The Preclinical Pharmacology and Therapeutic Activity of the Novel CHK1 Inhibitor SAR-020106

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Abstract

Genotoxic antitumor agents continue to be the mainstay of current cancer chemotherapy. These drugs cause DNA damage and activate numerous cell cycle checkpoints facilitating DNA repair and the maintenance of genomic integrity. Most human tumors lack functional p53 and consequently have compromised G1-S checkpoint control. This has led to the hypothesis that S and G2-M checkpoint abrogation may selectively enhance genotoxic cell killing in a p53-deficient background, as normal cells would be rescued at the G1-S checkpoint. CHK1 is a serine/threonine kinase associated with DNA damage-linked S and G2-M checkpoint control. SAR-020106 is an ATP-competitive, potent, and selective CHK1 inhibitor with an IC₅₀ of 13.3 nmol/L on the isolated human enzyme. This compound abrogates an etoposide-induced G₂ arrest with an IC₅₀ of 55 nmol/L in HT29 cells, and significantly enhances the cell killing of gemcitabine and SN38 by 3.0- to 29-fold in several colon tumor lines in vitro and in a p53-dependent fashion. Biomarker studies have shown that SAR-020106 inhibits cytotoxic drug-induced autophosphorylation of CHK1 at S296 and blocks the phosphorylation of CDK1 at Y15 in a dose-dependent fashion. Cytotoxic drug combinations were associated with increased γH2AX and poly ADP ribose polymerase cleavage consistent with the SAR-020106–enhanced DNA damage and tumor cell death. Irinotecan and gemcitabine antitumor activity was enhanced by SAR-020106 in vivo with minimal toxicity. SAR-020106 represents a novel class of CHK1 inhibitors that can enhance antitumor activity with selected anticancer drugs in vivo and may therefore have clinical utility. Mol Cancer Ther; 9(1); 89–100.

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Introduction

Normal cell division is a highly regulated process involving multiple cyclin-dependent kinases (CDK) and their appropriate cyclins that control a series of cell cycle checkpoints that ensure the fidelity of DNA replication (1). Genotoxic antitumor drugs cause DNA damage and activate cell cycle checkpoints at G1-S, S, and G2-M, which are required for DNA repair and the maintenance of genomic integrity (2). The transcription factor p53 is a critical checkpoint protein. In the presence of damaged DNA, p53 is stabilized and activated, causing the upregulation of the CDK inhibitor p21, which induces a subsequent delay in the cell cycle to facilitate DNA repair and/or apoptosis (2, 3). The observation that human cancers frequently have defects in p53 function, through either direct mutations or the disruption of its regulatory pathway, with a consequent loss of G1-S checkpoint control (3, 4) has led to the concept that G₂ checkpoint inhibitors combined with DNA-damaging agents may cause aberrant and lethal mitosis in such tumors, whereas normal tissue would be rescued at the p53-dependent G1-S checkpoint (2, 5–7). This strategy should therefore increase the therapeutic selectivity of several DNA-damaging anticancer drugs, which are still the mainstay of current cancer treatment.

In normal cells, DNA damage is sensed by a complex series of signal transduction pathways. The main proximal kinases are the phosphoinositide 3-kinase homologues ATM and ATR (2, 8). These transduce signals to the effector kinases CHK1 and CHK2, which then activate a series of cell cycle checkpoints. CHK1 is predominantly activated through phosphorylation on S317 and S345 catalyzed by ATR (9, 10), and undergoes autophosphorylation on S296 (11, 12). CHK1 is a serine/threonine kinase that is involved in the S-phase checkpoint through stabilizing replication forks, and in the G₂ checkpoint control through regulating the stability and availability of the CDC25 phosphatases (13–15).
Numerous studies have shown that UCN-01, a relatively nonselective inhibitor of CHK1, will preferentially sensitize p53-defective tumor cells to a variety of DNA-damaging anticancer drugs both in vitro and in vivo (16-18). More recently, inhibition of CHK1 function by knockdown of protein expression by small interfering RNA and several more selective CHK1 inhibitors have confirmed these early observations (19-21). As a part of the development of molecularly targeted kinase inhibitors, particularly those that might not have antitumor activity in their own right, it is important to identify appropriate biomarker readouts (22, 23). Consequently, the development of CHK1 inhibitors and the characterization of suitable biomarkers have become an important drug development objective (24-28).

In this study, we present the preclinical pharmacology and therapeutic utility of SAR-020106, a novel, selective, and potent inhibitor of CHK1. We show that SAR-020106 can potentiate SN38 and gemcitabine cytotoxicity in several colon cancer cell lines in vitro and that this potentiation occurs preferentially in cells that lack p53 function. We further show that SAR-020106 can abrogate an SN38-induced S-phase block and an etoposide-induced S and G2-M arrest in vitro. Biomarker studies showed that SAR-020106 selectively inhibited SN38 or gemcitabine induced autophosphorylation of CHK1 at S296, and that this was associated with a loss of CDK1 phosphorylation at Y15, a downstream target of CHK1. Increases in γH2AX and poly ADP ribose polymerase (PARP) cleavage were also observed, consistent with enhanced DNA damage and cell death. Finally, we present evidence of enhancement of irinotecan and gemcitabine therapeutic activity by SAR-020106 in association with CHK1 inhibition in human tumor xenografts.

Materials and Methods

Compounds

SAR-020106 was synthesized as described previously (29). Clinical formulations of gemcitabine and irinotecan were obtained from Eli Lilly (Gemzar, Eli Lilly and Pfizer, respectively). SN38, the active metabolite of irinotecan, was purchased from LKT Laboratories. Other reagents were obtained from Sigma-Aldrich Chemical Co.

Cells

HT29, SW620, and Colo205 human colon carcinoma cell lines were obtained from American Type Culture Collection (American Type Culture Collection lot no. 4487729, 3924081, and 57723824, respectively) and A2780 ovarian carcinoma cells were obtained from European Collection of Animal Cell Cultures (Sigma-Aldrich). Cells were grown in DMEM containing 10% fetal bovine serum (PAA “Gold”) and 5 mmol/L glutamine in a humidified atmosphere of 5% CO2 at 37°C and passaged ≤6 mo before the renewal from frozen, early passage

stocks indicated. A2780 cells were stably transfected with HPV16E6, and appropriate clones were screened and selected essentially as described previously (30). Cells were regularly screened for Mycoplasma using a PCR-based assay (VenorGem, Minerva Biolabs).

Kinase Assays

CHK1 and CHK2 assay conditions were as described previously (31). A kinase profile against 50 different human kinases was carried out using 10 μmol/L SAR-020106 with an ATP concentration equivalent to the Km for each enzyme (Millipore).

Cytotoxicity Assays

Cells were plated in 96-well plates (3–6 × 10³ per well) or tissue culture flasks (at 2–5 × 10⁵ per flask) and allowed to attach for 36 h to ensure exponential growth at the time of treatment. Cytotoxicity was determined using a 96-h (four cell doublings) SRB assay, and GI50 values were derived as described previously (32).

G2 Checkpoint Abrogation Assay

Measurements of intracellular CHK1 inhibition were carried out using HT29 and SW620 in a cell-based ELISA assay. Cells in 96-well plates (1–2 × 10⁴ per well) were treated with etoposide (50 μmol/L × 1 h) to induce a late S-G2 arrest and then were exposed to different concentrations of the test compound for a further 21 h in medium containing nocodazole (100 ng/mL) to trap cells in mitosis. Cells were then fixed in 4% formaldehyde in PBS for 30 min at 4°C and permeabilized with ice-cold methanol for a further 10 min. Cells were washed in PBS, blocked with 5% milk in PBS × 30 min at 37°C, and then treated overnight with an antibody to mitotic proteins (MPM-2, Upstate) in 5% milk in TBS. Cells were then rinsed in water containing 0.01% Tween 20 and were treated for 1 h with an Eu-labeled secondary antibody (Eu-N1, Perkin-Elmer) at 37°C. The MPM-2 epitope-expressing (i.e., mitotic) cells were quantified using a Wallac Victor 1420 multilabel counter (Perkin-Elmer). IC50 values for mitosis induction were determined using nocodazole as a positive control. The activity index was used as a measure of a compound’s ability to induce mitosis relative to its
cytotoxicity (i.e., ratio of the G2 checkpoint abrogation IC_{50} and SRB cytotoxicity GI_{50}).

**Potentiation Assay**

Cells were exposed to a fixed concentration of the cytotoxic agent that inhibited growth by 50% relative to untreated controls (GI_{50} dose) in combination with increasing concentrations of the CHK1 inhibitor in a 96-h SRB assay. The ability of SAR-020106 to enhance genotoxic cell killing was expressed as a potentiation index (PI), which was the ratio of GI_{50} for the CHK1 inhibitor alone and GI_{50} for the CHK1 inhibitor in combination with a cytotoxic agent.

**Immunoblotting**

Drug-treated cells were lysed in a buffer containing 50 mmol/L HEPES (pH 7.4), 250 mmol/L NaCl, 0.1% NP40, 1 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L NaF, 10 mmol/L β-glycerophosphate, 100 mmol/L NaVO₄, and 1× Complete proteinase inhibitor tablet per 10-mL buffer (Roche). Tumor samples were homogenized using a buffer containing 50 mmol/L Tris (pH 7.4),

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**Figure 2.** Effects of SAR-020106 on drug-induced cell cycle arrest in SW620 and HT29 colon cancer cells. **A,** effects of different concentrations of SAR-020106 alone (0.1, 0.5, and 1 μmol/L) or in combination with a fixed concentration of SN38 (20 nmol/L) for 24 h on cell cycle distribution in SW620 cells. Histograms, cell cycle distribution as measured by propidium iodide (PI) DNA staining; dot plots, DNA synthesis as measured by BrdUrd incorporation. Cell cycle distribution was assessed by BrdUrd distribution (G₁, S, S', and G₂-M) as shown and described in Materials and Methods. **B,** quantification of the effects of different concentrations of SAR-020106 on SN38-induced (20 nmol/L) cell cycle arrest in SW620 cells at 24 h. ▪, G₁-G₀ phase; □, S phase; △, S’ phase; ■, G₂-M phase. **C,** quantification of the effects of different doses of SAR-020106 on etoposide-induced (50 μmol/L × 1 h) cell cycle arrest in HT29 cells 23 h following cytotoxic exposure. Symbols as in **B.** Columns, mean derived from three independent experiments; bars, SD.
1 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L NaF, 1 mmol/L NaVO₄, 5 μmol/L Fenvalerate, 5 μmol/L Vbphen, 10 mg/mL TLCK, 1× Complete inhibitor tablet per 10-mL buffer (Roche), protease inhibitor cocktail, and phosphatase inhibitor 1 and 2 (Sigma-Aldrich; ref. 33). Protein concentrations were measured by Bradford assay. Proteins samples (50 μg) were denatured in Laemmli buffer and were separated on precast 10% or 16% Tris-glycine gels (Novex, Invitrogen). Samples were transferred to polyvinylidene difluoride membranes, which were incubated in a blocking buffer [5% dried milk in 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl and 0.1% Tween 20] for 1 h, probed with primary antibody in blocking buffer overnight at 4°C, and detected with HRPO conjugated secondary antibody (Bio-Rad). Proteins were visualized with enhanced chemiluminescence reagents (Pierce, Thermo Fisher) on Hyperfilm (GE Healthcare) using a Compact X4 developer (Xograft). Antibodies used were as follows: pS296 CHK1, pS317 CHK1, pY15 CDK1, total CDK1, cleaved PARP (CST), Total CHK1 (SC), pS139 H2AX, total H2AX (Upstate), and glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Millipore).

**Cell Cycle Analysis**

Cells were stained with bromodeoxyuridine (BrdUrd) and PI as described previously (34). Cells were pulsed with 10 μmol/L BrdUrd for 30 min before harvesting and fixation in 70% ethanol. Subsequently, cells were acid denatured in 2 mol/L HCl containing trypsin to obtain nuclei. These were stained with a primary antibody to BrdUrd (Clone Bu20a, DAKO) and then conjugated to a FITC-labeled secondary antibody (Sigma-Aldrich) and stained with PI overnight. Cells were analyzed on a BD LSR II flow cytometer (Becton Dickinson). Cell cycle analysis was carried out on gated single-cell events using WinMDI 2.8.

**Mice and Antitumor Studies**

BALB/c mice were obtained from Charles River. Nude mice (CrTacrCrr-Fox1nu) were bred in-house and mice ages 6 to 10 wk were used. Mice were allowed access to food (rodent diet no. 5002, Lab Diet) and water ad libitum. Tumor cells were inoculated s.c. in both flanks of the recipient mice and palpable tumors were treated at an average diameter of 0.38 to 0.47 cm. Animals were randomized into treatment groups of six to eight mice and drugs were administered either i.p. or i.v. at 0.01 mL/g as appropriate. SAR-020106 was dissolved in 10% DMSO and 5% Tween 20 in saline and was administered i.p. or i.v. as described in Materials and Methods. Points, mean for four replicates; bars, SD. Similar results were obtained in repeat experiments.

**Pharmacokinetics**

Drugs were extracted from plasma and tissue homogenates (25% w/v in water) using three volumes of acetonitrile containing internal standard. Drug concentrations...
were determined using reverse-phase high performance liquid chromatography and mass spectrometry analysis. Briefly, drugs were separated on a UPLC, BEH, C18 column (Waters), using gradient elution (9 mmol/L ammonium acetate in acetonitrile to 100% acetonitrile over 3 min and held for 1.6 min) with a flow rate of 0.4 mL/min at 55°C. SAR-020106 was identified and quantified by mass spectrometry analysis (MRM transition of 383.1→86.1 at 15 kV collision energy using an Agilent 1200 LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer with electrospray ionization in positive ionization mode). Noncompartmental pharmacokinetic parameters were determined using WinNonLin software version 5.2.

Statistics
Statistical significance (*, P < 0.05; **, P < 0.01; and ***, P < 0.001) was determined using unpaired, one-tailed, t tests as appropriate, with GraphPad Prism 4 software.

Results

Potent and Selective Inhibition of CHK1 by SAR-020106
SAR-020106 (Fig. 1) was shown to be a potent inhibitor of recombinant human CHK1 in vitro with an IC50 of 13.3 ± 1.3 nmol/L (mean ± SD, n = 3). Ki determination confirmed that the compound was acting as an ATP mimetic (Ki = 10.9 nmol/L). The corresponding IC50 values for CHK2 and CDK1 were >10 μmol/L. Kinase profiling with 10 μmol/L SAR-020106 showed minimal cross-reactivity with Aurora A, CDK1, CDK2, and CHK2 (>75% remaining activity compared with 2% for CHK1) and with 38 other kinases. Inhibition of Flt3, IRAK4, Met, MST2, p70S6K, Ret, RSK1, and Trk A (<20% activity remaining; see Supplementary Table 1) was observed.

SAR-020106 Abrogates Genotoxic-Induced Cell Cycle Arrest
A series of experiments were carried out to explore the ability of SAR-020106 to abrogate DNA damage–induced cell cycle checkpoints. The use of BrdUrd allowed the resolution of G1 from early S checkpoint and late S from G2-M checkpoints, which cannot always be resolved using simple PI-based analyses. Initial experiments were carried out in SW620 colon carcinoma cells following SN38 treatment. Figure 2A and B show that there were minimal effects of SAR-020106 alone on cell cycle distribution up to a concentration of at least 1 μmol/L. A concentration of 20 nmol/L SN38 alone caused a profound S-phase arrest at 24 hours (83.6%) with only 1.8% in G1 and 10.7% in G2-M with the remaining 3.9% cells in a noncycling S-phase (S†). The combination of SN38 with SAR-020106 (0.1 μmol/L) caused a marked abrogation of this SN38-induced S-phase block (35.8% S phase) with a subsequent increase in G2-M to 59.8%. Increasing concentrations of SAR-020106 up to 1 μmol/L seemed to cause only minor changes in this S-phase population but a slight decrease in G2-M with a correspondingly small increase in G1. Consequently, there was clear evidence that SAR-020106 caused abrogation of SN38-induced S-phase arrest. A summary of the cell cycle effects obtained in HT29 colon carcinoma cells treated with etoposide (50 μmol/L × 1 hour) and subsequently exposed to SAR-020106 for 23 hours is shown in Fig. 2C. Etoposide treatment alone induced a late S-phase and G2-M arrest relative to controls (52.8% and 49.3%,

<table>
<thead>
<tr>
<th>Cells</th>
<th>(G_{50}) (μmol/L)</th>
<th>Genotoxic</th>
<th>Combination (G_{50}) (μmol/L)</th>
<th>PI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>0.47 ± 0.19 (n = 11)</td>
<td>SN38</td>
<td>0.19 ± 0.10 (n = 5)</td>
<td>3.1 ± 1.6 (n = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gem</td>
<td>0.18 ± 0.089 (n = 7)</td>
<td>3.0 ± 1.2 (n = 7)</td>
</tr>
<tr>
<td>SW620</td>
<td>2.0 ± 0.93 (n = 4)</td>
<td>SN38</td>
<td>0.62 ± 0.12 (n = 4)</td>
<td>3.3 ± 0.81 (n = 4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gem</td>
<td>0.20 ± 0.10 (n = 4)</td>
<td>12 ± 4.9 (n = 4)</td>
</tr>
<tr>
<td>Colo205</td>
<td>3.5 ± 0.57 (n = 3)</td>
<td>SN38</td>
<td>1 ± 0.36 (n = 3)</td>
<td>4.1 ± 1.8 (n = 3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gem</td>
<td>0.13 ± 0.026 (n = 3)</td>
<td>29 ± 9.1 (n = 3)</td>
</tr>
<tr>
<td>A2780C</td>
<td>0.45 ± 0.09 (n = 5)</td>
<td>SN38</td>
<td>0.37 ± 0.12 (n = 4)</td>
<td>1.4 ± 0.59 (n = 4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gem</td>
<td>0.12 ± 0.0057 (n = 3)</td>
<td>3.6 ± 0.17 (n = 3)</td>
</tr>
<tr>
<td>A2780E6</td>
<td>0.29 ± 0.034 (n = 5)</td>
<td>SN38</td>
<td>0.11 ± 0.054 (n = 4)</td>
<td>3.2 ± 1.4 (n = 4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gem</td>
<td>0.02 ± 0.00581 (n = 3)</td>
<td>16.2 ± 5.8 (n = 3)</td>
</tr>
</tbody>
</table>

NOTE: Cytotoxicity (\(G_{50}\)) was determined by SRB assay. See Materials and Methods and Materials for further details.

*PI is the ratio of \(G_{50}\)/combination \(G_{50}\). Values are mean ± SD of n independent determinations.

1\(P < 0.05\) significantly different from unity.

2\(P < 0.01\) significantly different from unity.

3\(P < 0.001\) significantly different from unity.
respectively, in drug-treated cells) and 0.1 μmol/L SAR-020106 caused a profound abrogation of this S-phase block and a slight increase in the G2-M population (19.1% and 55.2%, respectively). An increase in concentration to 0.5 μmol/L SAR-020106 partially abrogated this G2-M block with a corresponding increase in G1 and S', with minimal changes in this distribution occurring at higher doses (1 μmol/L). These data clearly show that SAR-020106 abrogated an etoposide-induced S and G2 arrest.

G2 Checkpoint Abrogation and Potentiation of Cytotoxicity by SAR-020106

A cellular assay for measuring functional CHK1 inhibition and G2 checkpoint abrogation was developed. An acute etoposide exposure (50 μmol/L × 1 hour) was used to arrest cells in a late S-G2 checkpoint before abrogation with different concentrations of SAR-020106 in the presence of nocodazole. This allowed mitotic cells to be collected and quantified in a cell-based ELISA assay. This G2 checkpoint abrogation assay in HT29 and SW620 showed that SAR-020106 was capable of abrogating etoposide-induced cell cycle arrest with an IC50 of 55 ± 19 nmol/L and 91 ± 51 nmol/L, respectively (mean ± SD, n = 5; Fig. 3A). Table 1 shows that SAR-020106 was relatively nontoxic with a GI50 of 0.48 μmol/L in HT29 and 2 μmol/L in SW620, resulting in an activity index of 8.7 and 22, respectively. Furthermore, it enhanced the cytotoxicity of both SN38 and gemcitabine in HT29 cells to a similar degree (Table 1; Fig. 3B), giving a PI of 3. By contrast, greater potentiation occurred in SW620 and Colo205 with gemcitabine compared with SN38 (Table 1). The ability of SAR-020106 to selectively sensitize p53-deficient cells to genotoxic chemotherapy was assessed using an A2780 isogenic cell line in which p53 was functionally inactivated by the overexpression of HPV16E6. The PI values for parental A2780 (wild-type p53) with SN38 and gemcitabine were 1.4 and 3.6, respectively, whereas the corresponding values in A2780E6 (nonfunctional p53) were 3.2 and 16.2, giving a p53−/− selectivity of 2.3- and 4.5-fold, respectively.

Inhibition of CHK1 Autophosphorylation and the Cell Cycle Pathway by SAR-020106

The characterization of biomarker changes associated with target inhibition is an important aspect of molecularly targeted drug development (23). Figure 4A shows that phosphorylation at S296 of CHK1, a site of autophosphorylation, is barely detectable in unperturbed cells, but is markedly induced in response to SN38 treatment for 24 hours in SW620 cells. This response is thought to be catalyzed by the DNA damage-induced activation of CHK1 providing a potential biomarker of CHK1 activity (11, 12). The combination of SN38 with increasing concentrations of SAR-020106 was shown to decrease this signal in a concentration-dependent fashion with complete suppression at ≥1 μmol/L SAR-020106. These
changes in CHK1 phosphorylation were associated with minimal alterations in total protein expression. As shown by the previous data, a critical response of cells to DNA damage is cell cycle arrest. In particular, phosphorylation of tyrosine 15 on CDK1 (pY15) blocks the activity of this kinase, causing arrest at the G2-M transition. CHK1 controls this arrest through regulating CDC25 phosphatase activity (14, 36, 37), and consequently, pY15 CDK1 can be regarded as a downstream biomarker for CHK1 activity and G2-M arrest. Figure 4A shows that SN38 caused an increase in pY15 CDK1 consistent with a cell cycle arrest, whereas SAR-020106 alone had no effect. By contrast, the combination of SN38 and SAR-020106 blocked the induction of pY15 at ≥ 1 μmol/L SAR-020106, consistent with the loss of the pS296 CHK1 signal. In addition, the band shift of CDK1, which is indicative of Y15 phosphorylation, was also inhibited at concentrations of SAR-020106 that are ≥ 1 μmol/L. Interestingly, combinations of SN38 and SAR-020106 at ≥ 0.1 μmol/L caused an increase in S139 H2AX phosphorylation (γH2AX), a signal associated with DNA double-strand breaks (38) and there was a corresponding increase in PARP cleavage, a marker of cell death (39), at concentrations ≥ 1 μmol/L SAR-020106. Figure 4B shows that a concentration of 20 nmol/L gemcitabine (which induced pS296 CHK1 and pY15 CDK1) combined with SAR-020106 for 24 hours in SW620 cells caused similar effects to combinations with SN38, although S296 CHK1 phosphorylation...
Pharmacokinetics of SAR-020106 in Mice

The preliminary pharmacokinetic properties of SAR-020106 were determined in nontumor-bearing BALB/c mice to ensure that tissue drug concentrations were adequate for CHK1 inhibition in vivo. Figure 5A and Table 2 summarize the pharmacokinetic characteristics of SAR-020106 following administration at 5 mg/kg either i.v. or i.p. Peak plasma concentration was 1.35 μmol/L after i.v. administration with a relatively short half-life and high clearance and volume of distribution giving an i.v. bioavailability of 47.5%. Following i.p. administration, the plasma elimination time course was comparable with that following i.v. administration (data not shown), but the peak drug concentration was 3-fold lower, the clearance was 2-fold slower, and the volume of distribution was 4-fold higher (Table 2). This resulted in an AUC\(\text{∞}\) of 703 nmol/L h. Following i.p. administration, the muscle drug exposure was much higher than in plasma and there was evidence of tissue retention as shown by the 5- to 6-fold longer half-life and correspondingly lower clearance. Consequently, muscle drug exposure was much higher than in plasma with an AUC\(\text{∞}\) of 2615 nmol/L h and a tissue/plasma ratio of 8.1. Other tissues showed tissue/plasma ratios of 10:1 to 50:1 following i.v. administration, consistent with tissue drug accumulation and retention at this dose. Assuming linear pharmacokinetics, a dose of 40 mg/kg i.p. would be expected to result in tissue exposures of 21 to 130 μmol/L h, which equals or exceeds that required for biomarker modulation in vivo (24 μmol/L h; see Fig. 3). Figure 5B shows that SAR-020106 tumor concentrations greatly exceeded plasma drug concentrations with tumor/plasma ratios of 47:1 and 85:1 at 6 and 24 hours, respectively, following 40 mg/kg i.p. Moreover, this dose gave tumor drug concentrations that greatly exceeded those required for G2 checkpoint abrogation in vivo (IC\(_{50}\), 55 nmol/L) for at least 24 hours, consistent with checkpoint abrogation in tumor cells in vivo (see Fig. 5B). Plasma binding studies established that SAR-020106 was 94.4% bound to mouse plasma proteins.

Table 2. Summary of SAR-020106 pharmacokinetics in BALB/c mice following 5 mg/kg i.v. or i.p.

<table>
<thead>
<tr>
<th>Route</th>
<th>Tissue</th>
<th>Tmax (h)</th>
<th>Cmax (nmol/L)</th>
<th>T1/2 (h)</th>
<th>Cl (L/h)</th>
<th>Vz (L)</th>
<th>AUC(\text{∞}) (nmol/L h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.V.</td>
<td>Plasma</td>
<td>N/A</td>
<td>1350</td>
<td>0.658</td>
<td>0.371</td>
<td>0.353</td>
<td>703</td>
</tr>
<tr>
<td>I.P.</td>
<td>Plasma</td>
<td>&lt;0.083</td>
<td>415</td>
<td>0.995</td>
<td>0.781</td>
<td>1.12</td>
<td>334</td>
</tr>
<tr>
<td>I.P.</td>
<td>Muscle</td>
<td>0.5</td>
<td>644</td>
<td>5.59</td>
<td>0.100</td>
<td>0.806</td>
<td>2615</td>
</tr>
</tbody>
</table>

NOTE: Nonparametric pharmacokinetic parameters were determined using PC Nonlin software with six to seven time points and two to three mice per time point.
Abbreviation: N/A, not available.

Inhibition of DNA Damage–induced CHK1 Biomarkers by SAR-020106 In vivo

Having shown biomarker modulation in vitro and established that SAR-020106 exhibited pharmacokinetics that were consistent with potentially active tumor drug exposures, SAR-020106 was combined with the topoisomerase 1 inhibitor irinotecan to explore its effects on CHK1 biomarker expression in vivo. Figure 5C and D show that irinotecan at 25 and 50 mg/kg i.p. caused a marked increase in CHK1 autophosphorylation at S296 in SW620 tumors, 24 hours following drug administration, compared with controls. In addition, irinotecan also enhanced Y15 CDK1 phosphorylation consistent with a drug-induced cell cycle arrest in these tumor cells at 24 hours. The addition of SAR-020106 at 40 mg/kg caused a significant reduction in the pS296 CHK1 signal at 24 hours with minimal effects on total CHK1 levels consistent with CHK1 inhibition in vivo (Fig. 5D). Moreover, there was also a significant reduction in pY15 CDK1 signal intensity following SAR-020106 treatment, suggesting that irinotecan cell cycle arrest may have been abrogated in vivo (Fig. 5D).

Antitumor Effects of SAR-020106 in Combination with Irinotecan or Gemcitabine

The ability of SAR-020106 to potentiate the antitumor activity of irinotecan and gemcitabine in vivo was assessed in SW620 xenografts. Figure 6A shows that there were minimal effects of either irinotecan or SAR-020106 alone in SW620 tumors at the doses used. The mean time for control tumors to reach 300% of their initial treatment size was 6.2 days. By comparison SAR-020106 alone–treated tumors (40 mg/kg i.p.) and irinotecan alone–treated tumors (12.5 mg/kg i.p.) reached this size after a mean of 6.3 and 6.9 days, (\(P = 0.159\) and 0.460, respectively). However, there was a clear decrease in tumor growth associated with the combination with tumors reaching 300% by 12.5 days, resulting in a significant growth delay relative...
to irinotecan alone of 5.6 days \((P < 0.01)\) and an increase in growth delay of 5.5 days. Two mice were culled on days 15 and 18 in the SAR-020106 treatment alone group due to tumor growth. There was minimal body weight loss associated with this combination giving a nadir on day 11, and a mean body weight of 96.4% of the initial weight. The combination of gemcitabine with SAR-020106 in SW620 confirmed that SAR-020106 alone at 40 mg/kg i.p. had negligible antitumor activity (Fig. 6B), with a mean time for tumors to reach 300% of their initial treatment size of 7.0 compared with 6.2 days in controls \((P = 0.204)\).

Gemcitabine alone had a clear antitumor effect with tumors taking 10.1 days to reach 300%, giving a significant growth delay of 3.9 days relative to controls \((P < 0.001)\). The combination of gemcitabine and SAR-020106 was more potent when the two agents were administered simultaneously rather than with a 24-hour delay following the cytotoxic. The mean time for tumors to reach 300% of the initial treatment size was 14.7 and 12.5 days for the simultaneous and delayed combination, respectively, resulting in a growth delay of 3.8 days for the simultaneous treatment, which is a doubling of the antitumor effect of gemcitabine alone. One mouse died in the simultaneous combination group on day 18. Once again this syner-gistic antitumor interaction was associated with minimal weight loss with a nadir on day 16 and a mean body weight of 93.3% of the initial weight.

**Discussion**

These studies were undertaken to characterize the preclinical pharmacology of SAR-020106 and to identify genotoxic agents, which in combination with SAR-020106 exhibited enhanced antitumor activity in human tumor xenografts. As a part of these studies, it was important to identify and monitor suitable biomarkers of CHK1 inhibition and to establish any pharmacokinetic-pharmacodynamic relationships both *in vitro* and *in vivo*. This is a particularly important aspect of CHK1 inhibitor development as they are unlikely to exhibit any antitumor activity as single agents. The identification and use of these biomarkers allow the integrity of the CHK1 inhibitor/target pathway to be monitored and maintained throughout the preclinical and clinical evaluation process. Furthermore, this approach allows the construction of a "pharmacologic audit trail" that will facilitate further drug development and interpretation of clinical trials (23).

SAR-020106 is a novel, selective, and potent CHK1 inhibitor. This compound was initially assayed against recombinant human CHK1 and was shown to be a potent ATP competitive inhibitor. Kinase profiling data confirmed that there was minimal activity against the functionally related kinase CHK2 and the cell cycle kinase CDK1 (>7,000-fold selectivity). This was considered advantageous as the therapeutic utility of CHK2 is still unclear (40), and inhibiting CDK1 would interfere with cell cycle progression and prevent G2 checkpoint abrogation (41). There was some cross-reactivity with several receptor tyrosine kinases such as Flt3, Ret, Met, and Trk A, but it has been argued that sustained inhibition of
receptor tyrosine kinases is required for a pharmacologic effect and is therefore unlikely to occur with the relatively short exposures required for checkpoint abrogation (25).

Abrogation of an etoposide-induced G2 checkpoint and induction of mitosis was quantified in a cell-based ELISA assay. These data together with the cell cycle results (Fig. 2) confirmed that SAR-020106 abrogates an etoposide-induced G2 arrest in HT29 and SW620 cells with similar potencies, which were 9- and 22-fold lower than the corresponding GI50 values giving a potential therapeutic margin. The apparent discrepancy between the similar cellular CHK1 kinase inhibition and the different cytotoxicity of SAR-020106 in these two cell lines suggests that cytotoxicity is not directly associated with CHK1 inhibitory activity. Similar checkpoint abrogation activity was reported for PF00477736 (25), with the dual CHK1 and CHK2 kinase inhibitor AZD7762 giving a slightly lower value (24). The ability of SAR-020106 to potentiate the cytotoxicity of SN38 (the active metabolite of irinotecan, a topoisomerase 1 inhibitor) and gemcitabine (an antimetabolite) was evaluated in several colon tumor cell lines in vitro. In general, the cytotoxicity of gemcitabine was potentiated to a greater extent than SN38, although the degree of potentiation may depend on compound concentration and schedule as well as genetic background. Importantly, the use of an E6-transfected cell line with compromised p53 function, confirmed that CHK1 inhibition by SAR-020106 selectively enhanced genotoxic cell killing in a p53^-/- dependent fashion, with gemcitabine once again showing consistently greater sensitization than SN38 in this model. Other studies have also shown that several CHK1 inhibitors can potentiate SN38 and gemcitabine cytotoxicity in vitro with gemcitabine exhibiting the greatest potentiation (24–27).

At present, it is unclear which cytotoxic agents are most efficacious in combination with CHK1 inhibition. Nevertheless, the genotoxic agents that are most prominently reported in publications on CHK1 inhibition and show marked potentiation are irinotecan and gemcitabine. Both of these agents are reported to be involved in activating the intra-S-phase checkpoint through stalled replication forks (42, 43), and CHK1 is required for stabilizing these forks and preventing chromosomal breaks as well as reinitiating replication (13, 44). Our cell cycle studies showed that both SN38 and etoposide caused S-phase arrest that was completely abrogated by SAR-020106. Moreover, in vitro potentiating experiments and biomarker studies clearly show that this effect was associated with increased cell killing and induction of γH2AX and PARP cleavage with SN38. Although a correlation between CHK1 inhibition and premature mitosis and enhanced cell killing has been reported for camptothecins and gemcitabine (45, 46), there is still some controversy about the exact mechanism of increased cytotoxicity. For example, it has recently been reported that there is a better correlation between inhibition of the Rad 51 repair protein response and sensitization than checkpoint abrogation for gemcitabine and a CHK1 inhibitor (PD-321852) in pancreatic cancer cells (47). Consequently, the cellular response to a genotoxic agent and CHK1 inhibitor combination may depend on numerous factors including the type of genotoxic agent used, the checkpoint response involved, the DNA repair capacity, and the genetic background of the tumor cell. One fascinating corollary to this is the recent observation that tumor cyclin B1 expression may predict efficacy for CHK1 inhibitors (46).

The pharmacokinetics of SAR-020106 showed that the drug was concentrated and retained in tissues with a large AUC0-∞ relative to plasma. Consequently, tumor concentrations exceeding those required for CHK1 inhibition were readily achieved following a dose of 40 mg/kg i.p. The protein binding was moderately high but did not seem to prevent active drug concentrations from being achieved in vivo, and tissue drug accumulation seemed unimpaired. Despite these observations, it is possible that active drug concentrations may be required for at least one cell cycle in tumors and either repeat drug administration or drug infusion may be necessary for maximum antitumor effects in vivo.

Biomarker studies with SN38 and gemcitabine in vitro showed enhanced CHK1 autophosphorylation on S296 consistent with DNA damage–induced CHK1 activation (12). SAR-020106 inhibited this effect in a concentration-dependent manner with complete inhibition of >1 μmol/L × 24 hours in both colon tumor cell lines. Tyrosine 15 CDK1 phosphorylation was also modulated in a similar fashion, indicating that CHK1-mediated abolition of genotoxic drug induced cell cycle arrest. Biomarker changes consistent with CHK1 inhibition contributing to increased DNA damage and cell death specifically in the SAR-020106 treated combinations were also detected. Perhaps more significantly, irinotecan readily induced S296 CHK1 and pY15 CDK1 phosphorylation in tumor xenografts after 24 hours of exposure, confirming CHK1 activation and a cell cycle arrest. SAR-020106 was able to inhibit these effects, confirming that CHK1 inhibition and abrogation of the cell cycle arrest could be achieved in vivo under these conditions. Subsequent antitumor studies with relatively modest doses of irinotecan and gemcitabine showed that SAR-020106 could clearly potentiate the antitumor effects of these genotoxic agents in a synergistic fashion. These results support the use of pS296 CHK1 and 15Y CDK1 as biomarkers of CHK1 inhibition and confirm the relationship between CHK1 inhibition and the enhanced antitumor activity of SN38 in vivo. Reassuringly, there was minimal weight loss or antitumor effects of SAR-020106 alone, despite reports of the lethal effects of CHK1 knockouts in mouse embryos (48). The combination of either genotoxic agent with SAR-020106 was also well tolerated with minimal body weight loss, suggesting that intermittent inhibition of CHK1 may have minimal toxic side effects. Interestingly our studies showed that the simultaneous
combination of gemcitabine with SAR-020106 was more efficacious than gemcitabine followed 24 hours later by SAR-020106. Although we have shown that CHK1 inhibition by SAR-020106 gives a synergistic antitumor response with irinotecan and gemcitabine, an equally critical question relates to the ability of CHK1 inhibition to facilitate tumor cures. Other studies have shown cytotoxic anticancer drug potentiation with different CHK1 inhibitors (24–27) and there is also evidence of increased cure rates (24). The mechanism by which CHK1 inhibitors enhance anticancer drug cell killing is still unclear and the role and efficiency of checkpoint abrogation versus the inhibition of DNA damage repair remains to be resolved.

In conclusion, this study shows that SAR-020106 is a selective, potent CHK1 inhibitor. The cytotoxicity of SN38 and gemcitabine can be enhanced in different human tumor cell lines in vitro by SAR-020106 and this is associated with biomarker changes and cell cycle effects consistent with CHK1 inhibition. Perhaps most importantly, we show that SAR-020106 can enhance the antitumor effects of both irinotecan and gemcitabine in vivo with appropriate biomarker changes and minimal toxicity. These results support further CHK1 inhibitor development and clinical evaluation.

Disclosure of Potential Conflicts of Interest

M.I. Walton, P.D. Eve, A. Hayes, M. Valenti, A. De Haven Brandon, G. Box, K.J. Boxall, G.W. Aherne, S.A. Eccles, E.J. Raynaud, I. Collins, and M.D. Garrett are employees of The Institute of Cancer Research that has a commercial interest in CHK1 inhibitors. J.C. Reader is an employee of Sareum Ltd, which has a commercial interest in CHK1 inhibitors. Sareum PLC is a wholly owned subsidiary of Sareum Holdings PLC, of which J. C. Reader is a shareholder. D.H. Williams is a former employee of Sareum Ltd and is a shareholder of Sareum Holdings PLC. Both Sareum and the Institute of Cancer Research have been involved in a commercial collaboration with Cancer Research Technology Ltd to discover and develop inhibitors of CHK1.

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