Supplemental Information

MEK inhibitor PD-0325901 overcomes resistance to CK2 inhibitor CX-4945 and exhibits anti-tumor activity in head and neck cancer

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Supplemental Materials and Methods

Western Blot

Whole-cell lysates were obtained using an extraction kit from Active Motif. NuPAGE 4-12% Bis-Tris and Tris-Acetate precast gel was used for electrophoresis on the XCell surelock Mini-Cell (Invitrogen, Carlsbad, CA). A total of 30 μg of protein from each sample were denatured and then loaded in each lane. Proteins were then transferred on to a PVDF membrane. The signals were visualized using a horseradish peroxidaseconjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by ehemiluminescence detection (Pierce, Rockford, IL). Subsequently, blots were re-incubated with anti β-Actin antibody and developed similarly as loading controls. The following Cell Signaling Technology (Danvers, MA) antibodies were used: pan-Akt antibody (#2920) at 1:2000 dilution, pAkt (T308) antibody (#2965) at 1:1000 dilution, pAkt (S473) antibody (#4060) at 1:200 dilution, p-Erk1/2 (Thr202/Tyr204) antibody (#4376), p-S6 S235/236 (#4857) at 1:500, and total S6 (#2217), at 1:500, and Bcl-XL antibody (#2764) at 1:250. The anti-TP53 (sc-6243, 1:100) antibody was from Santa Cruz. The rest antibodies are from Abcam using at 1:200, including anti-p-Akt (S129) (ab133458), p-p21 (T145, ab47300), Erk (ab17942) antibodies. Anti-p21 antibody

(ab7960) was used at 1:500. β-Actin antibody from Millipore, Billerica, MA (# MAB1501) at 1:5000 was used as a loading control.

Immunohistochemical analyses of tumors

The OCT-embedded tissues were cut onto the silanated glass slides at 15 Am thickness with cryostat, air dried, and stored at -80°C. To process for immunohistochemistry staining, the cryosections were thawed at room temperature for 30 min. Serial frozen sections were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) at 4°C for 15 minutes. Slides were incubated in a 0.2% triton X-100/PBS solution for 5 minutes at room temperature for membrane permeation. Nonspecific binding sites were blocked with 5.5% serum (of secondary antibody host species)/tris buffered saline (TBS) and endogenous tissue peroxidase was quenched with a 0.6% H_2O_2/TBS solution. Excess solution was discarded and the sections were incubated with the first primary antibody in blocking solutions at 4°C overnight. After washing with PBS, the slides were sequentially incubated with the biotinylated secondary antibody (1:400; Vector Laboratories) for 1 h followed by avidin-biotin complex (Vector Labs, Burlingame, CA). Immune complexes were revealed via incubation with 3, 3'-(FASTDAB tablet; Sigma, St. Louis, MO) under microscopic control. The reaction was stopped in tap water, and the tissues were counterstained with hematoxylin, dehydrated through graded alcohols, cleared with xylenes, and mounted using Permount (Fisher, Waltham, MA). Whole slide images were acquired using an Aperio Scanscope at 200X magnification. Relevant areas were quantified as histological score using the Aperio Cell Quantification Software (Aperio, Vista, CA), and expressed as the percentage of positive cells with respect to the total number of cells in each histologically defined area. The following antibodies were used for immunostaning: p-Akt (S129) antibody (AP3020a, ABGENT, San Diego, CA). The following antibodies are from Cell Signaling: p-Akt

(S473) antibody (#3787), and pAkt (308) antibody (#2965) p-Erk1/2 (Thr202/Tyr204) antibody (#4370) p-p65 S536 (#3033), p-S6 S240/244 (#5364), BCL-XL (#2764) at 1:100 dilution. The following antibodies are from Abcam, anti-p-p65 S529 (ab47395) at 1:100), anti-BAX (ab7977) at 1:100 dilution, JunB (ab31421) at 1:250, and FosL-1 (ab117951) at 1:50. Anti-Ki-67 (TEC-3) antibody (DAKO, Glostrup, Denmark) was used at 1:500; anti-TP53 (FL-393) antibody was from Santa Cruz, using manufacturer's condition.

Deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Apoptotic cells in tumor tissues were quantified by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), using the in situ cell death detection kit, POD (Roche, Mannheim, Germany) according to the manufacturer's instructions. Then six representative high power field areas (200X) of each sections without necrosis were selected and both apoptotic cells and total cells were counted using Aperio Cell Quantification Software.
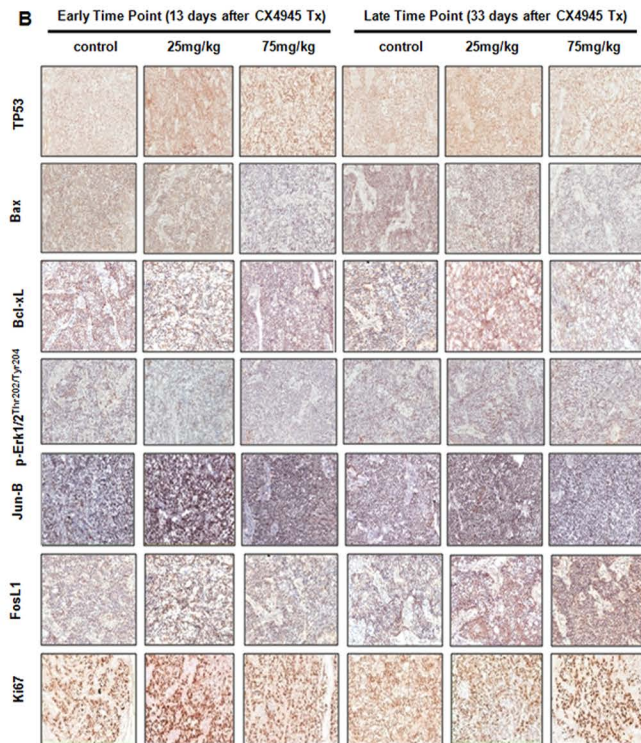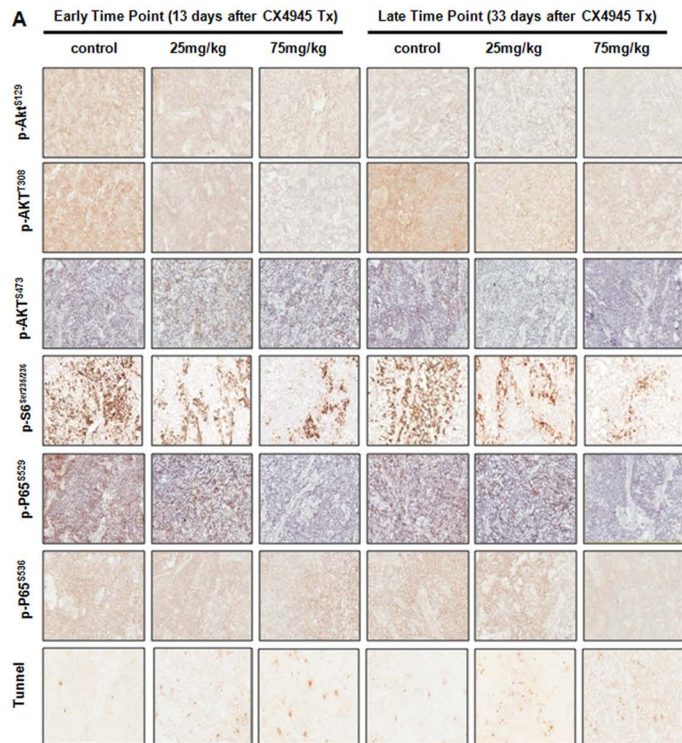
Supplemental Figure legends:

Supplemental Figure 1. Images of immunohistochemistry (IHC) of tumor specimens harvested from mice with CX-4945 treatment. Frozen tumor specimens were harvested after treatment at early time points (13 days) or late time point (33 day). IHC was performed and the images were acquired using an Aperio Scanscope at 200X magnification. Bars, 100 μM.

Supplemental Figure 2. A schematic of CX-4945 effects on signal regulated transcription factors that modulate cell growth, cell cycle, and apoptosis. Aberrant activation of growth factor receptors through growth factor stimulation or receptor mutations mediated CK2 and
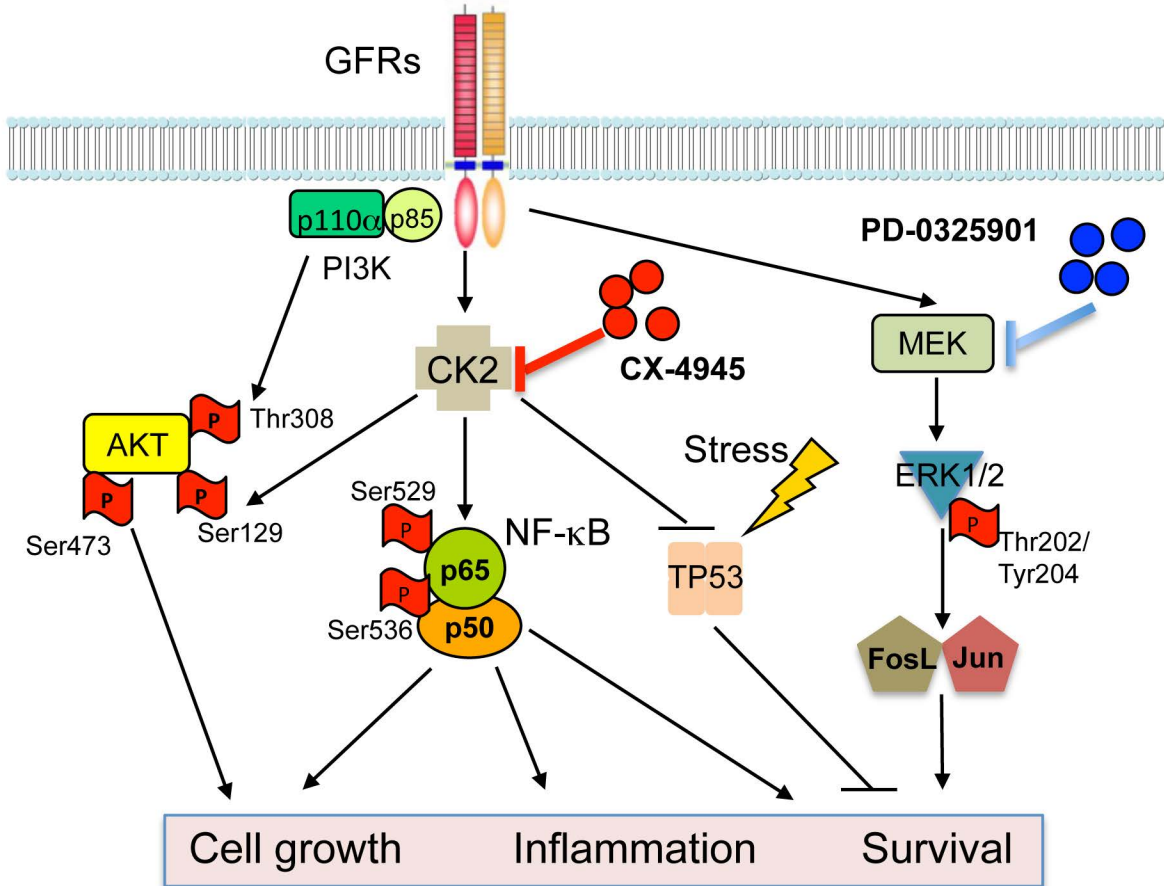
PI3/AKT signaling pathway activation. Activation of CK2 induces phophorylation of AKT S129 and NF-κB p65 s536 and S529, and suppresses TP53 induced by stress. PI3K activation induced phophorylation of AKT T308 and S473. CX-4945 Inhibited CK2 activity and suppressed AKT and NF-κB p65 phosphorylation and activation *in vitro* and *in vivo*. However, CX-4945 alone did not show significant anti-tumor activity *in vivo*, due to induced the compensatory activation of MEK/ERK/AP1 activation. We observed CX-4945 induced ERK1/2 phosphorylation of Thr202/Tyr204 and expression of FOSL and Jun subunits. Using MEK inhibitor PD-0325901, and in combination with CX-4549 inhibited ERK/AP1 activation and exhibited strong antitumor activity *in vivo* through blocking tumor cell growth, survival and inflammation.

Supplemental Table 1. Clinical information of nine UM-SCC cell lines and IC$_{50}$ of CX-4945 on cell proliferation measured by MTT assay

Supplemental Table 2. Copy number variations (CNV) of CK2 subunits in 279 HNSCC tumor specimens.

A

Early Time Point (13 days after CX4945 Tx) | Late Time Point (33 days after CX4945 Tx)

control | 25mg/kg | 75mg/kg | control | 25mg/kg | 75mg/kg

p-Akt^{S129}
p-AKT^{T308}
p-AKT^{S473}
p-S6$^{Ser235/236}$
p-P65^{S529}
p-P65^{S536}
Tunnel

B

Early Time Point (13 days after CX4945 Tx) | Late Time Point (33 days after CX4945 Tx)

control | 25mg/kg | 75mg/kg | control | 25mg/kg | 75mg/kg

TP53
Bax
Bcl-xL
p-Erk1/2$^{Thr202/Tyr204}$
Jun-B
FosL1
Ki67

Supplemental Figure 1

Supplemental Table 1. Tumor, treatment, outcome characteristics and CX-4945 IC50s of human HNSCC lines

Cell line	Age	Sex	Stage	TNM	Primary site	Prior Tx	Status	Survival (M)	IC50s (µM)
UM-SCC 1	72	M	I	T1N0M0	FOM	R	DWOD	15	4.1
UM-SCC 6	37	M	II	T2N0M0	BOT	N	LTF		3.4
UM-SCC 9	72	F	II	T2N0M0	Anterior tongue	R	DOD	15	10.6
UM-SCC 11A	65	M	V	T2N2aM0	Epiglottis	N	DOD	14	3.9
UM-SCC 11B				Persistent disease	Supraglottic Larynx	C			8.1
UM-SCC 22A	59	F	III	T2N1M0	Hypopharynx	N	DOD	10	5.2
UM-SCC 22B				Metastasis	LN metastasis	N			11.9
UM-SCC 38	60	M	IV	T2N2aM0	Tonsillar pillar	N	DOD	11	7.5
UM-SCC 46	57	F	III	NA	Epiglottis	R, S	DOD	6	3.4

UM-SCC lines (University of Michigan series of head and neck squamous cell carcinoma) and clinical information were provided by Drs. Thomas E Carey, and from previous publications. TNM, tumor-node-metastasis (staging system); Primary sites, the origin of the primary tumor; Rec, recurrence; Prior therapy, therapy given before the specimen used for culture was obtained; Survival, time in months from diagnosis to last follow up; FOM, floor of mouth; BOT, base of tongue; LN, lymph nodes; R, radiation; S, surgery; C, chemotherapy; N, no treatment; DOD, died with disease; DWOD, died without disease; LTF, lost to follow-up; NED: no evidence of disease; AWD: alive with disease. NA: not available.

Supplemental Table 2. Copy number variations (CNV) of CK2 subunits in 279 HNSCC tumor specimens.

Full names	Gene symbols	Cytoband	Diploid (%)	Amplification (%)		Deletion (%)		Statistics
				Hetero	Homo	Hetero	Homo	
Casein Kinase 2α	CSNK2A1	20p13	135 (48.4)	108 (38.7)	1 (0.3)	35 (12.5)	0 (0)	4.54E-38
Casein Kinase 2α'	CSNK2A2	16q21	173 (62.0)	61 (21.8)	2 (0.7)	43 (15.4)	0 (0)	1.54E-28
Casein Kinase 2β	CSNK2B	6p21.33	178 (63.8)	41 (14.7)	4 (1.4)	55 (19.7)	1 (0.3)	8.62E-32

CK2 copy number variation data were extracted from the HNSCC TCGA data set under "all_thresholded.by_genes.txt" downloaded from Firehose/Broad Institute. P-values are calculated as linear regression of gene expression on copy number variation. Hetero: heterozygous; Homo: homozygous.