Supplemental materials

Cell Cultures

Adherent cells were grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS). Cells were cultured at 37°C in humidified atmosphere with 5% CO_2. The cells were harvested using 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Invitrogen, USA). Cell growth was monitored under an inverted microscope with phase contrast.

In vitro and In vivo Stability Analysis

To evaluate the labeling stability in vitro, ^{99m}Tc-EGFR-PNA was incubated in normal saline or fresh human serum at 37°C for 1, 2, 4, 6, 12 and 24 h, respectively. At each time point, the radiochemical purities of the probe samples were analyzed by RP-HPLC using the experimental conditions described above.

The in vivo radiochemical stability was evaluated by RP-HPLC analysis of the urine and plasma from two groups of 5 SKOV3 tumor-bearing mice at 30 min and 2–3 h after injection with the ^{99m}Tc-EGFR-PNA or ^{99m}Tc-CTL-PNA. The urine and plasma samples were collected in Eppendorf vials and then centrifuged at 4,000 rpm for 10 min before analysis by RP-HPLC for the amount of radiolabeled probes as described above.

Cellular Uptake, Retention Kinetics, Internalization and Blocking Studies

Cellular uptake studies of ^{99m}Tc-EGFR-PNA and ^{99m}Tc-CTL-PNA were accomplished with SKOV3 and MDA-MB-435S cells plated at a density of 6×10^5 cells per well. Sextuplicate cell wells were used for each data point. The cells were washed twice with phosphate buffered saline (PBS), and 2 ml fresh RPMI 1640 medium containing 10% fetal bovine serum (FBS) was added, followed by 37 kBq of ^{99m}Tc-EGFR-PNA or ^{99m}Tc-CTL-PNA directly into each well. Cells were incubated

with the probes at 37°C and harvested using same trypsin-EDTA solution at 1, 2, 4, 6, 12 and 24 h as described above. Before cell lysis, the medium containing 10% FBS was removed, and the wells were washed with PBS twice. Counts containing radioactive medium and PBS were designated as C_{out}. After being lysed with 0.5 ml trypsin-EDTA, 0.5 ml medium containing 10% FBS was added to each well, and then cells were washed twice with PBS. Radioactivity counts of lysis solution and PBS were defined as C_{in}. The collected fractions were measured in an automated γ-counter. The cellular uptake ratio was calculated using the formula $C_{in} / (C_{in} + C_{out})$.

To determine cellular retention, ^{99m}Tc-EGFR-PNA or ^{99m}Tc-CTL-PNA was added directly into 6-well plates containing 6×10^5 SKOV3 or MDA-MB-435S cells and incubated at 37°C for 6 h. Subsequently, the medium was removed, and all wells were washed twice with PBS. After adding 2 ml of fresh RPMI 1640 medium containing 10% FBS into each well, the plates were incubated at 37°C for 1, 2, 4, 6, 12 or 24 h. Before cell lysis, the medium containing 10% FBS was removed, and the wells were washed with PBS twice. Counts containing radioactive medium and PBS were designated as C_{out}. After being lysed with 0.5 ml trypsin-EDTA, 0.5 ml medium containing 10% FBS was added to each well, and then cells were washed twice with PBS. Radioactivity counts of lysis solution and PBS were defined as C_{in}. The collected fractions were measured in an automated γ-counter. The cellular retention ratio was calculated using the same formula for determining cellular uptake kinetics given above.

For internalization studies, radiopharmaceutical uptake was performed as described. Several additional cell dishes were used during the binding study to separate the membrane-bound fraction of the conjugate from internalized radioactivity. The wells were washed with PBS twice. Then the isolated cell pellet was washed with

0.2 M acetic acid/0.5 M NaCl, pH 2.5, to remove surface-bound radioactivity at each time point. Radioactivity that was removed from cells by an acidic buffer was considered membrane bound and the rest was considered internalized.

For blocking experiments, 2.5μg (500times) or 5μg (1000 times) of unlabeled PNA was added simultaneously with the ^{99m}Tc-EGFR-PNA. After incubation for 2h, the EGFR mRNA expression of SKOV3 cells was measured by real-time quantitative reverse transcription-PCR (qRT-PCR) to determine the block results. Meanwhile, the control blocking experiments of unlabeled CTL-PNA in MDA-MB-435S cells were performed as above.

Measuring Cellular *EGFR* mRNA Expression by qRT-PCR

Total RNA was extracted from $1x10^6$ cell samples using a standard TRIzol (Invitrogen, USA) RNA isolation method and quantified by measuring UV absorbance at 260 nm. Reverse transcription of RNA was carried out according to the instructions of the Easy Script First-Strand cDNA Synthesis Super Mix kit (Trans Gen Biotech, CA). Real-time PCR was performed on an ABI PRISM 7300 PCR System (Applied Bio systems, USA) using SYBR Green I GoTaq® qPCR Master Mix (Promega, USA). The EGFR mRNA expression from each sample was analyzed in duplicates and normalized against GAPDH. The results were expressed as relative gene expression using the $2^{-\Delta\Delta Ct}$ method. The relative expression of EGFR mRNA in SKOV3 and MDA-MB-435S cells were obtained by the software SDS v1.3.2 attached with the PCR machine. The *EGFR* mRNA binding was blocked with excess of unlabeled EGFR-PNA in SKOV3 was also measured by qRT-PCR.

Animal Xenograft Model

Female BALB/c nude mice (mean weight ± SD; 20 ± 5 g; age, 4–6 wk; Department of Animal Center, Peking University) were used in this study. Mice were injected subcutaneously with 6×10^6 SKOV3 or MDA-MB-435S cells (200 μl of RPMI 1640 medium without FBS) in the right anterior superior limbs. The tumors were allowed to grow for 2–3 weeks and reached a diameter of 1.0–1.5 cm before the experiments were performed. The mice were maintained on a standard diet, bedding and environment, with free access to food and drinking water. All animal experiments were approved by local Animal Welfare Committee in accordance with Chinese legislation and performed in accordance to their guidelines, and the institutional review board approved this study in mice with human tumor xenografts.

Biodistribution in EGFR-expressing Tumor Model

For biodistribution studies, a 1,110 kBq dose of ^{99m}Tc-EGFR-PNA or ^{99m}Tc-CTL-PNA was injected through the tail vein into BALB/c nude mice bearing SKOV3 or MDA-MB-435S xenografts (n = 6 /group), which were sacrificed at 1, 2, 4 or 6 h after injection. Tumor and normal tissues were excised and weighed, and radioactivity was measured using a γ-well counter (Capintec, USA). The radioactivity uptake in the tumor and normal tissues was expressed as the percentage of the injected dose per gram of organ (% ID/g).

Molecular Imaging Procedure

Ten BALB/c nude mice with SKOV3 tumor xenografts were randomly divided into

2 groups of 5 mice each, 37 MBq (100 μL) of either ^{99m}Tc-EGFR-PNA or ^{99m}Tc-CTL-PNA (pH=7.0) was injected into each mouse via the tail vein. Moreover, five BALB/c nude mice with MDA-MB-435S tumor xenografts were injected with 37 MBq (100 μL) of ^{99m}Tc-EGFR-PNA. At 1, 2, 4, 6, 8 and 10 h after injection, mice were anesthetized and placed in a supine position near the center field of the view of the detector and imaged using a SPECT planar scanner (Siemens e.cam^{duet} SPECT) equipped with a pinhole collimator. A static anterior image (300,000 counts) was taken with a zoom factor of 1.78 and digitally stored in a 128 × 128 matrix. The energy window setting was 140 KeV, 15%. The ratio of radioactive counts in the tumor to that in the contralateral equivalent region (T/NT) was calculated by drawing regions of interest at each time point.