CC-223, a Potent and Selective Inhibitor of mTOR Kinase: In Vitro and In Vivo Characterization

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Abstract

mTOR is a serine/threonine kinase that regulates cell growth, metabolism, proliferation, and survival. mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2) are critical mediators of the PI3K–AKT pathway, which is frequently mutated in many cancers, leading to hyperactivation of mTOR signaling. Although rapamycin analogues, allosteric inhibitors that target only the mTORC1 complex, have shown some clinical activity, it is hypothesized that mTOR kinase inhibitors, blocking both mTORC1 and mTORC2 signaling, will have expanded therapeutic potential. Here, we describe the preclinical characterization of CC-223. CC-223 is a potent, selective, and orally bioavailable inhibitor of mTOR kinase, demonstrating inhibition of mTORC1 (pS6RP and p4EBP1) and mTORC2 [pAKT(S473)] in cellular systems. Growth inhibitory activity was demonstrated in hematologic and solid tumor cell lines. mTOR kinase inhibition in cells, by CC-223, resulted in more complete inhibition of the mTOR pathway biomarkers and improved antiproliferative activity as compared with rapamycin. Growth inhibitory activity and apoptosis were demonstrated in a panel of hematologic cancer cell lines. Correlative analysis revealed that IRF4 expression level associates with resistance, whereas mTOR pathway activation seems to associate with sensitivity. Treatment with CC-223 afforded in vivo tumor biomarker inhibition in tumor-bearing mice, after a single oral dose. CC-223 exhibited dose-dependent tumor growth inhibition in multiple solid tumor xenografts. Significant inhibition of mTOR pathway markers pS6RP and pAKT in CC-223–treated tumors suggests that the observed antitumor activity of CC-223 was mediated through inhibition of both mTORC1 and mTORC2. CC-223 is currently in phase 1 clinical trials. Mol Cancer Ther; 14(6); 1295–305. ©2015 AACR.

Introduction

mTOR is a serine/threonine kinase related to the lipid kinases of the PI3K family. It functions as a regulator of cell growth, metabolism, proliferation, and survival by integrating growth factor signaling with cellular nutritional status and energy use. The mTOR kinase exists within two distinct multi-protein complexes, mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2; ref. 1). Both complexes are critical mediators of the PI3K–AKT pathway. mTORC1 is responsible for regulating protein synthesis and growth (2) whereas mTORC2 has been shown to phosphorylate and activate AKT (3), a key kinase in the control of cell growth, metabolism, and survival. mTOR is activated by the PI3K–AKT pathway and as a result resides both downstream and upstream of AKT activation. The PI3K–AKT pathway is inappropriately activated in many cancers, through multiple mechanisms, including activating mutations of the PIK3CA oncogene or loss of function mutations or promoter hypermethylation of the tumor-suppressor PTEN (4). PI3K pathway dysregulation ultimately leads to hyperactivation of mTOR signaling (5, 6), making mTOR an attractive drug target.

Some clinical success has been achieved through targeting mTOR with rapamycin analogues, such as temsirolimus and everolimus, allosteric inhibitors that generally do not inhibit mTORC2 and have been shown to only partially inhibit the mTORC1 complex. Rapamycin analogues have been shown to stimulate the upstream kinase AKT by releasing the feedback inhibition of PI3K via p70S6 kinase and insulin receptor substrate 1. This may explain at least in part, the resistance to rapamycin analogues exhibited by the majority of cancer cell lines and tumors (7). It is hypothesized that mTOR kinase inhibitors, blocking both mTORC1 and mTORC2 signaling, will have expanded therapeutic potential (6).

The first generation of reported ATP-competitive mTOR kinase inhibitors to enter clinical trials presented as dual inhibitors of mTOR and the related lipid kinase, PI3K-alpha (8–10). Agents selectively targeting mTOR kinase, have since entered clinical investigation (8–10). We report here the in vitro and...
in vivo preclinical characterization of CC-223, a selective mTOR kinase inhibitor in phase I clinical development (11).

Materials and Methods
Chemicals
7-(6-(2-Hydroxypropan-2-yl)pyridin-3-yl)-1-((trans)-4-methoxy cyclohexyl)-3,4-dihydropyrazino[2,3-b]pyrazin-2(1H)-one (CC-223, Fig. 1A) was identified following a screening campaign and compound optimization effort. The synthesis of the compound has been reported (12). Please note that for in vitro studies, CC-223 DMSO stock solutions of 30 mmol/L were freshly prepared, within 4 hours of each assay. DMSO preparations were made and used at room temperature, but CC-223 DMSO solutions were not stored for future use. CC-223 was found to be stable after stock solutions were added to assay media and in the formulations used for in vivo studies.

Kinase assays
mTOR. Inhibition of mTOR kinase was determined using a standard kinase assay described previously (13).

DNA-dependent protein kinase. DNA-dependent protein kinase (DNA-PK) assays were run following procedures in the Promega DNA-PK Assay Kit (Promega). DNA-PK enzyme was purchased from and verified as being appropriate for DNA-PK (DNA-PK) assays were run following procedures in the Promega DNA-PK Assay Kit (Promega). DNA-PK enzyme was purchased from and verified as being appropriate for DNA-PK kinase assays.

ATR and SMG-1. HA-SMG-1 and FLAG-ATR were each transiently expressed in HEK293 cells and isolated by immunoprecipitation. Assays for ATR and SMG-1 were conducted in a P38 radioactive assay format, with ATP concentration of 100 μmol/L (3 μCi of P32-ATP) per well. Reaction times were 3 (SMG-1) or 4 (ATR) hours. CC-223 was tested in triplicate at 3 and 30 μmol/L and compared with a no inhibitor control.

Kinase panel. Counter screen against 246 protein kinases was outsourced and completed at a fixed CC-223 concentration (10 μmol/L). Follow-up IC50 value determinations for ephrin type-B receptor 3 kinase (EPHB3), colony stimulating factor 1 receptor tyrosine kinase (CSF1R or cFMS), and FMS-related tyrosine kinase 4 (FLT4) were outsourced to Invitrogen (Life Technologies).

Cell lines
Cell lines were purchased from and verified by the ATCC or Leibniz Institute DSMZ-German Collection of Microorganism and Cell Culture. Cells were cultured in growth media as recom mended by the vendor. Upon receipt, each line was expanded and frozen down at low passage. Cells were then used for less than 17 passages and over no more than 2 months. After 16 passages or 2 months, whichever came first, cells were disposed of and a fresh sample from original expansion was used.

Determination of mTOR pathway biomarkers in PC-3
PC-3 cells were treated for 1 hour with rapamycin or CC-223. Cells were lysed in radioimmunoprecipitation assay buffer and lysates were run on 4% to 12% gels. Western blotting was performed for the indicated pathway proteins.

Determination of phospho-4EBP1(T46). phospho-AKT(S473), and phospho-S6RP(S235/6) from cell lysates
Cells were plated at densities determined for each cell line and the following days were treated for 1 hour with CC-223. Cells were lysed and lysates were tested in the following assays according to the manufacturer’s protocol: Mesoscale (pS6RP and pAKT(S473)) and Invitrogen ELISA (p4EBP1). In HCT 116, MDA-MB-231 and HT29, in the pAKT(S473) and p4EBP1 assays, IGFI stimulation (500 ng/mL, recombinant human IGFI) for the last 10 minutes of incubation was used.

Cellular proliferation
Cell treatment and compound IC50 value determination for rapamycin sensitive/insensitive panel were completed as reported previously (13). For other cell panel proliferation assays, compound was spotted via an acoustic dispenser (EDCATS-100) into an empty 384-well plate. Cells were diluted to desired densities and added directly to the compound-spotted plates. Cells were allowed to grow for 72 hours. Viability was assessed using Cell Titer-Glo (Promega). All data were normalized and represented as a percentage of the DMSO-treated cells. Results were then expressed as GI50 and/or IC50 values.

Reverse phase protein array
Cell pellets were made for 37 cell lines without compound treatment and Reverse Phase Protein Array (RPPA) analysis, using 262 antibodies, was performed in collaboration with the Dr. Gordon Mills laboratory at MD Anderson Medical Center. The relative level of each protein in each sample was determined and normalized for protein loading. Protein levels were then transformed to log2 values and median centered by individual batch.

Determination of apoptotic cell death
A549 and a panel of 40 lymphoma cell lines were treated with CC-223 or rapamycin for 24 hours, and then assessed for Caspase 3/7 induction compared with DMSO control using Caspase 3/7-Glo (Promega). The concentration of compound required for a 2-fold induction of Caspase was determined (CalX).

In vivo studies
All animal studies were performed under protocols approved by Institutional Animal Care and Use Committees.

Animals. Female 6- to 8-weeks-old CB17 SCID mice were obtained from Charles River Laboratories. Mice were housed in a barrier facility in micro-isolator cages at 10 animals per cage. Mice were fed with Harlan-Teklad LM-485 Mouse/Rat Sterilizable Diet and autoclaved water ad libitum in a light–dark cycle. Animals were acclimatized to the animal housing facility for a period of 7 days before the beginning of the experiment.

Formulation. Suspensions of CC-223 were prepared in aqueous 0.5% carboxymethyl cellulose and 0.25% Tween-80. The formulations were homogenized using a Teflon pestle and mortar (Potter-Elvehjem tissue grinder). For multiday studies, the compound was freshly formulated every third day. Between doses, the formulated compound was stored under constant stirring using a
magnetic stirrer at 4°C in the dark. The test article and vehicle were administered by oral gavage.

PC-3 in vivo studies

Xenograft studies. Mice were inoculated s.c. with 2 × 10⁶ PC-3 cells. When tumors reached approximately 125 mm³, mice were randomized and treated once daily, twice daily, or every 2 days orally with vehicle or various doses of CC-223, at a dose volume of 5 mL/kg. The twice-daily doses were administered with a 10 hours separation between the morning and evening doses. Tumor volumes were determined before the initiation of treatment and were considered as the starting volumes. Tumors were measured twice a week for the duration of the study. The long and short axes of each tumor were measured using a digital caliper in millimeters. Tumor volumes were calculated using the formula: width² × length/2. The tumor volumes were expressed in cubic millimeters (mm³).

Pharmacokinetic/pharmacodynamic studies. Mice bearing PC-3 tumor ranging 300 to 500 mm³ were randomized and treated with a single dose of vehicle or CC-223. At predetermined time points following dosing, mice were euthanized and plasma and tumor samples were collected for pharmacokinetic/pharmacodynamic (PK/PD) determination. Compound concentrations in plasma and tumor were determined by LC/MS-MS. PD markers, pS6RP(S235/6), and pAKT(S473), were measured using MesoScale technology. Evaluation of PD marker p4EBP1(T37/46) in vivo was determined using MesoScale technology, following six daily doses of 5 mg/kg in mice bearing PC-3 tumor ranging between 300 and 500 mm³.

Mechanism of action studies. Mice bearing PC-3 tumors ranging 300 to 500 mm³ were dosed orally with vehicle or CC-223 at 25 mg/kg once daily for 6 days. Two hours after the last dose, mice were euthanized and tumors were dissected. Tumors were snap frozen in liquid nitrogen and processed for immunofluorescence and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as described previously (14).

Other in vivo studies

U-87 MG (5 × 10⁶), HCT 116 (2 × 10⁶), MDA-MB-231 (2 × 10⁶), and A549 (2 × 10⁶) studies were performed as described for PC-3, with the indicated number of cells s.c. inoculated on day 0. For the non–small cell lung carcinoma (NSCLC) patient-derived xenograft (PDX) model ST140, immunocompromised mice (Harlan; nu/nu) 4 to 6 weeks of age were housed in individual HEPA ventilated cages on a 12-hour light-dark cycle. Animals were fed water ad libitum and an irradiated standard rodent diet (Teklad 2919). Animals were implanted unilaterally on the right flank with tumor fragments harvested from 2 to 4 host animals. When tumors reached approximately 125 to 250 mm³, animals were matched by tumor volume into treatment and control groups and dosing initiated (day 0). Beginning day 0, tumor dimensions were measured twice weekly by digital caliper and data, including individual and mean estimated tumor volumes, recorded for each group. Tumor volume was calculated using the formula, \( TV = width^2 \times length \times 0.52 \).

Statistical analysis

Xenograft and in vivo biomarker data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism. A one-way ANOVA was performed for tumor volume and PD marker measurements. Post hoc analysis was performed using the Dunnett test where all treatment groups are compared with the vehicle control.

Results

CC-223 is a potent and selective inhibitor of mTOR kinase

CC-223 was identified following compound optimization effort that began with an imidazo[4,5-b]pyrazin-2-one screening hit (15). Optimization for potency, selectivity and PK properties led to the dihydropyrazin[2,3-b]pyrazin-2(1H)-one series and the discovery of CC-223 (Fig. 1A). CC-223 is a potent inhibitor of mTOR kinase, with an IC₅₀ value for mTOR kinase of 0.016 μmol/L. CC-223 is selective for mTOR kinase with >200-fold selectivity over the related PI3K-α (IC₅₀ = 4.0 μmol/L) of the PI3K related kinases tested. CC-223 shows no significant inhibition of ATR or SMG1 and inhibits DNA-PK with an IC₅₀ value of 0.84 μmol/L. When screened in a single-point assay against a commercially available panel of 246 kinases (16), only three kinases other than mTOR were inhibited >80% at 10 μmol/L by CC-223 (Supplementary Table S1). Upon follow-up IC₅₀ value determination, only two were inhibited by CC-223 with IC₅₀ values below 1 μmol/L: FLT4 (0.651 μmol/L) and cFMS (0.028 μmol/L). The exquisite kinase selectivity of CC-223 was confirmed upon evaluation in cellular systems using ActivX KinNavtiv profiling (17, 18). Other than mTOR kinase, no kinase target was identified when HCT 116 or A549 cells were treated for 1 hour with 1 μmol/L CC-223 and assayed for kinase activity.

CC-223 inhibits both mTORC1 and mTORC2 in cellular systems

Cellular inhibition of the PI3K–mTOR pathway by CC-223 was first assessed in PC-3 prostate cancer cells by Western blot analysis (Fig. 1B). Like rapamycin, CC-223 inhibits the direct and indirect mTORC1 substrates p-p70S6K and pS6RP. However, unlike rapamycin, CC-223 more fully inhibits mTORC1 activity, demonstrating a concentration-dependent reduction in the direct marker p-p70S6K (T46). CC-223 also demonstrated inhibition of the mTORC2 complex, as assessed by reduction in phosphorylation of the direct substrate AKT(S473) and the downstream markers PRAS40(T246) and GSK3β(S9). Rapamycin did not inhibit the kinase activity of TORC2, consistent with previous reports (19, 20). The IC₅₀ value for pS6RP(S235/S236), p4EBP1(T46), and pAKT(S473) in PC-3 cells was assessed using MesoScale assays following 1 hour of compound treatment (Fig. 1C). As observed by Western blot analysis, CC-223 showed a concentration-dependent reduction in each marker, with IC₅₀ values of 31 ± 2 nmol/L for pS6RP, 403 ± 47 nmol/L for p4EBP1, and 11 ± 10 nmol/L for pAKT(S473). Inhibition of these pathway biomarkers was investigated in additional cell types from a variety of tissue origins. CC-223 inhibited both mTORC1 (S6RP and 4EBP1) and mTORC2 [AKT(S473)] markers across the panel with IC₅₀ ranges of 27 to 184 nmol/L for pS6RP, 120 to 1,050 nmol/L for p4EBP1 and 11 to 150 nmol/L for pAKT(S473) (Table 1 and Supplementary Table S2).

Treatment of HCT 116 colon cancer cells with rapamycin results in the feedback loop mediated upregulation of pAKT(S473) to approximately 250% compared with the level of DMSO-treated cells (Fig. 1D). In contrast, treatment of HCT 116 cells with...
CC-223 leads to potent inhibition of pAKT(S473) and further, CC-223 is able to inhibit the increase induced by rapamycin when cells are cotreated with both compounds.

CC-223 inhibits cell growth across a number of cancer cell lines, including cell lines insensitive to rapamycin treatment.

Rapamycin effects on proliferation tend to plateau in most cell lines, making the determination of IC50 values problematic.

We classified a small panel of cell lines for sensitivity to rapamycin as determined by the level of inhibition where this plateau occurred: sensitive 100% to 55% inhibition; partially sensitive 54% to 31% inhibition and insensitive 0% to 30%. For example, treatment with 0.1 to 10 μmol/L rapamycin in PC-3 resulted in a flat growth inhibition of roughly 75%, classifying PC-3 as rapamycin sensitive. In HCT 116, the growth inhibition plateaus at roughly 30%, resulting in a classification...
of rapamycin insensitive (Supplementary Fig. S1A). Treatment with CC-223 in these two cell lines results in a more traditional dose–response curve and allows for the determination of IC_{50} values (Supplementary Fig. S1B). The effect of CC-223 on cellular proliferation was investigated in the panel of cell lines assessed for sensitivity to rapamycin. CC-223 was found to potentely inhibit cell growth across this cell panel, independent of the cell line’s rapamycin sensitivity, with IC_{50} values ranging from 92 to 1,039 nmol/L (Table 1 and Supplementary Table S3).

### Table 1. Cellular growth inhibition and TORC1 and TORC2 cellular biomarker inhibition for CC-223 across a panel of cell lines classified by rapamycin sensitivity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>Rapa sensitivity</th>
<th>Proliferation IC_{50} (µmol/L)</th>
<th>pS6RP IC_{50} (µmol/L)</th>
<th>p4EBP1 IC_{50} (µmol/L)</th>
<th>pAKT(S473) IC_{50} (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>Prostate</td>
<td>Sensitive</td>
<td>0.314</td>
<td>0.031</td>
<td>0.405</td>
<td>0.011</td>
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<tr>
<td>CAL-51</td>
<td>Breast</td>
<td>Sensitive</td>
<td>0.140</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AU565</td>
<td>Breast</td>
<td>Sensitive</td>
<td>0.092</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon</td>
<td>Inensitive</td>
<td>0.329</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Lung</td>
<td>Partially</td>
<td>0.200</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HepG2</td>
<td>HCC</td>
<td>Partially</td>
<td>0.321</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>Sensitive</td>
<td>0.208</td>
<td>0.036</td>
<td>0.330</td>
<td>0.177</td>
</tr>
<tr>
<td>T47D</td>
<td>Lung</td>
<td>Partially</td>
<td>0.555</td>
<td>0.184</td>
<td>1.05</td>
<td>0.150</td>
</tr>
<tr>
<td>U87MG</td>
<td>Glioma</td>
<td>Partially</td>
<td>0.358</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HEPG2</td>
<td>Glioma</td>
<td>Partially</td>
<td>0.555</td>
<td>0.184</td>
<td>1.05</td>
<td>0.150</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>Sensitive</td>
<td>0.669</td>
<td>0.027</td>
<td>0.120</td>
<td>0.036</td>
</tr>
<tr>
<td>NCI-H23</td>
<td>Lung</td>
<td>Insensitive</td>
<td>1.039</td>
<td>0.071</td>
<td>0.120</td>
<td>0.094</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

Analysis by tumor type showed that DLBCL, FL, and AML cells were more sensitive to CC-223 than MCL and ALCI lines. RPPA analysis identified that IRF4 protein level positively correlated with CC-223 GI_{50} values. These data suggest that cell lines with high IRF4 tend to be less sensitive CC-223 (Fig. 2D; Supplementary Table S5). Within the DLBCL lines, RPPA analysis identified mTOR pathway biomarkers that are associated with sensitivity to CC-223. A subgroup of DLBCL lines express high levels of mTOR biomarkers such as p-mTOR S2448, p-p70S6K T389, pGSK3β S9 and S21, pAKT T308 and S473, pTSC2 T1462, pS6 S235/236 and S240/244 and are more sensitive to CC-223 than the lines expressing low levels of those biomarkers (Fig. 2E).

### CC-223 inhibits cell growth and induces apoptosis in a panel of hematologic cancer lines

The antiproliferative effect of CC-223 was further investigated in a panel of 40 hematologic tumor cell lines. The panel was comprised of 23 diffused large B-cell lymphoma (DLBCL), 4 follicular lymphoma (FL), 5 acute myelogenous leukemia (AML), 6 mantle cell lymphoma (MCL), and two anaplastic large cell lymphoma (ALCL) lines. Potent growth inhibitory effects were observed with GI_{50} values below 700 nmol/L in 33 of 40 lines and GI_{50} Values between 1 and 5 µmol/L in the remaining seven lines (Fig. 2A; Supplementary Table S4). To evaluate induction of apoptosis, caspase 3/7 activation was determined after 24 hours of CC-223 treatment using the Caspase 3/7-Glo assay. Caspase 3/7 activation occurred in 10 cell lines with less than 2 µmol/L CC-223, suggesting that CC-223 caused apoptosis in these cell lines (Fig. 2B; Supplementary Table S4). In contrast, rapamycin caused a 2-fold caspase activation in only four lines at a concentration below 2 µmol/L. The concentration response curves for CC-223 and rapamycin in WSU-DLCL2 are shown as an example (Fig. 2C). The kinetics of apoptosis induction by CC-223 was investigated in WSU-DLCL2 and KARPAS-1106P using Annexin V and propidium iodide staining. Apoptotic cells were detected at 24 hours after treatment and the percentage of apoptotic cells increases at 48 and 72 hours with a corresponding decrease of viable cells (data not shown).

### Correlative analysis of CC-223 sensitivity in a panel of hematologic cancer lines

In an effort to identify predictive biomarkers that correlate with sensitivity to CC-223, correlative analyses were performed between CC-223 GI_{50} or CalX values and various baseline features of the cell lines such as tumor type, mutation, and protein expression. RPPA was used to evaluate 262 proteins or phosphoprotein levels at baseline in 37 of the 40 hematologic cell lines. Analysis by tumor type showed that DLBCL, FL, and AML cells were more sensitive to CC-223 than MCL and ALCI lines. RPPA analysis identified that IRF4 protein level positively correlated with CC-223 GI_{50} values. These data suggest that cell lines with high IRF4 tend to be less sensitive CC-223 (Fig. 2D; Supplementary Table S5). Within the DLBCL lines, RPPA analysis identified mTOR pathway biomarkers that are associated with sensitivity to CC-223. A subgroup of DLBCL lines express high levels of mTOR biomarkers such as p-mTOR S2448, p-p70S6K T389, pGSK3β S9 and S21, pAKT T308 and S473, pTSC2 T1462, pS6 S235/236 and S240/244 and are more sensitive to CC-223 than the lines expressing low levels of those biomarkers (Fig. 2E).

### CC-223 inhibits both mTORC1 and mTORC2 in vivo following a single oral dose in PC-3 tumor-bearing mice

The extent and duration of mTOR pathway inhibition and the relationship of the inhibition of pathway biomarkers to exposure (PK/PD relationship) was evaluated in PC-3 tumor-bearing mice. CC-223 was administered as a single dose of 1, 10, or 25 mg/kg. Plasma and tumor samples were collected at various time points for analysis. When CC-223 was dosed at 10 mg/kg, significant (P < 0.001) inhibition of pS6RP was maintained at 88% to 95% from 1 to 8 hours. Similarly, inhibition of pAKT (P < 0.001) from 1 to 8 hours was 64% to 93%. Beyond 8 hours when compound concentrations in the plasma and tumors were lower, the PD marker inhibition was diminished, but levels were still significantly (P < 0.01) lower than vehicle controls (Fig. 3A). At a lower dose of 1 mg/kg, pS6RP inhibition (P < 0.001) was observed up to 4 hours at 79% to 83%. Significant (P < 0.001) pAKT inhibition was observed at all time points, ranging from 67% to 72% through 4 hours (Fig. 3B). At 25 mg/kg, full inhibition of both pS6RP and pAKT was maintained through 16 to 24 hours (Supplementary Table S6). At early time points in all studies, the level of biomarker inhibition correlated well with the plasma and tumor compound levels (Supplementary Table S6). Using the data from the single dose in vivo biomarker studies at various dose levels, we built a PK/PD model on the correlation of biomarker inhibition with compound concentrations in plasma (Fig. 3C and Supplementary Fig. S2A). PK/PD modeling showed that the maximum effect (E_{max}) and 50% effective concentration (EC_{50}) values for pS6RP inhibition were 89.6% and 0.0478 µmol/L, respectively, and the E_{max} and EC_{50} values for pAKT(S473) inhibition were 88.1% and 0.174 µmol/L, respectively.
CC-223 inhibits mTORC1 marker p4EBP1(T37/46) in vivo in PC-3 tumor-bearing mice

Significant inhibition of p4EBP1 was observed at all time points collected when CC-223 was administered at 5 mg/kg once daily for 6 days. Inhibition of 71% \((P \leq 0.001)\) was observed at 1 hour, with 34% to 20% inhibition measured from 3 to 10 hours (Supplementary Fig. S2B).

CC-223 displays dose-dependent tumor growth inhibition in numerous solid tumor xenograft models

The antitumor activity of CC-223 in the PC-3 xenograft model was determined using a number of dosing paradigms. CC-223 significantly inhibited PC-3 tumor growth in a dose- and schedule-dependent manner. Dosing at 10 or 25 mg/kg, once daily, resulted in 46% \((P < 0.001)\) and 87% \((P < 0.001)\) reduction in tumor volume, respectively (Fig. 4A). Similar dose dependency was observed with twicedaily dosing at 5 or 10 mg/kg, corresponding to 65% \((P < 0.001)\) and 80% \((P < 0.001)\) tumor volume reductions (Fig. 4B). All dose levels were tolerated in the once-daily and twice-daily dosing studies, with only the 25 mg/kg/d group showing any significant body weight loss. These mice lost approximately 10% of their initial body weight after 3 weeks of dosing.

The effect of CC-223 on PC-3 tumor regression was evaluated in mice bearing large established tumors. Dosing was initiated on day 20 when average tumor volumes ranged between 450 and 486 mm\(^3\). CC-223 caused regression of the...
PC-3 tumors when dosed twice daily at 10 mg/kg (Fig. 4C). The average tumor volume on day 43 was significantly (P < 0.01) smaller than the average starting volume at dosing initiation (343.6 ± 32.6 mm$^3$ on day 43 vs. 482.2 ± 16.6 mm$^3$ on day 20). The twice daily dose of 5 mg/kg resulted in tumor stasis and the 1 mg/kg twice-daily dose significantly

Figure 3.
PK/PD relationship of CC-223 in mice with PC-3 tumors with a single oral dose of 10 mg/kg (A) or 1 mg/kg (B). Inhibition of pS6RP and pAKT$^{S473}$ in the tumors was correlated with the compound levels in both plasma and tumors. C, PK/PD model simulation of predicted biomarker inhibition at given plasma concentrations of CC-223.
(P < 0.01) slowed tumor growth. CC-223 at 25 mg/kg every 2 days caused regression of tumors initially during the first 2 weeks of dosing. Dosing once daily at 25 mg/kg was not tolerated in mice with the larger starting tumor size and this group was terminated on day 26 due to excessive weight loss. With the exception of the 25 mg/kg daily dosing group, no significant change in body weight was observed in any of the dosing groups in the regression study.

Dose-dependent antitumor activity was also observed in U-87 MG tumor xenografts with significant (P < 0.001) tumor volume reductions of 81%, 75%, 65%, and 49% after 5, 3, 1, and 0.5 mg/kg/d, respectively (Fig. 4D). CC-223 was evaluated in a patient-derived lung adenocarcinoma PDX model, ST140. CC-223 showed dose-dependent reduction of tumor growth, with 43% and 47% tumor growth inhibition at once-daily doses of 5 and 10 mg/kg (Fig. 4E). CC-223 has...
resulted in tumor growth inhibition in xenograft models of prostate, glioma, breast, lung, and colon. In the wide variety of models, treatment with CC-223 resulted in tumor growth inhibitions from 47% to 95% at tolerated doses (Supplementary Fig. S3A–S3C; Supplementary Table S7).

CC-223 inhibits proliferation and angiogenesis and induces apoptosis in PC-3 xenograft models

To investigate the mechanism of action of CC-223, PC-3 tumor-bearing mice were treated once daily for 6 days at 25 mg/kg and tumor samples were taken 2 hours following the sixth dose. Significant reduction, 84%, in the number of proliferating cells in the CC-223 treated tumors was determined by immunofluorescence staining for Ki67 (Fig. 5A). Staining with anti-CD31 antibody showed that CC-223–treated tumors had significantly fewer CD31-positive blood vessels (Fig. 5A). Induction of apoptosis was determined by TUNEL staining. When compared with vehicle control, the CC-223–treated tumors showed a 2-fold increase in TUNEL-positive cells in the PC-3 model (Fig. 5B).

Discussion

In this article, we describe the preclinical characterization of CC-223 as an orally available, potent and selective inhibitor of mTOR kinase. The impressive biochemical kinase selectivity was confirmed upon evaluation in cellular systems where binding interaction with no kinase other than mTOR was identified following treatment in cells at 1 μmol/L. CC-223 demonstrates inhibition of mTORC1 (pS6RP and p4EBP1) and mTORC2 [pAKT(S473)] and growth inhibitory activity was demonstrated, both in cells and in vivo, across a variety of tumor cell types.

In contrast with rapamycin, which has been shown to only partially inhibit the mTORC1 complex and generally does not inhibit mTORC2 function (19, 20), mTOR kinase domain inhibitors, such as CC-223, more fully inhibit the function of both
mTOR complexes. Although rapamycin has a profound inhibition of phosphorylation of p70S6K and 4E-BP1, there is little effect on the mTORC1 substrate 4E-BP1. CC-223, in contrast with rapamycin, shows a concentration-dependent inhibition of phosphorylation of both p70S6K and 4E-BP1 substrates of the mTORC1 complex and also potently inhibits the phosphorylation of the mTORC2 substrate AKT(S473). These added effects likely contribute to the antiproliferative properties of CC-223, observed even in cell lines that are relatively insensitive to rapamycin treatment. The results in rapamycin-insensitive cell lines, such as HCT 116, support the hypothesis that inhibition of both TORC1 and TORC2 complexes results in improved antiproliferative effects as compared with targeting only TORC1. In other cell lines with rapamycin insensitivity, such as NCI-H23 and MDA-MB-231, growth inhibition is observed with CC-223 treatment, but at higher concentration than observed in the more sensitive lines. The growth inhibition profiles in these lines, as compared with rapamycin treatment, supports the improved effect when targeting both mTOR complexes, but the higher IC_{50} values suggest something other than the mTOR pathway is likely driving cell survival. The potential therapeutic benefit of increased pathway inhibition by mTOR kinase inhibitors relative to rapamycin was also demonstrated in the lymphoma panel apoptotic study. CC-223 treatment caused apoptosis in a significant percentage of the lymphoma cell line panel where rapamycin caused cell death in only a small number of cases.

In the hematologic cell panel, CC-223 showed potent growth inhibition across the panel with GI_{50} values ranging from 25 to 645 nmol/L in 33 of the 40 lines. The remaining seven lines were less sensitive with GI_{50} values from 1 to 4.8 nmol/L. Although inhibitors of mTOR in general do not cause apoptosis in solid tumor lines, we found that CC-223–induced apoptosis in many hematologic cancer lines at concentrations less than 2 μmol/L. Given the exquisite kinase selectivity of CC-223, these data suggest that some hematologic cancer cells are dependent on mTOR activity for survival. Although the fact that rapamycin also causes apoptosis in some of the same lines further supports this notion, the apoptotic induction capability of rapamycin is limited to a small number of lines in comparison with CC-223, suggesting that more complete inhibition of TORC1 (p4EBP1) and inhibition of TORC2 activity is important. Bioinformatic analysis identified IRF4 and mTOR pathway biomarkers as potential markers to determine whether tumors may be resistant or sensitive to CC-223. IRF4 is a target gene of NFκB and an important player in B-cell function. The correlation of sensitivity to CC-223 and IRF4 level suggests a hypothesis that activation of the NFκB pathway may render cells less sensitive to CC-223. Cross-talk between the PI3K and the NFκB pathways have been reported previously (21). However, more studies are needed to confirm this hypothesis.

In the DLBCL subpanel, we found a group of DLBCL lines with high expression levels of mTOR pathway biomarkers, indicative of activation of both TORC1 and TORC2. These lines were more sensitive to CC-223 than lines with low expression of the mTOR pathway biomarkers. These data strongly suggest that activation of both TORC1 and TORC2 is associated with sensitivity to CC-223 in DLBCL lines. Therefore, biomarkers for mTOR activation may serve as predictive biomarkers for CC-223 in DLBCL.

In mouse xenografts, CC-223 was well tolerated in multiple xenograft studies, inducing dose-dependent tumor growth inhibition and/or regression across a number of tumor models, including a patient-derived xenograft model. In addition to effects on cell growth, the mTOR pathway also plays a role in angiogenesis (23). Our in vivo mechanistic study assessing blood vessel density by anti–CD-31 (24), shows CC-223 treatment results in a significant reduction in tumor vascularization. Taken together, the PK/PD and mechanistic studies in PC-3, suggest that mTOR pathway inhibition, leading to antiproliferative activity, apoptotic activity, and antiangiogenic activity are the potential mechanisms underlying the antitumor activity of CC-223 in vivo.

Although evidence of a small amount of apoptosis was observed in the in vivo models, in most solid tumor lines tested in vitro, treatment with CC-223 generally resulted in G1 cell-cycle arrest and inhibition of growth, but not induction of apoptosis (Supplementary Fig. S1C–S1D). This is consistent with other selective inhibitors of mTOR kinase (25), arguing that, although the mTOR pathway is important for cell growth, other pathways support survival. The lack of cell killing as a single-agent suggests that combination treatment, with standard of care agents or other targeted therapies, may be required to achieve cell death and tumor regression in patients with solid tumors. Other combinations with mTOR kinase inhibitors have been reported (26, 27) and the combination of CC-223 with Erlotinib or CC-486, an oral formulation of azacitidine, is under clinical study in NSCLC (28). In addition, the combination of CC-223 with CC-122 (immunomodulator), with and without rituximab, is being evaluated in DLBCL (29).

In summary, CC-223 is a potent and selective inhibitor of mTOR kinase with effects on both mTORC1 and mTORC2 substrates in cellular systems and in vivo xenograft studies. CC-223 has excellent PK properties and induces dose-dependent growth inhibition across a broad panel of tumor cell lines and xenograft models. In the hematologic cell panel,
potential markers to determine whether tumors may be resis-
tant or sensitive to CC-223 were identified. CC-223 is currently
being evaluated in phase I clinical studies.

Disclosure of Potential Conflicts of Interest
M. Hickman has ownership interest (including patents) Celgene Corpora-
tion. H.K. Raymon reports receiving commercial research support from and has
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