CX-4945, an Orally Bioavailable Selective Inhibitor of Protein Kinase CK2, Inhibits Prosurvival and Angiogenic Signaling and Exhibits Antitumor Efficacy


Abstract

Malignant transformation and maintenance of the malignant phenotype depends on oncogenic and non-oncogenic proteins that are essential to mediate oncogene signaling and to support the altered physiologic demands induced by transformation. Protein kinase CK2 supports key prosurvival signaling pathways and represents a prototypical non-oncogene. In this study, we describe CX-4945, a potent and selective orally bioavailable small molecule inhibitor of CK2. The antiproliferative activity of CX-4945 against cancer cells correlated with expression levels of the CK2α catalytic subunit. Attenuation of PI3K/Akt signaling by CX-4945 was evidenced by dephosphorylation of Akt on the CK2-specific S129 site and the canonical S473 and T308 regulatory sites. CX-4945 caused cell-cycle arrest and selectively induced apoptosis in cancer cells relative to normal cells. In models of angiogenesis, CX-4945 inhibited human umbilical vein endothelial cell migration, tube formation, and blocked CK2-dependent hypoxia-induced factor 1 alpha (HIF-1α) transcription in cancer cells. When administered orally in murine xenograft models, CX-4945 was well tolerated and demonstrated robust antitumor activity with concomitant reductions of the hypoxia-induced factor 1 alpha (HIF-1α) transcription in cancer cells.

Introduction

Cancer is a disease of cellular dysregulation arising from an accumulation of genetic errors and overexpression of elements required to maintain the transformed state (1, 2). Mutations arising in receptor tyrosine kinases and serine/threonine kinases are frequently implicated in oncogenesis and have consequently been the focus of extensive efforts to design molecularly targeted anticancer therapies (3). Other proteins, including kinases, that may not be directly transformative but serve to sustain the cancer phenotype have been less considered as anticancer targets until recently. A new model of cancer is emerging, whereby the establishment and maintenance of malignancy depends on the cooperation of deregulated oncoenes and an array of equally essential deregulated non-oncogenes (1, 4). Although oncogenes are critical for direct transformation, non-oncogenes are required for maintaining the transformed phenotype. The overexpression or deregulation of typically nonmutated non-oncogene products protects the cell from the increased burden of cellular stress that is an invariable hallmark of transformation resulting from oncogenic activation (1, 2, 4). As this overexpression of otherwise normal proteins is required to maintain oncogenic signaling, inhibition of non-oncogenic signaling represents an underexploited therapeutic approach for targeting processes essential for maintaining the cancer phenotype.

Among the proteins that sustain the transformed phenotype, protein kinase CK2 has recently become recognized as a prototypical non-oncogene (5). Protein kinase CK2 is a tetrameric enzyme composed of 2 catalytic (α and/or α′) subunits and 2 regulatory (β) subunits. CK2 is a highly conserved, constitutively active serine/threonine protein kinase that regulates multiple pathways including PI3K/Akt and WNT signaling cascades, NF-κB transcription, and the DNA damage response (reviewed in refs. 6 and 7). CK2 can be described as a promoter of the hallmarks of cancer, further emphasizing the known pleiotropy of this kinase. Although CK2 has long been known for its proproliferative and anti-apoptotic properties, an understanding of the extensive contribution of this protein kinase to tumor maintenance is just beginning to emerge (5).
In keeping with its non-oncogene profile, mutations in CK2 have not been reported. However, CK2 gene amplifications and an associated reduction in patient survival have been described in squamous cell lung cancer (8). Likewise, CK2 activity and expression levels are elevated in many cancers of diverse genetic background including breast (9), lung (8), prostate (10), colorectal (11), renal (12), and leukemias (13), and the overexpression of CK2 in cancer tissues has frequently been linked to disease progression and poor prognosis (reviewed in refs. 14 and 6). Collectively, these characteristics identify CK2 as a scientifically validated cancer target that remains unexploited as a therapeutic target.

Non-oncogenic proteins fall into 2 broad categories: intrinsic, which principally regulate prosurvival processes within the cancer cell, and extrinsic, which develop and support the tumor microenvironment (1). CK2 may be described as promoting both intrinsic and extrinsic non-oncogenic processes as a consequence of its impact on multiple signaling pathways. CK2 does not function simply as a signal transduction kinase in the classical sense, mediating "longitudinal signaling" from the cell surface to the nucleus, but rather plays a "lateral role" in multiple prosurvival signaling cascades (5, 6). These include intrinsic processes such as the regulation of cell-cycle proteins, PI3K/Akt signaling, and apoptosis pathways (reviewed in refs. 6, 7, and 15). The PI3K/Akt pathway is the most commonly altered signaling pathway in human cancers (16) and this pathway is susceptible to activation by CK2 (17, 18). Furthermore, CK2 has been characterized as a "master regulator" of angiogenesis (19), an extrinsic non-oncogene–driven process. CK2 is elevated under hypoxic conditions, regulates hypoxia-inducible transcription factor 1 alpha (HIF-1α) activity (20) and mediates the aberrant response of epithelial cells to growth factor signaling (21). Thus, CK2 drives both intrinsic and extrinsic processes to promote the cancer phenotype.

Several research groups have reported that inhibition of CK2, either by siRNA directed toward CK2α (22) or by using chemical CK2 inhibitors such as 4,5,6,7-tetram bromobenzotriazole (23) and 2-(dimethylamino)-4,5,6,7-tetram bromo–2H-benzimidazole (24), induced apoptosis and prevented the proliferation of cancer cells. Furthermore, antisense oligonucleotides directed toward CK2α have been shown to induce apoptosis and exhibit antitumor activity in xenograft models (22, 25). Given the generalized overexpression of CK2 in cancers, the central role of CK2 in multiple pathways that support the cancer phenotype and the evidence that CK2 is an essential protein for cancer cell survival, the development of selective CK2 inhibitors has emerged as an attractive targeted approach for the treatment of cancers (26).

Herein we describe CX-4945, a potent, selective, and ATP-competitive inhibitor of both isoforms of the CK2 catalytic subunits CK2α and CK2α′ with IC₅₀ values of 1 nmol/L. CX-4945 inhibited prosurvival and angiogenic signaling in cancer cell lines and exhibited antitumor activity in murine xenografts. Collectively, these studies indicate that the antitumor mechanism of action is mediated through inhibition of CK2-dependent intrinsic and extrinsic processes required to maintain the cancer phenotype. Finally, CX-4945 is the first orally bioavailable small molecule inhibitor of CK2 to advance into human clinical trials, thereby paving the way for an entirely new class of targeted treatment for cancer.

**Materials and Methods**

**Materials**

CX-4945 5-(3-chlorophenylamino)benzo[c][2,6]naphthyridine-8-carboxylic acid) was synthesized by Cylene Pharmaceuticals as a golden colored solid material (>99% pure by high-performance liquid chromatography assay). A 5 mmol/L stock solution in dimethyl sulfoxide was prepared and stored at −70°C. The drug was diluted directly into growth media immediately prior to use.

**Enzyme assays**

The 238 kinase selectivity panel was conducted using the Kinase Profiler service offered by Millipore, which utilizes a radiometric filter-binding assay. The percent inhibition of each kinase was estimated using 0.5 μmol/L CX-4945 at ATP concentrations equivalent to the Kᵢ value for ATP for each respective human recombinant kinase. The determination of IC₅₀ values was done at ATP concentrations equivalent to the Kᵢ for ATP for each kinase using 9 concentrations of CX-4945 over a range of 0.0001 to 1 μmol/L. The Kᵢ value (inhibition constant) for CX-4945 against recombinant CK2 was determined by graphing the IC₅₀ values of CX-4945 in the presence of various concentrations of ATP against the concentration of ATP. The Kᵢ value is equivalent to the Y-intercept according to the Cheng–Prusoff equation (ref. 27: Kᵢ = IC₅₀/(1+ [ATP]/Kᵢ), where Kᵢ is the inhibition constant and Kᵢ is the Michaelis constant.

**Cell culture**

SUM 149PT and SUM 190PT inflammatory breast cancer cells were purchased from Asterand. Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza Inc. All other cell lines were purchased from American Tissue Culture Collection. Cell lines were cultured according to the suppliers’ recommendations.

**Akt pathway analysis**

BT-474 (breast) or BxPC-3 (pancreatic) cancer or HUVEC cells were treated with a range of concentrations of CX-4945 for the durations stated in the figure legends. Cells (1–3×10⁵) were washed twice with cold PBS and lysed in 100 μL of lysis buffer [RIPA Buffer purchased from Sigma; 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 1× Serine Protease Inhibitor Cocktail Set I purchased from Calbiochem; 1× Phosphatase Inhibitor Cocktail Set II and Microcystin LR (1 mmol/L) purchased from Sigma]. Primary antibodies (PTEN, Akt-S129, p21-T145, and p21) were purchased from Abcam, and PTEN-S370, PTEN-S380, Akt, Akt-S473, and Akt-T308 were purchased from Cell Signaling.

**Alamar Blue cell viability assay**

Various cell lines were seeded at a density of 3,000 cells per well 24 hours prior to treatment, in appropriate media, and then treated with indicated concentrations of CX-4945. Suspensions cells were seeded and treated on the same day.
Following 4 days of incubation, Alamar Blue (20 μL, 10% of volume per well) was added and the cells were further incubated at 37°C for 4–5 hours. Fluorescence with excitation wavelength at 530–560 nm and emission wavelength at 590 nm was measured.

**CK2 levels in breast cancer cells**

Breast cancer cells were plated in 15-cm dish format overnight. Cells were collected at 50% confluence by scraping into ice-cold PBS. The RNA was isolated from cells using RNeasy kit (Qiagen) and relative levels of CK2α, CK2βα, CK2β, and β-actin mRNA were measured using Applied Biosystems’ proprietary primers-probe sets. Analyses were performed on a 7900HT Real-Time PCR System (Applied Biosystems). Protein levels of CK2α, CK2βα, CK2β, and β-actin were measured by Western hybridization. Primary antibodies (CK2α and CK2β) were purchased from Santa Cruz Biotechnology, CK2β from Millipore, and β-actin from Sigma.

**Cell-cycle analysis**

Untreated and CX-4945-treated BT-474 and BxPC-3 cells were harvested by trypsinization, washed with ice-cold PBS, and fixed with ice-cold 70% ethanol for 4 hours. Cells were washed with PBS and RNA was digested for 1 hour at 37°C in staining buffer (0.25 mg/mL RNase A, 0.025 mg/mL propidium iodide, in PBS). Cells were analyzed with a BD LSR II flow cytometer, histograms were generated with BD FACSDiva Software v4.1.2, and cell-cycle phases analyzed with ModFit LT 3.1 SP3.

**Caspase 3/7 activation**

Caspase 3/7 enzyme activity was evaluated over a range of CX-4945 concentrations in multiple cell lines with Caspase-Glo assay kit (Promega). The luminescence of each sample was measured in a plate-reading luminometer (SpectraMax M5; Molecular Devices) with parameters of 1-minute lag time and 0.5-second/well read time. Caspase activity was normalized to cell number as measured by CyQUANT (Invitrogen).

**In vitro models of angiogenesis**

HUVEC proliferation, migration and tube-formation experiments were performed by Southern Research Institute. Experimental details are provided in the supplementary materials.

**Hypoxia studies**

BT-474 and BxPC-3 cells were plated in 10-cm dish format overnight. On the following day, the cells were treated with CX-4945 in degassed CO2-independent media (Invitrogen) for 48 hours under normoxic or hypoxic (94% N2, 1% O2, and 5% CO2) conditions. At the end of treatment, HIF-1α transcriptional activity was measured by luciferase assay. The resulting data were normalized for cell number determined with CyQUANT (Invitrogen).

**In vivo studies in animals**

Female immunocompromised mice CrTac-Ncr-Boxntm (5–7 weeks old) were obtained from Taconic Farms. Animals were maintained under clean room conditions in sterile filter top cages. Animals received sterile rodent chow and water ad libitum. All procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide: The Care and Use of Laboratory Animals.

**Efficacy studies**

Xenografts were initiated by subcutaneous injection of BxPC-3 cells into the right hind flank region of each mouse or BT-474 cells were injected into the mammary fat pad of mice implanted with estrogen pellets. When tumors reached a designated volume of 150–200 mm3, animals were randomized and divided into groups of 9 to 10 mice per group. CX-4945 was administered by oral gavage twice daily at 25 or 75 mg/kg for 31 and 35 consecutive days for the BT-474 and BxPC-3 models, respectively. Tumor volumes and body weights were measured twice weekly. The length and width of the tumor were measured with calipers and the volume calculated using the following formula: tumor volume = (length × width²)/2. Percent tumor growth inhibition (TGI) values were calculated on the final day of the study for CX-4945–treated compared to vehicle-treated mice and were calculated as 100 × (1 − [(TreatedFinal day − TreatedDay 1)/(ControlFinal day − ControlDay 1)]). The significance of the differences between the treated versus vehicle groups were determined using 1-way ANOVA (Graphpad Prism).

**Pharmacodynamic studies**

Xenografts bearing BxPC-3 tumors (300–400 mm³) were randomized and divided into 3 groups of 5 mice. CX-4945 was administered by oral gavage twice daily at 25 or 75 mg/kg for 3 days. Four hours after the final dose of CX-4945, the animals were euthanized, and tumors were resected and fixed in 10% buffered formalin for 24 hours before transferring to 70% ethanol. Immunohistochemistry (IHC) for phospho-p21 (T145) was performed at Vel-lab Research. Stained tumor sections were visualized on a Zeiss Axiosvert 200 fluorescence microscope (Zeiss) and the degree of staining was quantified using Axiovision40AV.4.5.0.0 software.

**Results**

**CX-4945 is a potent and selective inhibitor of CK2 activity**

Utilizing molecular modeling techniques and medicinal chemistry, CX-4945 was rationally designed to be a low molecular weight (MW 350), ATP-competitive inhibitor of
CK2(Fig. 1A). A full description of its discovery and synthesis will be provided elsewhere. CX-4945 proved to be a potent and ATP-competitive (Fig. 1B) inhibitor of recombinant human CK2 (CK2α \( IC_{50} = 1 \) nmol/L and \( K_i = 0.38 \) nmol/L ± 0.02; CK2α′ \( IC_{50} = 1 \) nmol/L) and was found to be selective for CK2 when evaluated in a biochemical kinase screen. Using a single concentration of 500 nmol/L (500-fold greater than the \( IC_{50} \) of CK2), only 7 of the 238 kinases tested were inhibited by more than 90% and those 7 were further subjected to \( IC_{50} \) determination (Column 1, Table 1). It is well understood that enzymatic potency determined in cell-free systems does not always translate into biological activity, therefore, CX-4945 (10 \( \mu \)mol/L) was evaluated in relevant cell-based functional assays for FLT3, PIM1, and CDK1, and was found to be functionally inactive against these kinases (data not shown).

**Antiproliferative activity and CK2 expression**

When profiled against a panel of diverse cancer cell lines, CX-4945 exhibited a broad spectrum of antiproliferative activity. COMPARE analysis (http://dtp.nci.nih.gov/docs/compare/compare_methodology.html) of these data (Fig. 1C) indicated that the breast cancer cell lines displayed the widest range of sensitivity to CX-4945.

In addition to assessment of the antiproliferative activity, a panel of 11 breast cancer cell lines was further characterized to determine potential predictors of sensitivity to CX-4945. For this panel of cell lines, mRNA and protein expression levels of the CK2 subunits were determined (representative Western blot shown in Fig. 2). An apparent correlation was found between sensitivity to CX-4945 and CK2α mRNA, and protein levels (Table 2) but no correlations were observed between sensitivity to CX-4945 and either mRNA or protein levels of the other subunits.
CK2α catalytic subunit or the regulatory CK2β subunit (data not shown). Furthermore, the PI3K/Akt or PTEN mutational status for the breast cancer panel did not reveal any clear correlations with antiproliferative activity (Table 2).

CX-4945 suppresses PI3K/Akt signaling in cancer cells

Given the prominent role of CK2 as a regulator of the PI3K/Akt signaling pathway by direct phosphorylation of PTEN (28) and Akt itself (17), combined with the fact that CX-4945 was shown to be inactive against key kinases within this pathway (Column 2, Table 1), we examined the effects of CX-4945 on CK2-regulated PI3K/Akt activity. For this analysis we chose 2 models on the basis of their PI3K status: (1) BT-474 breast cancer cells that bear an activating PIK3CA mutation and (2) BxPC-3 pancreatic cells in which overexpression of the epidermal growth factor receptor activates wild-type PI3K signaling by heterodimerization with HER3 (29). Treatment of BT-474 cells and BxPC-3 cells with CX-4945 led to rapid dephosphorylation of the CK2 phosphorylation site, Akt (S129) (Fig. 3A), which was accompanied by significant reductions in the phosphorylation levels of both canonical regulatory sites Akt (T308) and Akt (S473). No significant reduction in PTEN phosphorylation at the CK2 phosphorylation sites, S370/S380, were observed at this 4-hour time point (data not shown) suggesting that CX-4945 suppresses PI3K/Akt signaling by directly blocking the phosphorylation of Akt at Serine 129 by CK2 rather than through activation of PTEN.

Cell-cycle arrest and caspase 3/7 activation

CK2 has been widely characterized as an important regulator of the cell cycle and has been found to regulate cell-cycle transition through multiple phases in a

![Figure 2. Protein levels of CK2 subunits in a panel of breast cancer cell lines.](image)

### Table 1. IC<sub>50</sub> determinations for CX-4945 against selected kinases from the 238-kinase selectivity panel

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</th>
<th>Kinase</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK2α</td>
<td>1</td>
<td>PI3Kβ</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CK2α'</td>
<td>1</td>
<td>PI3Kδ</td>
<td>&gt;500</td>
</tr>
<tr>
<td>DAPK3</td>
<td>17</td>
<td>PI3Kγ</td>
<td>&gt;500</td>
</tr>
<tr>
<td>FLT3</td>
<td>35</td>
<td>PDK1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>TBK1</td>
<td>35</td>
<td>AKT1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CLK3</td>
<td>41</td>
<td>AKT2</td>
<td>&gt;500</td>
</tr>
<tr>
<td>HIPK3</td>
<td>45</td>
<td>AKT3</td>
<td>&gt;500</td>
</tr>
<tr>
<td>PIM1</td>
<td>46</td>
<td>mTOR</td>
<td>&gt;500</td>
</tr>
<tr>
<td>CDK1/cyclin B</td>
<td>56</td>
<td>p70S6K</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

### Table 2. Antiproliferative activity of CX-4945, CK2α mRNA, protein levels, and mutational status of the PI3K pathway in a panel of breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>CX-4945 EC&lt;sub&gt;50&lt;/sub&gt; (µmol/L)</th>
<th>CK2α mRNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CK2α protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI3K pathway mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKBr3</td>
<td>1.71</td>
<td>2.96</td>
<td>1.87</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td>2.36</td>
<td>2.57</td>
<td>2.42</td>
<td>PIK3CA</td>
</tr>
<tr>
<td>BT-474</td>
<td>2.64</td>
<td>2.45</td>
<td>1.33</td>
<td>PIK3CA</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>4.99</td>
<td>1.85</td>
<td>2.00</td>
<td>PTEN</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>6.42</td>
<td>1.46</td>
<td>1.49</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>8.30</td>
<td>1.04</td>
<td>1.83</td>
<td>PTEN</td>
</tr>
<tr>
<td>T47D</td>
<td>8.39</td>
<td>1.66</td>
<td>1.72</td>
<td>PIK3CA</td>
</tr>
<tr>
<td>MCF 7</td>
<td>8.88</td>
<td>1.39</td>
<td>1.25</td>
<td>PIK3CA</td>
</tr>
<tr>
<td>HS578T</td>
<td>13.12</td>
<td>1.00</td>
<td>1.00</td>
<td>WT</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>15.32</td>
<td>1.33</td>
<td>1.32</td>
<td>PIK3CA</td>
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<tr>
<td>UACC-812</td>
<td>20.01</td>
<td>1.28</td>
<td>1.44</td>
<td>WT</td>
</tr>
</tbody>
</table>

<sup>a</sup>MRNA and protein levels normalized to levels found in Hs578T cells.

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cell-type–dependent manner (30–35). Activated Akt has been reported to phosphorylate and thereby decrease the proteosomal stability of the p21 and p27 cell-cycle inhibitor proteins allowing cell-cycle progression (36, 37). Treatment of cells with CX-4945 resulted in reduced phosphorylation of p21 (T145) and increased levels of total p21 and p27. Interestingly, treatment of BT-474 cells with CX-4945 induced a G2/M cell-cycle arrest, whereas treatment of BxPC-3 cells caused arrest to occur in G1, indicating that cell-cycle regulation by CK2 is cell-type dependent (Fig. 3B). Because both CK2 and its substrates such as Akt, may protect cells from apoptosis by phosphorylating a wide range of proteins involved in the apoptotic response (35, 38–40), we examined levels of caspase 3/7 activity in a set of transformed and normal cell lines in response to CX-4945 treatment. All cancer cell lines responded to the 24-hour treatment with CX-4945 by induction of caspase 3/7 activity in a concentration-dependent manner, whereas all normal cell lines failed to show a detectable change in caspase 3/7 activity at concentrations as high as 100 μmol/L (Fig. 3C).

**Angiogenesis**

**Normoxia.** CK2 has been described as a master regulator of angiogenesis due to its role in multiple molecular events that contribute to angiogenesis (19). This includes regulation of the PI3K/Akt pathway, which has a

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**Figure 3.** A, effects of 4 hour exposure to CX-4945 on Akt and p21 phosphorylation in BT-474 and BxPC-3 cells. B, cell-cycle arrest induced by CX-4945 in BT-474 and BxPC-3 cells (24 hours). C, caspase 3/7 activation in cancer cell lines and normal cells treated with CX-4945 (24 hours).
well-characterized role in mediating the response of HUVEC to growth factor signaling (41). Treatment of HUVEC with CX-4945 led to the rapid dephosphorylation of the CK2 phosphorylation site Akt (S129) in addition to both canonical regulatory sites, Akt (T308) and Akt (S473) and the downstream Akt effector, p21 (T145) (Fig. 4A). We also examined PTEN phosphorylation in response to CX-4945 treatment. We observed a reduction of phosphorylation at the CK2-phosphorylation sites on PTEN (S370/S380) and a concomitant reduction in total PTEN levels, but only after 24 hour exposure to CX-4945. Next we measured the effects of CX-4945 on the ability of HUVEC to proliferate, migrate, and form tube-like structures as a result of serum stimulation in vitro. CX-4945 inhibited HUVEC proliferation (IC_{50} = 5.5 μmol/L at 72 hour; data not shown), migration (IC_{50} = 2 μmol/L at 24 hour), and tube formation (IC_{50} = 4.0 μmol/L at 18 hour; Fig. 4B).

**Hypoxia.** Hypoxia, a condition often found in solid tumors, is known to increase CK2 activity leading to the upregulation of histone deacetylases (HDAC1 and HDAC2) that inhibit the expression of tumor-suppressor genes pVHL and p53, both of which control the activity of HIF-1α, a major regulator of proangiogenic signaling (20, 42, 43). Therefore, we investigated the effects of CX2 inhibition on HIF-1α-dependent transcription under hypoxic conditions. Using qRT-PCR (quantitative real-time PCR), we demonstrated that exposure of BT-474 and BxPC-3 cells to hypoxia for 48 hours resulted in the activation of HIF-1α transcriptional activity, as indicated by a 2- to 3-fold increase in levels of aldolase mRNA, a known target of HIF-1α transcription. Moreover, hypoxia reduced the levels of pVHL (2- to 3-fold) and p53 (2-fold). Treatment of BT-474 and BxPC-3 cells with CX-4945 under hypoxic conditions prevented downregulation of p53 and pVHL and reduced activation of HIF-1α transcription as measured indirectly by a reduction of aldolase expression (Fig. 4C). These data were corroborated by the results obtained with a pHIF-1α/Luc reporter system in which CX-4945 inhibited hypoxia-activated HIF-1α–driven expression of luciferase in a concentration-dependent manner (Fig. 4D).

**Antitumor efficacy of CX-4945**

Antitumor efficacy studies were conducted in BT-474 and BxPC-3 xenograft models in which CX-4945 showed dose-dependent antitumor activity in both models when administered orally twice a day (Fig. 5A and B). In the BT-474 model, CX-4945 (25 or 75 mg/kg) exhibited 88% and 97% TGI, respectively, with 2 of 9 animals in each group showing more than 50% reduction in tumor size compared with the initial tumor volume. In the BxPC-3 model, CX-4945 (75 mg/kg) showed 93% TGI with 3 animals having no evidence of tumor remaining at the end of the treatment period. Extensive preclinical and clinical pharmacokinetic evaluation of CX-4945 has been performed and will be described in a separate publication. In brief, the peak plasma concentration (C_{max}) of CX-4945 was 15 μmol/L occurring at 15 minute postdose, which rapidly decreased to submicromolar levels (Clearance, CL_{a} = 2.7 L/kg/h), suggesting that efficacy was driven by C_{max} rather than sustained exposure above a threshold. CX-4945 was well tolerated at all doses tested as indicated by minimal body weight loss and no overt toxicity.

**Pharmacodynamic studies**

CX-4945 was evaluated for its effect on p21 (T145) in BxPC-3 xenograft tissue using immunohistochemical methods. The data shown represent scoring derived from 4 representative, random images taken from each of 3 tumors per group (Fig. 5C). When compared to the vehicle group, the 25 and 75 mg/kg groups showed dose-dependent reductions in the phosphorylation of p21 (T145) consistent with the antitumor effects observed with these dose levels (representative images shown in Fig. 5C).

**Discussion**

In the reported studies we describe a novel, orally bioavailable inhibitor of protein kinase CK2 and the consequences of inhibiting this enzyme in a variety of biological assays. We have shown that CX-4945 modulated PI3K/Akt signaling, cell-cycle progression, regulatory CDK proteins p21 and p27, and ultimately apoptosis in cancer cells. Our findings are consistent with the results obtained with the relatively nonselective natural product CK2 inhibitors apigenin and emodin, which have previously been reported to increase levels of p21 and p27, induce cell-cycle arrest, and trigger apoptosis in cancer cells (44, 45). CK2 has been shown to regulate PI3K/Akt signaling at multiple junctions along the signaling cascade including the Akt protein (reviewed in ref. 6). The phosphorylation of Akt by CK2 (at residue S129) has been reported to hyperactivate Akt beyond the activity level achieved by the phosphorylations of the canonical regulatory sites, T308 and S473 (17, 46). We have shown that CX-4945 treatment of the BT-474 and BxPC-3 cancer cell lines resulted in significant reductions in the phosphorylation of Akt (S129) with corresponding decreases in the phosphorylation of the canonical regulatory sites Akt (T308) and Akt (S473). Although the extent of modulation of Akt (T308) and Akt (S473) compared with Akt (S129) dephosphorylation varied between cell lines, these results highlight the specificity of Akt (S129) phosphorylation by CK2 and suggest that cell-type–specific factors influence the degree to which Akt (S129) and hence CK2 regulates Akt signaling. Moreover, modulation of Akt (S129) may serve as a valuable mechanism of action biomarker to determine the extent of pharmacodynamic modulation of CK2. Although the antibody reagents and methods to detect Akt (T308) and Akt (S473) are available and used in the clinical setting, validation of Akt (S129) antibodies, IHC and other clinically relevant detection methods are under development.

CK2 has been described as a master regulator of angiogenesis (19). Indeed, chemical inhibitors of CK2 have been shown to inhibit proangiogenic activity in HUVEC and block retinal neovascularization in mice (19, 21, 47). PI3K/Akt signaling is known to regulate the response of endothelial cells to growth factor signaling including proliferation, migration, and tube formation (41), and thus represents a molecular mechanism through which CK2 may drive angiogenic signaling. CX-4945 inhibited HUVEC proliferation, migration, and...
Figure 4. A, effects of CX-4945 on proangiogenic signaling under normoxia and hypoxia. Western blots show the effects of CX-4945 on Akt phosphorylation in HUVEC. B, micrographs show the effects of CX-4945 on the tube formation and migration in HUVEC (24 hours). C, effects of CX-4945 on aldolase, pVHL, and p53 expression in BT-474 and BxPC-3 cells under hypoxic versus normoxic conditions (48 hours). D, effects of CX-4945 on HIF-1α–driven luciferase expression in HeLa cells under hypoxic versus normoxic conditions (48 hours).
tube formation. The treatment of HUVEC with CX-4945 led to rapid dephosphorylation of Akt (S129) and also Akt (T308) and Akt (S473). Dephosphorylation of the CK2 phosphorylation sites on PTEN (S370/S380) was observed only after 24 hour exposure to CX-4945 with concomitant reductions in total PTEN levels. Neither of the CK2 phosphorylation sites on PTEN or the levels of total protein were significantly affected after 4-hour treatment, yet dephosphorylation of Akt was readily observed, suggesting that the effects of CX-4945 on Akt activity are mechanistically and temporally independent of CK2-regulated PTEN activity.

Another mechanism by which CK2 may contribute to angiogenesis is via regulation of HIF-1α. Hypoxia, a condition that is often present in solid tumors, stimulates HIF-1α and increases the activity of CK2, thereby mediating proangiogenic responses required for adaptation to hypoxia (20, 42, 43). Elevated CK2 activity results in the upregulation of HDAC1/HDAC2 histone deacetylases that inhibit the expression of...
tumor-suppressor genes pVHL and p53, both of which downregulate the activity of HIF-1α (20, 42, 43). CX-4945 inhibited hypoxia-driven HIF-1α activity in cancer cells and prevented downregulation of pVHL and p53, potentially indicating that CX-4945 inhibits CK2 regulation of histone deacetylases. Collectively these data demonstrate that CX-4945 inhibits angiogenic signaling in HUVEC and inhibits HIF-1α transcription under hypoxia, suggesting that CK2 inhibition effectively disrupts important adaptive processes required to promote the tumor microenvironment.

The hypothesis that tumors rely on CK2 for survival was tested in murine xenografts. BT-474 breast cancer and BxPC-3 pancreatic xenografts were selected for antitumor efficacy studies. CX-4945 exhibited robust antitumor activity including partial and complete regressions. In vivo administration of CX-4945 resulted in decreased phosphorylation of the Akt substrate p21 (T145) in tumor xenograft tissue demonstrating a clear pharmacodynamic effect in mice. CX-4945 was well tolerated as indicated by minimal body weight loss and no evidence of overt toxicity.

The network of proproliferative signaling pathways supported by CK2 can be best described within the context of "intrinsic" and "extrinsic" non-oncogene–driven processes. Herein, we have characterized CX-4945 as an inhibitor of both "intrinsic" and "extrinsic" CK2-driven mechanisms and demonstrated that CX-4945 is efficacious and well tolerated in xenograft models. Furthermore, we have shown that sensitivity to CX-4945 correlates with the expression levels of CK2α in a panel of breast cancer cell lines suggesting a potential strategy for patient selection. We have also demonstrated that phospho-p21 (T145), serves as an effective mechanism of action biomarker for CK2 inhibition in vitro and in vivo, and may have immediate clinical relevance because there is an established relationship between Akt activation, phospho-p21 (T145) status, and clinical prognosis in breast cancers (48, 49). The pleiotropy and widespread distribution of CK2 in normal tissues have raised concerns that inhibition of CK2 might give rise to unacceptable toxicity. In practice, however, we have shown that the consequences of CK2 inhibition are context dependent and cancer cells are more vulnerable to CK2 inhibition than normal cells. CX-4945 represents the first orally bioavailable small molecule inhibitor of CK2 with acceptable pharmaceutical properties to warrant advancement into human clinical trials, thereby opening the door to an entirely new class of therapeutics for cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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References


27. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–106.


29. Buck E, Eyzaguirre A, Haley JD, Gibson NW, Cagnoni P, Ivata KK. Inactivation of Akt by the epidermal growth factor receptor inhibitor erlotinib is mediated by HER-3 in pancreatic and colorectal tumor cell lines and contributes to erlotinib sensitivity. Mol Cancer Ther 2006;5:2051–9.


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