently prescribed for multiple indications. These agents target the DNA of tumor cells and can activate one or more DNA repair response (DRR) mechanisms, potentially leading to the development of drug resistance (1). An emerging strategy to boost the effectiveness of these drugs is to combine them with inhibitors of corresponding DRR mechanisms (1, 2). Methods currently subject to clinical investigation include inhibitors of the checkpoint regulators CHK1 and CHK2, inhibitors of the direct repair enzyme MGMT and inhibitors of Poly (ADP-ribose) polymerase (PARP), a key mediator of base excision repair (1). Because different classes of DNA targeted anti-cancer drugs can trigger distinct DRR pathways, it would be advantageous to identify and inhibit a target protein to disrupt multiple DRR pathways simultaneously. Protein kinase CK2, a constitutively active serine/threonine kinase that is widely overexpressed in human cancers (3, 4), has recently emerged as a key regulator of the repair of both single and double strand breaks (5-7). Hence, by simultaneously disrupting multiple DNA repair pathways, inhibitors of CK2 have the potential to enhance the activity of a wide range of DNA targeted chemotherapeutics.

Recently validated as an anti-cancer drug target, CK2 regulates a diverse array of pro-survival cellular processes, including EGFR signaling, PI3K/AKT/mTOR signaling, Hsp90 machinery, hypoxia and IL-6 expression, all of which play important roles in resistance to various chemotherapeutics (3, 4, 8-11). Furthermore, protein kinase CK2 has emerged as a key participant in DRR, being essential for the surveillance and repair of both single and double strand breaks (6). Among the best characterized of the CK2-
dependent DRR substrates are the mediator/adaptor proteins XRCC1 and MDC1, which are essential components of the single strand break (SSB) and double strand break (DSB) repair machinery, respectively (Fig. 1).

XRCC1 is a key mediator of SSB repair, which includes both the base excision repair (BER) and nucleotide excision repair (NER) mechanisms (12). It exists in a tight complex with DNA ligase IIIα, which serves to re-ligate broken DNA single strands following the processing of damaged bases/nucleotides. XRCC1 is continuously phosphorylated by CK2 (13), an event that is required for its interaction with two proteins, aprataxin and polynucleotide kinase (PNK), which participate in DNA end-processing prior to ligation (Fig. 1). In addition, phosphorylation of XRCC1 by CK2 may also be required to maintain stability of the XRCC1-ligase IIIα complex itself (5, 14, 15).

MDC1 is a key mediator of homologous recombination (HR) DSB repair and is the principle binding partner of γ-H2AX that is anchored to DNA at sites of DSBs (16-18). Once bound to γ-H2AX, MDC1 recruits a key multiprotein complex 'MRN' that is required for DSB repair signaling. This interaction is dependent on the phosphorylation of MDC1 at multiple acidophilic sites by CK2 (16-18). The functional consequences of MRN complex binding to MDC1 include activation of both the S-phase and G2/M checkpoints following the treatment of cells with ionizing radiation (19-21) and amplification of ATM signaling (Fig. 1) (22). In addition, the phosphorylation of MDC1 by CK2 also promotes binding of aprataxin, implicating MDC1/CK2 in direct HR repair (6).
Following the discovery of CX-4945, a first-in-class clinical stage inhibitor of CK2 (23, 24), we sought to investigate whether the pharmacological targeting of CK2-dependent DRR functions could potentiate the ability of DNA targeted chemotherapeutic agents to kill tumor cells. For this purpose we selected cisplatin (or carboplatin) and gemcitabine in the context of ovarian cancer, where both drugs are used in combination chemotherapy (25). The primary mechanism involved in the repair of DNA-platinum adducts is NER, in which XRCC1/ligase IIIα complex plays a prominent role in re-ligating the broken DNA strand (Fig. 1) (12, 26). During DNA replication, unrepaired platinum adducts can stall the replication fork, triggering ATR-mediated repair. Likewise, the tri-phosphorylated form of gemcitabine is incorporated into DNA during DNA replication, also causing replication forks to stall. Failure to repair either cisplatin- or gemcitabine-induced stalled replication forks leads to replication fork collapse (DSBs), triggering the accumulation of CK2-phosphorylated MDC1 (bound to γ-H2AX), amplified ATM signaling and repair by HR (Fig. 1) (27-30). Thus, inhibition of CK2 could potentially synergize with cisplatin by disrupting XRCC1-dependent NER (SSB repair) and with cisplatin and gemcitabine by disrupting MDC1-mediated HR repair (DSB repair).
MATERIALS AND METHODS

Materials

CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid)) was synthesized by Cylene Pharmaceuticals (>99% pure by HPLC assay). Cisplatin, gemcitabine and carboplatin were purchased from Sigma-Aldrich (Fig. 2).

Cell culture

A2780 and SKOV-3 human ovarian carcinoma cell lines were purchased from American Tissue Culture Collection and used within six months with weekly monitoring for growth rates and morphology consistency. ATTC performs authentication testing of the cell lines using DNA profiling and cytogenetic analysis. Cell lines were cultured according to the suppliers’ recommendations.

Immunoprecipitation

Untreated or CX-4945 treated (10 μM, 24 h) cells were washed twice with PBS and lysed in 1X RIPA Buffer supplemented with PMSF and Protease Inhibitor Set 1 (EMD Chemicals). Samples were sonicated on ice and centrifuged at 14000 g for 10 min at 4 °C. Protein was quantitated using the Bradford protein assay. 10 μg of anti-MDC1 antibody (Bethyl Laboratories) was added to the cell lysate (2 mg) and 100 μL of 20%
protein A suspension. The immunoprecipitation reactions were rotated overnight at 4 °C. The samples were centrifuged and the resulting pellets were washed three times with 500 μl cold cell lysis buffer. Samples were analyzed by western blot. The antibody for the CK2 substrate consensus sequence (S*/T*D/EXD/E) was purchased from Cell Signaling.

**COMET assay**

SKOV-3 cells (1 x 10^5 cells) were combined with molten LMAgarose (Trevigen) at a ratio of 1:10 (v/v) and were immediately pipetted onto CometSlide (Trevigen). Slides were incubated at 4 °C in the dark for 10 min, then immersed in pre-chilled Lysis Buffer and incubated at 4 °C for 30 min. Slides were immersed in Alkaline Unwinding Solution, pH > 13 (200 mM NaOH, 1 mM EDTA) for 20 min at RT in the dark. Electrophoresis was done at 21 V for 30 min using Alkaline Electrophoresis solution (200 mM NaOH, 1 mM EDTA). The slides were washed twice in water for 5 min and once in 70% EtOH for 5 min then dried overnight and visualized by microscopy. Under these conditions the formation of “comet tail” is indicative of SSDs, DBSs and/or active excision repair of DNA crosslinks.

**Cell cycle analysis**

Cell cycle was analyzed by flow cytometry as described elsewhere (23). Data was analyzed with FlowJo software (TreeStar, Inc.).
Cell viability assay

A2780 and SKOV-3 cells were seeded at a density of 750 and 1000 cells per well, respectively, and treated 24 h later according to the pre-addition or post-addition schedule (Pre-addition schedule: 4h treatment with CX-4945 followed by 24 h treatment with cisplatin/gemcitabine. Post-addition schedule: 24 h treatment with cisplatin/gemcitabine followed 8 h treatment with CX-4945). Following completion of the treatment schedule, the media was replaced and the cells were cultured at 37 °C for the remainder of 96 h. Cell viability was analyzed by CyQuant assay (Invitrogen).

In vivo efficacy studies

Female immunocompromised mice CrTac:Ncr-Foxn1\textsuperscript{nu} (5-7 weeks old) were obtained from Taconic Farms. Animals were maintained under clean room conditions in sterile filter top cages. Animals received sterile rodent chow and water ad libitum. All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide: The Care and Use of Laboratory Animals. Xenografts were initiated by subcutaneous injection of A2780 cells into the right hind flank region of each mouse. When tumors reached a designated volume of 100-150 mm\textsuperscript{3}, animals were randomized and divided into groups of 9-10 mice per group. CX-4945 was administered by oral gavage twice daily (BID) at 75 or 100 mg/kg as indicated. Cisplatin (5 mg/kg) and carboplatin (75 mg/kg) were administered via intraperitoneal injection once a week. Gemcitabine (60 mg/kg) was administered via intraperitoneal injection according to a Q3D x 4 schedule. Tumor volumes and body weights were measured twice weekly. The
length and width of the tumor were measured with calipers and the volume calculated using the following formula: tumor volume = (length x width²)/2. Mean percent tumor growth inhibition (TGI) values were calculated on the final day of the study for drug-treated compared to vehicle-treated mice and were calculated as 100 x \{1-[(Treated_{Final day} – Treated_{Day 1})/(Control_{Final day} – Control_{Day 1})]\}. Time to endpoint (TTE) is defined as the median time taken for the tumors to reach a median of 2000 mm³.

**Pharmacodynamic studies**

Mice bearing A2780 tumors (300-400 mm³) were randomized and divided into 3 groups of 5 mice. Following a single dose of gemcitabine, CX-4945 was administered by oral gavage at 3.5 and 15.5 h. Animals were euthanized 24 h after administration of gemcitabine, tumors were resected, lysed and resulting protein extracts were subjected to western blot analysis.
RESULTS

**CX-4945 enhances the activity of cisplatin and gemcitabine in cell-based anti-proliferative experiments**

Gemcitabine and cisplatin are commonly used to treat patients with ovarian cancers (25). Since CK2 is involved in multiple processes that regulate the sensitivity of cancer cells to such DNA targeted anti-cancer drugs (10, 13, 16), we asked if combining CX-4945 with gemcitabine or cisplatin can improve the effectiveness of these chemotherapeutics. As p53 status is known to play an important role in the sensitivity of ovarian cancer cells to DNA targeted agents (31) and because of the high frequency of p53 mutations in ovarian cancer (32) we used both p53 WT A2780 cells and p53 null SKOV-3 cells for our mechanistic studies. To ensure inhibition of CK2 signaling throughout the experiment, cells were pretreated with CX-4945 four hours prior to addition of either gemcitabine or cisplatin, and the presence of CX-4945 was maintained throughout the treatment period. Under these conditions (Fig 3A), CX-4945 promoted synergistic antiproliferative effects in both cell lines (antiproliferative activity 8-17% greater than Bliss additivity (33).

In separate experiments using post-addition of CX-4945, we performed combination studies using a schedule wherein CX-4945 was added 24 hours after treatment with gemcitabine or cisplatin and then maintained in combination for only 8 h. Under these conditions, the chemotherapeutic agents have sufficient time to cause DNA strand breaks prior to the addition of CX-4945, while the presence of CX-4945 for only 8h
contributes minimal single agent antiproliferative activity. Using this schedule (Fig. 3B) CX-4945 significantly enhanced the antiproliferative effects of gemcitabine and cisplatin (antiproliferative activity 23-38% higher than Bliss additivity). These data are consistent with an enhancement of antiproliferative activity by CX-4945 as a consequence of inhibiting DRR mechanisms.

Stalled or collapsed replication forks produced by DNA-targeted drugs are known to cause cancer cells to arrest in S-phase (34). Indeed, we demonstrated that SKOV-3 cells and to a greater extent A2780 cells, can respond to gemcitabine or cisplatin treatment by accumulating in S-phase (data not shown). To determine the effects of CX-4945 on chemotherapeutic-induced cell cycle arrest, we evaluated the effects of gemcitabine alone or the combination of gemcitabine with CX-4945 in A2780 cells. After 28 h, A2780 cells treated with gemcitabine alone reached the maximal S-phase arrest and then began recovery from S-phase and progressed to G2/M by 36 h. However, the combination of CX-4945 with gemcitabine delayed replication recovery (Fig. 3C), while CX-4945 alone produced G2/M arrest, as previously described (24). These data suggest that by inhibiting DRR in ovarian cancer cells, the combination with CX-4945 inhibits replication recovery and increases cancer cell death induced by DNA targeted drugs.

**CX-4945 decreases XRCC1 and MDC1 phosphorylation and prevents DNA repair response in combination with cisplatin and gemcitabine**
To further define the mechanistic processes underlying the synergistic antiproliferative activity, we asked if CX-4945 alone or in combination with cisplatin or gemcitabine could reduce the phosphorylation of the DNA repair mediator/adaptor proteins XRCC1 and MDC1 thus inhibiting DRR. Treatment of A2780 or SKOV-3 cells with CX-4945 led to a significant decrease in the phosphorylation of XRCC1 at multiple CK2-specific sites (Fig. 4A and 4B). As specific antibodies for the CK2 phosphorylation sites on MDC1 were unavailable, we probed immunoprecipitated MDC1 from A2780 and SKOV-3 cells treated with CX-4945 using an antibody designed to bind to phospho-peptides with the CK2 substrate consensus sequence. Treatment of either cell type with CX-4945 led to significant reductions in MDC1 phosphorylation (Fig. 4C).

To determine if decreased XRCC1 and MDC1 phosphorylation prevented DNA repair after treatment with gemcitabine or cisplatin, we employed the alkaline comet assay to monitor the production of DNA strand breaks (35). At concentrations where neither CX-4945 nor gemcitabine or cisplatin caused significant comet formation in A2780 cells, the combination of CX-4945 with either gemcitabine or cisplatin produced prominent tails. Such tails are indicative of SSBs, DSBs and/or active excision repair of DNA crosslinks. This demonstrates that CX-4945 prevented DNA repair after gemcitabine or cisplatin treatment (Fig. 5A). Addition of the pan-caspase inhibitor zVAD-FMK did not reduce tail formation, indicating that the observed DNA strand breaks were not secondary to the induction of apoptosis. To confirm these findings, we monitored changes in the levels of γ-H2AX, a widely established marker of DNA strand breaks (36). Combining CX-4945 with either gemcitabine or cisplatin in either A2780 or SKOV-3 cells increased levels of
γ-H2AX compared to either agent used alone, confirming that levels of DNA strand breaks were increased in the cancer cells (Fig. 5B, 5C and S1).

Mechanistic data with CX-4945 presented thus far indicate that CK2, XRCC1 and MDC1, all act in a coordinated and essential fashion to facilitate the DRR that is triggered by cisplatin or gemcitabine treatment. This contention is corroborated by studies in which we utilized siRNA to knockdown CK2 (both the α and α' isoforms simultaneously), XRCC1 or MDC1 in SKOV-3 cells. Knockdown of XRCC1, MDC1 or simultaneous knockdown of CK2α/α' significantly increased the levels of γ-H2AX produced by either cisplatin or gemcitabine treatment alone (Fig. S2). These data confirm the relevance of CK2, XRCC1 and MDC1 in mediating gemcitabine or cisplatin-induced DRR, and highlight the utility of CK2 as a drug target to prevent the DRR triggered by such chemotherapeutic agents.

The presence of DNA strand breaks would be expected to trigger the activation of ATR and ATM, two kinases that play prominent roles in DRR signaling, which in turn phosphorylate CHK1 and CHK2 respectively, halting cell cycle progression to allow DNA repair to progress (1). In all combinations tested, addition of CX-4945 increased phosphorylation of CHK2 when compared to the cytotoxic agent used alone (Fig. 5B and 5C). The effects on CHK1 phosphorylation were mixed, particularly in A2780 cells where significant reductions of total CHK1 levels were also observed. Taken together, our data demonstrate that inhibition of CK2 by CX-4945 prevents activation of MDC1 and XRCC1 proteins and consequently suppresses the ability of cancer cells to repair DNA strand breaks caused by treatment with gemcitabine or cisplatin.
The combination of CX-4945 with cisplatin or gemcitabine results in apoptosis in p53 WT A2780 cells and mitotic catastrophe in p53 null SKOV-3 cells.

It has previously been reported that cisplatin-treated ovarian cancer cells undergo distinct modes of cell death which are dependent on the status of p53 (34). Therefore, we examined the mode of cell death of A2780 and SKOV-3 cells treated with CX-4945 and gemcitabine or cisplatin. First, we analyzed the change in levels of effector caspase-3/7 activity. In p53 WT A2780 cells, the combination of CX-4945 with either cisplatin or gemcitabine resulted in a significant increase in caspase-3/7 activity compared to either agent used alone (Fig. 6A). In p53 null SKOV-3 cells, the same combinations did not produce significant increases in caspase-3/7 activity. Consistent with these observations, in p53 WT A2780 cells a significant increase in cleaved PARP was seen upon combination treatment, confirming elevated apoptosis, while in p53 null SKOV-3 cells the levels of cleaved PARP were undetectable (Fig. 6B and S1).

Since p53 null SKOV-3 cells were previously shown to be susceptible to mitotic catastrophe in response to cisplatin treatment (34), we wanted to test if the combination of CX-4945 with cisplatin would lead to the same outcome. For this purpose we monitored nuclear morphology changes following drug treatment of SKOV-3 cells by DAPI staining (Fig. 6C). The nuclear morphology of SKOV-3 cells treated with cisplatin for up to 72 h was unchanged. Combination treatment of cisplatin with CX-4945 for 72 h produced enlarged multinucleated cells, a phenotypic response previously
characterized as cisplatin-induced mitotic catastrophe (34, 37, 38). Similar results were also seen when CX-4945 was combined with gemcitabine in SKOV-3 cells (Fig. 6C).

**CX-4945 synergizes with cisplatin, carboplatin and gemcitabine in xenograft models of ovarian cancer**

To determine whether the synergy observed between CX-4945 and cisplatin or gemcitabine in vitro could be translated into increased antitumor efficacy in vivo, we tested these combinations in a xenograft model. For this purpose we selected the A2780 model because it is aggressively tumorigenic (time to endpoint (TTE) ~ 15 days), and it allowed us to investigate cleaved PARP as a pharmacodynamic biomarker of combination activity in tumors. CX-4945 (dosed orally at 75 mg/kg twice a day) or cisplatin (dosed intraperitoneal at 5 mg/kg once every 7 days) had minimal antitumor effects as single agents in this model. However, combining CX-4945 with cisplatin produced robust tumor growth inhibition (TGI) and extended TTE to 30 days (Fig. 7A). Because cisplatin is known to cause negative effects on the body weights of treated animals, we combined CX-4945 with carboplatin, a cisplatin analog with a reduced side-effect profile and widely used in the treatment of ovarian cancer. Neither agent was effective when administered alone, while the combination resulted in robust TGI and extended TTE to 34 days. The body weight effects for combination were comparable to those produced by single agents (Fig. 7B). In combination with gemcitabine, CX-4945 improved the therapeutic benefit by increasing TGI to 99% and extending TTE to 39 days, while being well tolerated (Fig. 7C).
Our \textit{in vitro} antiproliferative studies suggested that transient exposure to CX-4945 was sufficient to produce synergy with gemcitabine or cisplatin. Therefore, we conducted an additional A2780 xenograft study wherein CX-4945 was dosed only on four occasions, each dose given 24 hr after administration of gemcitabine (Fig. 7D). This dosing schedule delivered significantly longer TTE than gemcitabine alone (51 days versus 37 days). Moreover, the enhanced efficacy using this dosing regimen provides \textit{in vivo} support that CX-4945 augments the antitumor effects of chemotherapeutic agents as a consequence of inhibiting DRR mechanisms.

To investigate the potential of cleaved PARP as a pharmacodynamic biomarker, we treated mice implanted with A2780 xenografts to a single dose of gemcitabine (60 mg/kg IP) followed by 2 subsequent doses of CX-4945 (75 mg/kg PO) at 3.5 and 15.5 h after administration of gemcitabine. Twenty four hours following gemcitabine addition, the tumors were resected, lysed and the resulting protein extracts were analyzed for levels of cleaved PARP using western hybridization (Fig. 7E). A clear increase in cleaved PARP levels was observed in tumors from mice treated with CX-4945 and gemcitabine in combination compared to either drug used alone, confirming that the antitumor effect of combining the two drugs resulted in increased apoptosis in A2780 xenografts \textit{in vivo}.
DISCUSSION

DNA targeted chemotherapeutics are commonly used as single agents or in combination for the treatment of various types of cancer. While these drugs are known for their robust initial efficacy, they are often limited by toxicity and inherent or acquired resistance (39). One of the mechanisms driving such resistance is DRR which limits the ability of DNA targeted chemotherapeutics to kill cancer cells (1). Several approaches aimed at combining DNA targeted chemotherapeutics with inhibitors of DRR are being investigated in the clinic. The majority of these inhibitors target highly specific DRR pathways; therefore, there is considerable need for DRR inhibitors that target a broader spectrum of DRR and potentially offer combinability with a greater number of anti-cancer chemotherapies. CK2, with its newly recognized role in the genomic surveillance and repair of both single and double strand breaks and its established overexpression in cancer cells, ideally fulfills these criteria.

We demonstrated that the presence of the CK2 inhibitor CX-4945 strongly enhances the antiproliferative activity of gemcitabine or cisplatin in ovarian cancer cells (Fig 3A and 3B). By varying dosing schedules we showed that relatively short exposure of cells already treated with gemcitabine or cisplatin to CX-4945 was sufficient to produce synergy. Cell cycle analysis established that CX-4945 prevented the replication recovery of cells treated with gemcitabine, indicating that the inhibition of DRR is the primary mechanism behind the observed synergy (Fig. 3C).

We demonstrated that inhibiting CK2 with CX-4945 decreased the phosphorylation of XRCC1 and MDC1 at CK2 specific phosphorylation sites (Fig. 3A), which prevented
efficient DRR in cisplatin or gemcitabine treated ovarian cancer cells, as judged by tail formation in the comet assay and increased H2AX phosphorylation (Fig. 5A, 5B and 5C). The combination treatments also resulted in amplification of DNA repair signaling as evident from the increased levels of phospho-CHK2. Interestingly, changes in the phosphorylation of CHK1 were cell line dependent, with relative levels of phospho-CHK1 being somewhat higher for the combination in SKOV-3 cells, but lower in A2780 cells, when compared to the effects of single agents (Fig. 5B and 5C). This can potentially be explained by the reduction of total CHK1 levels produced by both gemcitabine or cisplatin and combination treatments with CX-4945, which may be linked to the previously described caspase-mediated cleavage of CHK1 that can occur during apoptosis (40, 41). It is also possible that CK2 inhibition may directly regulate CHK1 stability in A2780 cells, since CHK1 is a client protein of HSP90 co-chaperone CDC37, which is known to depend on phosphorylation by CK2 for its activity (8).

Using siRNA knockdowns, we demonstrated that CK2α/α’, MDC1 and XRCC1 proteins were essential for efficient DRR resulting from cisplatin or gemcitabine treatment. Although XRCC1 has been implicated in cisplatin DRR in HepG2 cells (42), the role of XRCC1 in repairing gemcitabine induced DRR has not previously been described. One possible explanation is that XRCC1 has been shown to bind to RAD51, implicating XRCC1 in HR repair (43). An alternative explanation derives from the emerging role of XRCC1 in the coordination of DNA repair and replication during S-phase, mediated by its interaction with the p58 subunit of DNA Pol α-primase (44). This model would involve XRCC1 in the stabilization and repair of stalled replication forks created by gemcitabine, although the putative role of CK2 in this process remains to be investigated (Fig. 1).
The role of p53 in the cellular and clinical response to chemotherapies reflects the dual nature of p53 as an activator of both DNA repair and apoptosis (45). In the context of ovarian cancer, p53 mutations have been shown to confer resistance to cisplatin in vitro (46) and also correlate with resistance to platinum-based chemotherapy and poor prognosis in the clinic (31). While in antiproliferative experiments the combination of CX-4945 with gemcitabine or cisplatin was synergistic in both A2780 and SKOV-3 cells, the fate of these cells was found to be different, with p53 WT A2780 cells readily undergoing increased apoptosis, whereas p53 null SKOV-3 cells showed evidence of mitotic catastrophe. These data suggest that even though different cell death pathways are activated, the ability of CX-4945 to augment the antiproliferative activity of gemcitabine or cisplatin appears not to depend on the status of p53, suggesting that such combinations can be used successfully in a broad spectrum of patients with ovarian cancer, a disease characterized by a high frequency of p53 mutation (32).

In vivo studies with mice bearing A2780 xenografts confirmed that the combination of CX-4945 with cisplatin, carboplatin or gemcitabine increased the anti-tumor efficacy compared to the efficacy observed with single agents. We also demonstrated that administration of CX-4945 on an intermittent schedule, i.e. 24 h after each dose of gemcitabine could enhance the efficacy of gemcitabine in mice. These findings further illustrate that CX-4945 prevents CK2 from activating DRR mechanisms, thereby preventing replication recovery. Finally, we demonstrated that the increased anti-tumor activity of the CX-4945/gemcitabine combination correlates with enhanced apoptosis by measuring cleaved PARP as a pharmacodynamic biomarker in xenograft tumors.
CK2 potentially regulates multiple functions within DRR. However, a clearly prevailing mechanism is to facilitate the binding of signaling molecules and DNA end-processing factors to non-catalytically active mediator/adaptor proteins involved in both SSBR and DSBR. Remarkably, mirroring its function with XRCC1, phosphorylation by CK2 promotes the binding of both aprataxin and PNK to XRCC4, the mediator/adaptor binding partner of DNA ligase IV (7, 15). Because XRCC4/Ligase IV complex is an essential component of Non-homologous end-joining repair (NHEJ) of DSBs, the primary repair pathway triggered by ionizing radiation, it is possible that CK2 inhibition may synergize with radiotherapy in addition to the large number of DNA-targeted anti-cancer drugs that trigger SSBR, HR and NHEJ (Fig. S3).

Along with the DRR, CK2 positively regulates an extensive list of additional cellular processes that are also established effectors of sensitivity to DNA targeted chemotherapeutics as well as other anti-cancer drugs, including PI3K/AKT/mTOR signaling, NF-κB transcription, Hsp90 machinery activity, hypoxia, inhibition of apoptosis and IL-6 expression (3, 8-11). Indeed, sensitization of pancreatic cancer cells to gemcitabine and head and neck cancer cells to cisplatin by genetic manipulation of CK2 expression has been attributed to resulting suppression of Akt and NF-κB activity respectively (47, 48). Thus it is possible that in addition to the suppression of DRR, CX-4945 enhances activity of gemcitabine and cisplatin by modulating PI3K/Akt and NF-κB signaling. Our data combined with previous reports demonstrating enhancement of therapeutic activity by suppression of CK2 (e.g. (49)) demonstrates that CK2 represents a promising pharmacologic target that can be explored in combination therapy with multiple anti-cancer therapeutics.
Here we have described how targeting CK2 with CX-4945 can inhibit multiple DRR mechanisms by blocking phosphorylation of XRCC1 and MDC1 and synergize with the DNA-targeted anti-cancer drugs cisplatin and gemcitabine in ovarian cancer cells. These data provide a strong rationale for combining CX-4945 with gemcitabine and platinum-based chemotherapeutics in clinical trials for ovarian and possibly other cancers.
REFERENCES


Figure 1. Reported roles of CK2 in DNA repair mechanisms relevant to cisplatin and gemcitabine.

Figure 2. Structures of A, CX-4945. B, Gemcitabine. C, Cisplatin. D, Carboplatin.

Figure 3. Synergistic antiproliferative effects of combining CX-4945 with either cisplatin or gemcitabine in A2780 and SKOV-3 cells. The additivity line is equivalent to the theoretical combined antiproliferative effects of 2 drugs A and B (%A + %B*(100-%A)/100), where %A and %B are the percentage of cells killed by drug A and B respectively at given concentrations. In A2780 cells, 3 μM CX-4945, 300 nM cisplatin (Cis) and 3 nM gemcitabine (Gem) were used. In SKOV-3 cells, 10 μM CX-4945, 1 μM cisplatin (Cis) and 10 nM gemcitabine (Gem) were used. A, CX-4945 pre-addition schedule. B, CX-4945 post-addition schedule. C, Inhibition of replication recovery for A2780 cells treated with 10 nM gemcitabine and 10 μM CX-4945.

Figure 4. Western blot analysis of XRCC1 and MDC1 from CX-4945 combined with A, cisplatin or B, gemcitabine in A2780 or SKOV-3 cells (cells were treated with gemcitabine (10 nM) or cisplatin (3 μM) for 24 h followed by CX-4945 (10 μM) and the cells lysed 8 h after the addition of CX-4945; quantified phospho-XRCC1 levels (normalized to total XRCC1 levels). C, levels of Phospho-MDC1 in CX-4945 (10 μM, 24 h) treated A2780 and SKOV-3 cells by immunoprecipitation.
Figure 5. **A**, Comet assay demonstrating increased DNA strand breaks resulting from the combination of CX-4945 (10 μM) with gemcitabine (100 nM) or cisplatin (10 μM), with and without ZVAD (100 μM). Western blot analysis of γ-H2AX and CHK1/2 from CX-4945 combined with **B**, cisplatin or **C**, gemcitabine in A2780 or SKOV-3 cells (cells were treated with gemcitabine (10 nM) or cisplatin (3 μM) for 24 h followed by CX-4945 (10 μM) and the cells lysed 8 h after the addition of CX-4945; quantified phospho-CHK1/2 levels normalized to total CHK levels; quantified γ-H2AX levels are shown in Fig S4).

Figure 6. **A**, Increased Caspase 3/7 activation in ovarian cancer cells treated with indicated concentrations of CX-4945 and cisplatin or gemcitabine. **B**, Cleaved PARP levels measured in ovarian cancer cells treated with CX-4945 (10 μM) and cisplatin (3 μM) or gemcitabine (10 nM) (quantified cleaved PARP levels are shown in Fig S4). **C**, DAPI staining of SKOV-3 cells treated with cisplatin (10 μM), gemcitabine (100 nM) and/or CX-4945 (10 μM).

Figure 7. A2780 ovarian cancer xenograft efficacy studies. Mean tumor volumes, median times to end-point and changes in body weights are presented. **A**, CX-4945 was dosed 75 mg/kg PO BID for 21 days, Cisplatin was dosed 5 mg/kg IP Q7D for 21 days. **B**, CX-4945 was dosed 75 mg/kg PO BID for 21 days; carboplatin was dosed 75 mg/kg
IP Q7D for 21 days. C, CX-4945 was dosed 100 mg/kg PO BID for 21 days; gemcitabine was dosed 60 mg/kg IP Q3D for 10 days (days 1, 4, 7, 10). D, CX-4945 was dosed 100 mg/kg PO BID (days 2, 5, 8, 11); gemcitabine was dosed 30 mg/kg IP Q3D (days 1, 4, 7, 10). Combination treatments used combined single agent schedules. E, Analysis of cleaved PARP levels in A2780 tumor cells subjected to CX-4945 and gemcitabine alone or in combination.
Figure 2

A. 

B. 

C. 

D.
Figure 3
**Figure 5**

(A) Images showing cell viability assay results under different treatments: UTC, Gemcitabine, CX-4945, Gem + CX-4945, Gem + CX-4945 + ZVAD, UTC, Cisplatin, CX-4946, Cis + CX-4945, Cis + CX-4946 + ZVAD.

(B) Western blots and normalized intensity graphs for A2780 and SKOV-3 cells treated with CX-4945 and Cisplatin, showing γH2AX, CHK1, P-CHK1 (S345), CHK2, P-CHK2 (T68).

(C) Similar analysis for A2780 and SKOV-3 cells treated with CX-4945 and Gemcitabine.
Figure 6
Figure 7
Molecular Cancer Therapeutics

CK2 Inhibitor CX-4945 Suppresses DNA Repair Response Triggered by DNA Targeted Anticancer Drugs and Augments Efficacy: Mechanistic Rationale for Drug Combination Therapy

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