CX-4945 Blocks the "Master Regulator CK2" in Multiple Intracellular Signaling Pathways Revealing Significant Anti-Proliferative and Anti-Tumor Activities

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Abstract

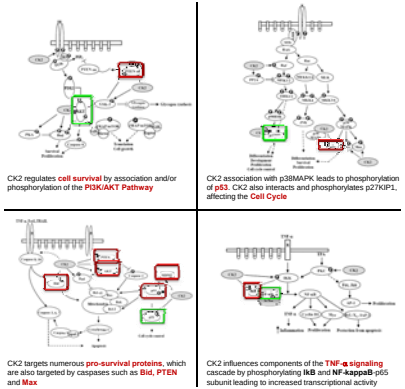
There is considerable evidence supporting a multifunctional role of the constitutively active serine/threonine protein kinase CK2 (casein kinase II) in a myriad of cellular events contributing to the cancer phenotype. The elevation of CK2 activity is due to overexpression of the molecule. Unlike other signaling molecules such as PI3K, PTEN, RAF, RAS, where genetic alterations lead to an altered molecule concomitant with a deregulation of their pathways, in the case of CK2, only its high expression levels have been associated with a disease state and no mutations have been found to date. CX-4945, a synthetically derived small molecule, was optimized to selectively inhibit CK2 activity (IC_{50} of 2nM). Here we describe the biological characterization of CX-4945 including potential biomarkers. CX-4945 showed broad spectrum anti-proliferative activity in multiple cancer cell lines including inflammatory breast cancer. CK2 is required for transition through all phases of the cell cycle through its association with cell cycle regulatory proteins $p21^{WAF1/CIP1}$ and $p27^{Kip1}$. CK2 promotes cell survival signaling via the PI3K/AKT.

A series of experiments were performed to:

1) evaluate the activity of CX-4945 on cell cycle regulatory proteins $p21^{WAF1/CIP1}$ and $p27^{Kip1}$
2) characterize the effects of CX-4945 on cell cycle distribution
3) evaluate the activity of CX-4945 on PI3K/AKT
4) characterize CX-4945 modulation of apoptosis via caspase activation
5) characterize the effects of CX-4945 on anti-angiogenic signaling via HIF-1α and pVHL under hypoxic conditions

CX-4945 inhibits CK2 activity, resulting in concentration and time dependent dephosphorylation of $p21^{WAF1/CIP1}$ at T145 in multiple cell lines allowing $p21^{WAF1/CIP1}$ to re-enter the nucleus and bind to CDK/cyclin complexes and promote cell cycle arrest in G1 or G2/M depending on cell type. CX-4945 inhibits CK2 activity resulting in induction of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor in BxPC3 cells. CK2 regulates cell survival by association and/or phosphorylation of various components of the PI3K/AKT pathway. CX-4945 inhibits CK2 activity, resulting in dephosphorylation of AKT at T308 and S473 and silencing of this intracellular signaling pathway. CX4945 induces apoptosis via activation of caspase 3/7 in a concentration and time dependent manner. Further, CK2 activity is linked to key signaling pathways involved in angiogenesis. CK2 is elevated under hypoxic conditions and positively regulates HIF-1 transcriptional activity. CX-4945 inhibits new blood vessel formation in HUVECs, and under hypoxic conditions commonly seen in tumors, CX-4945 inhibits the activity of HIF-1α, a key driver of neoangiogenesis while activating the tumor suppressor pVHL. CX4945 demonstrated robust antitumor activity, including 70% tumor free survival, in BxPC3 xenografts. Our findings suggest the antiproliferative and antitumor activity of CX4945 is a result of disruption of cell cycle control, inhibition of AKT mediated cell survival signaling, induction of apoptosis and anti-angiogenic activity. Potential biomarkers include p21-T145, AKT-T308 and AKT-S473, and apoptosis. CX4945 is positioned to initiate a multi-center phase 1 trial in 2008.

CK2 is "Multi-Tasking" Kinase



CK2 regulates **cell survival** by association and/or phosphorylation of the **PI3K/AKT Pathway**

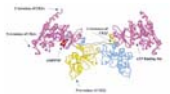
CK2 association with p38MAPK leads to phosphorylation of **p53**. CK2 also interacts and phosphorylates p27KIP1, affecting the **Cell Cycle**

CK2 targets numerous **pro-survival proteins**, which are also targeted by caspases such as **Bid, PTEN** and **Max**

CK2 influences components of the **TNF-α signaling** cascade by phosphorylating **IκB** and **NF-kappaB**-p65 subunit leading to increased transcriptional activity

Barbara Guerra and Olaf-Georg Issinger, Current Med. Chemistry (2008) 15

The CK2 Holoenzyme shown with AMPPNP docked in the ATP Binding Site

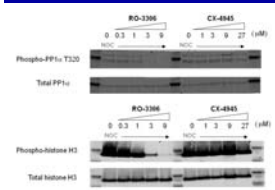


D.W. Litchfield, J.Biochem. (2003) 369

KINASE	IC_{50} (nM)	KINASE	IC_{50} (nM)
CK2 α'	1	HIPK3	45
CK2 α	3	PIM1	46
DAPK3	17	CDK1/cyclin B	56
FLT3	35	DYRK2	91

- CX-4945 (10 μM) was evaluated against a panel of over 145 protein kinases.
- IC_{50} determinations for CX-4945 against subset of kinases shown in table above.
- No significant inhibition at all other kinases.

No CDK Inhibition with CX-4945



Nocodazole-blocked A375 cells were treated for 1 hour with RO-3306 (CDK1 inhibitor) or CX-4945 and subjected to Western blot analysis using antibodies for Phospho-PP1α T320, total PP1α, phospho-histone H3, and histone H3.

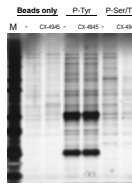
Phospho-PP1α T320 and histone H3 are direct substrates for CDK1.

CX-4945 was found to inhibit CDK1 activity (in nM range) in the molecular screen, but does not inhibit CDK1 activity in the cell. This further demonstrates selectivity for CK2

CX-4945 Shows Antiproliferative Activity in Alamar Blue Assay

Cell Line	Cancer Type	Cell Viability EC_{50} (μM)	Cell Line	Cancer Type	Cell Viability EC_{50} (μM)
A375	Melanoma	3.9	MCF7	Breast	6.0
A549	Lung	3.0	MDA-MB-231	Breast	1.3
BxPC3	Pancreas	4.4	MDA-MB-468	Breast	5.1
Colo205	Colon	2.0	Mia^{PaCa} 2	Pancreas	1.1
H1299	Lung	2.4	PANC1	Pancreas	15.3
H460	Lung	10.3	PC3	Prostate	2.1
HCT-116	Colon	2.2	LNCaP	Prostate	4.7
HL60	Leukemia	1.1	SK-MES-1	Lung	6.8
HT29	Colon	1.6	SK-OV-3	Ovarian	9.0
Jurkat	Leukemia	4.2	SUM 149PT	Breast (IBC)	1.3
K-562	Leukemia	3.0	SUM 190PT	Breast (IBC)	1.7
RPMI 8226	Myeloma	4.0	Hs578T	Breast	0.67

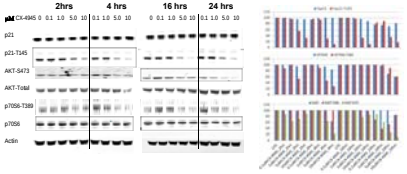
CX-4945 Selectively Blocks Phosphorylation of Ser/Thr Substrates



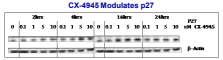
- BxPC3 cells were treated with or without CX-4945 for 2.5 hrs.
- Whole cell lysate were prepared and subjected to immunoprecipitation using antibodies against phospho-Tyr, phospho-Ser/thr(ArgXXS/T), and phospho-Thr proteins.
- The antibodies/beads-bound proteins were then eluted, and applied to 4-12% SDS-PAGE.
- The SDS-PAGE gel was silver-stained.

Phospho-protein immunoprecipitations in BxPC3 +/- CX-4945

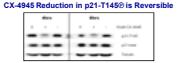
CX-4945 Blocks P13K/AKT Signaling BxPC3 Cells

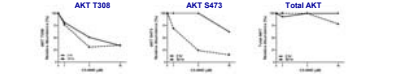


CX-4945 Modulates p27

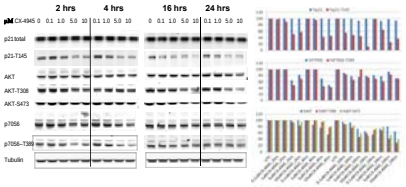


CX-4945 Reduction in p21-T145© is Reversible

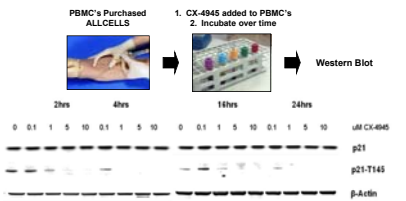




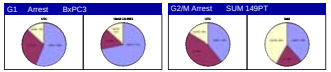
CX-4945 Blocks PI3K/AKT Signaling SUM149 Cells



CX-4945 Modulates p21-T145© Biomarker in Human PBMC's

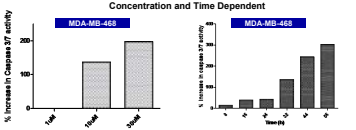


CX-4945 Induces Cell Cycle Arrest



Cell line	Tumor Type	Phase	Untreated (% of cells)	CX-4945 (1μM) (% of cells)
MDA-MB-468	Breast	G1	44	28
		S	36	33
		G2/M	20	39
MDA-MB-231	Breast	G1	53	64
		S	35	26
		G2/M	13	10
*SUM 149PT	IBC	G1	39	39
		S	44	19
		G2/M	16	42
SUM 190PT	IBC	G1	57	44
		S	24	20
		G2/M	20	36
BxPC3	Pancreas	G1	51	71
		S	36	15
		G2/M	13	13
PC3	Prostate	G1	33	24
		S	39	10
		G2/M	28	66
HCT 116	Colon	G1	34	38
		S	40	32
		G2/M	27	30
A549	Lung	G1	59	40
		S	26	33
		G2/M	15	18
A375	Melanoma	G1	47	28
		S	33	29
		G2/M	71	43

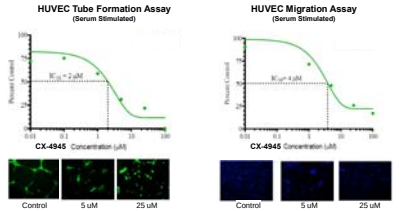
CX-4945 Activates Caspase



CX-4945 Caspase Activation in Multiple Cell Lines

Cell Line	CX-4945 uM	% Increase Caspase 3/7 Activation vs UTC
MDA-MB-468	10	245
SUM 149PT	5	61
SUM 190PT	5	93
BxPC3	10	86

CX-4945 Shows Anti-angiogenic Activity *In Vitro*

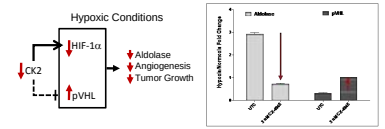


CX-4945 Inhibits Angiogenic Signaling

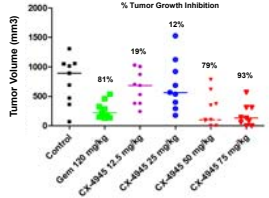
Inactivation of the von Hippel-Lindau tumor suppressor protein (pVHL) and activation of HIF-1α are associated with tumor growth and vasculogenesis

- **CK2 is elevated under hypoxic conditions**
- **CK2 positively regulates HIF-1α transcriptional activity**
- **CK2 promotes silencing of pVHL transcriptional activity**

CX-4945 Reduces Aldolase mRNA and Induces pVHL mRNA in Hypoxic BxPC3 cells

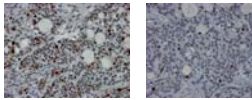


CX-4945 Shows Anti-tumor Activity in BxPC3 Xenografts



Nu/nu mice bearing subcutaneous BxPC3 tumors were administered vehicle (DI water/50mM sodium phosphate buffer), CX-4945 (12.5, 25, 50 or 75 mg/kg, p.o., b.i.d.) or gemcitabine (120 mg/kg q3d x 4). CX-4945 administered orally twice a day for 35 days demonstrated dose-dependent tumor growth inhibition compared to vehicle-treated control.

CX-4945 Reduces p21-T145 in BxPC3 Tumors



Vehicle — CX-4945 (75 mg/kg)

Summary

- CX-4945 shows potent inhibition of CK2 enzymatic activity.
- CX-4945 kinase profile is highly selective for CK2 vs other kinases.
- CX-4945 showed broad spectrum antiproliferative activity in cancer cell lines.
- CX-4945 inhibits phosphorylation of the cell cycle regulatory protein $p21^{WAF1/CIP1}$ at Thr145.
- CX-4945 inhibits phosphorylation of AKT at Thr 308 and Ser 473.
- CX-4945 induces cell cycle arrest and apoptosis.
- CX-4945 shows in vitro anti-angiogenic activity in HUVECs.
- CX-4945 under hypoxic conditions, inhibits the activity of HIF-1α, while activating the tumor suppressor pVHL.
- CX-4945 shows potent antitumor activity in BxPC3 xenografts.
- *An IND for CX-4945 was submitted October 2008.