Supplementary Data

RESEARCH DESIGN AND METHODS

Cell growth inhibition

Feline SCC cells (SCCF1) were generously provided by T. Rosol (Ohio State University, Columbus, OH) and cultured as previously described.[1] Human UM-SCC-11a cells were obtained from the University of Michigan (Ann Arbor, MI), and cells were plated in three-dimensional culture using 0.5-mg/cm^2 tenascin C:fibronectin 1-coated nanofiber scaffolds to improve cell surface lipid raft density and to facilitate nanocapsule uptake, as we have previously described.[2] Both SCCF1 and UM-SCC-11a cells were passaged individually fewer than 20 times before being used in experiments. Both cell lines were treated with 7.5 and 15 μM concentrations of TBG-RNAi-CK2 nanocapsules, with TBG-sugar nanocapsules as a control, and cell growth was measured by neutral red uptake after 48 hr. Neutral red (67 μg/ml) was added to the cells in cell growth medium for 2 hr, and the assay was performed essentially as described.[3] Percent cell proliferation in the treated cells, measured relative to control treated cells, was compared with 100 for each dose, using one-sample Wilcoxon signed-rank tests. SCCF1 and UM-SCC-11a data were combined for analyses; data were similar when separate although sample size was too small to run formal statistical analyses.

TBG-RNAi-fCK2$\alpha\alpha'$ preparation and characterization

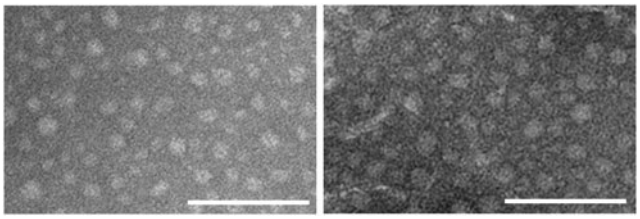
Sequences for the chimeric oligonucleotides targeting feline CK2α and CK2α' are shown in Supplementary Table S1. TBG nanocapsules containing the oligonucleotides were manufactured as previously described.[4,5] Transmission electron micrographs of TBG-RNAi-fCK2$\alpha\alpha'$ are shown in Supplementary Fig. S1 and confirmed the uniform size range of nanocapsules as less than 50 nm. Surface charge was determined to be -2.25 ± 4.0 meV and was measured by dynamic light scattering from across a 20-V potential in 1 mM KCl at 2 mg/ml. Preparations were tested for endotoxin with a commercial kit (Pierce chromogenic LAL endotoxin quantitation kit; Thermo Fisher Scientific, Waltham, MA). The endotoxin level was lower than the limits advised by the Food and Drug Administration at every planned dose level in this study. Once prepared, TBG-RNAi-fCK2$\alpha\alpha'$ was divided into aliquots and stored in 10% lactitol at –80°C. No more than 1 hr before each administration, a new aliquot was thawed at room temperature and diluted to either 10 μg/ml for cats treated at 2 μg/kg or 100 μg/ml for cats treated at 20 μg/kg with a sterile, neutral pH solution (Plasmalyte A; Baxter Healthcare, Deerfield, IL) before filtration. The final volume to be administered was drawn into a sterile syringe.

Supplementary Table S1. *Sequences of oligonucleotides targeting feline CK2α and CK2α'*

Target	*Sequence*
CK2α	5′-ATACAACCCAAGCT-2′-*O*-methyl(ccacau)-propyl-3′
CK2α'	5′-ATACAGCCCAAACT-2′-*O*-methyl(ccacau)-propyl-3′

Ethics statement

This study was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Bethesda, MD). The treatment protocol was approved by the Institutional Animal Care and Use Committees at the University of Minnesota (IACUC protocol 1207A16861) and the University of Tennessee (IACUC protocol 2227), and written informed consent was obtained from owners before enrollment. Surgical biopsies were performed under anesthesia (premedication with butorphanol [0.3 mg/kg intramuscularly], anesthesia induction and maintenance with propofol to effect up to 6 mg/kg intravenously) and all animals were prescribed buprenorphine (0.005–0.02 mg/kg sublingually every 8 hr) for analgesia after biopsy. Between study visits, cats were housed and cared for at their owners' homes.



Supplementary Figure S1. Transmission electron micrograph of the two batches of TBG-RNAi-fCK2$\alpha\alpha'$ nanocapsules used in the study, showing uniform size. Scale bar: 100 nm.

Inclusion and exclusion criteria

Cats presenting to the University of Minnesota Veterinary Medical Center and the University of Tennessee Small Animal Hospital between March 2013 and December 2014 with a histologic diagnosis of squamous cell carcinoma confined to the oral cavity ± regional lymph nodes were eligible for the study. Before enrollment, complete blood count, serum chemistry panel, serum total thyroxine (T_4), urinalysis, thoracic radiographs, and abdominal ultrasound were performed. Cats were excluded if there was evidence of metastatic disease beyond regional lymph nodes; other malignancy; severe systemic disease based on examination, laboratory or imaging tests; if life expectancy was less than 30 days; or if general performance score was >1 using Veterinary Cooperative Oncology Group (VCOG) criteria.[6] Clinical signs at diagnosis were determined on the basis of recorded history and physical examination findings in the medical record.

Dose selection and escalation

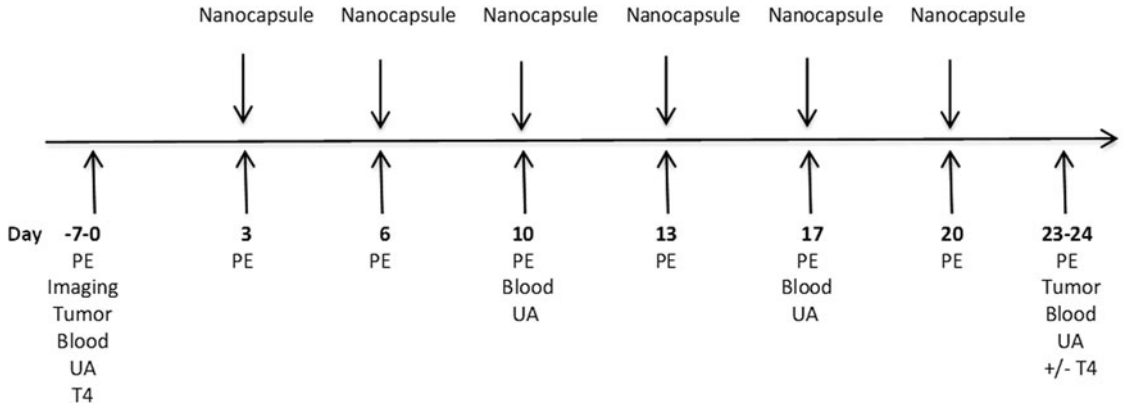
Planned dose levels were 2, 20, and 200 μg/kg, with a total of nine cats to be enrolled. The initial dose level was chosen on the basis of work in rodent models showing safety and efficacy at 10 μg/kg, and scaled by a factor of 5, based on the estimated relative body surface area of mice and cats.[7,8] Three cats were to be enrolled at the first dose level. If no dose-limiting toxicity (DLT), defined as a hematologic adverse event ≥ grade 4 or other adverse event ≥ grade 3 (using VCOG criteria), was observed, then the next three cats were to be enrolled at the higher dose. If DLT was observed in one of three cats in a cohort, this cohort would be expanded to six cats. If no additional DLTs were seen, the dose would be escalated. Once nine cats were enrolled, enrollment ceased regardless of dose level.

Treatment protocol

The protocol for treatment and diagnostic tests is outlined in Supplementary Fig. S2. Owners were required to complete a history form before every visit, and treatments were planned twice weekly (i.e., every 3–4 days), for six treatments in total. Each treatment was administered via intravenous catheter over approximately 5 min. Cats could be withdrawn at any time at investigators' or owners' discretion. In cats in which T_4 was elevated pretreatment, this was tested at the posttreatment evaluation (days 23–24). Before treatment (days –7 to 0) and posttreatment (days 23–24), two 2- to 4-mm punch biopsies of tumor and normal oral mucosa distant from the tumor were collected. Tumors were measured with calipers and digital photographs were taken before biopsy. All cats were prescribed buprenorphine postbiopsy (0.005–0.02 mg/kg sublingually every 8–12 hr) if they were not already receiving it. Alternative opioids, additional pain medications excluding nonsteroidal antiinflammatory drugs (NSAIDs), or other supportive care medications were prescribed at the discretion of the treating clinician. Tumor stage was classified according to World Health Organization criteria,[9] based on pretreatment assessment.

Adverse events assessment

Adverse events were determined on the basis of physical examination findings, history forms completed by the owner at each visit, and laboratory evaluation (CBC, serum chemistry, urinalysis) throughout the protocol. Severity of adverse events and relationship of the adverse event to the experimental drug (unrelated, unlikely, possible, probable, or definite) were assigned by one investigator (C.M.C.) after study completion and data review, using VCOG criteria.[6] Per these criteria,



Supplementary Figure S2. Study protocol timeline for cat enrollment, treatment, and assessment of response and toxicity. Nanocapsule, TBG-RNAi-fCK2$\alpha\alpha'$ administration; PE, physical examination; Imaging, thoracic radiographs and abdominal ultrasound; Tumor, tumor measurements and biopsies; Blood, complete blood count and serum chemistry; UA, urinalysis; T_4, total serum thyroxine.

grade 1–2 adverse events are generally considered mild and are usually self-limiting or require minor interventions, grade 3–4 adverse events are more severe, and a grade 5 adverse event is death.

Tumor response assessment

Tumor response was assessed by comparing pretreatment and posttreatment longest tumor diameter (or sum of tumor and lymph node longest diameters if metastatic disease was present) from caliper measurements, using RECIST (Response Evaluation Criteria in Solid Tumors) criteria.[10] Complete response (CR) is defined as resolution of all target lesions, partial response (PR) as ≥30% reduction in sum of longest diameters of target lesions from baseline, progressive disease (PD) as ≥20% increase in sum of longest diameters of target lesions or appearance of new lesion(s), and stable disease (SD) as insufficient change to qualify as either PR or PD.

Immunohistochemistry for CK2α in feline oral SCC and normal oral mucosa

Tissue specimens were fixed in 10% neutral buffered formalin before being routinely processed and embedded in paraffin. Histologic sections were cut at 4 μm and stained with hematoxylin and eosin or prepared for immunohistochemistry (IHC).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of tissue were deparaffinized and rehydrated. Antigen retrieval was performed with 10 m*M* citrate buffer, pH 6.0, in a steamer. After endogenous peroxidase blocking, a protein block was applied. Immunohistochemistry for CK2α was performed with an anti-CK2α rabbit monoclonal primary antibody (EP1963Y; Abcam, Cambridge, MA) at a 1:100 dilution on an autostainer. This antibody has been previously validated by our group for cross-reactivity with feline CK2α in Western blot and immunohistochemical applications.[11] Detection was achieved with a commercial detection system (EnVision+ anti-rabbit–HRP [horseradish peroxidase] detection system; Dako, Carpinteria, CA) with diaminobenzidine as the chromogen. Sections were counterstained with Mayer's hematoxylin. Feline testes were used as positive control tissue. For negative control slides the primary antibody was substituted with appropriate isotype control antibody (rabbit IgG, EPR25A; Abcam). Tissue sections were imaged at magnifications of $\times$200 and $\times$400, using a Nikon E800M microscope, Nikon DSRi2 camera, and Nikon Elements D version 4 software. Each section was imaged with the same white balance and shading correction settings. Whole image sharpness was uniformly adjusted, and minor background blemishes were removed with Photoshop Elements version 11. All samples were stained in the same run and each sample was scored for CK2α expression by two independent pathologists (M.G.O'S. and I.C.). The scoring system was semiquantitative, with a score of 0–4 assigned to each sample based on the percentage of immunoreactive cells, overall intensity of nuclear and cytoplasmic labeling, and intensity of labeling in the most immunopositive and immunonegative regions of the section. Final scores were arrived at by consensus for each sample. Pathologists were blinded to cat identification and pre- or posttreatment time point when scoring samples.

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