

Design of a functional cyclic HSV1-TK reporter and its application to PET imaging of apoptosis

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Positron emission tomography (PET) is a sensitive and noninvasive imaging method that is widely used to explore molecular events in living subjects. PET can precisely and quantitatively evaluate cellular apoptosis, which has a crucial role in various physiological and pathological processes. In this protocol, we describe the design and use of an engineered cyclic herpes simplex virus 1–thymidine kinase (HSV1-TK) PET reporter whose kinase activity is specifically switched on by apoptosis. The expression of cyclic TK (cTK) in healthy cells leads to inactive product, whereas the activation of apoptosis through the caspase-3 pathway cleaves cTK, thus restoring its activity and enabling PET imaging. In addition to detailing the design and construction of the cTK plasmid in this protocol, we include assays for evaluating the function and specificity of the cTK reporter in apoptotic cells, such as assays for measuring the cell uptake of PET tracer in apoptotic cells, correlating doxorubicin (Dox)-induced cell apoptosis to cTK function recovery, and *in vivo* PET imaging of cancer cell apoptosis, and we also include corresponding data acquisition methods. The time to build the entire cTK reporter is ~2–3 weeks. The selection of a stable cancer cell line takes ~4–6 weeks. The time to implement assays regarding cTK function in apoptotic cells and the *in vivo* imaging varies depending on the experiment. The cyclization strategy described in this protocol can also be adapted to create other reporter systems for broad biomedical applications.

INTRODUCTION

Conventional imaging modalities, such as computed tomography (CT), are primarily used to obtain anatomical information, whereas molecular imaging is used to study biological events at the cellular and subcellular levels, revealing complicated biological cascades and enabling early detection of diseases and therapy response monitoring¹. PET is a powerful molecular imaging tool that can be used to noninvasively and sensitively monitor cellular and molecular events that occur in the progression of diseases such as cancer². Using a radioisotope-labeled target-specific tracer, which has unlimited signal penetration within organs and tissues, PET provides sufficient spatial and temporal resolution for exploring biological events in animal models and humans³. In this protocol, we describe the design and use of a PET reporter system to monitor apoptosis; however, this reporter system can be adapted using a similar rationale to monitor alternative enzyme activities.

Imaging-based methods to monitor apoptosis

Programmed cell death, or apoptosis, is an important cellular event that is involved in biological development and many pathological processes⁴. For effective anticancer therapy, most chemotherapeutic agents induce cancer cell death, primarily via apoptosis. In drug discovery and development, chemical compounds are usually screened for their ability to target specific cytosolic proteins or enzymes at different apoptotic stages (either to enhance or to inhibit activity). However, endogenous mutations of cancer cells counteract the apoptosis-induction effect of chemotherapeutics, which leads to unwanted drug resistance in anticancer therapy^{5,6}. It is thus highly important to develop sensitive molecular imaging methods to evaluate apoptosis during anticancer treatment in order to monitor therapy response from an early stage and to improve the medical management of patients⁷. In addition, the development of imaging probes for

monitoring the progression of apoptosis in living organisms enables a better understanding of the complicated pathological changes that occur during treatment.

Several imaging strategies have been developed to noninvasively monitor apoptosis^{8,9}. One strategy is the measurement of phosphatidylserine accumulation on apoptotic cell membranes via its binding affinity with annexin V, which is either labeled with isotopes for radionuclide imaging or conjugated with fluorophores for optical imaging^{10–12}. An alternative approach is to use a radionuclide-labeled small molecule, ML10, which binds to the surface of apoptotic cells for PET imaging¹³. However, it can be difficult to distinguish apoptosis from necrosis using cell surface markers, as necrotic and apoptotic cells usually share identical surface characters¹⁴. To circumvent this problem, the imaging of specific intracellular apoptotic mediators can be used to study intracellular activities during apoptosis, especially in the early stages of this process.

The cytosolic caspases, a family of cysteine proteinases, have essential roles in apoptosis^{15,16}. Caspase-3, the ‘executioner’ of apoptosis, is a highly potent target molecule for apoptosis imaging^{17,18}. Various activatable probes have been developed to monitor caspase-3 activity under microenvironmental cues *in vivo*^{19–22}. However, most probes are associated with high background noise owing to nonspecific cleavage²³ and low cell membrane permeability. Genetically coded fluorescent or bioluminescent reporter systems have also been constructed to detect caspase-3 activities²⁴. These reporter systems have become valuable tools to investigate spatiotemporal caspase activities in living cells²⁵ or animals²⁶. However, optical reporter systems are limited by photon penetration attenuation and lack of quantitative and tomographic outputs. It is thus highly desirable to develop a sensitive, quantitative and translational reporter system for real-time apoptosis imaging.

PROTOCOL

HSV1-TK (encoded by the *HSV1-TK* gene), along with its radiolabeled specific substrates, is an enzyme-based reporter system that has been extensively used for PET imaging^{27–29}. After phosphorylation by HSV1-TK, purine and pyrimidine nucleoside analogs become highly positively charged and accumulate in cells, allowing signal detection by single-photon emission computed tomography (SPECT) or PET³⁰. Over the past decade, our laboratory has used an HSV1-TK PET reporter system for real-time tracking of embryonic stem cells after cardiac injection³¹, for tumor-targeted imaging of modified virus particles²⁹ and for the evaluation of new PET tracers^{32,33}.

Recently, we have developed and used a genetically encoded functional HSV1-TK in truncated and cyclic form for sensitive PET imaging of caspase-3 activities during apoptosis³⁴. The major applications of this cTK are preclinical screening of potential apoptosis inducers or inhibitors in living animals and real-time monitoring of cell apoptosis in a noninvasive and quantitative manner by PET. By changing the substrate recognition sites in cTK structure, this cTK-based PET imaging platform can be used to detect the activities of other cellular enzymes, such as cathepsin B, in real time. The cTK system can also be combined with other engineered enzyme systems, such as *cre-loxP*³⁵, for inducible reporter gene expression or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (CRISPR/Cas9)^{36,37}, enabling sensitive functional PET imaging in transgenic mice^{38,39}.

Here we describe the design and development of cTK and an application in PET imaging of Dox-induced apoptosis in cancer cells (*in vitro* and *in vivo*). This cTK reporter system can be combined with other imaging modalities or artificial enzyme systems to broaden its range of applications.

Experimental design

This protocol describes the rationale and experimental procedures for the construction of the cTK plasmid (Steps 1–29), synthesis of ¹⁸F-FHBG tracer (Steps 30–42) as the functional TK substrate, verification of cTK function in apoptotic cells and the use of it for *in vivo* PET imaging of tumor apoptosis (Step 43A–43C). The successful construction of the cTK plasmid is the major task in this protocol. Adapting the following rationale should enable the development of cyclic reporters to detect other enzyme activities; however, it is important to bear in mind that not all enzymes are suitable for cyclization. One should screen the specific enzyme sequences and test the function recovery rate when using the described cyclization strategy to check suitability. All the subsequent procedures are used to serve and validate the function and application of the cTK plasmid. The success of this protocol is based on the integration of molecular biology, PET radiochemistry and small-animal imaging.

cTK reporter construct design

Figure 1 illustrates the principle of cyclic TK reporter design and preselected permutation sites for effective PET imaging. The two fragments of DnaE intein (split DnaE as DnaEc and DnaEn; PCC6803; ref. 40) are fused to the flank ends of HSV1-TK (TK) connected by a caspase-3 recognition substrate sequence, Asp-Glu-Val-Asp (DEVD), on the N and C termini of the TK sequence. Upon translation into a single polypeptide in the cytosol, the split fragments of TK undergo ligation through intein protein splicing, resulting in a closed circular polypeptide. We initially engineered the whole HSV1-TK sequence into a truncated version by deleting the first 45 amino acids, which showed reduced cytotoxicity in comparison with the full-length TK⁴¹.

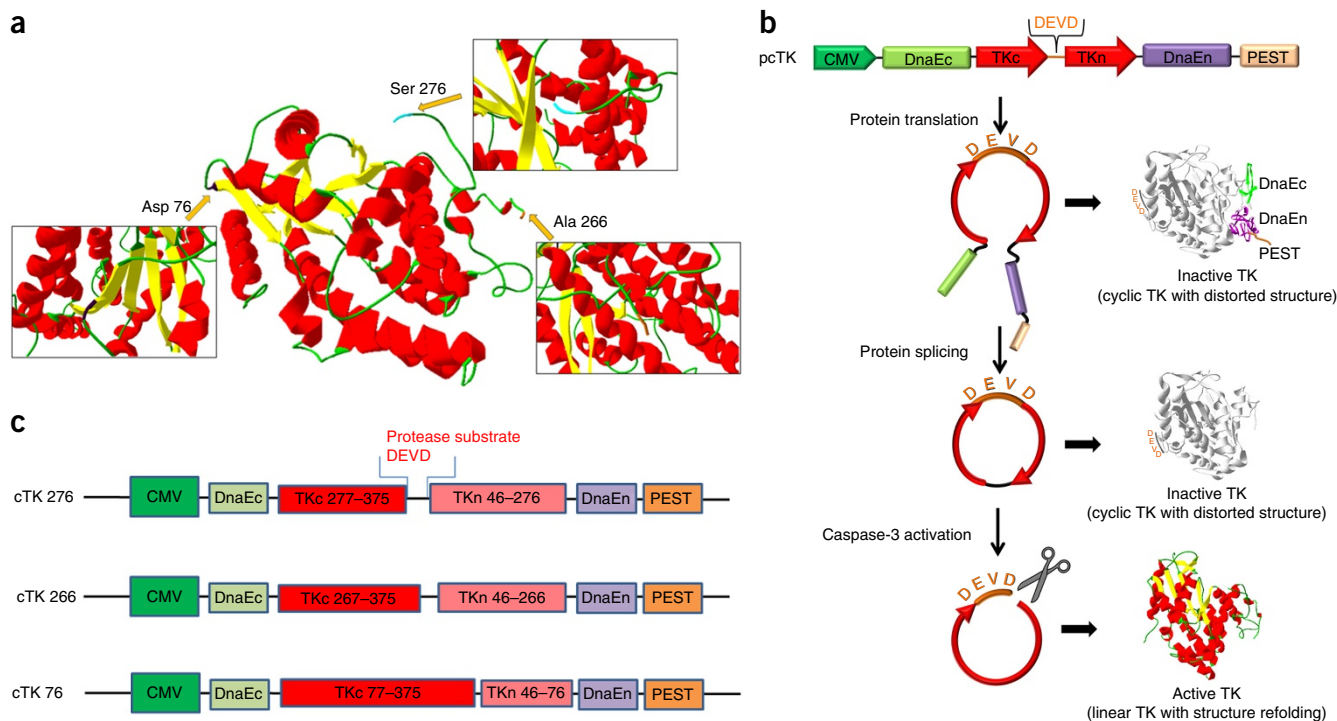


Figure 1 | cTK reporter system design for apoptosis PET imaging. **(a)** Crystal structure of 1–45 amino acid-truncated HSV1-TK, highlighting the split sites for protein engineering. **(b)** Schematic illustration of cTK design principle. The N and C termini of the truncated HSV1-TK were linked by a sequence, which is the caspase-3 substrate. During cellular apoptosis, the caspase-3 pathway is activated. The linking sequence, DEVD, is cleaved and the activity of cTK is restored. **(c)** Illustration of cTK variant plasmid constructs at different split sites. This figure is not drawn to scale. CMV, cytomegalovirus promoter.

More importantly, the truncation reduces the structurally flexible region of the entire TK structure that restrains the tension of cyclic protein structure, prohibiting the accessibility of catalytic center to its substrates. A PEST sequence, a peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T), was introduced to the cTK structure to accelerate the degradation of the unspliced protein. Hence, only the cyclic form of TK was retained in the cytosol^{42,43}. Upon caspase-3 activation, the DEVD sequence was cleaved, and the linear form of cTK was released to restore TK activity to catalyze the phosphorylation of its tracer substrates trapped in cells for PET imaging²⁷.

To successfully develop the functional cTK, it was crucial to identify the specific sites on the primary TK polypeptide sequence, from which the TK sequence can be split into two fragments in the cTK construct. The wild TK is a homodimer with 376 residues per subunit displaying the general $\alpha\beta$ folding pattern⁴⁴ (Fig. 1a). The amino acid sequences from 161 to 192 serve as the substrate-binding domain. The amino acid at site 163 critically determines the enzymatic activity of TK, and site 162 is responsible for coordination with Mg^{2+} . The amino acids at sites 317 and 318 are essential in TK homodimer formation. All the above sites on the TK sequence are not appropriate candidate positions for splitting. In addition, it has been reported that the mutation of valine at site 119 into cysteine ($V^{119}C$) led to a higher K_m value in ATP binding⁴⁵. This site-directed mutation was included in the construct, and this site was not chosen in protein fragmentation splitting design. On the basis of the analysis of protein crystal structure of TK, we identified three potential splitting sites to construct cTK. Among them, one (Asp^{76}) is within the catalytic domain of TK, and the other two sites (Ala^{266} and Asn^{276}) are out of the catalytic active region, while functioning as connecting sequences in α -helices (Fig. 1b). Hence, the three identified molecularly engineered cTK reporters, designated as cTK76, cTK266 and cTK276, were constructed through linking the N and C termini of truncated TK with DEVD peptide, which is flanked by DnaE intein at each end (Fig. 1c). The results from cell uptake experiments indicated that the Ala^{266} is the optimal split site to form cyclic TK with relatively high tracer cell uptake and low background noise (Supplementary Fig. 1).

For the absolute radioactivity test (Fig. 2a), $cTK276 > cTK266 > cTK76$, whereas cTK266 showed the most prominent radioactivity fold change after normalizing with control cells (Fig. 2b). This comparison suggests that cTK276 may result in higher background noise for imaging, and cTK76 is not appropriate for imaging study. Among the three different split sites in cTK vector construction, we chose cTK266, which maintains good enzyme function after caspase-3 cleavage, and presents the lowest background noise in ^{18}F -FHBG uptake. Thus, all the procedures described in this protocol use the cTK266 reporter.

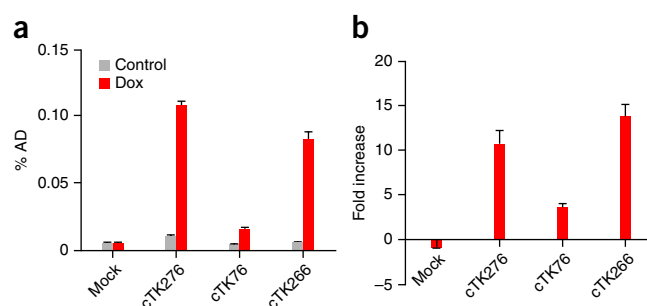


Figure 2 | ^{18}F -FHBG uptake assay from different cTK variants. (a) ^{18}F -FHBG uptake assay results from three cTK variant-transfected cell colonies (cTK76, cTK266 and cTK276). The highest transgene expression cell colony was chosen in each cTK variant. Wild-type cell without transfection was used as the mock (control). All the cells were treated using 1 μ g/ml Dox 24 h before the experiment ($n = 3$). (b) Fold change in ^{18}F -FHBG of Dox-treated cells over untreated control cells ($n = 3$). AD, added dose. Error bars are S.D. Adapted from ref. 34 with permission.

Animal model and cell line

It is strongly recommended that the selected cell line used in relevant studies go through drug-sensitivity testing before use in this assay. It is well known that cancer cells may present drug resistance to particular chemotherapeutic drugs by endogenous or exogenous routes⁴⁶. The success of functional cTK in apoptosis PET imaging relies much on the high sensitivity of selected cancer cells to chemotherapeutic agents, which ultimately leads to significant apoptosis through caspase-3 signaling pathway activation^{47–49}. In addition, some cancer cells, such as MCF-7 breast cancer cells, undergo caspase-3-independent apoptosis⁵⁰, and they are thus not appropriate for cTK PET imaging, whereas they can be used as the negative control cell line to confirm the cTK specificity in apoptosis. Moreover, it is highly recommended that cancer cells used in the relevant studies independently express another reporter system, such as firefly luciferase (fLuc) or renilla luciferase (RLuc), to serve as a cell proliferation indicator *in vitro* and *in vivo*. The signal from cTK PET imaging can be normalized to the signal generated from the additional reporter system for comparison.

Before *in vivo* imaging, an appropriate xenograft tumor model should be well established. The tumor volume should be well controlled to be 150–200 mm^3 (calculated as length \times width²/2, ~7–8 mm in diameter). Larger tumor sizes may have necrosis inside the tumors, which may lead to high background noise or false positive signal. Small tumor sizes may have immature tumor-associated blood vessels and an undeveloped tumor microenvironment.

For the animal tumor model described in this protocol, it is strongly recommended to have six mice in each group to obtain statistically meaningful results. However, if one uses a different experimental model, such as a different cell line, transgenic mice and so on, the animal size number per group should be adjusted on the basis of pilot studies.

MATERIALS

REAGENTS

- ^{18}F **CAUTION** It is a radioisotope. Personnel involved in this study should have received appropriate radiation safety training. Radioactive chemical waste should be handled strictly in accordance with institutional radiation safety protocols.
- Acetonitrile, anhydrous (ACN; Sigma-Aldrich, cat. no. 271004)

- Agarose (Bio-Rad, cat. no. 161-3102EDU)
- Ammonium acetate (NH_4OAc ; Sigma-Aldrich, cat. no. A1542)
- Annexin V: FITC apoptosis detection kit I (BD Bioscience, cat. no. 556547)
- Athymic nude mice (female, 5–6 weeks old; Harlan Laboratories)
- **CAUTION** All animal experiments should be conducted in accordance



PROTOCOL

with relevant guidelines and protocols approved by institutional animal research review committees.

- cTK stable transgenic cell line: established as described in **Box 1** (see also **Supplementary Fig. 2**)
- Deionized water (Millipore, Milli-Q-Q POD)
- *N,N*-dimethylformamide (DMF; Sigma-Aldrich, cat. no. 270547)

! CAUTION It is a volatile, flammable and harmful organic chemical that may cause skin and eye irritation.

- Dichloromethane (Sigma-Aldrich, cat. no. 650463) **! CAUTION** This organic chemical is volatile and harmful. It can cause serious skin and eye irritation and respiratory sensitization. Handle the compound carefully.

Box 1 | Generation of a cTK stable transgenic cell line ● TIMING 4–6 weeks

To facilitate the establishment of tumor xenograft in nude mice for PET imaging of apoptosis, a stable transgenic cell line should be first established. The UM-SCC-22B (or 22B) cell line was chosen because of its high sensitivity to Dox-induced apoptosis in our laboratory³⁴ (**Supplementary Fig. 2**).

MATERIALS

Human head and neck squamous carcinoma UM-SCC-22B cell line (University of Michigan) ▲ **CRITICAL** Other cell lines can be used if apoptosis can be induced.

PROCEDURE

Day 1:

1. Culture 1×10^6 22B cells in a 10-cm tissue culture dish in DMEM supplemented with 10% (vol/vol) FBS, 100 U/ml penicillin and 100 mg/ml streptomycin tissue culture medium. Prepare two cell culture dishes so that you can use one dish as a negative control for Zeocin selection.

▲ **CRITICAL STEP** The cultured cell density per dish is crucial for subsequent transfection efficiency. In a typical experiment, one should estimate the cell number to ensure 70–80% confluency on the day of transfection (day 2). Both high and low cell density can adversely affect transfection efficiency. It is strongly recommended that a cell growth curve is calculated before performing stable transfection.

Day 2:

2. Transfect one plate with 20 μ g of cTK plasmid with Lipofectamine 2000 according to the manufacturer's instructions. One can use 60 μ l of Lipofectamine 2000 solution as the starting condition. Do not transfect the negative control plate.

Day 3:

3. Replace the medium in both plates with fresh tissue culture medium (DMEM) containing 100 μ g/ml Zeocin.

! CAUTION Zeocin is a strong antibiotic, and the optimal selection concentrations vary in different cell lines. For a given cell line, one should optimize the selection concentration before the experiment.

Day 4:

4. Split transgenic cells 1/3, 1/5 and 1/10 in fresh 10-cm tissue culture dishes, and add tissue culture medium with 100 μ g/ml Zeocin. Handle the negative control plate in the same manner.

▲ **CRITICAL STEP** Cells should be suspended homogeneously in the dish to facilitate colony picking.

Day 5:

5. Check cells daily, with a medium change every other day. For the negative control cells, all cells should die in 5–7 d.

? TROUBLESHOOTING

6. Most of the cells die over the selection period (1–2 weeks), whereas sporadic cell colonies become resistant to Zeocin owing to prior successful transfection. Pick up colonies into a six-well plate, one colony for each well, when colonies become large and visible. To pick up the colonies, warm up 0.25% (wt/vol) trypsin in a 37 °C water bath. Aspirate the medium and wash the cells with PBS two times. Withdraw 20 μ l of trypsin solution using a yellow tip, and then apply it on cell colonies. Scrape each colony gently with the yellow tip and aspirate the colony into the yellow tip. Transfer the aspirated cells to one well of a six-well plate containing 1 ml of tissue culture medium with 100 μ g/ml Zeocin.

▲ **CRITICAL STEP** Avoid picking up cells from close or overlapped colonies. Only pick up the cells in the central part of each colony. During the scraping and transferring operations, try to minimize the time cells are exposed to air. Long exposure to air without medium substantially lowers cell viability and could induce apoptosis.

? TROUBLESHOOTING

7. Continue to culture the cells in selection medium to confluence. Split the cells in each well into six single wells of a six-well plate.

? TROUBLESHOOTING

8. When the cells become confluent, freeze one well of cells, continue to expand one well of cells and test the third well of cells by real-time PCR to examine the cTK expression level, as described in the following steps.

▲ **CRITICAL STEP** The fLuc-expressing 22B cells can be selected by following the same procedure as that used in steps 1–8, but 500 μ g/ml G418 instead of Zeocin should be used for selection. In doing fLuc selection, the bioluminescence test of selected cells is conducted by adding 10 μ l of *D*-luciferin (33 mg/ml) to cultured cells in a 24-well plate.

9. Choose three colonies randomly to test the cTK expression level in stable selected cells. To run real-time PCR, lyse the cells with TRIzol reagent to isolate total RNA according to the manufacturer's instructions. Reverse-transcribe an equal amount of total RNA (1 ng) into cDNA using the First-Strand Synthesis kit according to the manufacturer's instruction. To run real-time PCR, amplify synthesized cDNAs using the power SYBR Green PCR master mix kit according to the manufacturer's instructions. Use *GAPDH* as the housekeeping gene.

- Methanol (Sigma-Aldrich, cat. no. 34860) **! CAUTION** It is a highly volatile, flammable and harmful organic chemical that may cause serious skin and eye irritation, respiratory sensitization and acute toxicity.
- Diethyl sulfoxide (DMSO, Sigma-Aldrich, cat. no. D8148) **! CAUTION** This chemical should be kept in a desiccator to avoid moisture.
- 1 N hydrochloric acid (1 N HCl; Sigma-Aldrich, cat. no. 71763) **! CAUTION** It is toxic and corrosive, and it causes severe skin burns, eye damage and respiratory irritation.
- Isoflurane (RxElite, cat. no. NDC60307-120-25) **! CAUTION** It is a highly volatile liquid, and it has profound respiratory depressant properties. Wear goggles and a face mask during experiments. Bottles containing this chemical should be securely sealed after use.
- Geneticin antibiotic (G418; Invitrogen, cat. no. 10131027)
- Zeocin (Life Technologies, cat. no. R25005)
- Potassium carbonate (Sigma-Aldrich, cat. no. 209619)
- Kryptofix 222 (EMD Chemicals, cat. no. 810647)
- N²-(p-Anisylidiphenylmethyl)-9-[(4-tosyl)-3-p-anisylidiphenylmethoxymethylbutyl]guanine (FHBG, ABX; Radeberg, cat. no. 2970.0002)
- C-18 BondElut (Agilent Technologies, cat. no. 12105102B)
- Ethanol (Sigma-Aldrich, cat. no. 459844)
- Luna C18 column 10 × 250 mm 5 μm 100 Å (Phenomenex, cat. no. 00G-4041-N0)
- MCF7 human adenocarcinoma breast cancer cell line (ATCC, cat. no. HTB-22), to be used as a negative control
- Doxorubicin hydrochloride (2 mg/ml, Pfizer, cat. no. 0069-303-20)
- D-Luciferin (Goldbio.com, cat. no. L-123-1)
- pcDNA3.1/Zeo(+; Invitrogen, cat. no. V860-20)
- pCMV-HSV1-TK plasmid (deposited in our laboratory)
- cDNA of DnaE and PEST
- pcDNA3-Casp3 plasmid was obtained from Addgene (plasmid no. 11814)
- Restriction enzymes (New England BioLabs): Hind III-HF (cat. no. R3104); BamHI-HF (cat. no. R3136); PstI-HF (cat. no. R3140); NotI-HF (cat. no. R3189); XhoI (cat. no. R0146); EcoRI (cat. no. R0101T)
- T4 ligase (New England BioLabs, cat. no. M0202T)
- Ampicillin (Sigma-Aldrich, cat. no. A9393)
- Expand high-fidelity PCR system (Roche, cat. no. 11732650001)
- 1-kb DNA ladder (New England BioLabs, cat. no. N3232S)
- 6× DNA loading dye (New England BioLab, cat. no. B7021S)
- GelRed nucleic acid stain (Biotium, cat. no. 41001) **! CAUTION** This staining reagent potentially binds with DNAs. Handle it with care.
- dNTP mix (10 mM each, Fermentas, cat. no. R0191)
- QIAprep spin miniprep kit (Qiagen, cat. no. 27106)
- MinElute PCR purification kit (Qiagen, cat. no. 28104)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- One Shot Top 10 chemically competent DH5α *Escherichia coli* (Life Technologies, cat. no. 12297016)
- DMEM (Life Technologies, cat. no. 11965-092)
- S.O.C. medium (Life Technologies, cat. no. 15544-034)
- Penicillin/streptomycin 100× stock solution (Invitrogen, cat. no. 15140122)
- Luria-Bertani broth (LB medium, KD Medical, cat. no. BLF-7030)
- LB agar with 100 μg/ml ampicillin (Quality Biological, cat. no. 340-108-231)
- Polypropylene round-bottom tubes, 14 ml (BD Falcon, cat. no. 352059)
- Glycerol (molecular biology grade, Sigma-Aldrich, cat. no. G5516)
- Ultrapure water (Invitrogen, cat. no. 10977-023)
- β-Mercaptoethanol (Sigma-Aldrich, cat. no. M6250) **! CAUTION** This chemical is corrosive, causes acute toxicity and respiratory sensitization and is hazardous. Handle it with care and dispose of it properly.
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. 221465) **! CAUTION** It is a highly corrosive chemical. Handle it with necessary laboratory goggles and gloves.
- FBS (Life Technologies, cat. no. 10437-077)
- N²-(p-Anisylidiphenylmethyl)-9-[(4-tosyl)-3-p-anisylidiphenylmethoxy-methylbutyl]guanine (Tosyl-FHBG, ABX, cat. no. 2960.003)
- Caspase-3 colorimetric assay kit (Sigma-Aldrich, cat. no. CASP3C-1KT)
- IDN6556 (Emricasan-MedChem Express, cat. no. HY-10396)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- Lipofectamine RNAiMAX transfection reagent (Invitrogen, cat. no. 13778030)
- SignalSilence caspase-3 siRNA I (Cell Signaling, cat. no. 6466)
- SignalSilence scramble siRNA (Control siRNA, Cell Signaling, cat. no. 6201)
- SuperScript III first-strand synthesis system (Invitrogen, cat. no. 18080-051)
- TRIzol RNA isolation reagents (Invitrogen, cat. no. 10296010)
- Power SYBR Green PCR master mix (5 ml; AB Applied Biosystems, cat. no. 4367659)
- HSV1-TK (Santa Cruz Biotechnology, cat. no. sc-28037)
- β-Actin (Cell Signaling Technology, cat. no. 4970)
- Anti-rabbit IgG (Jackson ImmunoResearch Laboratories, cat. no. 711-035-152)
- Anti-goat IgG (Jackson ImmunoResearch Laboratories, cat. no. 705-035-003)

EQUIPMENT

- Xenogen IVIS Lumina II system (Caliper Life Sciences)
- Inveon PET scanner (Preclinical Solutions, Siemens)
- γ-counter (1480 Wizard 3, PerkinElmer)
- ECL X-ray film developer (Amersham Biosciences)
- Multiwell plate reader (Synergy II, BioTek)
- UV-visible spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific, cat. no. 840-208100)
- HPLC with diode array detector and with a NaI crystal equipped radioactivity detector (Ultimate 3000, Dionex)
- Benchtop 2UV transilluminator (UVP)
- Gel documentation system (Biospectrum 810, UVP)
- Epifluorescence microscope (X81, Olympus)
- NanoDrop ND-2000 spectrophotometer (Thermo Scientific, cat. no. ND-2000)
- Orbital shaker (Lab Campaion, cat. no. SI-600)
- UV incubator (UVP, cat. no. SI-950)
- Benchtop centrifuge (Eppendorf centrifuge 5424, cat. no. 5424 0000.410)
- Autoclave (Tuttnauer 3545EP Autoclave)
- PCR thermal cycler (Techne, TC-512)
- Real-time PCR system (Applied Biosystems, 7300 real-time PCR system)

REAGENT SETUP

Glycerol bacteria storage solution (30%, vol/vol) Dilute 30 ml of pure glycerol in 70 ml of Milli-Q water in a 250-ml volume screw cap glass bottle. Autoclave this solution at 121 °C for 15 min. This stock solution can be stored at room temperature (22–24 °C) for up to one month under sterile conditions. **! CAUTION** Always loosen the cap when you are autoclaving. Handle the hot glass bottle with appropriate protective gloves.

Tris-acetate-EDTA stock buffer (50× TAE buffer) Weigh 242 g of Tris base into 750 ml of Milli-Q water. Add 57.1 ml of acetic acid with 100 ml of 0.5 M EDTA (pH 8.0). Adjust the solution to pH 8 and top it up with Milli-Q water to a final volume of 1 liter. This stock solution can be stored at room temperature for up to 1 year. If precipitation is observed, dissolve the precipitate thoroughly before use.

Tris-acetate-EDTA buffer (1× TAE) Dilute 20 ml of 50× stock buffer with Milli-Q water (vol/vol) into 1 liter. This buffer can be stored at room temperature for several months.

PROCEDURE

Plasmid construction round 1: cloning DnaEc cDNA into pcDNA3.1(+)/Zeo vector to produce VectorR1 ● TIMING 3–4 d

! CAUTION All chemical reactions should be carried out with caution in designated fume hoods. Most of the reagents in this protocol are toxic or harmful, and they require the use of lab goggles, gloves and a lab coat. In some conditions, a face mask will also be needed for protection.

1| Amplify the cDNA fragment of DnaEc by PCR using high-fidelity polymerase. Use the designated oligonucleotide primers shown in **Table 1**. Set up the PCR and thermocycling conditions as follows:



PROTOCOL

Reagents	Volume (μl)	Final concentration
Deoxynucleotide mix (dNTP) 10 mM	1	200 μM
Forward primer	1.5	300 nM
Reverse primer	1.5	300 nM
DNA template	1	100 ng
Expand high-fidelity buffer (10 \times)	5	1 \times
Expand high-fidelity enzyme	0.75	2.6 U
ddH ₂ O	39.25	
Final reaction volume	50	

Cycle number	Denaturation	Annealing	Polymerization	Extension
1	2 min at 94 °C			
2–36	30 s at 94 °C	45 s at 62 °C	1.5 min at 72 °C	
37				15 min at 72 °C

? TROUBLESHOOTING

2| Purify the PCR-amplified cDNA fragments using a PCR purification kit according to the manufacturer's instructions. Elute in 20 μl of pure H₂O.

■ **PAUSE POINT** The PCR-amplified cDNA fragments can be stored at 4 °C overnight. Longer storage is not recommended.

3| Run the PCR product on a 0.8% (wt/vol) agarose gel in 1 \times TAE buffer to check the quality of cDNAs (should be a single band) and to check that the size is ~1.3 kbp.

4| Digest the cDNA of DnaEc and pcDNA3.1/Zeo(+) (vector) by Hind III-HF and BamH I-HF (buffer 4, BSA) in 20 μl at 37 °C overnight. Purify the digested DnaEc cDNA using the Qiagen PCR purification kit according to the manufacturer's instructions.

▲ **CRITICAL STEP** NEB has changed its buffer names. In this protocol, we use the company's previous buffer name, buffer 4. Currently, the CutSmart buffer is used for all high-fidelity restriction enzyme reactions. In our experimental test, both buffers yielded identical DNA digestion products with the same ligation results.

5| Mix the digested vector with the appropriate volume of 6 \times loading dye. Run the digested vector on a 1% (wt/vol) agarose gel with GelRed nucleic acid stain using TAE buffer. Set the voltage to 100 V for 30 min. Use a 1-kb DNA ladder as the marker. Cut the target linear vector (size 5 kbp) using a sharp scalpel under UV light. Minimize the size of the cut gel to include the linear plasmid only, but not smears.

! **CAUTION** In this step, the investigator is subjected to intense UV exposure. Wear a UV-protective head shield, gloves and lab coat to cover all the skin exposed to UV irradiation. Dispose of the gel in an appropriate waste box.

▲ **CRITICAL STEP** Minimize the UV exposure time to prevent mutation of the DNA or unexpected damage.

6| Combine the digested DNA fragments of DnaEc and vector from Steps 3 and 4 at a ratio of 3:1, respectively. Ligate the digested DNA fragments of DnaEc and vector using T4 DNA at 16 °C overnight. For fast ligation, this ligation step can be done at room temperature in 4 h. In a typical experiment, we use 1 μl of ligase and 2 μl of ligation buffer to give a total volume of 20 μl .

? TROUBLESHOOTING

7| Transform the ligation product into chemically competent *E. coli* according to the manufacturer's instructions. After incubation of transformed *E. coli* on ice, transfer the transformed *E. coli* into 250 μl of S.O.C. medium and incubate it in the orbital shaker at 37 °C at 250 r.p.m. for 1 h.

8| Warm up an agar plate (with 100 µg/ml ampicillin) in an UV incubator at 37 °C before streaking the cultured *E. coli* on a plate.

! CAUTION Handle all materials carefully to avoid possible workspace contamination with other bacteria. Keep all items sterile all the time.

▲ CRITICAL STEP High-quality competent *E. coli* cells should be used to ensure maximal transformation efficiency.

? TROUBLESHOOTING

■ PAUSE POINT The bacterial colonies on the agar plate can be sealed with Parafilm and stored at 4 °C for up to 1 month.

9| On the next day, pick up a single colony into 3 ml of LB medium with 100 µg/ml ampicillin in a 14-ml round-bottom tube, and culture it in the orbital shaker at 37 °C at 250 r.p.m. overnight. After overnight culturing, the LB medium should become cloudy with an optical density of ~0.6–0.8 as measured by a UV-visible spectrophotometer.

10| Transfer the broth into 1.5-ml sterile microcentrifuge tubes, 1 ml per tube, and spin down the *E. coli* pellet using a benchtop centrifuge at 3,500g for 10 min at room temperature.

▲ CRITICAL STEP Avoid excessive centrifugation, as it may make the pellet difficult to resuspend.

■ PAUSE POINT Cultured bacteria in LB medium can be stored at 4 °C for up to 2 weeks. Alternatively, bacteria in LB medium can be aliquotted into several 1.5-ml sterile microcentrifuge tubes and mixed with 30% (vol/vol) glycerol solution (autoclaved for sterilization) for long-term storage in a –80 °C freezer.

11| Extract DNA from *E. coli* using the QIAprep spin miniprep kit according to the manufacturer’s instructions. The final eluate should be 20 µl. Measure the concentration of extracted DNA with a NanoDrop spectrophotometer.

12| Digest the eluted DNA using Hind III-HF and BamH I-HF enzymes with buffer 4 and BSA (NEB products). Use a total volume of 20 µl containing 1 U of each enzyme and 2–5 µl of DNA. After extensive digestion at 37 °C overnight, analyze the digested product on an 0.8% (wt/vol) agarose gel in 1× TAE buffer. Dilute 2 µl of digested DNA with 3 µl of ultrapure water into a total volume of 5 µl. Mix 1 µl of 6× loading dye (NEB) with the above DNA and load it on agarose gel. Run the gel at 125 V for 20 min. Visualize the DNA bands using a UV lamp (UVP). Two sharp and bright bands should be visible at 5 kbp (vector) and 1.3 kbp (DnaEc). If desired, perform plasmid sequencing to ensure the accuracy of the cloned sequence in the vector.

▲ CRITICAL STEP It is important to separate the digested DNA mixture completely, as low amounts of digested fragments may not be visualized if the bands are not fully resolved. If necessary, adjust the image contrast to ensure that all DNA bands are visible. After picking up the correct clone, extract and purify a sufficient amount of DNA for the next rounds of the experiment, and keep a portion of the plasmid for long-term storage either in bacteria (–80 °C freezer) or in solution (–20 °C freezer). At this stage, we named the temporal pcDNA3.1(+)/Zeo with the insertion of DnaEc as VectorR1.

■ PAUSE POINT Plasmid can be stored for the long term either in bacteria (–80 °C freezer) or in solution (–20 °C freezer).

Round 2: cloning the C-terminal TK fragment (TKc) into VectorR1 to produce VectorR2 ● TIMING 3–4 d

13| Perform PCR amplification of the TKc cDNA fragment using high-fidelity polymerase with oligonucleotide primers shown in **Table 1**. Refer to Step 1 for the reagents required for PCR. Perform PCR using the following thermocycling conditions:

TABLE 1 | Oligonucleotide sequences used in this protocol.

Primer name	Sequence (5'–3')
DnaEc forward	CACAAGCTT <u>G</u> CCACCATGGTTAAAGTTATCGGTCTG
DnaEc reverse	GAAGGATCCGCGGCATCGCCCC
TKc forward (77–375)	ACAGGATCCATCGTCTACGTACCCGAGCC
TKc forward (267–375)	ACAGGATCCGTGCCGCCACGGGTGCCGAGC
TKc forward (277–375)	ACAGGATCCAACGCGGGCCACGACCCCATATC
TKc reverse	AATGAATTCGTTAGCCTCCCCATCTCCC
TKn forward	TAAGAATTCATGCCACGCTACTGC
TKn reverse (46–76)	GAACTGCAGATCGTCGCGGAACCCAGG
TKn reverse (46–266)	GAACTGCAGGGCCGTCCCCGAAAGCTGTC
TKn reverse (46–276)	GAACTGCAGGCTCTGGGGCTCGGCAC
DnaEn forward	ATACTGCAGCGGCACCGAAATTTAACC
DnaEn reverse	AAAGCGGCCGCTAAGTTAATTGTCCCAGCGTCAAG
PEST forward	ATAGCGGCCGACGCATGGCTTCCC GCCG
PEST reverse	CAACTCGAGCTACACATTGATCTAGC
CTK forward (real-time PCR)	TACCCGAGCCGATGACTTAC
CTK reverse (real-time PCR)	CCGATTAGAGGAGCCAGAAC
GAPDH forward	GCGAGATCCCTCCAAAATCAA
GAPDH reverse	GT TCACACCCATGACGAACAT

Note: The underlined oligonucleotide sequences indicate the restriction enzyme sites used for construction of cTK.



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Cycle number	Denaturation	Annealing	Polymerization	Extension
1	2 min at 94 °C			
2–36	30 s at 94 °C	45 s at 62 °C	1.5 min at 72 °C	
37				15 min at 72 °C

▲ **CRITICAL STEP** The length of the TKc fragment depends on the protein split site. In our experiment, a 90-s extension time was sufficient for all three predetermined split sites (76, 266 and 276).

? TROUBLESHOOTING

14| Purify the TKc PCR product, as described in Step 2.

15| Perform double digestion of the cDNA of purified TKc and VectorR1 by Hind III-HF and BamHI-HF (buffer 4, BSA) enzymes in 20 µl at 37 °C overnight. Purify the digested TKc cDNA fragment using the Qiagen PCR purification kit according to the manufacturer's instructions.

16| Repeat Steps 4–11 to purify digested VectorR1, and insert the TKc cDNA fragment into the VectorR1 plasmid to produce VectorR2.

Round 3: cloning the N-terminal TK fragment (TKn) into VectorR2 to produce VectorR3 ● TIMING 3–4 d

17| Perform PCR amplification of the TKn cDNA fragment using high-fidelity polymerase using the oligonucleotide primers shown in **Table 1**. Refer to Step 1 for the reagents required for PCR. Perform PCR using the following thermocycling conditions:

Cycle number	Denaturation	Annealing	Polymerization	Extension
1	2 min at 94 °C			
2–36	30 s at 94 °C	45 s at 62 °C	1.5 min at 72 °C	
37				15 min at 72 °C

▲ **CRITICAL STEP** The length of the TKn fragment depends on the protein split site. In our experiment, a 90-s extension time was sufficient for all the three predetermined split sites (76, 266 and 276).

? TROUBLESHOOTING

18| Purify the PCR product of TKn according to the method in Step 2.

19| Perform double digestion of the cDNA fragment of purified TKn and VectorR2 by EcoRI and PstI-HF (buffer 4, BSA) enzymes in 20 µl at 37 °C overnight. Purify the digested TKc cDNA fragment using the Qiagen PCR purification kit according to the manufacturer's instructions.

20| Repeat Steps 4–11 to purify digested VectorR2, and then insert the cDNA fragment of TKn into VectorR2 plasmid to produce VectorR3.

Round 4: cloning DnaEn into VectorR3 to produce VectorR4 ● TIMING 3–4 d

21| Perform PCR amplification of the DnaEn cDNA fragment using high-fidelity polymerase with the oligonucleotide primers shown in **Table 1**. Refer to Step 1 for the reagents required for PCR. Perform PCR using the following thermocycling conditions:

Cycle number	Denaturation	Annealing	Polymerization	Extension
1	2 min at 94 °C			
2–36	30 s at 94 °C	45 s at 60 °C	3 min at 72 °C	
37				15 min at 72 °C

? TROUBLESHOOTING

22| Purify the DnaEn PCR product according to the method in Step 2.

23| Perform double digestion of the cDNA fragment of purified DnaEn and VectorR3 by NotI-HF and PstI-HF (buffer 4, BSA) enzymes in 20 μ l at 37 °C overnight. Purify the digested cDNA fragment of DnaEn using the Qiagen PCR purification kit according to the manufacturer's instructions.

24| Repeat Steps 4–11 to purify digested VectorR3, and then insert the cDNA fragment of DnaEn into VectorR3 plasmid to produce VectorR4.

Round 5: cloning the PEST fragment into VectorR4 to produce complete cTK plasmid ● **TIMING 3–4 d**

25| Perform PCR amplification of the cDNA fragment of PEST using high-fidelity polymerase with oligonucleotide primers shown in **Table 1**. Refer to Step 1 for the reagents required for PCR. Perform PCR using the following thermocycling conditions:

Cycle number	Denaturation	Annealing	Polymerization	Extension
1	2 min at 94 °C			
2–36	30 s at 94 °C	45 s at 62 °C	1 min at 72 °C	
37				15 min at 72 °C

? TROUBLESHOOTING

26| Purify the PEST PCR product according to the method in Step 2.

27| Perform double digestion of the cDNA fragment of purified PEST and VectorR4 by NotI-HF and XhoI (buffer 4, BSA) enzymes in 20 μ l at 37 °C overnight. Purify the digested PEST cDNA fragment using the Qiagen PCR purification kit according to the manufacturer's instructions.

28| Repeat Steps 4–11 to purify the digested VectorR4, and insert the PEST cDNA fragment into VectorR4 plasmid to produce the complete cTK plasmid.

29| Sequence the entire cTK plasmid to check the accuracy of cloning from round 1 to round 5.

■ **PAUSE POINT** The cTK plasmid can be stored at –80 °C for long-term storage.

Synthesis of the HSV1-TK substrate: ¹⁸F-FHBG ● **TIMING 1–1.5 h**

! **CAUTION** As radioactive chemicals will be used throughout this experiment, the personnel involved in this study should have received appropriate radiation safety training. Radioactive chemical waste should be handled strictly in accordance with the institutional radiation safety protocol.

30| Add 60 μ l of 0.1 M potassium carbonate and 100 μ l of Kryptofix 222 (4.5 mg in ACN) in a 1-ml V-vial.

31| Add ~3.7 GBq (100 mCi) of no-carrier-added aqueous H¹⁸F from the cyclotron target into the above mixed solution.

! **CAUTION** High levels of radioactivity are used in this step. Handle radioactivity with extreme care. Wear radiation-protective devices.

32| Remove the solvent under a stream of argon while heating it in a 105 °C hot block. Remove residual water by azeotropic drying with 100 μ l of ACN three times (100 μ l \times 3).

33| Dissolve 1 vial (3 mg) of Tysol-FHBG into 100 μ l of anhydrous DMF.

34| Add the Tysol-FHBG dissolved in DMF into the anhydrous potassium fluoride/Kryptofix 222 complex.

▲ **CRITICAL STEP** The anhydrous condition of the reaction system is very important for labeling and final yield.

35| Vigorously vortex the mixture from Step 34 and heat it at 160 °C for 15 min, followed by dilution with Milli-Q water (10 ml).



PROTOCOL

36| Equilibrate 100 mg of C-18 BondElut cartridge with 2 ml of ethanol and 4 ml of water before adding the above intermediate product into the cartridge.

37| Wash the cartridge with 2 ml of water, and elute the product from the cartridge with 20% (vol/vol) methanol in dichloromethane.

38| Evaporate the organic solvent. Treat the residue with 200 μ l of 1 N HCl in methanol and 200 μ l of 1 N HCl in water and heat the mixture at 105 °C for 10 min.

▲ **CRITICAL STEP** The order in which the organic and aqueous phases are added into the residue can substantially affect the final product.

39| Remove the bulk of the methanol under an argon stream at room temperature.

! **CAUTION** Control the velocity of the argon stream to prevent splashing of chemicals.

40| Add 600 μ l of 50 mM NH_4OAc to the residue.

41| Apply the solution from Step 48 to semipreparative HPLC for final product separation. Use a mobile phase of 7% (vol/vol) ethanol in 50 mM NH_4OAc and a flow rate of 5 ml/min. The elution time of ^{18}F -FHBG is ~10–11 min (column Luna C18, 10 \times 250 mm, Phenomenex). Measure the purity of eluted ^{18}F -FHBG by radio-HPLC; it should be >97%.

? TROUBLESHOOTING

42| Dilute the eluate with saline to the desired concentration for further application.

! **CAUTION** The decay half-life of fluorine-18 is 110 min. The radioisotope labeling of Tysol-FHBG should be done as efficiently as possible. The synthesized ^{18}F -FHBG should be used immediately.

? TROUBLESHOOTING

Activity assays

43| Test cTK function via a cell uptake assay (option A), a caspase activity assay (option B) and/or an *in vivo* imaging assay (option C).

(A) ^{18}F -FHBG uptake assays ● TIMING 2–3 h

- Culture stably selected 22B cells on a 24-well plate at a 1×10^5 density 48 h before the experiment.
- Treat the cells with compounds of interest and appropriate controls. We use the wide-spectrum chemotherapeutic drug Dox at the predetermined concentration of 1 $\mu\text{g}/\text{ml}$ for 24 h as a positive control to induce apoptosis.
- Aspirate the culture medium after the desired treatment period. We aspirate the medium after 24 h of treatment with 1 $\mu\text{g}/\text{ml}$ Dox.
- Wash the cell monolayer two times with ice-cold PBS.

▲ **CRITICAL STEP** At this time point, most cells are under apoptotic conditions. They are fragile and likely to detach. Handle the cells carefully and wash the cell monolayer gently.

- Add 5 $\mu\text{Ci}/\text{ml}$ ^{18}F -FHBG into each well and culture the cells at 37 °C, 5% CO_2 for 1 h.
 - Replace the medium with 500 μl of fresh medium and incubate it for an additional 1 h.
 - Aspirate the medium, bearing in mind that this will contain ^{18}F -FHBG. Wash the cell monolayer two times with PBS (pH 7.2).
- ▲ **CRITICAL STEP** Handle it with care and wash the cells gently.
- Add 200 μl of 0.1 N NaOH into each well to lyse the cells.
 - Transfer the entire cell lysate into a 1.5-ml volume Eppendorf tube. Measure the cell-associated radioactivity using the γ -counter.

? TROUBLESHOOTING

(B) Caspase-3 activity and specificity tests ● TIMING ~9 d

- Part I: ^{18}F -FHBG uptake in Dox-induced apoptotic cTK-22B cells after transfection of anti-caspase-3 siRNA.* Culture the cells on a 24-well plate at a cell density of 0.8×10^5 per well 24 h before siRNA transfection.
- Transfect the cells with anti-caspase-3 siRNA at 0, 25, 50 and 100 nm using Lipofectamine RNAiMAX according to the manufacturer's protocol.
- 24 h after transfection, add 2 $\mu\text{g}/\text{ml}$ of Dox into the transfected cells. Incubate the drug with cells for 24 h.
! **CAUTION** Dox is a highly toxic chemotherapeutic agent. Handle it with care and dispose of it properly.
- Wash the cells with ice-cold PBS two times, and then follow Step 43A(iv–ix) for ^{18}F -FHBG uptake study and analysis.
- Part II: ^{18}F -FHBG uptake in Dox-induced apoptotic MCF7 cells after transfection of caspase-3 plasmid.* Culture the cells on a 24-well plate at a cell density of 0.8×10^5 per well 24 h before transfection.

- (vi) Transfect the cells with caspase-3 plasmid at 1 µg per well using Lipofectamine 2000 according to the manufacturer's protocol.
- (vii) 24 h after transfection, add the drug of interest (for example, 5 µg/ml Dox), into the transfected cells. Incubate the cells with the drug for the desired time period: for Dox, 24 h.
- (viii) Wash the cells with ice-cold PBS two times, and then follow Steps 43A(iv–ix) for ¹⁸F-FHBG uptake study and analysis.
- (ix) *Part III: ¹⁸F-FHBG uptake in Dox-induced apoptotic cTK-22B cells with caspase inhibitor.* Culture cTK stably transfected cells on a 24-well plate at a cell density of 1 × 10⁵ per well 24 h before transfection.
- (x) Add the drug of interest, for example, 0.5 µg/ml Dox with or without the caspase inhibitor IDN6556 (final concentration 20 µM), into each well for 24 h.
 - ▲ **CRITICAL STEP** The optimal concentration of the caspase inhibitor should be investigated before this experiment. The most effective inhibitory effect may vary in different cell lines.
- (xi) Wash the cells with ice-cold PBS two times, and then follow Step 43A(iv–ix) for ¹⁸F-FHBG uptake study and analysis.

(C) In vivo imaging ● **TIMING 2–3 weeks**

- (i) Establish a suitable xenograft tumor model. For example, inject 5 × 10⁶ cTK-22B cells subcutaneously (in 100 µl of sterile saline or PBS) into nude mice (5–6 weeks old). Wait for tumor growth for ~2–3 weeks, while keeping the mice in a regular animal housing facility.
 - ▲ **CRITICAL STEP** The tumor volume should be well controlled to be 150–200 mm³ volume (calculated as length × width²/2, ~7–8 mm in diameter).
- (ii) Randomize the mice into an appropriate number of groups. For example, for the treatment schedule given here, divide the mice into two groups with six mice per group.
- (iii) Induce apoptosis in the treatment group. For example, inject one group of mice with 10 mg/kg Dox intravenously. Inject the second group of mice with an equal amount of PBS (100 µl). Keep the mice in an animal holding facility for 72 h to induce extensive cancer cell apoptosis before *in vivo* imaging.
- (iv) *Part I: in vivo bioluminescence imaging (BLI).* On the day of BLI imaging, intraperitoneally inject 150 mg/ml D-luciferin into each mouse.
- (v) Anesthetize mice with a 2% (vol/vol) isoflurane/oxygen mixture.
- (vi) Wait for the mice to be completely anesthetized and breathing smoothly, this should take 5–10 min.
- (vii) Conduct multiple series of BLI on mice at 5-min intervals using the Xenogen Lumina II system. We use the following settings: Acquisition time = 5 min; Acquisition times = 5–6; F/stop (aperture) = 1 (Fully open); Binning = Medium; Emission filter = Open; Field of View (FOV) = D, 12.5 cm; stage temperature: 37 °C. Keep the mouse anesthetized with 1.5–2% (vol/vol) isoflurane/oxygen through a nose delivery system.
- (viii) After imaging, keep the animal under lamp light with free air inhalation until it is awake. Next, transfer the animal back to the transportation box.
- (ix) Analyze the data. We use Live Image software provided by the manufacturer (PerkinElmer). Draw regions of interest (ROIs) by drawing a circle around the tumor periphery in each mouse. Use the average radiance (p/s/cm²/sr) unit to present the imaging signal intensity. The absolute photon counting values can be used to generate plots and figures.
- (x) *Part II: in vivo micro-PET imaging.* Reanesthetize the mice after BLI with 2% (vol/vol) isoflurane/oxygen.
- (xi) Inject ¹⁸F-FHBG intravenously at 300 µCi per mouse.
- (xii) Keep the mice awake in a warm place for 2 h. Next, anesthetize the mice with 1% (vol/vol) isoflurane/oxygen.
- (xiii) Transfer the mice onto the PET machine bed, and apply static PET scanning to the mice for 10 min. For PET scanning, perform the scan protocol with the Inveon Acquisition Workstation.
- (xiv) Recover the mice with free air inhalation under lamp light before transferring them into the transportation box.
- (xv) Reconstruct the acquired images with a 2D order-subset expectation maximization (2D OSEM) algorithm. Do not apply any correction for attenuation or scatter.
- (xvi) For each scan, draw the ROI. We used the vendor software (ASI Pro 5.2.4.0) on decay-corrected whole-body coronal images. Obtain the radioactivity concentrations (accumulation) within the tumors from mean pixel values within the multiple ROI volumes and then convert them to MBq per milliliter. Divide these values by the administered activity to obtain (assuming a tissue density of 1 g/ml) an image ROI-derived percent injected dose per gram (%ID/g).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

PROTOCOL

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1, 13, 17, 21, 25	No PCR products	Annealing temperature is not appropriate for each specific pair of primers	Adjust the annealing temperature to between 60 and 65 °C
6	Ligation failure	The gel is cut too large or the quality of the purified gel is poor	Keep the cut gel as small as possible. Add a 1/3 volume of isopropanol after dissolving the gel in the kit-provided buffer. Avoid chemical contamination
8	No colony appears	Poor ligation	Try to use freshly digested and purified fragment and vector for ligation Be sure to keep the ligase at –20 °C all the time. Use a table-top cooler (–20 °C) to handle enzymes Minimize the number of freeze-thaw cycles for ligation buffer, as ATP is contained in the buffer. Thaw the buffer completely before use Increase both the insert and vector amount in the ligation step, especially for blunt-end ligation
Box 1, step 5	Negative control cells are not killed	Zeocin selection failure	Check the expiry date of the Zeocin to make sure that the drug is active and effective The drug concentration used in cell selection is not optimal; use a higher Zeocin concentration
Box 1, step 6	All the transfected cells die	The Zeocin concentration is too high or cells are not transfected properly	Lower Zeocin concentration Repeat the transfection step
Box 1, step 7	Cells do not grow normally	Transfection failure or cells are dying owing to membrane damage during scraping and transferring	Repeat the transfection step Repeat the scraping step gently but efficiently
41, 42	Low purity and low yield	Low radiolabeling efficiency or contamination with other chemical residues	Re-distill all relevant organic solvents to prepare anhydrous ones. Re-prepare all buffers. Dry glassware thoroughly. Re-flush the HPLC column with 90% (vol/vol) ethanol/50 mM ammonium acetate, and then with 10% (vol/vol) ethanol/50 mM ammonium acetate thoroughly. Check the purity of Tysol-FHBG
43A(ix)	No or low radioactivity	Apoptosis is not induced properly or apoptotic cells are washed off	Adjust the drug concentration or drug/inhibitor ratio to induce apoptosis Handle and wash the cells carefully

● TIMING

Steps 1–12, plasmid construction round 1: cloning DnaEc into pcDNA3.1(+)/Zeo vector to produce VectorR1: 3–4 d

Steps 13–16, round 2 cloning TKc into VectorR1 to produce VectorR2: 3–4 d

Steps 17–20, round 3 cloning TKn into VectorR2 to produce VectorR3: 3–4 d

Steps 21–24, round 4 cloning DnaEn into VectorR3 to produce VectorR4: 3–4 d

Steps 25–29, round 5 cloning PEST into VectorR4 to produce complete cTK plasmid: 3–4 d

Steps 30–42, synthesis of HSV1-TK substrate: ¹⁸F-FHBG: 1–1.5 h

Step 43, activity assays: 3–4 weeks

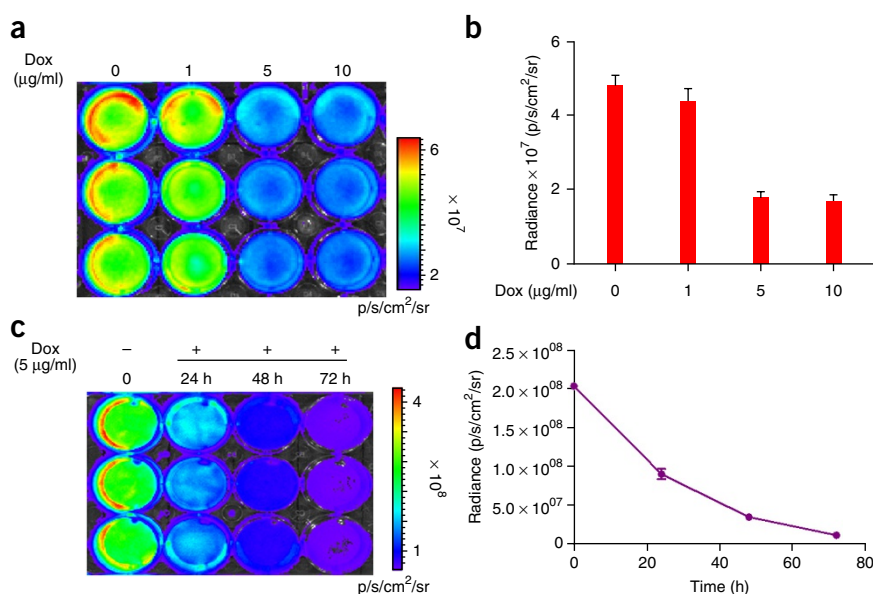
Box 1, generation of cTK stable transgenic cell line: 4–6 weeks

ANTICIPATED RESULTS

Expression of cTK variants in 22B cancer cells

After cTK plasmid construction and transfection into 22B cells, we recommend that a real-time PCR test be performed to screen for the cells expressing the highest cTK variant expression (as described in **Box 1**; Step 9 of the PROCEDURE).

Figure 3 | Transfection of cTK266-containing cells with firefly luciferase (fLuc) as the cell proliferation indicator. **(a)** Bioluminescence imaging (BLI) of different Dox concentration-treated cTK266-22B-fLuc cells after 24 h in a 24-well plate. **(b)** Quantitative analysis of BLI results from **a** ($n = 3$); error bars are means \pm s.d. **(c)** BLI results of cells treated with 5 $\mu\text{g/ml}$ at different time points ($n = 3$) in a 24-well plate. **(d)** Quantitative analysis of BLI results from **c** ($n = 3$); error bars are means \pm s.d. Reproduced from ref. 34 with permission.



These cells should subsequently be chosen for future *in vivo* PET imaging studies. **Figure 2** shows the expression level of different cTK variants after transfection and real-time PCR test in 22B cells. We chose the highest expression cells for our further studies: cTK76(4), cTK266(1), cTK276(3). For ease of identification, cTK76 = cTK76(4), cTK266 = cTK266(1) and cTK276 = cTK276(3).

Stable transfection of cTK-22B cells with fLuc as the cell proliferation indicator

fLuc is transfected into cells to facilitate real-time imaging of cell proliferation when cells are undergoing apoptosis. Typical experimental results from cTK-22B cells are shown in **Figure 3**. At different Dox dosages, cTK-22B cells showed gradual proliferation reduction compared to the untreated control (**Fig. 3a,b**). In most cases, 5 $\mu\text{g/ml}$ Dox was able to induce extensive cell apoptosis after 24 h of incubation. A longer incubation time (72 h) resulted in markedly low cell proliferation (**Fig. 3c,d**).

In vitro characterization of the cTK266 reporter

In cTK266 stably transfected cTK-22B cells, the ^{18}F -FHBG uptake increment was proportional to Dox dosage (**Fig. 4a**). Western blot analysis (**Fig. 4b**) showed increased concentration of linear TK protein with increased Dox dosages compared to untreated controls, whereas the concentration of cyclic TK protein decreased accordingly. Results from **Figure 4** indicate that cell apoptosis condition can be monitored through ^{18}F -FHBG radiotracer, via cTK cleavage by caspase-3.

Caspase-3 specificity of cTK266 function in cTK-22B cancer cells

The specificity of an imaging reporter is essential for precise molecular imaging. To evaluate the specificity of the cTK266 reporter in apoptosis imaging via the caspase-3 signaling pathway, we first used a caspase inhibitor, IDN6556, to study the ^{18}F -FHBG uptake. The result (**Fig. 5a**) showed statistically significant ($P < 0.01$) ^{18}F -FHBG cell uptake inhibition in the presence of IDN6556. Next, we used anti-caspase-3 siRNA to downregulate caspase-3 in cTK-22B cells, followed by a ^{18}F -FHBG uptake study. **Figure 5b** showed gradually lowered ^{18}F -FHBG cell uptake with increasing concentrations of anti-caspase-3 siRNA. To further confirm the caspase-3 specificity of the cTK266 reporter, we used a special cell line, MCF-7, whose apoptosis is naturally independent of the caspase-3 signaling pathway. Upon transfection of the caspase-3 plasmid followed by Dox treatment, there was a statistically higher ^{18}F -FHBG cell uptake than in all other control groups (**Fig. 5c**). All the above assays solidly demonstrated high caspase-3 specificity of our cTK reporter, and they should be used to confirm the specificity of the reporters generated using this protocol.

In vivo PET scanning and BLI of apoptosis induced by Dox treatment

Dox treatment in a cTK-22B-fLuc stably transfected 22B cell xenograft tumor model can lead to extensive apoptosis in tumors.

Figure 6a shows the PET imaging of apoptosis via cTK reporter after ^{18}F -FHBG administration (**Fig. 6b**). Extensive

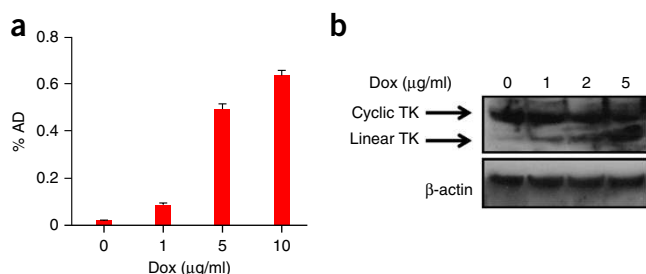
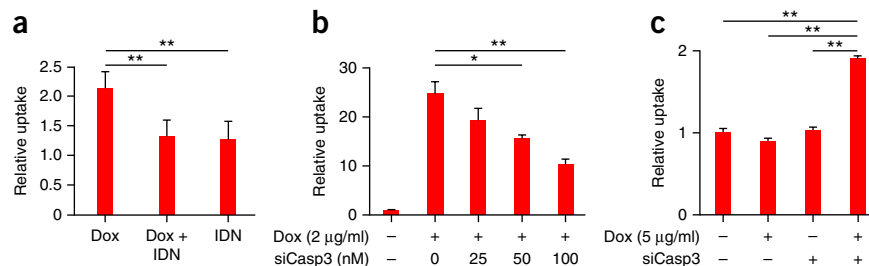


Figure 4 | Characterization of cTK266 reporter in 22B cells. **(a)** ^{18}F -FHBG uptake assay of cTK266-transfected 22B cells treated with different dosages of Dox (1, 5 and 10 $\mu\text{g/ml}$) for 24 h ($n = 4$). Error bars are means \pm s.d. **(b)** Western blot analysis of gradient cTK activation with different Dox treatment concentrations (1, 5 and 10 $\mu\text{g/ml}$) for 24 h. The primary antibodies we used were HSV1-TK and β -actin. The secondary antibodies used were: anti-rabbit IgG and anti-goat IgG. Reproduced from ref. 34 with permission.

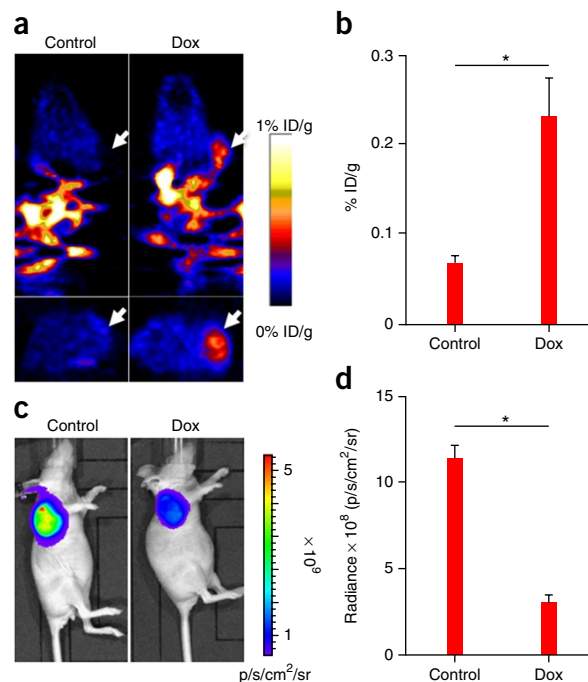
PROTOCOL

Figure 5 | Caspase-3 specificity evaluation of cTK266 in cells. **(a)** The ^{18}F -FHBG uptake is prohibited after adding the caspase inhibitor IDN6556 during 0.5 $\mu\text{g}/\text{ml}$ Dox treatment for 24 h. IDN, IDN6556, $**P < 0.01$, ($n = 4$); error bars are means \pm s.d. **(b)** The ^{18}F -FHBG uptake in cells treated with anti-caspase-3 siRNA. The downregulation of caspase-3 in apoptotic cells lowers the ^{18}F -FHBG uptake. $*P < 0.05$, $**P < 0.01$, $n = 4$, error bars are means \pm s.d.



(c) Caspase-3-dependent ^{18}F -FHBG uptake in MCF7 cells. MCF7 cells were transfected with cTK266 or with cTK266 and caspase-3 together for 24 h. Next, the cells were treated with 5 $\mu\text{g}/\text{ml}$ for an additional 24 h before the ^{18}F -FHBG uptake assay. The data were normalized with the same total protein amount of each cell lysate. $**P < 0.01$ ($n = 3$), error bars are means \pm s.d. Reproduced from ref. 34 with permission.

Figure 6 | *In vivo* PET imaging and BLI of apoptotic cells after Dox treatment. **(a)** Representative images of coronal (top) and transaxial (bottom) PET imaging of nude mice bearing cTK266-22B-fLuc tumors at 2 h after injection of ^{18}F -FHBG at $\sim 300 \mu\text{Ci}$ per mouse, treated with 10 mg/kg Dox 72 h before PET imaging ($n = 6$). The arrows indicate the tumor areas. **(b)** Quantitative analysis of the PET signal at the tumor site. Data are represented as the mean percentage injected tracer dose per gram (%ID/g); error bars are means \pm s.d. **(c)** Representative BLI imaging of the same group of mice showing markedly low cell proliferation after Dox (10 mg/ml) treatment for 72 h. **(d)** Quantitative analysis of BLI imaging. $*P < 0.05$, $n = 6$, error bars are means \pm s.d. All the animal studies were conducted in accordance with the principles and procedures outlined in the guide for care and use of laboratory animals and approved by the NIH animal care and use committee of clinical center. ID, injected dose. Reproduced from ref. 34 with permission.



PET signal was detected in the tumor after Dox treatment. In BLI imaging of the corresponding cell proliferation, Dox treatment led to a significantly lower cell proliferation than that seen in the untreated control, as indicated by low BLI signal (Fig. 6c,d), which was consistent with PET imaging data showing extensive apoptosis after chemotherapeutic agent treatment. Dox treatment thus represents a suitable positive control when using this assay to look at alternative chemotherapeutic agents.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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