Supporting Information

Nephrotoxicity Assessment with Human Kidney Tubuloids using Spherical Nucleic Acid-based mRNA Nanoflares

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Materials and Methods

All materials, except when mentioned otherwise, were purchased from Sigma-Aldrich. NF oligonucleotide strands and primers for RT-qPCR were obtained from Integrated DNA Technologies. Dulbecco's modified eagle medium (DMEM), DMEM with F-12 nutrient mixture (DMEM/F-12), Roswell Park Memorial Institute (RPMI) 1640 medium, Geltrex LDEV-Free Membrane Matrix and NucBlue Live ReadyProbes Reagent, trypsin–EDTA (0.05%), fetal bovine serum (FBS), and penicillin–streptomycin (10 kU/ml) were obtained from Thermo Fisher Scientific. iScript cDNA Synthesis Kit and iQ SYBRGreen Supermix were obtained from Bio-Rad. TRIzol reagent was obtained from Invitrogen.

Culture of kidney cells: Human primary proximal tubule cells (PTCs) were obtained from ATCC or isolated from the unutilized portion of removed kidneys during the nephrectomy of renal cell carcinoma (RCC) patients in Brigham and Women's Hospital (IRB number 2009P001527; Boston, US), with modifications from previous methodology.[1] Briefly, the renal cortex was cut and digested in collagenase solution (0.5 mg/ml). The reaction was later stopped through the addition of FBS. Subsequently, the tubules were separated from the glomeruli and other tissue clumps by decanting after 2 mins of gravity sedimentation. Afterwards, the remaining samples was rinsed twice, and the tubules were then suspended in tubular culture medium (composed of DMEM/F12 and 5% bovine serum albumin (BSA), 5 µg/ml transferrin, 20 ng/ml hydrocortisone, 10 µg/ml insulin, 6.7 ng/ml selenium, and supplemented with 10 ng/ml epidermal growth factor (EGF). Isolated tubule cells were left incubated for an additional 7-10 days prior to further usage.[2]

To check for signal localization and specificity, adenoviruses carrying human KIM-1 and EGFP gene were prepared and utilized to infect PTCs as described previously.[3] The LLC-PK1 porcine

kidney cell line was obtained from the ATCC. KIM-1-PK1 cells were prepared by modifying LLC-PK1 cells with full length human KIM-1 cDNA, as described previously.[1] As controls, PC-PK1 cells were modified with empty pcDNA3 vector.

Tubuloid preparation and drug treatment: The manuscript describing the formation of renal tubuloids from patient cells is currently being prepared. To summarize, isolated human PTCs were seeded on ultra-low adherent well plates and cultured with RPMI supplemented with 5% FBS. 3 days later, Matrigel (10% v/v) was introduced, and the media was replaced with tubuloid media: RPMI supplemented with 5% FBS and containing 10ng/ml bFGF, EGF, and HGF.[2] Culture media was replaced twice a week, and the tubuloids were used for subsequent experiments after 2 weeks of culture. In checking NF specificity, prepared tubuloids were incubated with adenovirus carrying KIM-1 and EGFP genes for 24 hours before NF labeling. For drug treatment, drug stocks in their respective solvents (Table S1) were diluted with tubuloid media to the designated concentration and incubated with the tubuloids for up to 3 days.

NF synthesis, hybridization assay and labeling: KIM-1 and GAPDH NFs were synthesized using a method reported in a previous study with slight modifications.[4] Briefly, the recognition strand and flare strand (Table S2, 100 μM) were first annealed at 2:1 molar ratio, slowly cooled to room temperature over 1 hour from initial heating at 95°C. Annealed duplexes were then subjected to reduction with excess Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride (5 mM) for 1 hour followed by purification with Micro Bio-Spin 6 Columns (Bio-Rad) to allow thiol activation of the recognition strand. Activated duplexes were then added to 13 nm citrate-capped gold nanoparticles (5 nM) and stored overnight at -20 °C, followed by salt aging every 1 hour with 1M NaCl

solution until a final concentration of 0.3 M. The solution was sonicated for 30 seconds after each salt addition. Finally, NFs were collected by centrifugation at 15,000 g for 15 mins, rinsed twice with PBS, resuspended to 100 nM gold nanoparticle concentration, and stored at 4 °C until needed.

Synthesized NFs were mixed with target strands (complementary & non-matching target, 1 μM) and incubated for 15 mins at 37 °C. Fluorescence intensity of the mixture was read at 550/575 nm for KIM-1 NF and 650/675 nm for GAPDH NF using a Synergy H4 microplate reader (BioTek). Measurement was done in triplicate and repeated thrice.

For cellular labeling, KIM-1 and GAPDH NFs were diluted in cell culture medium to a final concentration of 1 nM gold nanoparticles. 2D seeded cells or prepared tubuloids were incubated overnight with NF labeling medium. Excess NFs were removed by rinsing the cells and tubuloids twice with PBS prior to imaging or subsequent processing.

Epifluorescence and confocal microscopy imaging: NF fluorescence was visualized on a LX71 inverted fluorescence microscope (Olympus) with a Lumenera Infinity 3-3URF digital camera. Fluorescence images were taken with consistent exposure times of 250 milliseconds and 5x gain. Confocal imaging was performed with LSM710 confocal microscope (Zeiss). Tubuloids were stained with NucBlue reagent for confocal imaging, according to the manufacturer's protocol. Subsequent image processing including background subtraction and fluorescence signal quantification was done using ImageJ software.

Quantitative polymerase chain reaction (qPCR) analysis: Untreated or drug-treated kidney tubuloids were retrieved at designated timepoints and lysed with TRIzol reagent. Following the RNA

extraction, cDNA conversion was performed with iScript™ cDNA Synthesis Kit (Bio-Rad), as per manufacturer's protocol. Primer sequences utilized are listed in Table S2. Then, CT values of KIM-1 and clusterin (CLU) mRNA were obtained through qPCR (iQ™ SYBR® Green Supermix with CFX ConnectTM PCR System, Biorad). Finally, the $2^{-\Delta\Delta CT}$ formula was adopted to quantify mRNA fold expressions (normalized to untreated tubuloid group).

Statistical analysis: Data points are reported as average ± standard deviation (stdev). Each experiment was tested in triplicates, except when indicated otherwise in the figure captions. To assess statistical significance, analysis of variance (ANOVA) with associated post-hoc analysis was done using a web-based statistics calculator from astatsa.com (http://astatsa.com/One-Way_Anova_with_TukeyHSD/). Student's t-test was applied in determining a significant difference between two groups. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$. N.S represents non-significant difference. N=4, except when specifically mentioned otherwise.

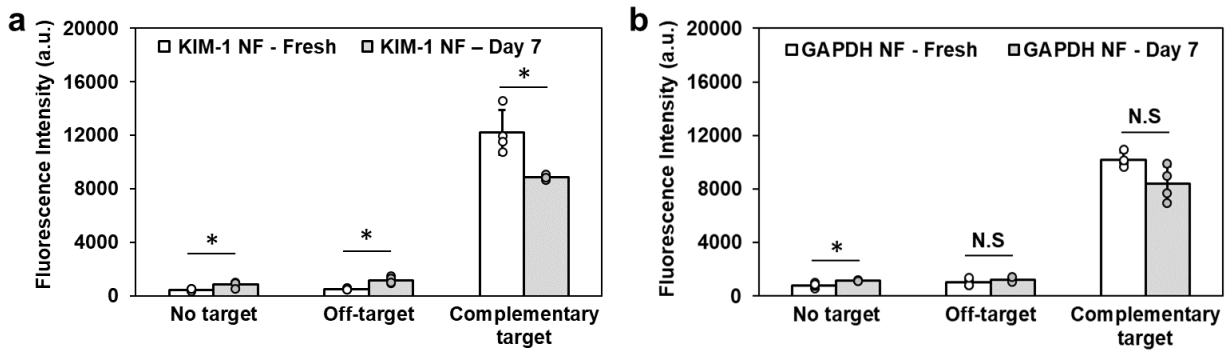

Figure S1. Hybridization assay comparing freshly prepared NFs (white column) or those post 7-day storage at 4 °C (grey column) for (a) KIM-1 NFs and (b) GAPDH NFs. *: $p<0.05$, N.S: non-significant. N=4.

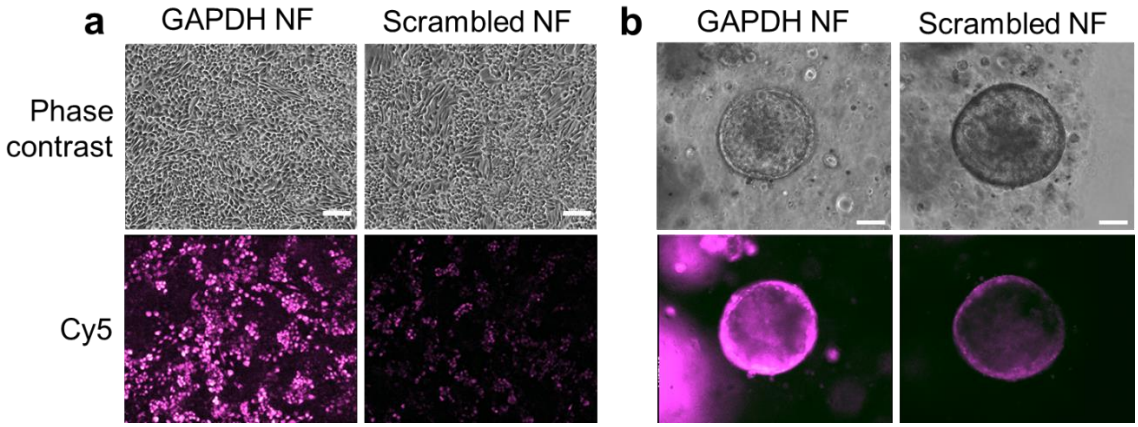

Figure S2. Fluorescence images (a) primary proximal tubule cells and (b) primary tubuloids post labeling with GAPDH NFs and scrambled NFs (Cy5-tagged, 650/675 nm). Scale bars: 100 µm.

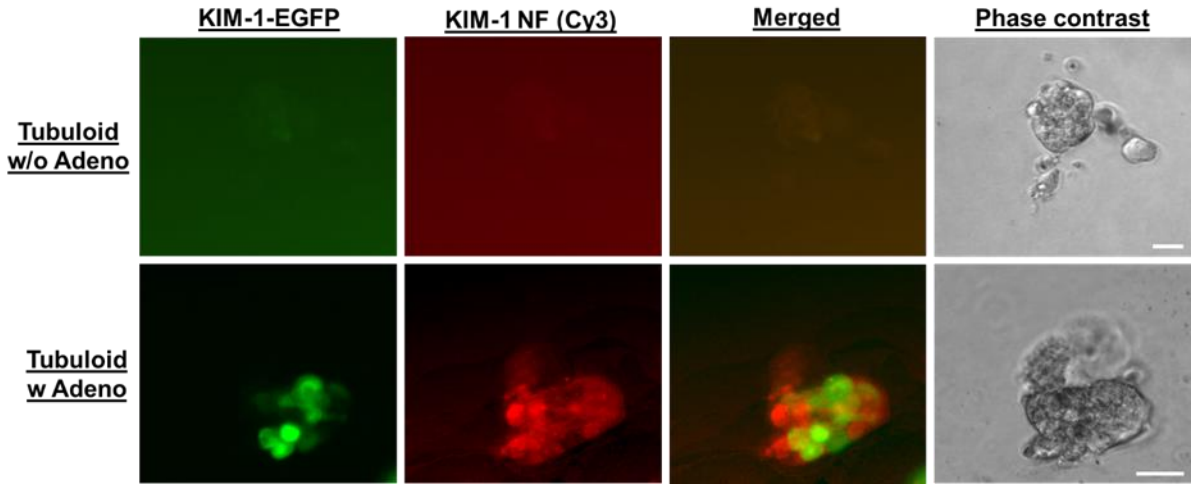

Figure S3. Fluorescence images of KIM-1 NF-labeled primary tubuloids infected with KIM-1/EGFP adenovirus. Scale bars: 100 μm.

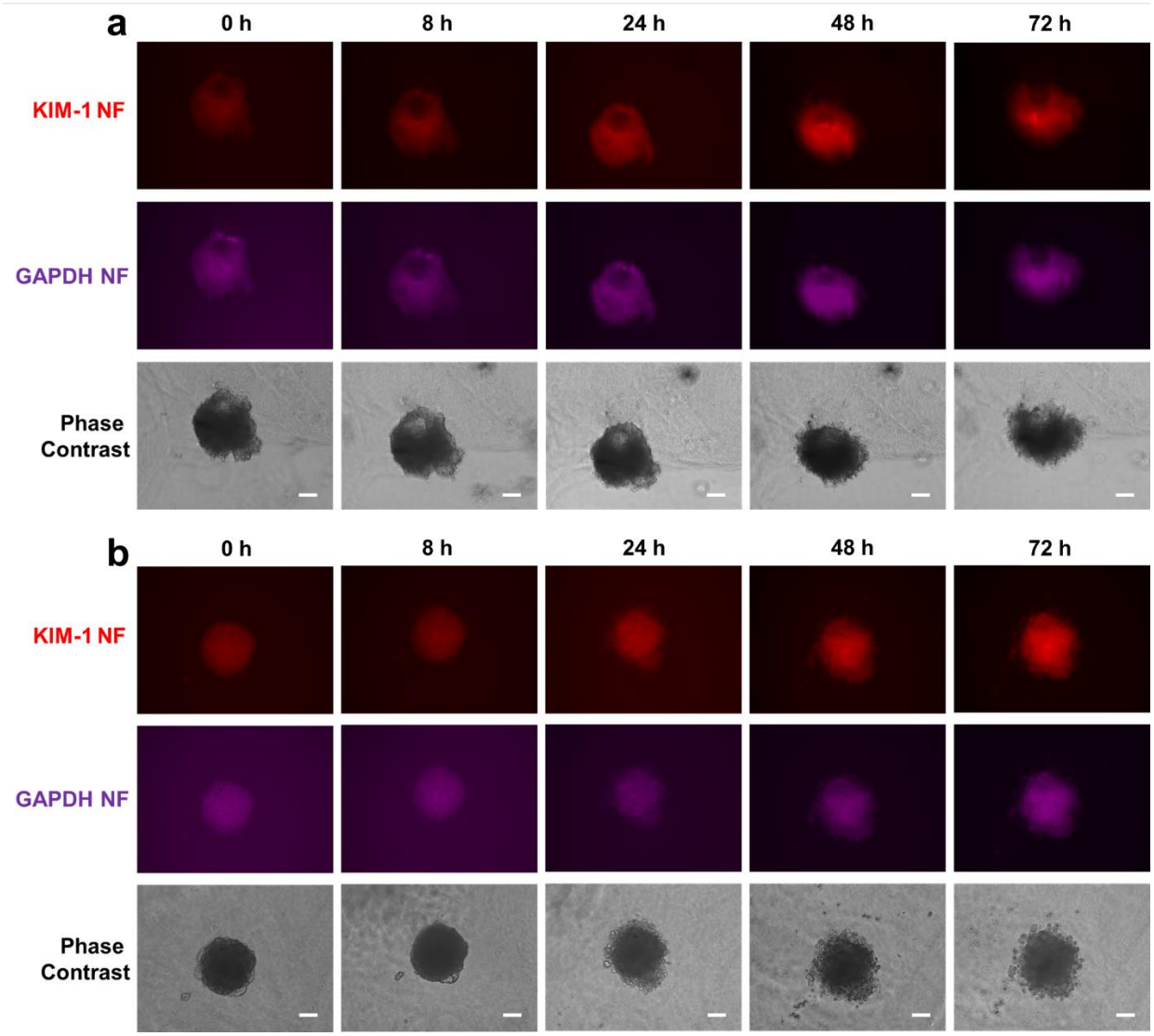

Figure S4. Representative images of KIM-1 NF and GAPDH NF labeled primary tubuloids which were treated with (a) 20 μM and (b) 40 μM AA. Scale bar: 100 μm.

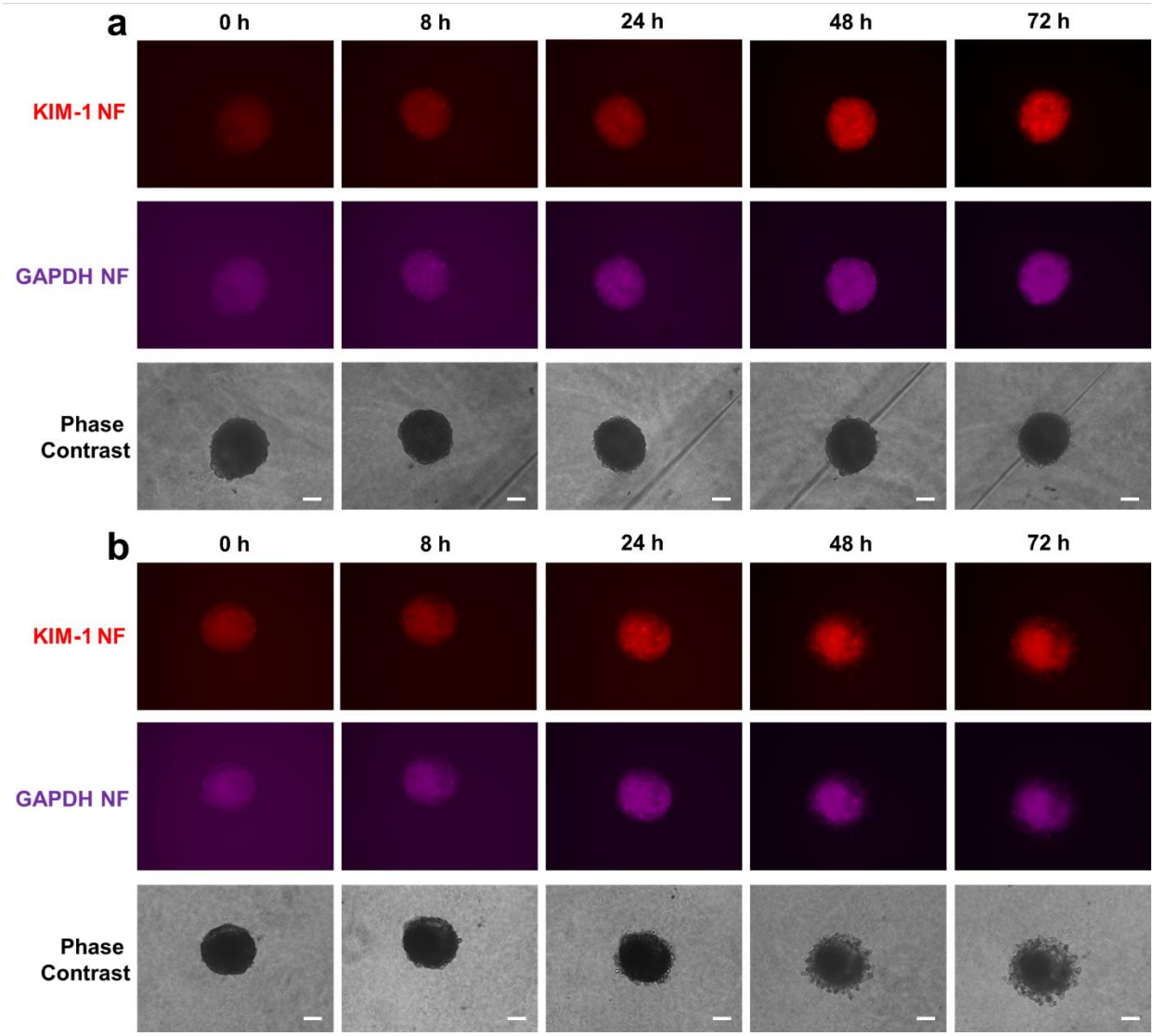

Figure S5. Representative images of KIM-1 NF and GAPDH NF labeled primary tubuloids which were treated with (a) 10 µg/ml and (b) 20 µg/ml cisplatin. Scale bar: 100 µm.

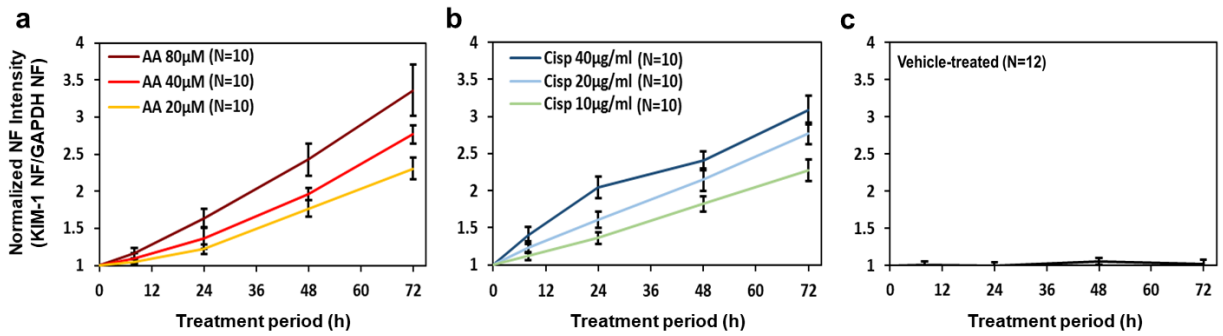

Figure S6. Averaged signal quantification profile by normalizing KIM-1 NF signal against that of GAPDH NF in (a) AA-treated tubuloids (20, 40, 80 μM concentration, N=10 each), (b) cisplatin-treated tubuloids (10, 20, 40 μg/ml concentration, N=10 each), and (c) vehicle-treated tubuloids (N=12).

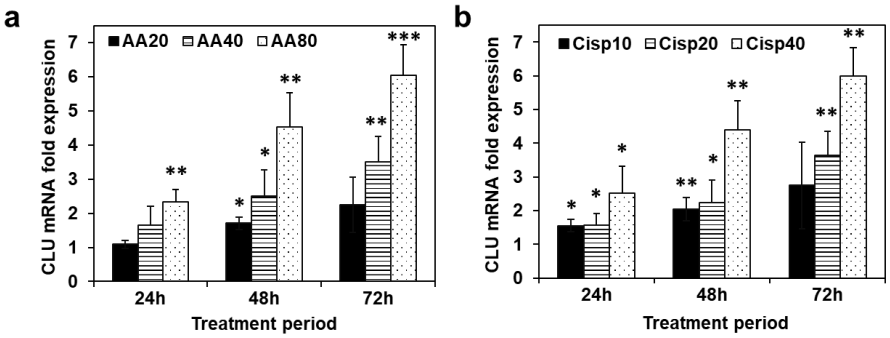

Figure S7. Changes in tubuloid clusterin (CLU) mRNA expression from qPCR following (a) AA and (b) cisplatin treatments for 24, 48 or 72 hours. N=4. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Table S1. Tested Anti-cancer Drugs for Tubuloid Nephrotoxicity

Classification	Drug	Solvent	Concentration tested
Alkylating agents	Ifosfamide (IFO)	Dimethyl sulfoxide	100 µM, 500 µM
	Cyclophosphamide (CP)	Dimethyl sulfoxide	100 µM, 250 µM, 1mM
Anti-metabolites	5-Fluorouracil (5-FU)	Dimethyl sulfoxide	1 µM, 5 µM, 20 µM
	Methotrexate (MTX)	Dimethyl sulfoxide	1 µM, 5 µM, 20 µM
	Pemetrexed (PEM)	Phosphate-buffered saline	5 µM, 20 µM, 50 µM
Anti-microtubular	Paclitaxel (PTX)	Dimethyl sulfoxide	100 nM, 500 nM, 5 µM
Cyclin dependent kinase (CDK) 4/6 inhibitors	Abemaciclib mesylate (ABM)	Dimethyl sulfoxide	500 nM, 2 µM, 5 µM
Demethylation agents	5-Azacytidine (5-AzaC)	Dimethyl sulfoxide	5 µM, 10 µM, 20 µM
Checkpoint inhibitors (PD-1)	Nivolumab (Nivo)	Phosphate-buffered saline	500 nM, 2 µM
Platinum agents	Cisplatin (Cisp)	Saline solution (0.9%)	4 µg/ml, 10 µg/ml, 20 µg/ml, 40 µg/ml
Tyrosine kinase inhibitor	Imatinib mesylate (IM)	Dimethyl sulfoxide	5 µM, 10 µM, 20 µM

Table S2. List of Oligonucleotides for NF Synthesis and qPCR analysis

Oligonucleotides	**Sequences (5' → 3')**
KIM-1 NF - recognition	GAGCAAGAAGCACCAAGACAGAAAAAAAA/3ThioMC3-D/ (Thiolated 3' modification)
KIM-1 NF - flare	/5Cy3/CTGTCTTGGTGCTTC
KIM-1 NF - target	TCTGTCTTGGTGCTTCTTGCTC
GAPDH NF - recognition	GATGGCATGGACTGTGGTCATGAGTCCTAAAAAA /3ThioMC3-D/
GAPDH NF - flare	/5Cy5/AGGACTCATGACCAC
GAPDH NF - target	AGGACTCATGACCACAGTCCATGCCATC
GAPDH primer - forward	GAAGGTGAAGGTCGGAGT
GAPDH primer - backward	GAAGATGGTGATGGGATTTC
KIM-1 primer - forward	ACCATGAACCAGTAGCCACT
KIM-1 primer - backward	GCAAGAAGCACCAAGACAGA
CLU primer - forward	GCCGGGAGATCTTGTCTGTGG
CLU primer - backward	TCCTCCCGGTGCTTTTTGCGG

References

(1) Ichimura, T.; Asseldonk, E. J.; Humphreys, B. D.; Gunaratnam, L.; Duffield, J. S.; Bonventre, J. V. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J. Clin. Invest.*, **2008**, 118, (5), 1657-1668.

(2) Mori, Y.; Ajay, A. K.; Chang, J.-H.; Mou, S.; Zhao, H.; Kishi, S.; Li, J.; Brooks, C. R.; Xiao, S.; Woo, H.-M.; Sabbisetti, V. S.; Palmer, S. C.; Galichon P.; Li, L.; Henderson, J. M.; Kuchroo, V. K.; Ichimura, T.; Bonventre, J. V. KIM-1 mediated tubular fatty acid uptake leads to progressive diabetic kidney disease. *Cell Metab.*, **2021**, 33, (5), 1042-1061.e7.

(3) Zhang, Z.; Humphreys, B. D.; Bonventre, J. V. Shedding of the urinary biomarker kidney injury molecule-1 (KIM-1) is regulated by MAP kinases and juxtamembrane region. *J. Am. Soc. Nephrol.*, **2007**, 18, (10), 2704-2714.

(4) Liu, B.; Liu, J. Freezing Directed Construction of Bio/Nano Interfaces: Reagentless Conjugation, Denser Spherical Nucleic Acids, and Better Nanoflares. *J. Am. Chem. Soc.*, **2017**, 139, (28), 9471-9474.