Supporting Text

Supporting Methods

Synthesis of Cypate (NIR Dye). We recently described a general method for the synthesis of cypate (Fig. 1) in high purity (>99%) (1). Briefly, a mixture of 1,1,2-trimethyl-[^{1}H]benz[e]indole (40.0g, 19.11 mmol) and 3-bromopropanoic acid (40.0 g, 26.15 mmol) in 1,2-dichlorobenzene (200 ml) was heated at 110°C for 18 h. After the resulting mixture was cooled to room temperature, the precipitate was collected by filtration, followed by trituration in dichloromethane (DCM) to remove the unreacted material, and then dried under vacuum to afford 1,1,2-trimethyl-[^{1}H]benz[e]indole-3-propanoic acid. A solution of acetic anhydride (1.20 g, 11.75 mmol) in DCM (5 ml) was added dropwise to a cooled suspension of glutaconaldehyde dianil monohydrochloride (2.84 g, 9.97 mmol) and diisopropylethylamine (DIEA, 2.60 g, 20.11 mmol) in DCM (20 ml). The resulting clear solution was stirred for 1 h and added dropwise to a refluxing solution of 1,1,2-trimethyl-[^{1}H]benz[e]indole-3-propanoic acid (8.2 g, 22.64 mmol) and sodium acetate (3.2 g, 39.01 mmol) in acetonitrile/water (95/5 ml). The mixture was refluxed for 16 h, cooled, filtered, and washed with acetonitrile, 5% HCl solution and ethyl ether. About 6 g (85%) of cypate was obtained. It was further purified by recrystallization from 30% acetonitrile in water. Mass calculated: 625; observed (ESI-MS): 625.34 $[M]^+$.

Linear peptide synthesis and conjugation with fluorescent probes: The peptides were prepared with ACT APEX 396 peptide synthesizer by standard fluorenylmethyl (Fmoc) protocol (2), as described (3, 4). Briefly, a Wang resin preloaded with Fmoc-Lys-OH on a 25-μmol scale was placed in a reaction vessel. Subsequent Fmoc-protected amino acids (75 μmol) were sequentially coupled to the resin-bound amino acid from the carboxyl to amino terminus. Fmoc removal was accomplished with 20% piperidine in dimethyl formamide (DMF). The coupling reagents *N*-hydroxybenzotriazole (HOBt, 75 μmol) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate (HBTU, 75 μmol) were used for the peptide assembly in the presence of DIEA (150 μmol). Peptide

cleavage from the resin and concomitant removal of the amino acid side-chain protecting groups were accomplished by adding a cleavage mixture (1 ml) of TFA (85%), distilled water (5%), phenol (5%), and thioanisole (5%) to the resin and gently mixing the content for 3 h. The crude peptide was precipitated in cold *t*-butyl methyl ether (MTBE) and lyophilized in acetonitrile/water (2:3) mixture. The resulting powder was purified by HPLC and analyzed by ES-MS.

Conjugation of the dyes with the peptides was performed on solid supports. After peptide assembly (25 μmol), the *N*-terminal Fmoc group was removed with 20% piperidine in DMF. Cypate or cypate-3 (75 μmol) was preactivated with diisopropyl carbodiimide (DIC, 125 μmol) in DMF (2 ml) for 20 min and added to the resin-bound peptide. The mixture was swirled for 12 h, filtered, washed with DMF and methanol. The resulting green resin was treated as described above for peptide cleavage and workup. The following compounds were prepared by this general method:

Gly-Arg-Asp-Ser-Pro-Lys-OH (GRD): 9 mg; 14 μmol; 40% isolated yield; mass (calculated): 658.35; observed (ES-MS): 659.36 $[MH]^+$.

Cypate-Gly-Arg-Asp-Ser-Pro-Lys-OH (Cyp-GRD): 8.1 mg; 6.40 μmol; 25.6% isolated yield; mass (calculated): 1265.3; observed (ES-MS): 1265.3 $[M]^+$.

Cypate-Gly-Arg-Gly-Asp-Pro-Lys-OH (Cyp-RGD): 6.4 mg; 4.84 μmol; 19.4% isolated yield; mass (calculated): 1322.37; observed (ES-MS): 1322.55 $[M]^+$

Synthesis of Cypate-cyclo[Arg-Gly-Asp-D-Phe-Val-Lys(ε-cypate)] (Cyp-cyclo-RGD). This compound was prepared in three steps: solid phase peptide synthesis, intramolecular lactamization in solution, and dye conjugation.

(a) H-Lys(Dde)-Arg(Pbf)-Gly-Asp(OBu^t)-D-Phe-Val-OH: The linear peptide was assembled on Fmoc-Val-2-chlorotrityl resin (0.6 g, substitution 0.33 mmol/g) by conventional Fmoc chemistry and cleaved with TFA/DCM mixture (1:99), as described

above. The filtrate was added to a solution of pyridine (20 ml) and methanol (80 ml), concentrated, washed with water, and dried to afford the desired compound (142.0 mg; 60%). ES-MS: $[MH]^+$ 1193.51

(b) Cyclo[Arg(Pbf)-Gly-Asp(OBu^t)-D-Phe-Val-Lys]: To a solution of the above linear peptide (130.0 mg, 0.11 mmol), HOBt (23.0 mg, 0.17 mmol), and PyBOP (88.4 mg, 0.17 mmol) in DMF (15 ml) was added DCM (500 ml), followed by DIEA (44.0 mg, 0.34 mmol). The mixture was stirred at room temperature for 24 h, concentrated under vacuum, and re-dissolved in DCM. The solution was washed with water, 5% HCl, and 5% K_2CO_3, dried, and concentrated to afford a crude cyclic product, cyclo[Arg(Pbf)-Gly-Asp(OBu^t)-DPhe-Val-Lys(Dde)] (ES-MS: $[MH]^+$ 1175.47). The crude product was stirred in a 30 ml solution of 5% hydrazine in acetonitrile for 30 min, concentrated, and purified by HPLC to afford 30.0 mg (23%) of the cyclic compound containing free ε-amino lysine group. This product was dissolved in DCM and washed with 5% sodium carbonate solution for subsequent conjugation with cypate. Mass (calculated): 1011.2; observed (ES-MS): 1011.3$[M]^+$, 506.1$[M + 2H]^{2+}$.

(c) Conjugation with cypate: A mixture of cypate (42.3 mg, 0.06 mmol), 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI; 11.5 mg, 0.06 mmol), HOBt (16.0 mg, 0.12 mmol), and the above cyclic peptide (25.0 mg, 0.02 mmol) in DMF (3.0 ml) was stirred for 24 h, concentrated, re-dissolved in DCM, washed with 5% aqueous HCl solution and water, and concentrated to give the protected peptide (ES-MS: 1618.30 $[MH]^+$). The crude product was stirred in 15 ml of TFA/water (95:5) at room temperature for 3 h to remove the side-chain protecting groups, concentrated, and precipitated in cooled MTBE solution. The precipitate was purified by HPLC to afford 3.7 mg (12%) of the desired compound. ES-MS: 1309.47 $[MH]^+$; 655.29 $[M + 2H]^{2+}$.

Synthesis of DOTA-Gly-Arg-Asp-Ser-Pro-Lys-OH (DOTA-GRD). The peptide was prepared as described above at a 25-μmol scale. After removal of the N-terminal Fmoc, orthogonally protected tri-*tert*-butyl *N',N'',N''',N''''*-dodecyltetraacetic acid (DOTA; 75 μmol, Macrocylics, Inc) was coupled automatically with the free amino group in the

presence of HOBt/HBTU/DIEA in DMF. TFA cleavage, workup, and purification were performed as described above to afford DOTA-GRD as a colorless powder (9.2 mg; 35%). Mass (calculated): 1044.8; observed (ES-MS): 1045.69 $[MH]^+$.

Absorption and Emission Properties. The spectral properties of Cypate and their peptide conjugates were determined in 20% aqueous dimethyl sulfoxide (DMSO), which solubilized all of the compounds. This solvent mixture has also been shown to be biocompatible for both *in vitro* and *in vivo* applications (4, 5). Absorbance measurements were performed on a Beckman Coulter DU 640 spectrophotometer. The fluorescence spectra were recorded on Fluorolog-3 fluorometer (JOBIN YVON/HORIBA, Edison, New Jersey). To prepare the stock solutions, a high precision analytical balance was used to weigh >2 mg of each compound, which was dissolved in DMSO. The DMSO solutions were then diluted with water to obtain the stock solution of 20% aqueous DMSO. Absorbances of a serial dilution of the stock solution were used to determine the molar absorptivity of dye-peptide conjugates. For fluorescence measurements, we used probe solutions at 0.1 absorbance units to minimize inner filter effects.

Cell Culture. The human non-small cell carcinoma cells A549 were purchased from ATCC and grown in 75 cm tissue culture flasks or on Lab-Tek chambered slides in Ham's F12K medium with 2 mM L-glutamine supplemented with 1.5 g/liter sodium bicarbonate, 10% FCS, 100 units/ml penicillin and 100 units/ml streptomycin.

Immunohistochemistry. A549 cells grown on Lab-Tek microscope slides were fixed in 3.5% paraformaldehyde for 5 min. After washing in PBS, cells were treated with 0.3% H_2O_2 for 10 min to block endogenous peroxidase activity. Cells were blocked with 5% rabbit serum in PBS for 1 h at room temperature and incubated with a 1:100 dilution of primary antibody (goat anti-α_v or goat anti-β_3, Santa Cruz) overnight at 4°C. After washing three times for 5 min each with PBS, the cells were incubated with a 1:200 dilution of biotinylated rabbit anti-goat secondary antibody (Vector) for 1 h at room temperature. Slides were treated with the Vecstastain ABC Peroxidase Reagent kit and signal developed using the 3,3'-diaminobenzidine (DAB) Substrate Kit(Vector).

Western Blot Analysis. A549 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 1 mM PMSF) for 15 min on ice and centrifuged at 10,000 × *g* for 10 min at 4°C to remove insoluble material. Protein concentration was determined by the Bio-Rad protein assay per manufacturer's instructions. Cell lysate containing 20 μg of protein was mixed with two volumes of sample buffer (Bio-Rad) and boiled for 3 min. The cell lysates were resolved by electrophoresis on a 7.5% Tris·HCl polyacrylamide gel at 150V for 45 min. The protein was transferred to poly(vinylidene difluoride) (PVDF) membranes and nonspecific binding was blocked by incubating in SuperBlock (Pierce) for 1 h at room temperature. The membranes were then incubated with a 1:5000 dilution of primary antibody (goat anti-α_v or goat anti-β_3, Santa Cruz Biotechnology) in SuperBlock for 1 h at room temperature. After washing in TBS-Tween, the membranes were incubated with a 1:500,000 dilution of horseradish peroxidase-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology). Bands were visualized by treating the membranes with SuperSignal West Dura chemiluminescent substrate (Pierce) according to manufacturer's instructions and detecting the bioluminescence with Kodak Multistation scanner.

Cytotoxicity MTT Assay. Cytotoxicity assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) *in vitro* toxicology assay kit per the manufacturer's protocol (Sigma). Briefly, A549 cells were grown in 96 well plates to 75% confluence in phenol red free Dulbecco's Modified Eagle's Medium supplemented with 10% FCS, 100 units/ml Penicillin and 100 units/ml Streptomycin. A concentration range (0-25 μM) of the compound to be tested was added to each well in a final volume of 100 μl/well and the cells were incubated for 24 h at 37°C in 5% CO_2. After incubation, the media was removed and replaced with 100 μl/well phenol red free medium. Absorbances at 570 nm and 690 nm were measured. MTT (10 μl) was added to each well and the cells were incubated for 2 h. MTT solubilization solution (100 μl, formazan crystals dissolved in 0.04 M HCl in 2-propanol) was added to each well and formazan crystals were dissolved by vigorous mixing. Absorbances at 570 nm and 690 nm were measured. The Δ570 and Δ690 were determined by taking the difference of the

absorbances before and after the addition of MTT. The percent of viable cells *B* was determined according to the formula

$$B = \frac{(\lambda_{\Delta 570} - \lambda_{\Delta 690})_{treated}}{(\lambda_{\Delta 570} - \lambda_{\Delta 690})_{untreated}} \times 100,$$

where $\lambda_{\Delta 570}$ is the absorbance of formazan crystals at 570 nm and $\lambda_{\Delta 690}$ is background at 690 nm.

Animal Preparation and Administration of Molecular Probes. All *in vivo* studies were performed in compliance with the Washington University Animal Study Committee's requirements for the care and use of laboratory animals in research. Nude mice (18-22 g) were anesthetized with xylazine/ketamine mixture via i.p. injection and placed in a supine position. The mice were injected s.c. in the lower back of the mice with 1×10^6 A549 cells. Tumor masses were palpable at 5-7 days after implant, which reached 5 mm in 10-15 days. Before injection of the probe, the mice were anesthetized as described above. A 29-gauge needle was used to inject the probes into the mouse via the lateral tail vein.

Whole-Body Small Animal Planar Optical Imaging. We used a noninvasive *in vivo* continuous wave fluorescence imaging apparatus to visualize the distribution and preferential tissue uptake of the cypate-peptide molecular beacons in nude mice (6). Light from two laser diodes of nominal wavelength 780 nm and nominal power of 50 mW was launched into a fiber optic bundle. We used two laser diodes to produce as much uniform illumination of the whole animal or organ parts as possible. The nominal 50 mW of incident power was reduced to about 44 mW at the output of the fiber optic bundle. A de-focusing lens in position after the bundle expanded the beam such that nearly the whole rat was illuminated. A Photometrics CCD camera (16 bit, 1024 × 1024 pixel, back illuminated, thermoelectric Peltier cooled with forced air) was used to capture the emitted photons. An interference filter (830 nm) was mounted in front of the CCD camera input lens such that only emitted fluorescent light from the contrast agent was imaged. Real-

time acquisition, display and data processing software (WinView/32) was used to obtain the optical image. The samples were dissolved in 20% aqueous DMSO and animals were injected with doses of 0.3 μmol/kg body weight via the lateral tail vein. Injected volumes were 100 μl for each mouse. Data analysis consists of subtracting (pixel by pixel) the preinjection image from the post injection images, and displaying the false color results. Integration of the relative fluorescence intensities of tumor vs. surrounding tissues indicates the extent of selective uptake of the contrast agent. At the completion of the study, the mice were euthanized and some organ parts were excised and rinsed with water. The tissues were placed under the CCD camera and the fluorescence emission from each organ was measured after excitation with the 780 nm laser sources. Tissue parts, instead of whole organs, were used to minimize problems associated with depth-dependent non-linear fluorescence emission. A statistical program in the WinView package was used to estimate the mean fluorescence intensity per organ part.

High-Resolution 3D Tumor Tissue Imaging with a Low-Temperature Fluorometric Redox Scanner. High resolution spatial distribution of the Cyp-GRD molecular beacon and mitochondrial markers of cellular metabolic activity (NADH and flavoprotein, FP) was obtained by a snap-freeze method, as described previously (7). After performing planar fluorescence imaging, the animal under anesthesia was immersed in liquid nitrogen for 10 min and the tumor tissue was surgically excised, embedded in an ethanol-glycerol-water mixture (10:30:60, -30°C) and mounted in a redox scanner for 3D surface fluorometric scanning. The frozen tumor sample was imaged in the *Z* direction by chipping the tumor tissue at 100 μm intervals. A bifurcated optical fiber bundle (7 quartz fibers, 70 μm core diameter for each, 1 fiber for emission in center, 6 fibers for excitation around the emission fiber) was stepped across the tissue surface at a fixed distance from the tissue surface (70 μm). The filters for the fluorescence excitation and emission of the probe, NADH and FP were chosen based on the absorption and emission spectra of each fluorophores. Using a Mercury Arc lamp and a 785 nm laser diode as the excitation light sources, the fluorescent signals of FP (filters: Ex: 440DF20, Em: 520DF40), NADH (filters: Ex: 365HT25, Em: 455DF70) and Cyp-GRD beacon (filters: Ex: 780DF10, Em: 830DF20) were imaged at various depths of the tumor. The scanning was performed at

128 × 128 steps that covered 1.024 × 1.024 cm^2 area (80 μm per step). The fluorescence of FP, NADH and Cyp-GRD were recorded digitally on a PC and reconstructed with MATLAB software. The redox ratio of NADH/(FP+NADH) calculated with MATLAB represented the reduced state of the mitochondria.

Supporting Results and Discussion

Spectral Properties of the Molecular Probes Are Minimally Altered After Conjugation with Peptides. We determined the absorption and emission spectral properties of Cypate and its peptide conjugates in 20% aqueous DMSO. Previous studies have shown that this solvent mixture boosts fluorescence intensity and is well tolerated by rodents (4, 5). Additionally, Cypate and its peptide conjugates are soluble in this solvent mixture, providing a common medium for spectral measurements. Table 1 summarizes the spectral properties of the fluorescent compounds. Interestingly, conjugation of Cypate with the peptides did not alter its spectral properties in dilute solutions. This feature allowed us to study Cypate and the conjugates *in vivo* by using the same excitation and emission wavelengths, as demonstrated in previous studies (5).

Western Blot and Immunohistochemistry. We used immortalized human non-small cell lung cancer cells (A549) for this study because the cell line has been reported to express ABI. Western blot analysis (Fig. 6) and IHC (Fig. 7) showed that the A549 cell line is ABI-positive.

Cytotoxicity Assay. The result of the MTT assay shows that cypate-GRD is not cytotoxic up to 100 μM solution in 20% aqueous DMSO (Fig. 8).

High-Resolution 2D Imaging of Probe Distribution and Redox State of Tumor Tissue *ex Vivo*. Fig. 9 shows the stacked image of the distribution of mitochondrial flavoproteins, mitochondrial NADH, and Cyp-GRD. It demonstrates the heterogeneity of the probe distribution at different tumor depths in the *Z* direction.

Fig. 10 shows the heterogenous 3D fluorescence images of mitochondrial FP, NADH, and Cyp-GRD at the same tumor depth. A remarkable spatial correlation between the reduced state (high NADH) and Cyp-GRD distribution was observed at different tumor depths (Fig. 11a and 11b). In contrast, oxidized FP expression (oxidized state), and the overall oxidized (FP/(FP + NADH)) and reduced (NADH/(FP + NADH)) state ratios of the tumor are less spatially correlated to Cyp-GRD distribution.

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