CHIR-124, a Novel Potent Inhibitor of Chk1, Potentiates the Cytotoxicity of Topoisomerase I Poisons In vitro and In vivo


Abstract

Purpose: Chk1 kinase is a critical regulator of both S and G₂-M phase cell cycle checkpoints in response to DNA damage. This study aimed to evaluate the biochemical, cellular, and antitumor effects of a novel Chk1 inhibitor, CHIR124.

Experimental Design: CHIR-124 was evaluated for its ability to abrogate cell cycle checkpoints, to potentiate cytotoxicity, and to inhibit Chk1-mediated signaling induced by topoisomerase I poisons in human tumor cell line and xenograft models.

Results: CHIR-124 is a quinolone-based small molecule that is structurally unrelated to other known inhibitors of Chk1. It potently and selectively inhibits Chk1 in vitro (IC₅₀ = 0.0003 μmol/L). CHIR-124 interacts synergistically with topoisomerase poisons (e.g., camptothecin or SN-38) in causing growth inhibition in several p53-mutant solid tumor cell lines as determined by isobologram or response surface analysis. CHIR-124 abrogates the SN-38-induced S and G₂-M checkpoints and potentiates apoptosis in MDA-MD-435 breast cancer cells. The abrogation of the G₂-M checkpoint and induction of apoptosis by CHIR-124 are enhanced by the loss of p53. We have also shown that CHIR-124 treatment can restore the level of cdc25A protein, which is normally targeted by Chk1 for degradation following DNA damage, indicating that Chk1 signaling is suppressed in the presence of CHIR-124. Finally, in an orthotopic breast cancer xenograft model, CHIR-124 potentiates the growth inhibitory effects of irinotecan by abrogating the G₂-M checkpoint and increasing tumor apoptosis.

Conclusions: CHIR-124 is a novel and potent Chk1 inhibitor with promising antitumor activities when used in combination with topoisomerase I poisons.

To ensure high-fidelity transmission of genetic material, dividing cells are equipped with surveillance mechanisms known as cell cycle checkpoints, which function to delay cell cycle progression when DNA damage is present. Dysregulation of checkpoint function results in genomic instability and represents a pathologic hallmark of neoplasia.

Chk1 is a serine/threonine kinase that is required for both S and G₂-M phase checkpoints (for review, see refs. 1, 2). In response to a variety of genotoxic stressors, including replicative block from UV light and hydroxyurea and DNA strand breakage from ionizing radiation and topoisomerase poisons, Chk1 is activated by phosphorylation at Ser172 and Ser345 by upstream kinases (3). Current evidence indicates that ATR is the primary kinase responsible for Chk1 activation, although ATM has also recently been implicated as the activating kinase in the case of ionizing radiation–induced DNA damage (4). Chk1 regulates checkpoints by targeting the cdc25 family of dual-specificity phosphatases, cdc25A at the S phase checkpoint, and both cdc25A and cdc25C at the G₂-M checkpoint, respectively. Phosphorylation of cdc25A by Chk1 at multiple sites results in increased proteosomal degradation of the phosphatase and inability of cdc25A to interact with its cyclin/cyclin-dependent kinase substrates (5–7). Chk1 phosphorylates cdc25C at Ser216, leading to complex formation with 14-3-3 proteins and cytoplasmic sequestration of the phosphatase (8). Earlier cell cycle studies have identified a role for cdc25A in promoting G₁-S transition, whereas cdc25C and cdc25B (another member of cdc25 phosphatase family) are critical for mitotic entry (8). However, recent data have indicated that cdc25A also possesses promitotic function (9). Surprisingly, mice lacking both cdc25C and cdc25B are viable and had no demonstrable cell cycle abnormalities, strongly suggesting that cdc25A alone may be sufficient to promote mitotic entry (10).
Mounting evidence has indicated that abrogation of the Chk1-mediated cell cycle checkpoints results in increased chemosensitivity and radiosensitivity of tumor cells. Because of the intrinsic checkpoint defect(s) associated with malignant transformation, pharmacologic disruption of additional checkpoints may render tumor cells more susceptible to DNA damage by cytotoxic chemotherapy or ionizing radiation (4, 11, 12). Theoretically, this can be achieved by targeting a different checkpoint of the cell cycle (e.g., inhibiting the G2-M checkpoint in tumors that lack a normal G1 checkpoint) or by blocking complementary pathways of the same checkpoint (ref. 13; this report). p53, the tumor suppressor found to be mutated in >50% of human cancers (14), is a critical regulator of both the G1 and G2 checkpoints (15). We and others have shown that inhibition of the Chk1-dependent G2-M checkpoint using the staurosponine analogue UCN-01 (7-hydroxystaurosponine) selectively sensitizes tumors lacking p53 to the cytotoxic effect of chemotherapy (13, 16). Time-lapse microscopy studies using GFP-H2B-expressing cells revealed that tumor cells that had escaped the G2-M checkpoint entered an aberrant mitosis (mitotic catastrophe) resulting in either apoptosis or fragmentation of chromatins into micronuclei (13).

The clinical development of UCN-01 has been hampered by its unfavorable pharmacokinetic properties and toxicities, such as hyperglycemia, hypotension, and pulmonary dysfunction, most of which are likely due to effects of the drug that are unrelated to Chk1 inhibition (17). Thus, identification of alternative small-molecule Chk1 inhibitors represents a high priority in this area of drug development. We now present preclinical data on a novel, potent, and specific inhibitor of Chk1 [CHIR-124, (E)-3-(1H-benzo[d][imidazol-2-yl]-6-chloro-4-[(quinolin-3-ylamino)quinolin-2(1H)-one]]. We show that CHIR-124 abrogates the S and G2-M checkpoints induced by topoisomerase I poisons and selectively sensitizes tumors lacking p53 function to undergo mitotic death. Furthermore, CHIR-124 enhances the antitumor effect of irinotecan in tumor xenografts by inhibiting the G2-M checkpoint and inducing apoptosis.

Materials and Methods

Reagents. CHIR-124 was synthesized at Chiron Corp. (18). Irinotecan (CPT-11; Camptosar) was obtained from Pharmacia Upjohn, Inc. (Kalamazoo, MI), and doxorubicin (Adriamycin) and cisplatin (cisplatin injection) were purchased from American Pharmaceutical Partners, Inc. (Schaumburg, IL). Camptothecin was purchased from Sigma (St. Louis, MO). SN-38 was a generous gift from Dr. J. Patrick McGovern (formerly at Pharma ia and Upjohn, Inc.) and from Liangsu Hengui Medicine Co. (Lianyungang, China). UCN-01 was kindly provided by Dr. Robert Schultz (National Cancer Institute, Bethesda, MD). All drugs were dissolved in dimethyl sulfoxide and stored in aliquots at −20°C.

Cell culture. MDA-MB-435 cells were obtained from Dr. Joshua Fidler of the M.D. Anderson Cancer Center and from the American Type Culture Collection (Manassas, VA). Cultures were maintained in 50% DMEM-high glucose, 50% F-12 medium or 100% DMEM, containing 4-(quinuclidin-3-ylamino)quinolin-2(1H)-one. The parental HCT116 colon carcinoma cell line and its p53-null derivative (kindly provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD) were grown in RPMI 1640 supplemented with 10% fetal bovine serum. The MDA-MB-231, SW620, and COLO 205 cell lines used for isobologram studies were obtained from the American Type Culture Collection and cultured as recommended.

Kinase selectivity assays. For the CHK1 assay, the kinase domain was expressed in Sf9 insect cells, and a biotinylated cdc25c peptide containing the consensus Chk1/Chk2 phosphorylation site ([biotin-H][SGSG*GLYRSPSMP-ENLNRPR(CONH2)]) was used as the substrate. A dilution series of CHIR-124 was mixed with a kinase reaction buffer containing a final concentration of 30 mmol/L Tris-HCl (pH 7.5), 10 mmol/L MgCl2, 2 mmol/L EDTA, 5 mmol/L MnCl2, 0.01% bovine serum albumin, 3.35 mmol/L [32P]γ-label [specific activity = 2,000 Ci/mmol]. Reactions and detection of the phosphate transfer were carried out by a radioactive method, as previously described (19).

For all other kinase assays, the enzymes were expressed in insect cells or purchased from Upstate Biotechnology (Charlottesville, VA) or New England Biolabs (Ipswich, MA). Reactions were carried out generally as previously described (19). See Supplemental Material 1 for details.

Drug interaction by isobologram analysis. MDA-MB-231, MDA-MB-435, SW-620, and COLO 205 cells in log-phase were plated into 96-well microplates. CHIR-124 was serially diluted in the presence of six different concentrations of camptothecin or 0 mmol/L camptothecin. Camptothecin was also serially diluted in the absence of CHIR-124. The compounds were added to cells in 96-well dishes and incubated at 37°C for 48 h. Each treatment condition was done in triplicate. Cell proliferation was monitored by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), inner salt (Promega Corp., Madison, WI) assay. MTS inner salt was added to the microplates, which were incubated for another 3 h, and absorbance at 490 nm was read on a plate reader. The concentrations of each drug in the combinations required to produce 50% inhibition were plotted to generate the isoboles. Isobologram analysis of drug interaction is based on the equation of Loewe additivity (1=1/C0,A + 1/C0,B, where IC50,A and IC50,B are the concentrations of drugs to result in 50% inhibition for each drug alone, and Dλ and Dβ are concentrations of each drug in the combination that yield 50% overall inhibition (20). A diagonal line indicating Loewe additivity is included in each graph. Data points that fall below the line indicate synergy, whereas those that fall above the line (data not shown) would indicate antagonism.

Drug interaction by response surface analysis. Response surface analysis determines additivity or degree of interaction between two drugs via exposure of cells to a combinatorial matrix of concentration (reviewed in ref. 21). MDA-MB-435 cells were plated into black 384-well plates (Greiner Bio-One, Inc., Longwood, FL). Cells were exposed to a matrix of drug combinations comprised of 10 CHIR-124 concentrations and 8 SN-38 concentrations, for a total of 80 combinations. A dilution series of each drug with vehicle was also run. The CHIR-124 and SN-38 concentrations consisted of a series of 1.4-fold stepwise dilutions from 3.3 × 10−6 to 1.6 × 10−8 mol/L and 4-fold stepwise dilutions from 2.7 × 10−6 to 1.6 × 10−10 mol/L, respectively. Each drug was also run in combination with itself as a control.

Compounds were added either 24 or 48 h after plating, for a 72- or 48-h drug exposure, respectively. CHIR-124 was given as a single 48-h treatment, whereas SN-38 was applied every 24 h. Daily reapplication of SN-38 was required to generate a meaningful dose-response curve (data not shown) due to the short half-life of the drug in serum-containing media (22). This did not significantly change the concentration of CHIR-124. Drug combinations were tested following either simultaneous or sequential addition. In the case of simultaneous addition, cells were treated with both SN-38 and CHIR-124 for 48 h (including a second application of SN-38 24 h later). In the case of sequential addition, a SN-38 application was given 24 h before simultaneous addition of both drugs (i.e., a 72-h exposure of SN-38 (three total applications) plus a 48-h exposure of CHIR-124). Viable cell

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number was determined 96 h after plating using the Cell Titer-Glo assay kit (Promega) and luminescence intensity read with a Victor luminometer (Perkin-Elmer, Wellesley, MA). Cell plating, drug addition, PBS washes, and the addition of Cell Titer-Glo were all done with an FX robot (Beckman-Coulter, Durate, CA). The data were analyzed by plotting the deviation from additivity: the difference between the observed inhibition effect and the effect predicted by the Loewe additivity model (21).

\[ A = E_{\text{obs}} - E_{\text{add}} \]

where \( A \) is the deviation from additivity, \( E_{\text{obs}} \) is the observed cell number normalized to the untreated controls, and \( E_{\text{add}} \) is the additive effect predicted by the Loewe additivity model. The variables for the additive model, the \( IC_{50} \) values and Hill slopes for the individual drugs, were determined using custom software developed by E. Moler (Chiron). A key feature of the Loewe additivity model is that by definition, a drug is additive in combination with itself, providing a well-defined control for additivity. Drug interaction is considered synergistic or antagonistic when the response surface deviation from additivity is greater than +10% or −10%, respectively. The results are illustrated graphically in three dimensions as plots of data points within a color-coded surface. Additive data points are in green (ranging from blue-green at +10% deviation to green at the zero point and chartreuse at −10%); synergy is represented from blue to violet; and antagonism is represented as orange or red. CHIR-124 and SN-38 drug concentrations are on the x- and y-axes, respectively, and the deviation from additivity is along the z-axis.

Assessment for apoptosis and micronucleation in vitro. Log-phase cells were plated onto 60-mm dishes in duplicate and allowed to adhere for 36 to 48 h. Following drug treatment, both drug adherent and nonadherent cells were harvested, fixed in 3% paraformaldehyde, and stained with 4,6-diamidino-2-phenylindole (Sigma). In experiments involving sequential therapy, floating cells were collected after incubation with the first drug and were added back to the plate for subsequent treatment. The incidence of apoptosis and micronucleation was determined by examining the nuclear morphology of cells under fluorescence and scored for those with condensed fragmented chromatin and multiple (2–3) interphase nuclei, respectively. At least 400 cells were counted for each sample.

Cell cycle analysis. Biparameter flow cytometry was done as described previously (13). Samples were analyzed on a FACSscan (Becton Dickinson, Franklin Lakes, NJ) for DNA content and mitotic index following labeling with the mitosis-specific antibody MPM-2 (Becton Dickinson, Franklin Lakes, NJ) for DNA content and mitotic index following labeling with the mitosis-specific antibody MPM-2.

Immunoblot analysis. Twenty-five to 50 μg of lysate protein were fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride Immobilon membranes (Millipore, Billerica, MA). Blots were blocked with 5% nonfat milk and probed with antibodies against Chk1, cdc25A (both from Santa Cruz Biotechnology, CA), phospho-Chk1 (Ser317), phospho-cdc25C (Ser216, both from Cell Signaling, Beverly, MA), cdc25A, and α-tubulin (both from Sigma). Bound primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies (ICN/Jackson ImmunoResearch, West Grove, PA) and visualized with enhanced chemiluminescence reagent (Amersham Pharmacia, Piscataway, NJ). The levels of expression were quantified using Kodak 1D Image Analysis Software (Rochester, NY).

In vivo antitumor activity studies. Severe combined immunodeficient mice harboring MDA-MD-435 tumor xenografts were randomized into the following treatment groups of 10: vehicle (captisol) alone, 5 mg/kg CPT-11, 10 mg/kg CHIR-124, 20 mg/kg CHIR-124, 5 mg/kg CPT-11 plus 10 mg/kg CHIR-124, or 5 mg/kg CPT-11 plus 20 mg/kg CHIR-124. Treatment was initiated on the day after randomization (day 1). CPT-11 was given i.p. daily (four times daily) × 5 on days 1 to 5, whereas CHIR-124 was given orally four times daily × 6 on days 2 to 7 in captisol. Percent tumor growth inhibition was defined as % %T/C, where \( T \) is the treatment group mean, and \( C \) is the control group mean. In a similar study, tumors harvested from mice sacrificed on day 4 of treatment were examined for apoptosis by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining and for mitotic index by immunofluorescence labeling with phospho-histone H3 antibody. See Supplementary Material 2 for details.

### Results

**CHIR-124 is a potent and selective inhibitor of Chk1 kinase.** CHIR-124 is a lead member of a novel series of quinolone derivatives identified upon screening a diverse chemical library for Chk1 inhibitors (Fig. 1A; ref. 18). CHIR-124 is structurally distinct from the prototypical Chk1 inhibitor

<p>| Table 1. Kinase selectivity profile of CHIR-124 |
|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle kinases</td>
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<tr>
<td>CHK1 KD</td>
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</tr>
<tr>
<td>CHK2</td>
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<tr>
<td>Cdk4/cyclin D</td>
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<tr>
<td>CDC2/cyclin B</td>
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<tr>
<td>Cdk2/cyclin A</td>
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</tr>
<tr>
<td>Receptor tyrosine kinases</td>
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<tr>
<td>c-MET</td>
<td>≥3</td>
</tr>
<tr>
<td>bFGFR</td>
<td>2.01</td>
</tr>
<tr>
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</tr>
<tr>
<td>VEGFR1 FLT1</td>
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</tr>
<tr>
<td>PDGFR</td>
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<tr>
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</tr>
<tr>
<td>Serine/threonine kinases</td>
<td></td>
</tr>
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<tr>
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<tr>
<td>PKCβ II</td>
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<tr>
<td>PKCγ</td>
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</tr>
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<tr>
<td>PKA</td>
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<tr>
<td>GSK3</td>
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<tr>
<td>Non-receptor tyrosine kinases</td>
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<td>LCK</td>
<td>0.1583</td>
</tr>
<tr>
<td>FYN</td>
<td>0.0988</td>
</tr>
</tbody>
</table>

Abbreviations: KD, kinase domain; Cdk2, cyclin-dependent kinase 2; bFGFR, basic fibroblast growth factor receptor; PKC, protein kinase C; VEGFR, vascular endothelial growth factor receptor; ERK, extracellular signal-regulated kinase.
UCN-01 (Fig. 1B). In an in vitro kinase assay, CHIR-124 showed potent inhibitory activity against the kinase domain of recombinant Chk1 with an IC_{50} of 0.0003 μmol/L, a value that was 2,000-fold lower than that against Chk2 (0.70 μmol/L; Table 1). Co-crystal structure of the kinase domain of Chk1 in complex with CHIR-124 shows occupation of the ATP-binding site of the enzyme by the inhibitor (18). Assuming a competitive mode of inhibition and using a K_{m} of 105 μmol/L of the enzyme for ATP, the K_{i} of CHIR-124 was 0.0003 μmol/L. In addition, CHIR-124 is 500- to 5,000-fold less active against other cell cycle kinases, such as cyclin-dependent kinase 2/ cyclin A (0.19 μmol/L), cdc2/cyclin B (0.51 μmol/L), and cyclin-dependent kinase 4/cyclin D (2.1 μmol/L; Table 1). When tested against a panel of representative kinases, CHIR-124 showed good selectivity against members of the receptor tyrosine kinases, non–receptor tyrosine kinases, and serine/threonine kinases, with the exception of FLT3 and platelet-derived growth factor receptor, which were inhibited at an threonine kinases, with the exception of FLT3 and platelet-tyrosine kinases, non–receptor tyrosine kinases, and serine/threonine kinases, with the exception of FLT3 and platelet-derived growth factor receptor, which were inhibited at an.

**Growth inhibition by topoisomerase I poisons in combination with CHIR-124 is synergistic in a variety of cancer cell lines.** Treatment of cells with type I topoisomerase poisons, such as camptothecin and its derivatives, generate double-stranded DNA breaks in a replication-dependent manner (24, 25), resulting in activation of both the S and G_{2}-M checkpoints where Chk1 plays a critical role. We therefore studied the in vitro effect of a matrix of camptothecin and CHIR-124 combinations in a number of human cancer cell lines, including breast carcinoma (MDA-MB-231 and MDA-MB-435) and colon carcinoma (SW-620 and Colo205), all of which are mutant for p53. The antiproliferative effect of treatment was evaluated using an MTS inner salt assay, and drug interaction was assessed by the isobologram method. A synergistic interaction between camptothecin and CHIR-124 was shown in all four cell lines (Fig. 2A). The IC_{50} of the combined drugs represent lower concentrations of each drug than would be predicted by Loewe additivity equation. In addition, we observed synergy between CHIR-124 and cisplatin in MDA-MB-435 cells and with doxorubicin in MDA-MD-231 cells (data not shown).

To further evaluate the combined effect of a topoisomerase I poison and CHIR-124 on MDA-MB-435 cells, we did response surface (21) analysis with CHIR-124 and SN-38, an active metabolite of the clinically important drug irinotecan (Fig. 2B). This methodology allows a more rigorous evaluation of drug interactions than the isobologram technique. When cells were simultaneously exposed to a matrix of 80 different concentration combinations of CHIR-124 and SN-38 for 48 h, significant synergy or >10% deviation from additivity was observed (Fig. 2B, e) in the concentration ranges of ≥4.2 × 10^{-8} mol/L for SN-38 and ≥6.0 × 10^{-8} mol/L for CHIR-124. These values overlap and fall below the IC_{50}s for SN-38 (1.2 × 10^{-7} mol/L) and CHIR-124 (2.2 × 10^{-7} mol/L), respectively. Sequential addition of SN-38 followed 24 h later by CHIR-124 (72- and 48-h exposure, respectively), resulted in >35% deviation from additivity (Fig. 2B, f). This occurred in the concentration range of ≥4.2 × 10^{-8} for SN-38, close to the IC_{50} for this drug (8.5 × 10^{-8} mol/L), and over the entire dilution series of CHIR-124. Thus, synergy was most pronounced at higher concentrations of SN-38 and over a wide range of concentrations of CHIR-124 (Fig. 2B, f). Importantly, control plates of SN-38 plus SN-38 at 48 or 72 h (Fig. 2B, g and h) or CHIR-124 plus CHIR-124 at 48 h (Fig. 2B, g and h) show a flat surface of data points within 10% deviation from additivity, as expected.

**CHIR-124 abrogates the SN-38–induced S and G_{2}-M phase cell cycle checkpoints.** Having established that there is synergy between CHIR-124 and topoisomerase I poisons in inhibiting the proliferation of human cancer cells, we set out to determine the mechanistic basis for this interaction. A 24-h treatment of MDA-MB-435 cells with 100 nmol/L CHIR-124 or 100 nmol/L UCN-01 resulted in no appreciable changes in the cell cycle distribution of these cells (Fig. 3A). Treatment with 20 nmol/L SN-38 for 24 h caused an S phase arrest as evidenced by the accumulation of cells with >2N and ≤4N DNA content as well as the loss of MPM-2 positive mitotic cells (Fig. 3A). However, when cells were exposed concurrently to SN-38 and CHIR-124, this S-phase delay was abolished as the majority of cells had completed replication and appeared as cells with 4N DNA content at 24 h (Fig. 3A). As expected, UCN-01, the known Chk1 inhibitor, also abrogated the S-phase checkpoint induced by SN-38 (Fig. 3A). Following removal of SN-38 and incubation in drug-free medium (ND) over time (SN → ND_{24 h}), these cells progressed through the S phase but eventually arrested in G_{2} without mitotic entry (Fig. 3B). However, when MDA-MB-435 cells were treated sequentially...
with SN-38 for 24 h followed by graded concentrations of CHIR-124 (20, 50, and 100 nmol/L), a dose-dependent increase in mitotic index was observed, which was most apparent at the 8 h time point, indicating an abolishment of the SN-38–induced G2-M checkpoint by CHIR-124 (Fig. 3B). The extent of G2-M checkpoint abrogation induced by 100 nmol/L CHIR-124 was comparable with that seen with an equimolar concentration of UCN-01 (Fig. 3B). All mitotic (MPM-2 positive) cells had a 4N DNA content, indicating that cells that had escaped the G2-M checkpoint and entered mitosis did so only after completion of DNA replication (data not shown).

Enhancement of apoptosis by sequential treatment with SN-38 followed by CHIR-124. We next examined whether G2-M checkpoint release observed in MDA-MB-435 cells treated with SN-38 and CHIR-124 resulted in apoptotic cell death. Single-agent treatment with 20 nmol/L SN-38 or 100 nmol/L CHIR-124 for 24 h was ineffective in causing apoptosis in these cells (Fig. 3C). Sequential treatment with SN-38 followed by CHIR-124 caused a marked increase in apoptotic cells (up to 32 ± 2% for SN → CHIR24 h; Fig. 3C) in a time-dependent manner. The extent of apoptosis observed was comparable with that induced by SN-38 followed by 100 nmol/L UCN-01 (Fig. 3C). Sequential treatment with either CHIR-124 or UCN-01 also resulted in a mild increase in interphase cells with micronuclei, consistent with mitotic catastrophe previously described in HCT116 colon cancer cells treated sequentially with SN-38 and UCN-01 (Fig. 3C; ref. 13).

Selective abrogation of the DNA damage–induced G2-M checkpoint and induction of cell death by CHIR-124 in p53-null HCT116 cells. We have previously shown that HCT116 colon cancer cells that lack p53 are more prone to undergoing G2-M checkpoint abrogation and apoptosis by UCN-01 than parental cells containing wild-type p53 (13). We therefore compared the integrity of the G2-M checkpoint between parental HCT116 and its isogenic derivative that lacked p53 following combined treatment with SN-38 and CHIR-124. Our data indicated that p53-null cells were selectively more susceptible to undergoing abrogation of the G2-M checkpoint after sequential treatment with SN-38 followed by CHIR-124 when compared with parental cells (51% versus 15 % mitosis at 8 h following the addition of CHIR-124; Supplementary Material 3A). Furthermore, under our experimental conditions, single-agent SN-38, CHIR-124, or SN-38 followed by drug-free medium resulted in minimal apoptosis (<5%) in both cell lines (Supplementary Material 3B). However, sequential treatment with SN-38 followed by CHIR-124 resulted in enhanced induction of apoptosis and micronucleation in p53-null cells (23% and

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Fig. 2 Continued. E, simultaneous addition of SN-38 and CHIR-124 for 48 h resulted in significant synergy, as determined by a >10 % deviation from additivity. The degree of drug interaction is color coded (see Materials and Methods). F, sequential addition of SN-38 for 24 h followed by a further 48-h exposure to SN-38 plus CHIR-124 resulted in synergy as indicated by the >30% deviation from additivity at higher concentrations of SN-38 and over a wide range of CHIR-124 concentrations. G, H, and I, additivity controls for SN-38 plus SN-38 for 48 and 72 h, respectively, and for CHIR-124 plus CHIR-124 for 48 h.

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Phosphorylation of cdc25A by Chk1 on several NH2-phospho-Chk1 and cdc25A in HCT116 of the phosphatase (5, 6). We therefore examined the levels of terminal sites resulted in accelerated proteosomal degradation cytotoxic effect of combined SN-38 and CHIR-124.

loss of p53 also rendered these cells more susceptible to the CHIR-124 in p53-null cells (Supplementary Material 3A), the Chk1 is activated by phosphorylation at Ser317 and Ser345 by response to DNA damage induced by topoisomerase I poison, ATR (3). An important downstream target of activated Chk1 is the G2-M checkpoint by CHIR-124. A, asynchronous MDA-MD-435 cells were treated for 24 h with single agent 20 nmol/L SN-38, 100 nmol/L CHIR-124, or 100 nmol/L UCN-01, concurrently with SN-38 and CHIR-124, or concurrently with SN-38 and UCN-01. Cell cycle distribution was analyzed by biperarameter flow cytometry for DNA content and mitotic index (M) as described in Materials and Methods.

Fig. 3. Abrogation of cell cycle checkpoints and induction of apoptosis by CHIR-124 in MDA-MD-435 cells. A, asynchronous MDA-MD-435 cells were treated for 24 h with single agent 20 nmol/L SN-38, 100 nmol/L CHIR-124, or 100 nmol/L UCN-01, concurrently with SN-38 and CHIR-124, or concurrently with SN-38 and UCN-01. Cell cycle distribution was analyzed by biperarameter flow cytometry for DNA content and mitotic index (M) as described in Materials and Methods.

20%, respectively) compared with parental cells (14% and 3%, respectively; Supplementary Material 3B). Thus, consistent with the results of selective abrogation of the G2-M checkpoint by CHIR-124 in p53-null cells (Supplementary Material 3A), the loss of p53 also rendered these cells more susceptible to the cytotoxic effect of combined SN-38 and CHIR-124.

Suppression of the Chk1 signaling pathway by CHIR-124. In response to DNA damage induced by topoisomerase I poison, Chk1 is activated by phosphorylation at Ser317 and Ser345 by ATR (3). An important downstream target of activated Chk1 is the dual-specificity phosphatase cdc25A, which promotes cell cycle progression through both the G1-S and G2-M transitions (26). Phosphorylation of cdc25A by Chk1 on several NH2-terminal sites resulted in accelerated proteosomal degradation of the phosphatase (5, 6). We therefore examined the levels of phospho-Chk1 and cdc25A in HCT116 cells treated with the SN-38 and CHIR-124 combination. As expected, SN-38 treatment resulted in a 2.5-fold increase in activating phosphorylation of Chk1 at Ser317 (ref. 3; Fig. 4). Total Chk1 protein levels remained fairly constant under these conditions. Treatment with SN-38 alone for 24 h or followed by a drug washout for serial time points resulted in a marked decrease of cdc25A to 20% of untreated control level (Fig. 4). Incubation with 200 nmol/L CHIR-124 led to a 2.5-fold elevated level of cdc25A above that of the untreated control (Fig. 4), a finding that is in accord with the concept that some basal level of Chk1 activity is present during an unperturbed cell cycle to promote cdc25A turnover (4, 6). The down-regulation of cdc25A induced by SN-38 was completely restored by concurrent or sequential treatment with CHIR-124, providing biochemical evidence that CHIR-124 inhibited the Chk1-mediated destruction of cdc25A in whole cells (Fig. 4). Although it is frequently stated that Chk1 phosphorylates Ser216 of cdc25C following DNA damage, this site is constitutively phosphorylated even in untreated cells (Fig. 4). Treatment with SN-38 only increased Ser216 phosphorylation further by 30% when its expression was normalized to total cdc25C (Fig. 4). The specificity of the phospho-antibody has been confirmed using HCT116 cells that overexpress GFP-fused wild-type cdc25C or cdc25C mutated at Ser216 (Ser → Ala; data not shown). When compared with SN-38 followed by drug-free medium at 3 h (SN24 h → ND3 h), we did not observe any appreciable decrease in Ser216 phosphorylation following the addition of CHIR-124 (SN24 h → CHIR3 h). Similarly, there was only a modest 30% reduction of phosphorylation at a later time point (SN24 h → CHIR6 h) when cells had already entered mitosis as evidenced by an increase in phospho-histone H3, raising the possibility that the loss of Ser216 phosphorylation may represent the consequence rather than the cause of mitotic entry. Conversely, the effect of CHIR-124 on cdc25A levels was apparent as early as 3 h (SN24 h → CHIR3 h), providing a temporal linkage between restoration of cdc25A level and abrogation of the G2-M checkpoint (Fig. 4). Whereas total cdc25C levels did not change with treatment, the protein became hyperphosphorylated during mitosis, resulting in an upward mobility shift on the gel, consistent with previous report on cdc25C hyperphosphorylation during mitosis (Fig. 4, arrow; refs. 8, 27).

CHIR-124 in combination with CPT-11 shows potentiation of tumor growth inhibition and increased apoptosis in vivo. To determine whether CHIR-124 enhances the effect of topoisomerase 1 poisons in vivo, we evaluated the Chk1 inhibitor in combination with CPT-11 in an orthotopic breast carcinoma model. Severe combined immunodeficient mice implanted in the mammary fat pad with MDA-MB-435 cells were randomized into the following treatment groups: vehicle alone, daily dosing with the single-agent CPT-11 at 5 mg/kg on days 1 to 5, daily dosing of 10 or 20 mg/kg CHIR-124 alone on days 2 to 7, or 5 mg/kg CPT-11 in combination with either dose of CHIR-124. Thus, in the two combination groups, CPT-11 was given alone on day 1, and CHIR-124 was given 4 h following CPT-11 on subsequent dosing days. CHIR-124 at either 10 or 20 mg/kg did not have a significant effect on tumor growth when compared with the vehicle-treated group at the end of the study (day 36; Fig. 5A). However, when animals were treated sequentially with CPT-11 followed by CHIR-124, there was potentiation of tumor growth inhibition relative to CPT-11 treatment alone. At the end of the study, there was a significant difference between mean tumor volumes of the CHIR-124 alone groups and the CPT-11 plus CHIR-124 dose-matched combination groups (P < 0.05; Fig. 5A). The % tumor growth inhibition on day 36 in the groups dosed with 5 mg/kg CPT-11 and the combination groups dosed with 5 mg/kg CPT-11 plus 10 mg/kg CHIR-124 or 5 mg/kg CPT-11 plus 20 mg/kg
CHIR-124 was 14%, 34%, and 53%, respectively. Finally, animals in all groups were healthy and did not manifest any signs of toxicity or significant body weight loss (defined as >5% of body weight).

Apoptosis induction in MDA-MB-435 xenograft tumors from mice treated with vehicle alone, 5 mg/kg CPT-11, 20 mg/kg CHIR-124 alone, or the combination of 5 mg/kg CPT-11 plus 20 mg/kg CHIR-124 was assessed by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining on day 4. Occasional terminal deoxynucleotidyl transferase–mediated nick-end labeling–positive cells were observed in the tumors from the vehicle and CHIR-124–treated animals (1.8 ± 0.4%...
and 2.0 ± 0.6%, respectively, group; Fig. 5B and D). The percentage of labeled cells was similar in tumors obtained from CPT-11–treated animals (2.3 ± 1.0%; Fig. 5B and D) but was markedly increased in tumors derived from animals treated with the drug combination (12.4 ± 1.9%; Fig. 5B and D). The percentage of positive staining in these tumors was significantly different from that in tumors from animals treated with either single agent (P < 0.01; Fig. 5D). These results indicate that the potentiation of the tumor growth inhibitory effect of CPT-11 by CHIR-124 is associated with an increase in apoptosis induction in the tumors.

To assess the pharmacodynamic effect of CHIR-124 in vivo, we also examined the mitotic index in tumor sections obtained from animals treated with either agent or the combination of CPT-11 and CHIR-124, using the antibody against the mitotic marker phospho-histone H3. Treatment with CPT-11 resulted in a decrease in the number of mitoses per low power field compared with control (17 versus 28, P = 0.06), consistent with activation of the G2-M checkpoint (Fig. 5C and D). Importantly, cotreatment with CHIR-124 reversed the suppression of phospho-H3 staining induced by CPT-11 (34 versus 17, P = 0.005; Fig. 5B and D), indicating abrogation of the G2-M checkpoint by CHIR-124 in the xenografts.

Discussion

Dysregulation of cell cycle checkpoints is now recognized as a salient feature of the malignant transformation process. Checkpoint dysfunction in tumors provides an opportunity for developing a therapeutic strategy that combines conventional cancer treatment with inhibitors of cell cycle checkpoints. This is based on the proposition that pharmacologic disruption of checkpoint function may selectively sensitize tumors with intrinsic checkpoint defects to genotoxic stress imparted by chemotherapy or radiation. Chk1 is considered a potential target for such a combination approach. In this report, we present data on the molecular pharmacology of a potent and selective Chk1 inhibitor (CHIR-124) in combination with topoisomerase I poisons.

Our data indicate that CHIR-124 is a biologically active Chk1 inhibitor. First, in an in vitro biochemical assay, CHIR-124 shows potent inhibitory activity against the kinase domain of recombinant human Chk1 and is highly selective for Chk1

![Fig. 3. Continued. C, enhancement of apoptosis by sequential treatment with SN-38 followed by CHIR-124. MDA-MD-435 cells were treated with single agent 20 nmol/L SN-38, 100 nmol/L CHIR-124, or 100 nmol/L UCN-01 for 24 h, or sequentially with SN-38 for 24 h followed by drug-free medium (ND). CHIR-124, or UCN-01 for the indicated times. Apoptosis and micronucleation were determined by examining the nuclear morphology of fixed cells under fluorescence after 4′,6′-diamidino-2-phenylindole staining. Points, averages of three independent experiments (mean); bars, SD.](image)
relative to several other kinases, including Chk2. Second, CHIR-124 inhibits Chk1 function in cells. It abrogates the S and G2-M checkpoints induced by SN-38 in the p53-defective MDA-MB-435 cell line at concentrations comparable with that of the prototypical Chk1 inhibitor UCN-01. Third, CHIR-124 treatment reverses the suppression ofcdc25A by SN-38 both in combination and sequentially, indicating that cellular signaling of Chk1 is altered by CHIR-124. Interestingly, although CHIR-124 effectively abolishes the G2-M checkpoint induced by DNA damage, we did not observe consistent inhibition of the level of Ser216 phosphorylation in cdc25C. This is in contrast to what investigators have previously reported with UCN-01 (28) but is consistent with a recent study using the selective Chk1 inhibitor CEP-3891, which also abrogates the G2-M checkpoint without altering Ser216 phosphorylation (29). Overall, it seems that reversal of DNA damage–induced suppression of cdc25A protein level by CHIR-124 represents a more reliable pharmacodynamic indicator of Chk1 inhibition.

We have shown that CHIR-124 interacts synergistically with camptothecin in four different p53-deficient carcinoma cell lines. The combined effect of CHIR-124 and a topoisomerase I poison was further examined in vivo in MDA-MB-435 xenografts. CHIR-124 alone was well tolerated by the animals, as expected, but did not inhibit tumor growth. However, CHIR-124 potentiated the antitumor activity of CPT-11 and enhanced induction of apoptosis. To our knowledge, these results represent the first demonstration of potentiation of the antitumor activity of a cytotoxic agent by a specific Chk1 inhibitor in vivo. A significant, although

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**Fig. 5. Potentiation of the antitumor effect of CPT-11 by CHIR-124 in vivo.**

A. CHIR-124 potentiates the growth inhibitory effect of CPT-11 in a human breast carcinoma xenograft model. MDA-MB-435 cells were implanted in the mammary fat pad of 8- to 10-week-old female severe combined immunodeficient mice. Animals were randomized into the indicated groups after reaching a mean tumor volume of 200 mm$^3$ ± 10%. CPT-11 and CHIR-124 were dosed either alone or in combination. CPT-11 was given daily at 5 mg/kg on days 1 to 5 (four times daily × 5; filled arrows), whereas 10 mg/kg CHIR-124, 20 mg/kg CHIR-124, or vehicle were dosed daily on days 2 to 7 (four times daily × 6; open arrows).

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**Fig. 5 Continued.** B. Increased apoptosis in tumor xenograft from animals treated with both CPT-11 and CHIR-124 relative to each single agent. Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated nick-end labeling staining in tumors harvested from mice on day 4 receiving the indicated treatments daily. Black arrows, terminal deoxynucleotidyl transferase–mediated nick-end labeling–positive cells. Representative sections at a magnification of ×400.
less profound, abrogation of the G2-M checkpoint, as measured by the induction of mitotic entry, was observed in MDA-MD-435 xenografts derived from mice treated with CPT-11 and CHIR-124 compared with that obtained in the same cells treated in culture with SN-38 and CHIR-124. This discrepancy may stem from differences in the timing of tumor sampling or in drug exposure between in vivo and in vitro studies.

We have previously shown that the cytotoxicity induced by disruption of the Chk1-mediated S and G2-M checkpoints involves disparate mechanisms (13). Thus, abrogation of the S-phase checkpoint leads to an amplified DNA damage response characterized by an enhanced induction of p53, p21, and γ-H2AX, whereas inhibition of the G2-M checkpoint results in an aberrant mitosis and increased apoptosis (13). Pharmacologic manipulation of the latter checkpoint is of particular interest because p53-deficient cells are more sensitive than wild-type cells in undergoing premature G2-M entry, aberrant mitosis, and subsequent apoptosis (ref. 13 and this report).

Current evidence has indicated that following mitotic failure, the ensuing reproductive cell death can proceed by several mechanisms: (a) apoptosis induction either during aberrant mitosis or in micronucleated cells that have exited mitosis (13), (b) sustained cell cycle arrest due to activation of the p53-dependent endoreduplication checkpoint (30, 31), or (c) unknown mechanisms leading to demise of polyloid giant cells, which can possibly involve cellular senescence.6 Notably, our current results show that cell fate after G2-M checkpoint abrogation seems to differ between cell lines. For example, HCT116 p53−/− cells that have escaped the G2-M checkpoint develop either apoptosis or micronucleation, whereas MDA-MB-435 cells that undergo checkpoint abrogation culminate primarily in apoptosis.

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6 A. N. Tse and G. K. Schwartz, unpublished data.

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Fig. 5 Continued. C, abrogation of the CPT-11 induced G2-M checkpoint by CHIR-124. Mitotic cells were labeled using the rabbit polyclonal antibody against phospho-H3 (green), and chromatin was stained with 4',6'-diamidino-2-phenylindole (blue). Representative fluorescence micrographs at a magnification of ×100.

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Fig. 5 Continued. D, quantitation of apoptotic and mitotic cells in tumor sections. Percentage apoptosis and mitotic index per low-powered field in different treatment groups were determined as described in Materials and Methods. Differences between groups were compared using paired t test. Columns, mean; bars, SD.
Several unresolved issues remain regarding the use of Chk1 inhibitors in cancer therapy. First, because Chk1 is an essential checkpoint kinase required for maintaining genomic stability, one theoretical concern is whether Chk1 inhibition might produce any deleterious effects in the renewal of normal stem cells in various tissues. The consequence of Chk1 inhibition in an unperturbed cell cycle has recently been addressed (32). Interference of Chk1 function using small-molecule inhibitors or by small interfering RNA resulted in increased initiation of DNA replication, an ATR-dependent DNA damage response, and frank DNA breaks (32). However, our mouse xenograft studies have indicated that CHIR-124, when given as a single agent for five consecutive days, is well tolerated and results in no appreciable tumor growth inhibition, yet enhances the antitumor activity of CPT-11 in animals. These results suggest that the effect of Chk1 inhibition on S-phase cells in the absence of DNA damage, if any, does not seem to affect the short-term proliferative potential of both normal and tumor cells in vivo.

Second, it is generally believed that the function of checkpoint delay following genotoxic stress is to allow time for repair before any DNA damage becomes irreversibly fixed after cell division. However, the relationship between checkpoint abrogation and chemosensitivity or radiosensitivity is not entirely clear. A recent report has shown a direct linkage between Chk1 signaling and the homologous recombination repair pathway, suggesting that the enhanced cytotoxicity observed with Chk1 inhibition following DNA damage may also be mediated by regulation of the homologous recombination repair system by Chk1 (33).

Third, as with all combination therapies, the sequence of administration of the individual agents may critically affect treatment outcome. The importance of schedule was shown in our in vitro studies in which concurrent treatment with a topoisomerase I poison and Chk1 inhibitor targeted primarily the S-phase checkpoint, whereas sequential exposure favored inhibition of the G2-M checkpoint. Of note, in our in vivo studies in MDA-MD-435 xenografts where potentiation of the antitumor effect of CPT-11 by CHIR-124 was shown, CPT-11 was given alone on the 1st day and 4 h before CHIR-124 thereafter. We have not examined other treatment sequences or varied the time interval between drug administrations; additional studies are required to determine the optimal treatment schedule in vivo.

In summary, we have shown that CHIR-124 is a potent and selective Chk1 inhibitor. CHIR-124 disrupts the S and G2-M checkpoints by interfering with Chk1 intracellular signaling and enhances the antitumor activity of topoisomerase I poisons both in vitro and in vivo. Furthermore, tumor cells that lack p53 function are more susceptible to undergoing G2-M checkpoint abrogation upon treatment with CHIR-124, validating the concept of combining Chk1 inhibitors with DNA-damaging agents as a therapeutic strategy to sensitize tumors with intrinsic checkpoint defects. Newer generations of Chk1 inhibitors using different chemical scaffolds (34) and alternate means to down-regulate Chk1 function (35) will be reported and are poised for clinical testing. Our current data show that CHIR-124 is a promising Chk1 inhibitor that warrants further investigation to understand its therapeutic potential.

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CHIR-124, a Novel Potent Inhibitor of Chk1, Potentiates the Cytotoxicity of Topoisomerase I Poisons In vitro and In vivo

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