Supplementary Material for

Scintigraphic Imaging of Mutant *KRAS* mRNA in Human Pancreas Cancer Xenografts with [^{111}In]DOTA$_n$-Poly(diamidopropanoyl)^m-*KRAS* Peptide Nucleic Acid-D(Cys-Ser-Lys-Cys) Nanoparticles

Nariman V. Amirkhanov[1,2], Kaijun Zhang[3], Mohan R. Aruva[3], Mathew L. Thakur[3,4], Eric Wickstrom[2,4]

[1]Laboratory of Nucleic Acids Chemistry, Institute of Chemical Biology & Fundamental Medicine, Novosibirsk 630090, Russia; [2]Biochemistry & Molecular Biology, [3]Radiology; [4]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Corresponding author: Dr. Eric Wickstrom, Department of Biochemistry & Molecular Biology, Thomas Jefferson University, 233 S. 10th Street, Suite 219, Philadelphia, PA 19107 voice: 215-955-4578; fax: 215-955-4580; e-mail: eric@tesla.jci.tju.edu

SUPPLEMENTARY MATERIALS AND METHODS

Assembly of (DOTA-AEEA)$_n$-PDAP^m-AEEA$_2$-*KRAS* PNA-AEEA-IGF1 analog hybridization probes on polymer supports [from (*1*)]

Hybridization probes were assembled by Fmoc coupling chemistry as described (*1*) on NovaSyn TG Sieber resin (9-Fmoc-amino-xanthen-3-yloxy TG resin), 0.12-0.17 mmol/g (01-64-0092, NovaBiochem, San Diego CA) on a PS3 automated solid phase peptide synthesizer (Protein Technologies, Tucson AZ), as illustrated in Schemes 1. Coupling was activated by N-[(dimethylamino)-1H-1, 2, 3-triazolo[4, 5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide (HATU) (GEN076523, Applied Biosystems, Foster City CA) in anhydrous $(CH_3)_2NCHO$ (227056-2L, Sigma-Aldrich, Milwaukee WI) containing 0.4 M N-methylmorpholine (PS3-MM-L, Protein Technologies). The coupling time for all monomers was 30 min. After each coupling step unreacted free amino groups were capped by acetylation with 20% $(CH_3CO)_2O$ in $(CH_3)_2NCHO$ containing 0.4 M N-methylmorpholine, then Fmoc groups were deprotected with 20% piperidine in $(CH_3)_2NCHO$ (PS3-PPR-L Protein Technologies), 3 times for 3 min. each. Deprotection and wash solutions were collected and combined after each coupling for quantitation of the released dibenzofulvene-piperidine adduct by absorbance (ε_{301} = 7800 $M^{-1}cm^{-1}$). Fmoc yields after each coupling were 95-100%. The typical overall Fmoc yield after 17 coupling steps was 60-70%, which corresponds to 97-98% coupling on average at each step.

First, D(Cys-Ser-Lys-Cys) was extended from the polymer support, starting from the C-terminus, using Fmoc-D-Cys(Acm), Fmoc-D-Lys(Boc)-OH, and Fmoc-D-Ser(tBu)-OH (Novabiochem, San Diego CA), followed by the linker 8-Fmoc-amino-3,5-dioxo-octanoic acid (Fmoc-aminoethoxyethoxyacetate, Fmoc-AEEA) (Peptides International, Louisville KY). Next, Fmoc-PNA monomers (Applied Biosystems) were extended sequentially from the N-terminus of the linker-peptide-resin to create the mutant *KRAS* G12D antisense PNA sequence, N-GCCA<u>T</u>CAGCTCC-C, complementary to codons 10-13 of *KRAS* Asp12 mRNA or 1-mismatched mutant *KRAS* Val12D antisense PNA sequence, N-GCCA<u>A</u>CAGCTCC-C. Finally,

the dendrimer was assembled by coupling of two Fmoc-AEEA residues and an $(Fmoc)_2$-diaminopropanoate (DAP) (B-2265, Bachem Bioscience, King of Prussia PA), yielding two available amines (generation 1) (Scheme 1). The dendrimer cycle was repeated to obtain four available amines, (generation 2). The dendrimer cycle was repeated yet again to obtain 8 available amines (generation 3) (Scheme 1). 1,4,7,10-tetra(carboxymethylaza)cyclododecane *ter*t-butyl ester (DOTA-3tBu) (Macrocyclics, Richardson, TX) was then coupled to samples of generation 1 and generation 3 resin beads.

Cyclization, cleavage, and chromatography of $(DOTA\text{-}AEEA)_n$-$PDAP^m$-$AEEA_2$-*KRAS* PNA-AEEA-IGF1 analog hybridization probes [from (*1*)]

After the final coupling step, the cysteine residues on the polymer support were cyclized on solid phase with 10 equivalents of I_2 in $(CH_3)_2NCHO$ for 4 h at room temperature (*2*). Then the hybridization probes were cleaved from the polymer support and deprotected with 80% CF_3CO_2H, 20% m-cresol cocktail, for 4 h at room temperature. The deprotection filtrates plus washes were combined and precipitated in cold $(C_2H_5)_2O$. The precipitates were washed with $(C_2H_5)_2O$, dried, and dissolved in water. PNA in hybridization probes was quantitated by absorbance (ε_{260} = 108400 $M^{-1}cm^{-1}$ for G12D mutant N-GCCATCAGCTCC-C or ε_{260} = 115400 $M^{-1}cm^{-1}$ for G12V mutant N-GCCAACAGCTCC-C) (*3*).

Crude hybridization probes were purified by reversed phase liquid chromatography at 50°C on Alltima C_{18} columns (Alltech, Deerfield IL) eluted with linear gradients from aqueous 0.1% CF_3CO_2H to 30% CH_3CN in aqueous 0.1% CF_3CO_2H. Effluents were monitored at 260, 295 or 300 nm. Peak fractions were lyophilized by centrifugation under vacuum. The molecular mass of each purified compound was measured either on a SELDI (Ciphergen, Fremont, CA) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer from an α-cyano-hydroxycinnamic acid matrix excited at 338 nm, and calibrated with porcine neuropeptide Y, or on an API SciEx 3000 (Applied Biosystems) electrospray ionization (ESI-MS) mass spectrometer.

Electrospray ionization mass spectroscopy of purified $(DOTA\text{-}AEEA)_n$-$PDAP^m$-$AEEA_2$-*KRAS* PNA-AEEA-IGF1 analog hybridization probes [from (*1*)]

Electrospray ionization mass spectroscopy (ESI-MS) of positively charged ions was carried out on an ABI Sciex 3000 spectrometer. A Q1 scan was programmed for three seconds and approximately 10 scans were averaged to obtain the final spectrum. The spectra of multiply charged ions were deconvoluted by hand (until software was available) and the molecular masses were derived accordingly. Samples were diluted to approximately 20 pmoles/μL in a diluent of aqueous 0.2 % HCO_2H in 50% CH_3CN. Samples were infused at 10 μL/min into the Ionspray source at a voltage of 5000 V.

Thermodynamic stability of $(DOTA\text{-}AEEA)_n$-$PDAP^m$-$AEEA_2$-*KRAS* PNA-AEEA-IGF1 analogs hybridized to RNA [from (*1*)]

The temperature dependence of light absorbance at 260 nm of antisense and mismatch DOTA-PDAP dendrimer *KRAS* PNA-peptide probes hybridized to a *KRAS*D12 20-mer RNA: 5'-AGUUGGAGCUGAUGGCGUAG- 3' (from DHARMACON) was monitored to determine the relative stabilities of the antisense probe and the mismatch probes. To provide the maximum likelihood of a two-state pattern for the duplex association/dissociation transition, the sequence of the RNA was designed to avoid the formation of a hairpin or slipped duplex structure, and to

limit the duplex length to 12 bp, leaving a 4 nt overhang on either end of the duplex. Melting curves were recorded at 260 nm on a Cary 3 spectrophotometer (Varian) fitted with a thermoelectric temperature-controlled cell holder. 10 mm pathlength cuvets with Teflon stoppers were used for absorbance measurements in different temperatures. The actual temperature inside the cuvet was monitored using a temperature probe dipped inside one reference cuvet containing buffer. For each temperature ramp, both the 20-mer *KRAS*D12 RNA (20 mer) and the chelator-PDAP dendrimer-PNA-peptide radiohybridization probe were dissolved in buffer containing 10 mM Na_2HPO_4, pH, 7.4, 0.14 M NaCl to a final concentration of 1 μM and the volume was made up to 1 mL. The duplexes were annealed by heating at 1°C/min to 90°C, and the temperature was held for 3 minutes at that temperature to dissociate any residual structures before the strands were allowed to re-anneal during cooling to 20°C at 0.4°C/min. Thermal melting of the duplexes was carried out by heating at 0.5°C/min up to 90 or 95 °C, measuring absorbance at 260 nm, with a spectral bandwidth of 1 nm, every 0.2°C, averaging 2 points collected over 1 sec. The melting curves were blanked against buffer alone to account for any changes in buffer absorbance with temperature. The melting temperatures (T_m) were calculated automatically as the peak of the 1st derivative plot, and were measured in triplicate.

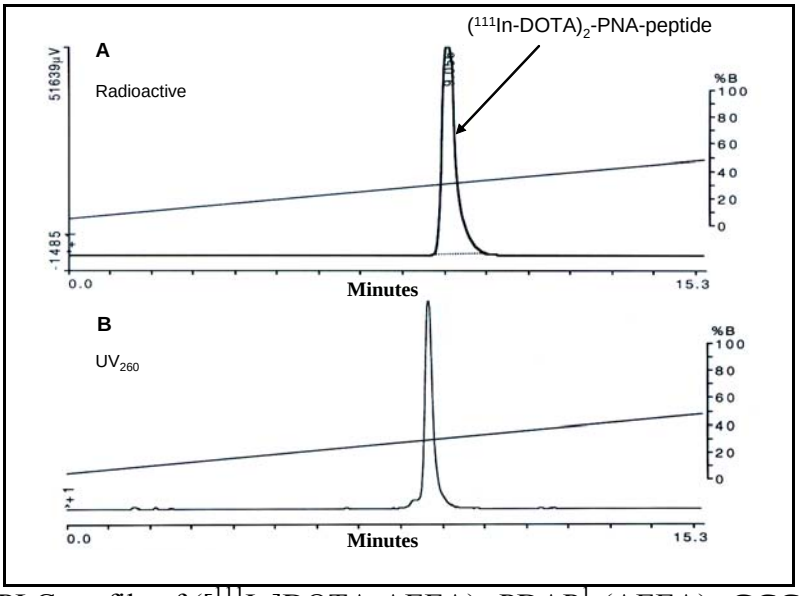


Figure S1. C_{18} HPLC profile of ([^{111}In]DOTA-AEEA)$_2$-PDAP[1]-(AEEA)$_2$-**GCC AtC AGC TCC**-AEEA-D(Cys-Ser-Lys-Cys), *KRAS* G12D hybridization probe, **WT5195**, after $^{111}InCl_3$ labeling as described in Materials and Methods.

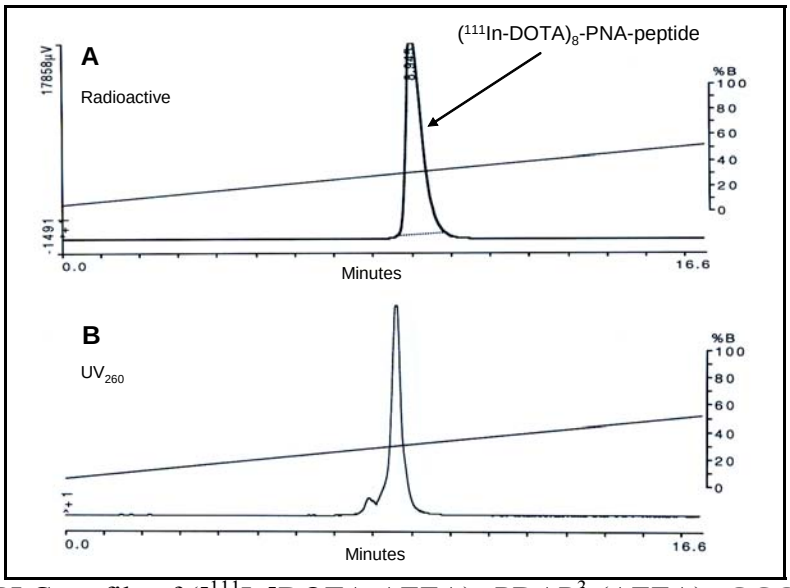
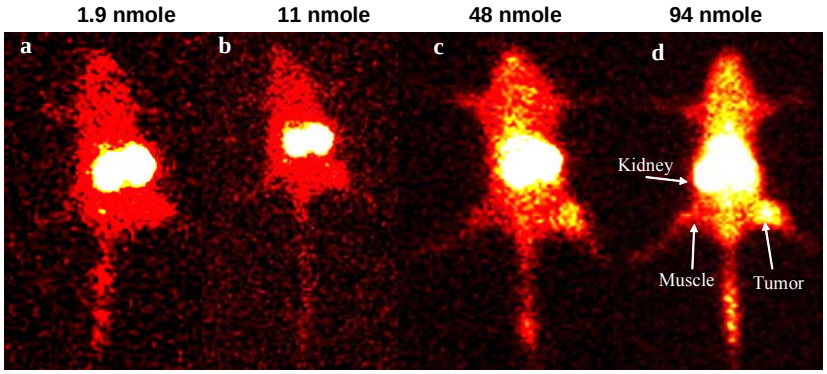


Figure S2. C_{18} HPLC profile of ([^{111}In]DOTA-AEEA)$_8$-PDAP[3]-(AEEA)$_2$-**GCC AtC AGC TCC**-AEEA-D(Cys-Ser-Lys-Cys), *KRAS* G12D hybridization probe, **WT8900**, after $^{111}InCl_3$ labeling as described in Materials and Methods.

Scintigraphic imaging of mice at 24 h after probe administration



DOTA$_2$-PNA injected doses	1.9 nmole	11 nmole	48 nmole	94 nmole
T/M (gamma)	3.9± 0.4	3.9± 0.4	4.3±0.4	5.7±0.6
K/T (tissue distribution)	132±13	108±11	41±4	20±2

Figure S3. Scintigraphic images of immunocompromised mice bearing human CAPAN2 pancreas cancer xenograft at 24 h after tail vein administration of ~2 nmol (300-350 µCi, 11-13 MBq) of fully matched ([^{111}In]DOTA-AEEA)$_2$-PDAP[1]-AEEA$_2$-*KRAS* G12V PNA-AEEA-IGF1 analog, **WT5204,** mixed with 1.9 nmol (a), 11 nmol (b), 48 nmol (c), or 94 nmol (d) of non-radioactive (Gd-DOTA-AEEA)$_2$-PDAP[1]-AEEA$_2$-*KRAS* G12V PNA-AEEA-IGF1 analog. Tumor/Muscle (T/M) ratios were calculated from γ-particle intensity data. Kidney/Tumor (K/T) ratios were calculated from tissue distribution data.

Table S1. Tumor/Muscle γ-particle intensity ratios of the [^{111}In]*KRAS* G12D fully matched probes with [^{111}In]$DOTA_2$, **WT5195,** [^{111}In]$DOTA_8$, **WT8900,** and [^{111}In]$DOTA_{16}$, **WT13842,** and free [^{111}In]Cl_3 and [^{111}In]DOTA controls at 4, 8 and 24 h after tail vein administration to immunocompromised mice bearing human AsPC1 pancreas cancer xenografts (n=3)

Probes	4 h	8 h	24 h
[^{111}In]$DOTA_2$	2.37 ± 0.10	3.06 ± 0.25	3.08 ± 0.18
[^{111}In]$DOTA_8$	3.60 ± 0.37	3.85 ± 0.20	4.06 ± 0.34
[^{111}In]$DOTA_{16}$	5.21 ± 0.30	5.81 ± 0.17	6.19 ± 0.42
[^{111}In]Cl_3	2.74 ± 0.18	3.62 ± 0.20	3.43 ± 0.24
[^{111}In]DOTA	1.82 ± 0.13	2.03 ± 0.14	2.06 ± 0.14

Tumor/Muscle (T/M) ratios were calculated from γ-particle intensities in each pixel of the scintigraphic images.

Table S2. Tissue distribution (% injected dose/g) at 24 h after tail vein administration of fully matched ([^{111}In]DOTA-AEEA)$_n$-PDAP^m-AEEA$_2$-*KRAS* G12V PNA-AEEA-IGF1 analogs, **WT5204** and **WT8910,** mixed with increasing doses of non-radioactive (Gd-DOTA-AEEA)$_8$-PDAP^3-AEEA$_2$-*KRAS* G12V PNA-AEEA-IGF1 analog, vs. [^{111}In]DOTA, in immunocompromised mice bearing human CAPAN2 (G12V) pancreas cancer xenografts (n=3)

	[^{111}In]$_2$				[^{111}In]$_8$			[^{111}In]DOTA	
Tissues	1.9 nmole	11 nmole	48 nmole	94 nmole	1.9 nmole	11 nmole	94 nmole	6 pmole	1000 nmole
Muscle	0.028 ± 0.002	0.023 ± 0.002	0.073 ± 0.005	0.102 ± 0.009	0.028 ± 0.002	0.023 ± 0.002	0.080 ± 0.006	0.012 ± 0.003	0.013 ± 0.003
Intestine	0.046 ± 0.003	0.043 ± 0.003	0.224 ± 0.016	0.296 ± 0.027	0.032 ± 0.003	0.031 ± 0.003	0.171 ± 0.012	0.018 ± 0.003	0.024 ± 0.003
Heart	0.023 ± 0.002	0.028 ± 0.002	0.106 ± 0.007	0.193 ± 0.017	0.019 ± 0.002	0.022 ± 0.002	0.092 ± 0.006	0.008 ± 0.002	0.011 ± 0.003
Lungs	0.047 ± 0.003	0.062 ± 0.005	0.200 ± 0.014	0.371 ± 0.033	0.045 ± 0.004	0.047 ± 0.004	0.162 ± 0.011	0.038 ± 0.009	0.029 ± 0.007
Blood	0.014 ± 0.001	0.022 ± 0.002	0.183 ± 0.013	0.259 ± 0.023	0.006 ± 0.001	0.009 ± 0.001	0.129 ± 0.009	0.013 ± 0.003	0.005 ± 0.001
Spleen	0.074 ± 0.005	0.123 ± 0.010	0.421 ± 0.029	0.664 ± 0.060	0.048 ± 0.004	0.057 ± 0.005	0.338 ± 0.024	0.032 ± 0.003	0.044 ± 0.004
Kidneys	20.83 ± 1.46	14.44 ± 1.16	13.64 ± 0.95	12.71 ± 1.14	21.80 ± 1.74	13.47 ± 1.21	9.37 ± 0.656	0.500 ± 0.099	0.818 ± 0.162
Liver	0.167 ± 0.012	0.179 ± 0.014	0.557 ± 0.039	0.654 ± 0.059	0.068 ± 0.005	0.094 ± 0.008	0.413 ± 0.029	0.055 ± 0.005	0.073 ± 0.007
Tumor	0.120 ± 0.008	0.136 ± 0.011	0.396 ± 0.028	0.491 ± 0.044	0.125 ± 0.010	0.102 ± 0.009	0.305 ± 0.021	0.041 ± 0.005	0.070 ± 0.009
Total, %	21.35 ± 1.49	15.06 ± 1.20	15.80 ± 1.11	15.74 ± 1.42	22.17 ± 1.77	13.85 ± 1.25	11.06 ± 0.774	0.72 ± 0.14	1.09 ± 0.21
T/M ratio	4.32 ± 0.43	6.03 ± 0.60	5.39 ± 0.54	4.83 ± 0.48	4.48 ± 0.45	4.39 ± 0.44	3.80 ± 0.38	3.40 ± 0.74	5.60 ± 1.22
T/B ratio	8.42 ± 0.84	6.24 ± 0.62	2.16 ± 0.22	1.9 ± 0.19	20.96 ± 2.10	11.9 ± 1.19	2.36 ± 0.24	3.18 ± 0.45	13.90 ± 1.97
K/T ratio	172.98 ± 17.30	105.83 ± 10.58	34.45 ± 3.45	25.88 ± 2.59	174.81 ± 17.48	132.32 ± 13.23	30.76 ± 3.08	12.12 ± 3.62	11.67 ± 3.49
T/M (gamma)	3.94 ± 0.39	3.94 ± 0.39	4.32 ± 0.43	5.56 ± 0.56	3.87 ± 0.39	4.19 ± 0.42	6.29 ± 0.63	2.06 ± 0.14	-

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