# Bioisosteric discovery of NPA101.3, a second generation pan-RET/VEGFR2 inhibitor, optimized for single-agent polypharmacology

## Supporting Information

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**Supplementary Methods**

**RET DFG-out Homology Model Development.** A VEGFR2 DFG-out crystal structure (PDB# 2OH4) and the amino acid sequence of RET (PDB# 2IVU) were obtained. Using SWISS-MODEL Automatic Modelling Mode (swissmodel.expasy.org), the RET sequence was employed to build a RET DFG-out homology model using the VEGFR2 DFG-out structure as a template. The resulting RET DFG-out homology model was used to complete molecular modeling studies. The RET DFG-out homology model is available in the Supplemental Information.

**Molecular modelling.** Using AutoDock Tools, all hydrogens were added as 'Polar Only' and a grid box for the ATP binding site was created (center x = -25.881, center y = 9.55, center z = -10.927 / size x = 16, size y = 44, size z = 18) in the RET DFG-out homology model. Compounds to be computationally modelled were assigned appropriate rotatable bonds using AutoDock Tools. AutoDock Vina was employed to computationally model the compounds. AutoDock Vina reports receptor affinity in terms of $\Delta G$ of the receptor/ligand complex. The modelling results were visualized and analysed with Discovery Studio 3.5.

**Kinome scan.** NPA101.3 (100 nM) was subjected to a kinome scan against a 96 kinases panel representing major kinome clusters. The screening (KINOMEscan) was outsourced to DiscoverX (Fremont, CA, United States)

**Synthesis of ethyl 2-(4-((4-bromo-2-nitrophenyl)amino)phenyl)acetate (1a)**. Ethyl 4-aminophenyl acetate (3.67 g, 20.45 mmol) was added to a 20 mL microwave vial along with 4-bromo-1-fluoro-2-nitrobenzene (3.00 g, 13.64 mmol) and DMA (10 mL). The reaction was sealed and placed under microwave irradiation for 30 minutes at 160 °C. The crude reaction was added to water and extracted with EtOAc. The organic extract was washed with brine 1X, acidified water (pH ~4) 2X, and brine 2X. The organic layer was collected, dried with $MgSO_4$, and adsorbed on silica. The reaction was purified using flash chromatography with hexanes/EtOAc to afford **1a** as blood-red oil that eventually solidified (4.2 g, 81%). $^1$H NMR (400 MHz, Chloroform-*d*) δ 9.41 (s, 1H), 8.34 (d, J = 2.4 Hz, 1H), 7.41 (ddd, J = 9.2, 2.4, 0.6 Hz, 1H), 7.34 (d, J = 8.3 Hz, 2H), 7.21 (d, J = 8.3 Hz, 2H), 7.10 (d, J = 9.2 Hz, 1H), 4.18 (q, J = 7.2 Hz, 2H), 3.63 (s, 2H), 1.28 (t, J = 7.2 Hz, 3H). ESIMS *m*/*z* $[M+H]^+$ 379.

**Synthesis of ethyl 2-(4-((2-amino-4-bromophenyl)amino)phenyl)acetate (1b)**. Compound **1a** (2.03 g, 5.35 mmol) was placed into a 250 mL round bottom flask. EtOH (80 mL) and zinc (2.450 g, 37.5 mmol) were added to the flask and the reaction was placed in an ice bath. Acetic acid (2.145 mL, 37.5 mmol) diluted with EtOH (40 mL) was added dropwise to the reaction over the course of 1 hour. After the addition, the reaction was stirred at 0 °C for 5 hours. The reaction was filtered and EtOH was

evaporated. The reaction was slowly basified with aqueous $NaHCO_3$ and extracted with diethyl ether. The reaction was washed 3X with aqueous $NaHCO_3$ and the organic layer was collected, dried with $MgSO_4$, and evaporated to yield **1b** as a slight purple solid (1.834 g, 98%). $^1$H NMR (400 MHz, Chloroform-*d*) δ 7.12 (d, *J* = 8.5 Hz, 2H), 6.95 (d, *J* = 8.3 Hz, 1H), 6.92 (d, *J* = 2.2 Hz, 1H), 6.83 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.67 (d, *J* = 8.5 Hz, 2H), 5.07 (s, 1H), 4.14 (q, *J* = 7.1 Hz, 2H), 3.82 (s, 2H), 3.51 (s, 2H), 1.25 (t, *J* = 7.1 Hz, 3H). ESIMS *m*/*z* $[M+H]^+$ 349.

**Synthesis of ethyl 2-(4-(5-bromo-1H-benzo[d]imidazol-1-yl)phenyl)acetate (1).** Compound **1b** (2.01 g, 5.76 mmol) was added to a 150 mL round bottom flask followed by TMOF (50 mL) and a stir bar. After, pTSA (59.6 mg, 0.314 mmol) was added and the reaction was stirred at room temperature for about an hour or until complete conversion based on TLC. After complete consumption of the starting material, the reaction was extracted with EtOAc and washed with $NaHCO_3$ 3X and brine 3X. The organic layer was collected, dried with $MgSO_4$, and condensed to yield **1** as a brown solid (2.04 g, 99%). $^1$H NMR (400 MHz, Chloroform-*d*) δ 8.08 (s, 1H), 8.02 (dd, J = 1.7, 0.6 Hz, 1H), 7.51 (d, J = 8.7 Hz, 2H), 7.47 – 7.42 (m, 3H), 7.39 (dd, J = 8.7, 0.6 Hz, 1H), 4.21 (q, J = 7.1 Hz, 2H), 3.72 (s, 2H), 1.30 (t, J = 7.1 Hz, 3H). ESIMS *m*/*z* $[M+H]^+$ 359.

**Synthesis of N-(5-(tert-butyl)isoxazol-3-yl)-2-(4-(5-(4-(methylsulfonyl)phenyl)-1H-benzo[d]imidazol-1-yl)phenyl)acetamide (NPA-101.3).** Compound **1** (150 mg, 0.418 mmol) was placed into a 20 mL microwave vial along with 4:1 DMF/Water (5 mL). (4-(methylsulfonyl)phenyl)boronic acid (109 mg, 0.543 mmol) was added to the vial along with $Na_2CO_3$ (175 mg, 1.670 mmol). The reaction vessel was degassed with $N_2$ for 10 minutes, followed by the addition of $Pd(dppf)Cl_2$ (1.169 mg, 0.004 mmol). The reaction vessel was sealed under $N_2$ and microwaved at 130 °C for 20 minutes. After, upon solvent evaporation, the crude product was dissolved in DCM and washed with $NaHCO_3$ 3X and brine 3X. The organic layer was collected, dried with $MgSO_4$, and condensed to yield crude product (120 mg). The crude product was dissolved in 1:1 THF/Water (4 mL). LiOH (26.5 mg, 1.105 mmol) was added to the reaction and the reaction was heated under microwave irradiation for 10 minutes at 100 °C. The reaction was subsequently acidified to a pH of ~3-4 and extracted 5X with 4:1 chloroform/IPA. The organic layer was dried with $MgSO_4$ and condensed to yield the acid (90.7 mg). The acid was added to a 5 mL vial. Anhydrous DMF (3 mL) was added to the vial, followed by EDC (52.5 mg, 0.338 mmol) and DMAP (8.10 mg, 0.0.066 mmol). 5-(tert-butyl)isoxazol-3-amine (40.4 mg, 0.288 mmol) was added and the reaction was sealed under $N_2$ and was stirred at room temperature for 12 hours. The reaction was quenched with water, extracted with 4:1 chloroform/IPA, and washed with saturated $NaHCO_3$ 5X. The organic layer was collected, dried with $MgSO_4$, adsorbed onto silica, and purified by flash chromatography using a DCM/MeOH to generate NPA-101.3 (**2**) (39.7 mg, 18.0%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.29 (s, 1H), 8.63 (s, 1H), 8.17 (s, 1H), 8.08 – 7.91 (m, 5H), 7.72 (s, 2H), 7.67 (d, J = 8.5 Hz, 2H),

7.56 (d, J = 8.5 Hz, 2H), 3.78 (s, 2H), 3.25 (s, 3H), 1.26 (s, 9H). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 144.97, 128.15, 128.08, 124.04, 123.40, 119.09, 111.86, 93.54, 44.06, 32.95, 28.76, 128.25, 180.96, 169.61, 158.35, 146.00, 131.28, 145.07, 139.49, 135.56, 134.89, 133.84, 133.60. ESIMS *m*/*z* $[M+H]^+$ 529. LC-MS Purity, >95%.

**Patch-Clamp assay.** The patch-clamp assay has been outsourced to Aptuit (Verona, Italy). Briefly, HEK293 cells expressing an inducible hERG were kept in cryogenic storage. After thawing, cells were maintained in culture using minimum essential medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 2mM L-glutamine, 1% pen-strep, 15μg/ml blasticidin and 100 μg/ml hygromycin. hERG channel expression induction was obtained by adding 10 μg/ml tetracycline 48 h before the experiment. For the assay, cells were washed with extracellular solution, treated with vehicle followed by 0.1-1-10 μM concentration of NPA101.3, by positive control E-4031 or vehicle, and finally washed with extracellular solution. Current amplitude was measured and IC50 was calculated.

**LCMS plasma analysis**. NPA101.3 (10 mg/kg) was orally dosed to mice. Blood plasma samples were collected at 1h and 4h after administration and analyzed by liquid chromatography–mass spectrometry (LC-MS) (Thermo Surveyor LCMS System with Thermo Finnigan LCQ Deca) equipped with a Phenomenex Kinetex XB-C18 column (50 x 2.10 mm, 5 μm). A 5-minute gradient elution method using water and methanol (0.1% of formic acid was added in both solvents) was applied. The ratio of methanol increased from 30% to 100% in 4 minutes and returned to 30% for column equilibration.

**Toxicity.** NPA101.3 (10 mg/kg) was orally dosed to 4 mice for 7 days; mice (n.4) treated with vehicle alone were used as controls. Plasma was subjected to biochemical analysis. Alkaline Phosphatase, Glucose, Alanine Transaminase, Total Proteins, Albumin, Globulins, Calcium, Blood Urea Nitrogen (BUN) and Phosphorus were measured by using automated biochemical analyzer Biotecnica Instrument BT1500 including proper calibration and QC. In particular, the following methods were used: IFCC for Alkaline Phosphatase and Alanine Transaminase, GOD-POD for Glucose, Buriet for Total Proteins, Bromocresol Green for Albumin, Arsenazo III for Calcium, UV for Urea/BUN and Phosphorus. Globulin was: Total proteins minus Albumin. The analysis was outsourced to Biogem Scarl, Ariano Irpino (AV), Italy.

## Supplementary Tables

### Supplementary Table S1: ΔG values of NPA101.3 in the RET DFG-out Homology Model with various (Valine 804) Gatekeeper Mutations

| Protein | NPA101.3 |
|---|---|
| RET Wild Type | -11.9 kcal/mol |
| RET V804M | -9.9 kcal/mol |
| RET V804L | -10.5 kcal/mol |

**Supplementary Table S2: NPA101.3 KinomeScan Panel screen.**

NPA101.3 was screened at a single concentration of 100 nM. Hits values lower than 10% of control were considered strongly positive.

| DiscoveRx Gene Symbol | Entrez Gene Symbol | Percent Control | Compound Concentration (nM) |
|---|---|---|---|
| ACVR1 | ACVR1 | 92 | 100 |
| AKT1 | AKT1 | 100 | 100 |
| ALK | ALK | 93 | 100 |
| ASK1 | MAP3K5 | 95 | 100 |
| AURKA | AURKA | 100 | 100 |
| AURKB | AURKB | 49 | 100 |
| AXL | AXL | 62 | 100 |
| BMX | BMX | 94 | 100 |
| BRAF | BRAF | 98 | 100 |
| BRSK2 | BRSK2 | 100 | 100 |
| BTK | BTK | 93 | 100 |
| BUB1 | BUB1 | 100 | 100 |
| CAMK1 | CAMK1 | 95 | 100 |
| CAMK2A | CAMK2A | 98 | 100 |
| CDK5 | CDK5 | 97 | 100 |
| CDK7 | CDK7 | 28 | 100 |
| CDK9 | CDK9 | 100 | 100 |
| CHEK2 | CHEK2 | 79 | 100 |
| CLK1 | CLK1 | 82 | 100 |
| CSF1R | CSF1R | 0.05 | 100 |
| CSNK1A1 | CSNK1A1 | 69 | 100 |
| CTK | MATK | 56 | 100 |
| DLK | MAP3K12 | 100 | 100 |
| DMPK | DMPK | 99 | 100 |
| DRAK1 | STK17A | 75 | 100 |
| DYRK1A | DYRK1A | 95 | 100 |
| EGFR | EGFR | 96 | 100 |
| EIF2AK1 | EIF2AK1 | 99 | 100 |
| EPHA1 | EPHA1 | 39 | 100 |
| ERK1 | MAPK3 | 100 | 100 |
| FAK | PTK2 | 86 | 100 |
| FER | FER | 96 | 100 |
| FGFR3 | FGFR3 | 100 | 100 |
| FRK | FRK | 1.1 | 100 |
| FYN | FYN | 27 | 100 |
| GAK | GAK | 42 | 100 |
| GSK3B | GSK3B | 100 | 100 |
| HCK | HCK | 6.5 | 100 |
| HIPK2 | HIPK2 | 98 | 100 |
| IGF1R | IGF1R | 100 | 100 |
| IKK-beta | IKBKB | 100 | 100 |
| INSR | INSR | 100 | 100 |

| | | | |
|---|---|---|---|
| IRAK4 | IRAK4 | 100 | 100 |
| ITK | ITK | 72 | 100 |
| JAK1(JH2domain-pseudokinase) | JAK1 | 93 | 100 |
| JAK2(JH1domain-catalytic) | JAK2 | 100 | 100 |
| JNK2 | MAPK9 | 78 | 100 |
| LIMK2 | LIMK2 | 94 | 100 |
| LKB1 | STK11 | 81 | 100 |
| LRRK2 | LRRK2 | 100 | 100 |
| LTK | LTK | 61 | 100 |
| LYN | LYN | 2.8 | 100 |
| MAP4K3 | MAP4K3 | 95 | 100 |
| MEK1 | MAP2K1 | 92 | 100 |
| MELK | MELK | 83 | 100 |
| MET | MET | 79 | 100 |
| MKNK2 | MKNK2 | 0.15 | 100 |
| MLK2 | MAP3K10 | 100 | 100 |
| MLK3 | MAP3K11 | 100 | 100 |
| MTOR | MTOR | 97 | 100 |
| MUSK | MUSK | 23 | 100 |
| MYLK4 | MYLK4 | 89 | 100 |
| MYO3A | MYO3A | 77 | 100 |
| NDR1 | STK38 | 86 | 100 |
| NEK2 | NEK2 | 100 | 100 |
| PAK1 | PAK1 | 100 | 100 |
| PCTK1 | CDK16 | 89 | 100 |
| PDPK1 | PDPK1 | 96 | 100 |
| PIK3C2B | PIK3C2B | 100 | 100 |
| PIK3CA | PIK3CA | 100 | 100 |
| PIM1 | PIM1 | 94 | 100 |
| PIM2 | PIM2 | 94 | 100 |
| PKAC-beta | PRKACB | 82 | 100 |
| PLK1 | PLK1 | 99 | 100 |
| PLK4 | PLK4 | 100 | 100 |
| PRKD2 | PRKD2 | 100 | 100 |
| PYK2 | PTK2B | 95 | 100 |
| RIPK4 | RIPK4 | 100 | 100 |
| ROCK2 | ROCK2 | 100 | 100 |
| RPS6KA4(Kin.Dom.2-C-terminal) | RPS6KA4 | 100 | 100 |
| S6K1 | RPS6KB1 | 100 | 100 |
| SLK | SLK | 35 | 100 |
| STK33 | STK33 | 83 | 100 |
| SYK | SYK | 100 | 100 |
| TAK1 | MAP3K7 | 13 | 100 |
| TIE2 | TEK | 30 | 100 |
| TLK1 | TLK1 | 100 | 100 |
| TRKA | NTRK1 | 1.3 | 100 |
| TRKC | NTRK3 | 0.25 | 100 |

| | | | |
|---|---|---|---|
| TSSK1B | TSSK1B | 96 | 100 |
| TTK | TTK | 66 | 100 |
| TXK | TXK | 85 | 100 |
| ULK1 | ULK1 | 100 | 100 |
| ULK2 | ULK2 | 100 | 100 |
| WNK1 | WNK1 | 93 | 100 |
| YANK2 | STK32B | 98 | 100 |

**Supplementary Table S3: NPA101.3 inhibition of hERG.**

NPA101.3 was tested in a patch-clamp assay to evaluate percentage of hERG inhibition at 0.1, 1 and 10 μM with respect to vehicle. $IC_{50}$ dose is reported

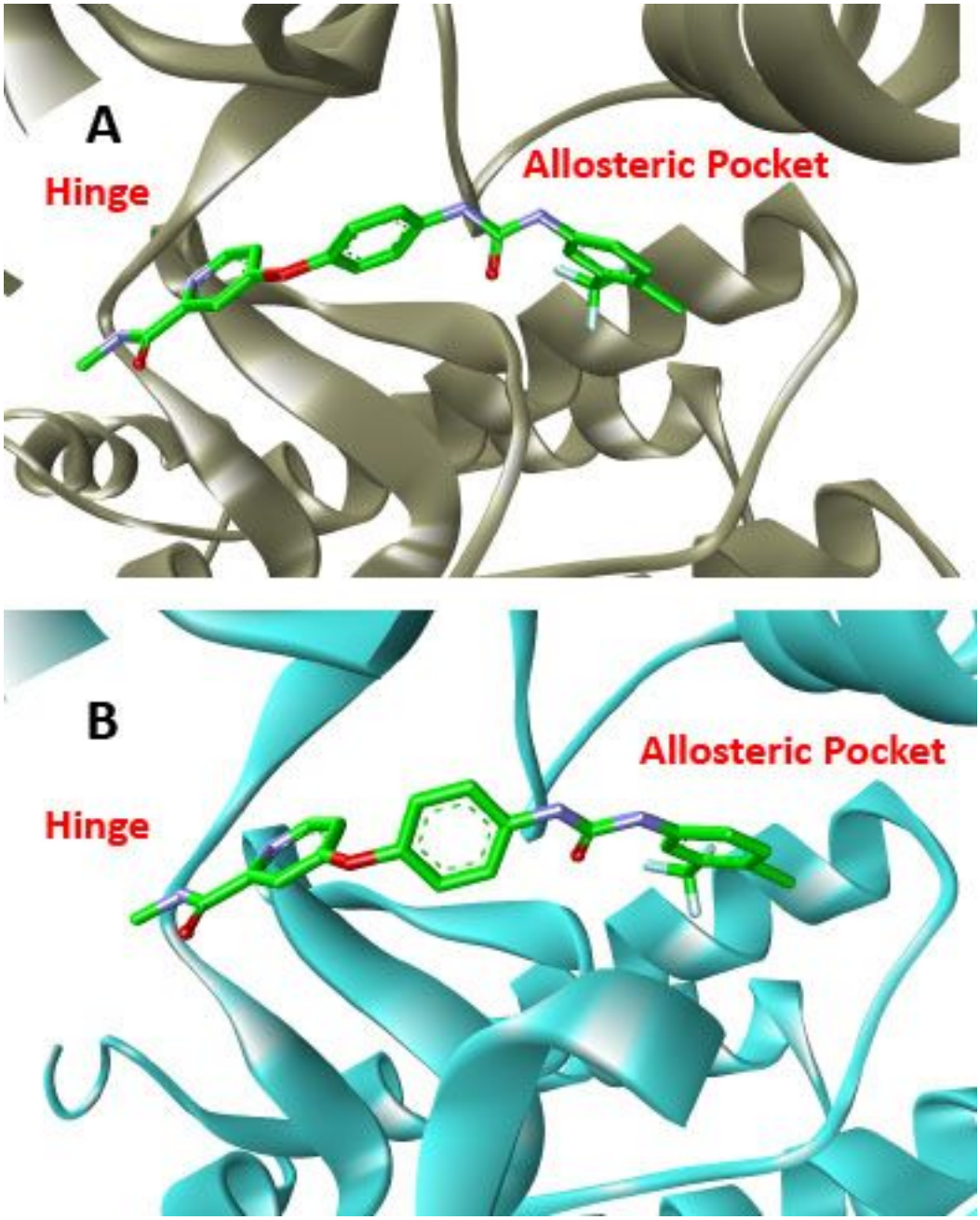
| | **% Mean Inhibition** | | | **$IC_{50}$ (μM)** |
|---|---|---|---|---|
| NPA101.3 | 0.1 μM | 1 μM | 10 μM | 7.57 |
| | 0.41 | 14.55 | 55.94 | |
| E-4031 | 0.003 μM | 0.03 μM | 0.3 μM | 0.027 |
| | 2.35 | 53.90 | 93.36 | |

**Supplementary Table S4: NPA101.3 growth inhibitory effect on human cells.**

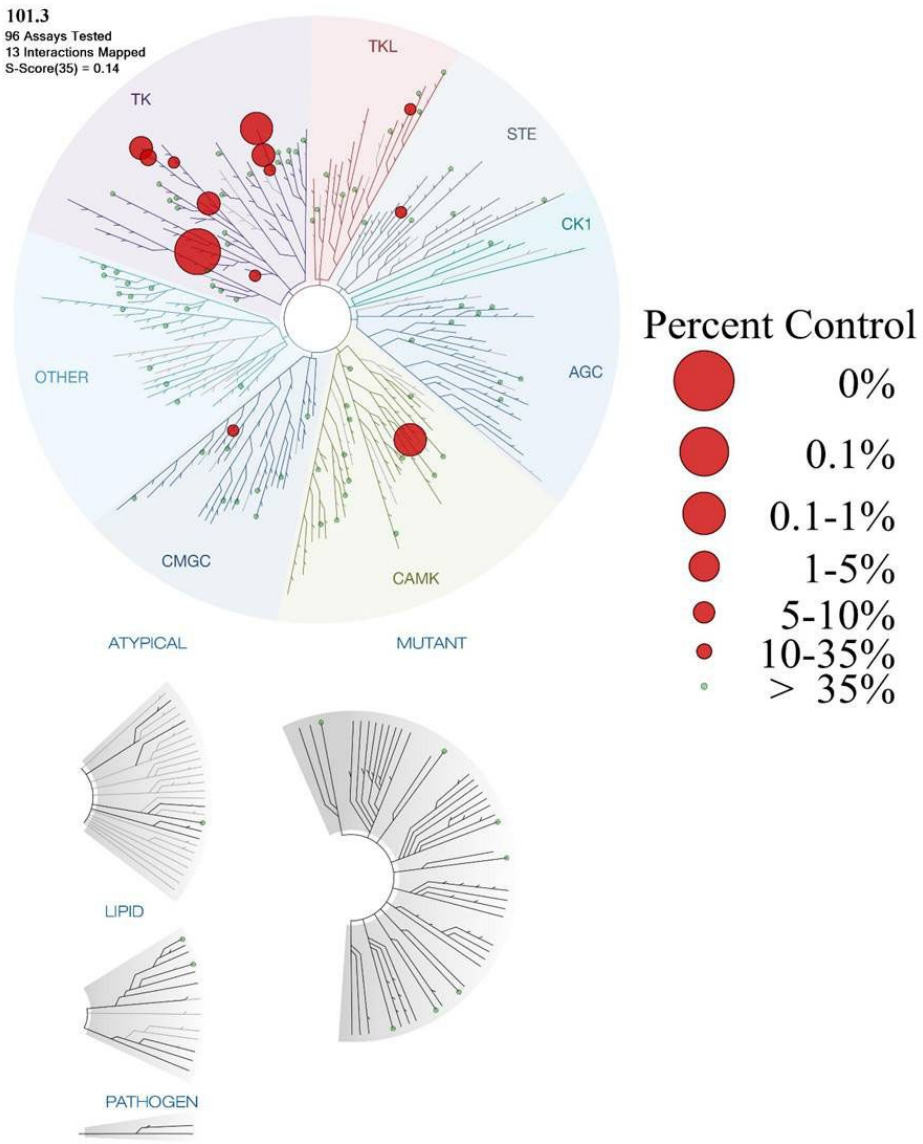
| Cell line | Origin* | Oncogene mutation | $IC_{50}$ nM (95% CI) |
|---|---|---|---|
| TT | MTC | RET C634W | 2.19 (1.79-2.68) |
| MZ-CRC-1 | MTC | RET M918T | 2.21(1.19-4.1) |
| TPC-1 | PTC | CCDC6-RET | 0.67 (0.03-132) |
| Nthy-ori-3-1 | thyroid | SV40 LT | > 100 |
| BCPAP | PTC | BRAF V600E | > 100 |
| 8505-C | ATC | BRAF V600E | > 100 |
| Lc-2/ad | LADC | CCDC6-RET | 3.6 (0.9-13.9) |
| CALU-1 | LADC | KRAS G12C | > 100 |
| A549 | LADC | KRAS G12S | > 100 |
| PC-9 | LADC | HER1 delLREA(747-750) | > 100 |

*MTC (medullary thyoroid carcinoma); PTC (papillary thyroid carcinoma); ATC (anaplastic thyroid carcinoma); LADC (lung adenocarcinoma)

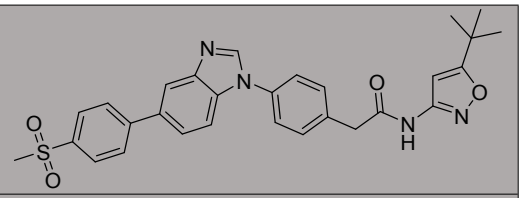
**Supplementary Figures**



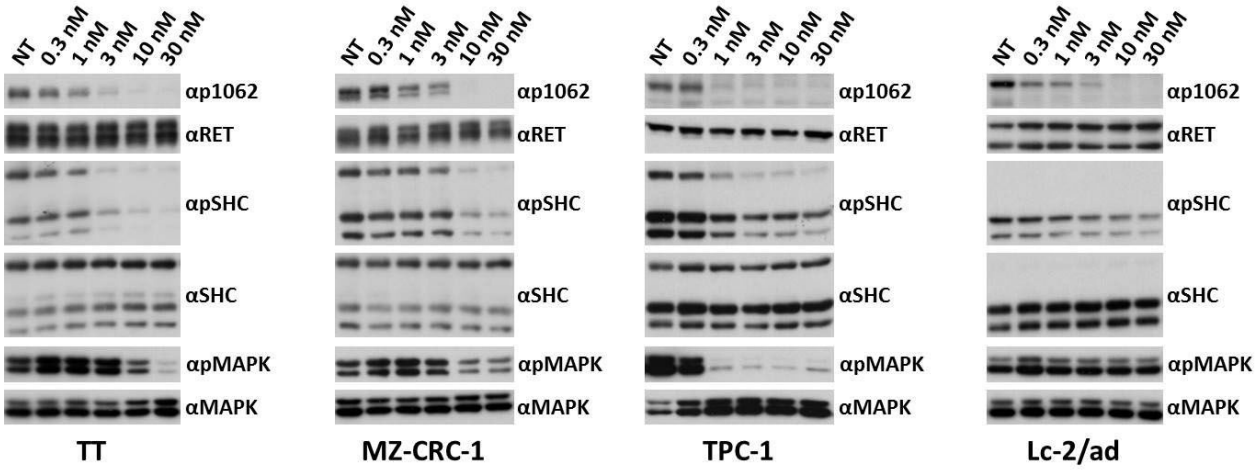

**Supplementary Figure S1: Docking pose of sorafenib in the RET DFG-out model compared to X-Ray crystal structure of Sorafenib in VEGFR2 (PDB #3WZE) —** Sorafenib binds to the RET DFG-out homology model (A) in a very similar pose to that of the VEGFR2 structure (B). The pose is also similar to NPA101.3 (Fig. 2), which further suggests NPA101.3 as a Type-II RET inhibitor.



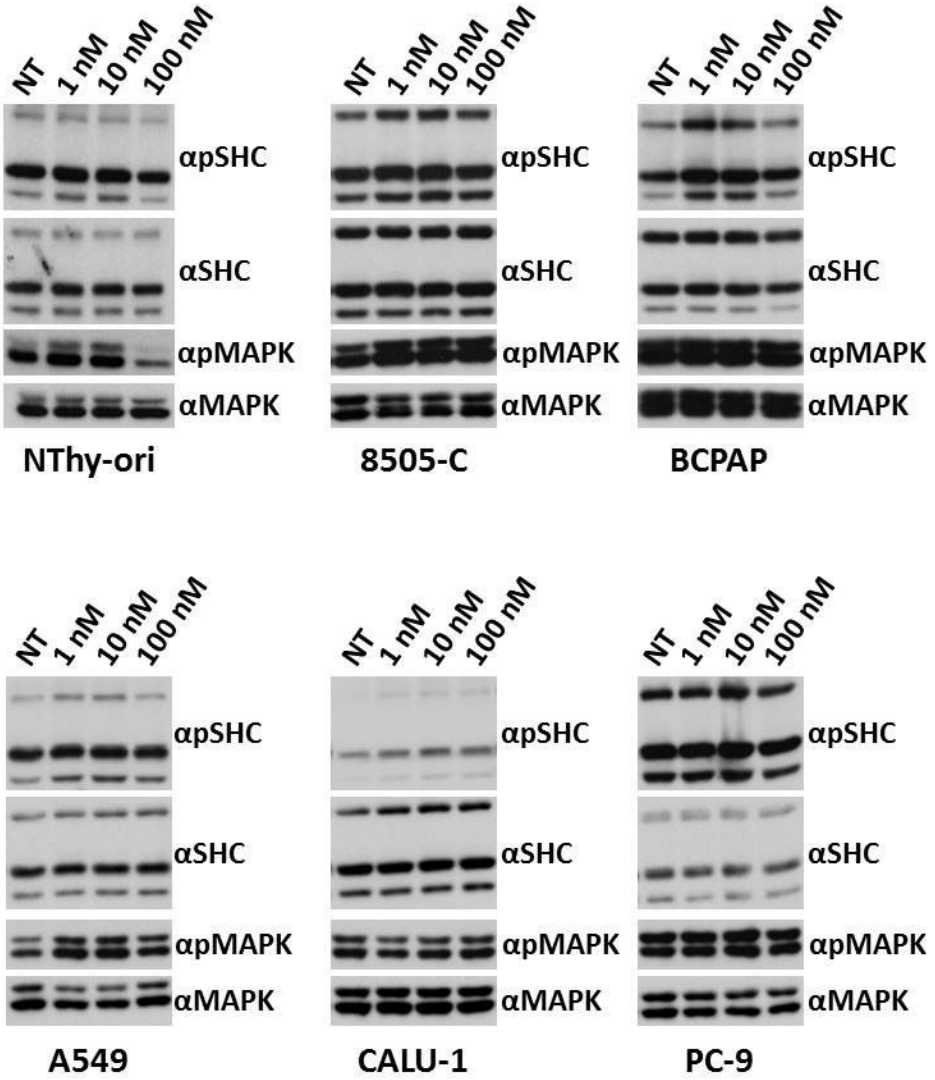

**Supplementary Figure S2: NPA101.3 binding selectivity**—NPA101.3 was screened against a panel of 96 kinases at a concentration of 100 nM. RET and VEGFR2 were not included in the screen because NPA101.3 inhibition of both was previously determined.

| |  |
|---|---|
| Kinase | NPA 101.3 $IC_{50}$ (nM) |
| CSF1R | 46 |
| TRKA | 32 |
| RET | 1 |

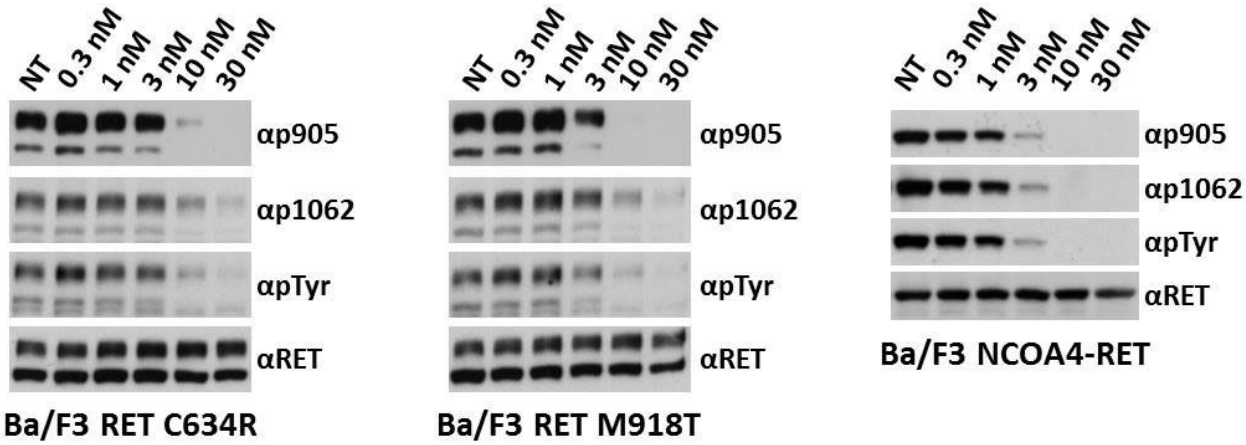
**Supplementary Figure S3: RET, TRKA and CSF1R kinase inhibition by NPA101.3**



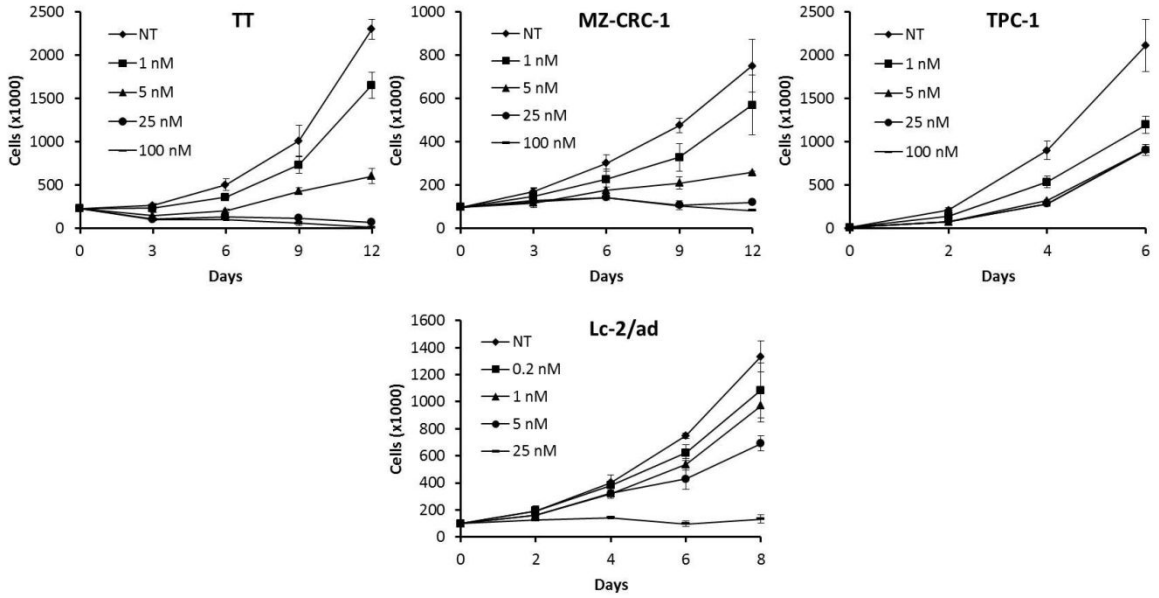

**Supplementary Figure S4: NPA101.3-mediated inhibition of phosphorylation and signalling of oncogenic RET proteins endogenously expressed in human cancer cells —** Serum-starved RET mutant human cancer cell lines were treated for 2 hours with the indicated concentrations of the drug. Total cell lysates (50 μg) were subjected to immunoblotting with αp1062 RET, anti-phospho-MAPK (αpMAPK) and anti-phospho-SHC (αpSHC) antibodies. The blots were normalized using anti-RET (αRET), anti-MAPK (αMAPK) and anti-SHC (αSHC).



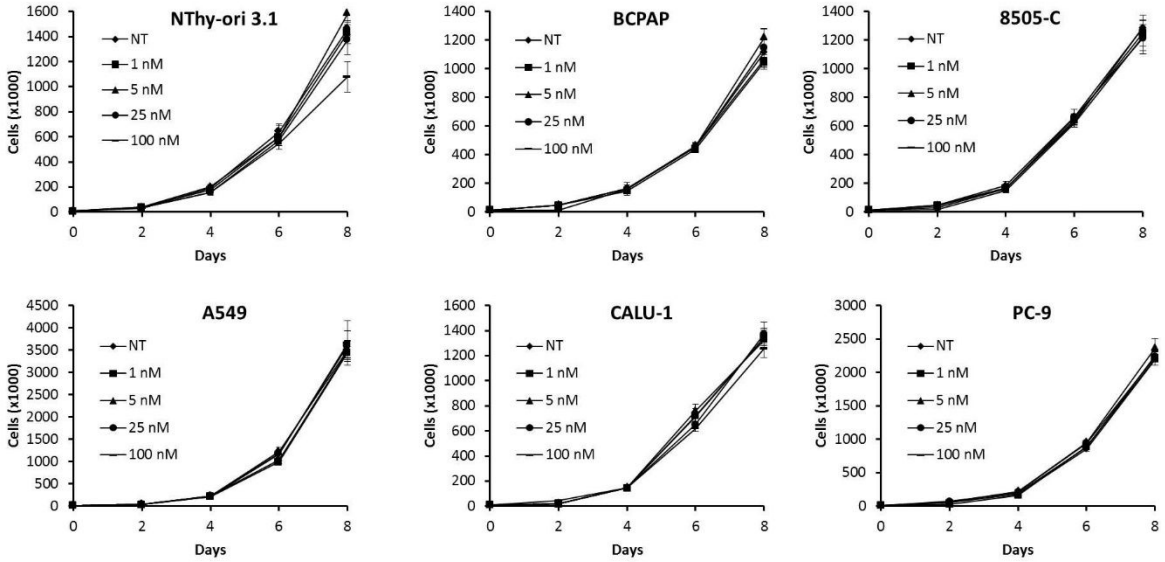

**Supplementary Figure S5: Effects of NPA101.3 on RAS/MAPK signalling pathway in RET-negative human cancer cells —** The indicated cell lines were serum-starved and treated for 2 hours with indicated concentrations of the drug. Total cell lysates (50 μg) were subjected to immunoblotting with indicated antibodies.



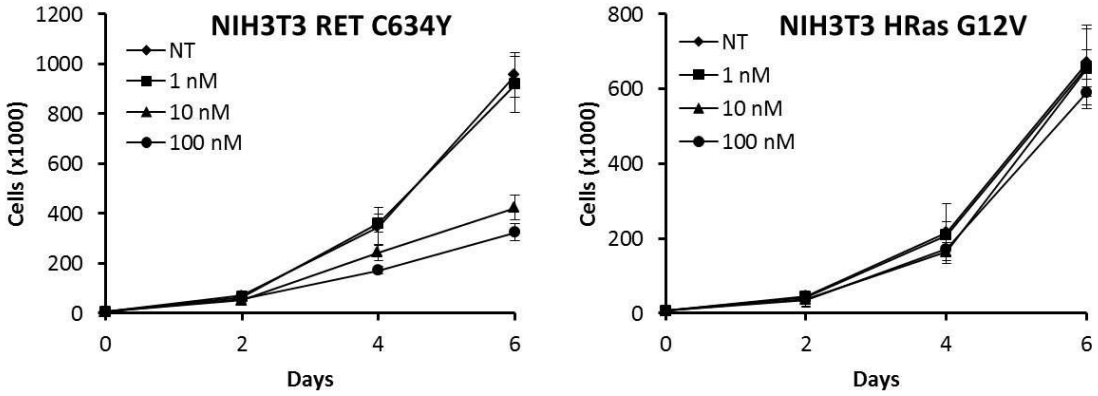

**Supplementary Figure S6: NPA101.3-mediated inhibition of phosphorylation of oncogenic RET proteins expressed in Ba/F3 cells** — The indicated Ba/F3 transfectants were serum starved and treated for 2 hours with the indicated concentrations of the drug. Total cell lysates (50 μg) were subjected to immunoblotting with indicated antibodies.




**Supplementary Figure S7: NPA101.3-mediated inhibition of proliferation of RET mutant thyroid and lung cancer cells —** The indicated cell lines were incubated with vehicle (NT: not treated) or the indicated concentrations of the compound and counted at the indicated time points. Data are the mean ± SD of a single experiment performed in triplicate.



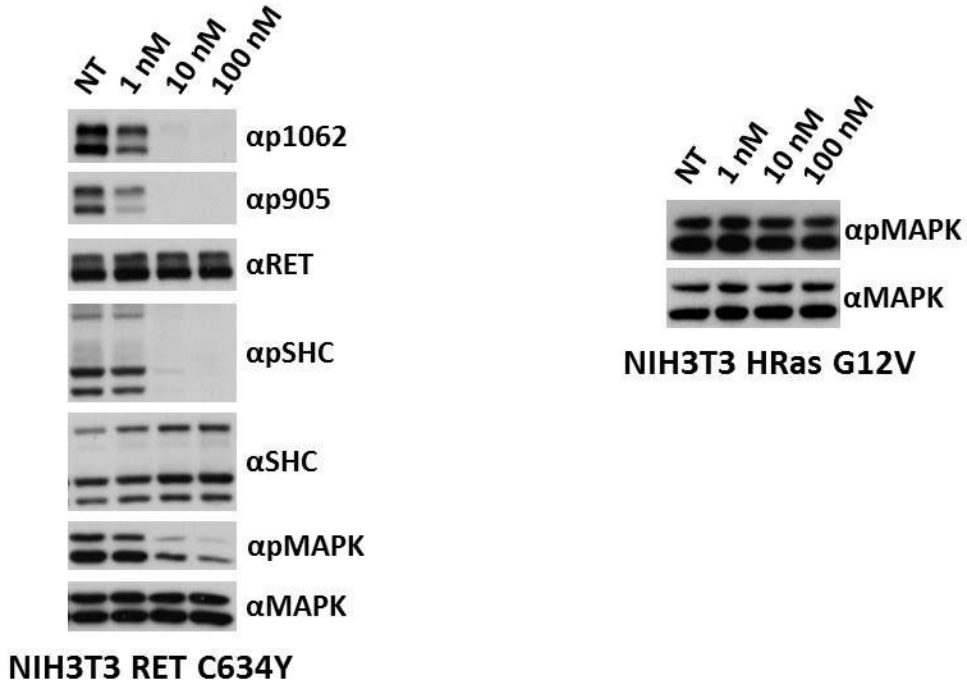

**Supplementary Figure S8: Effects of NPA101.3 on proliferation of RET-negative cancer cells —** The indicated cell lines were incubated with vehicle (NT: not treated) or the indicated concentrations of the compound and counted at the indicated time points. Data are the mean ± SD of a single experiment performed in triplicate.



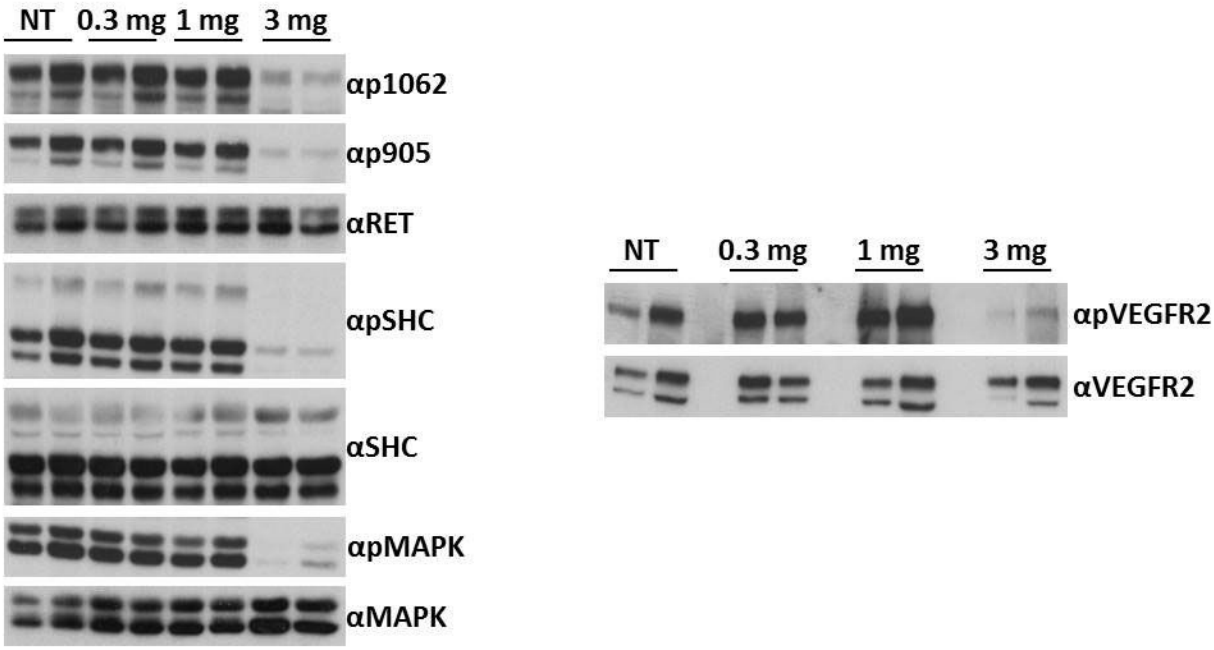

$IC_{50}$ dose

| | |
|---|---|
| **NIH3T3 RET C634Y** | 4.17 nM (1.16-11.19 nM) |
| **NIH3T3 HRas G12V** | Not measurable |

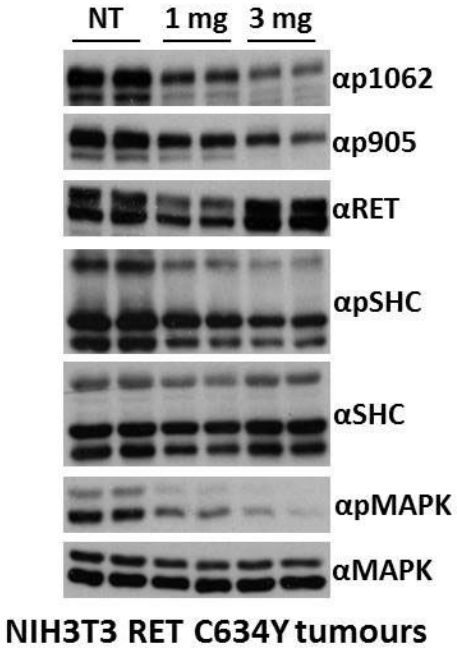
**Supplementary Figure S9: NPA101.3-mediated inhibition of proliferation of RET-transformed NIH3T3 cells —** Serum-starved NIH3T3 cells exogenously expressing the RET/C634Y or HRAS/G12V oncogenes were incubated with vehicle (NT: not treated) or the indicated concentrations of the compound and counted at the indicated time points. Data are the mean ± SD of a single experiment performed in triplicate. Growth inhibition $IC_{50}$ doses of the compound are reported. 95% CI are indicated in brackets.



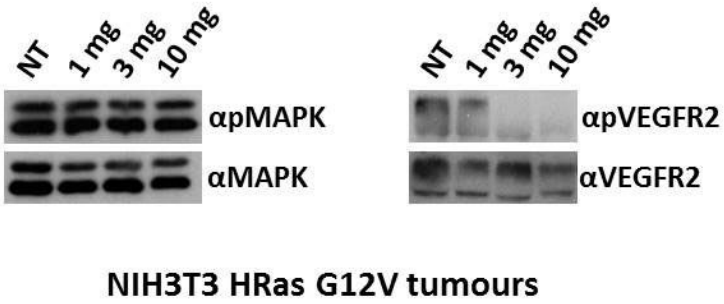

**Supplementary Figure S10: NPA101.3-mediated inhibition of phosphorylation and signalling of oncogenic RET proteins expressed in NIH3T3 cells —** Serum-starved RET/C634Y or HRAS/G12V-transformed NIH3T3 cells were treated for 2 hours with the indicated concentrations of the drug. Total cell lysates (50 μg) were subjected to immunoblotting with with indicated antibodies.



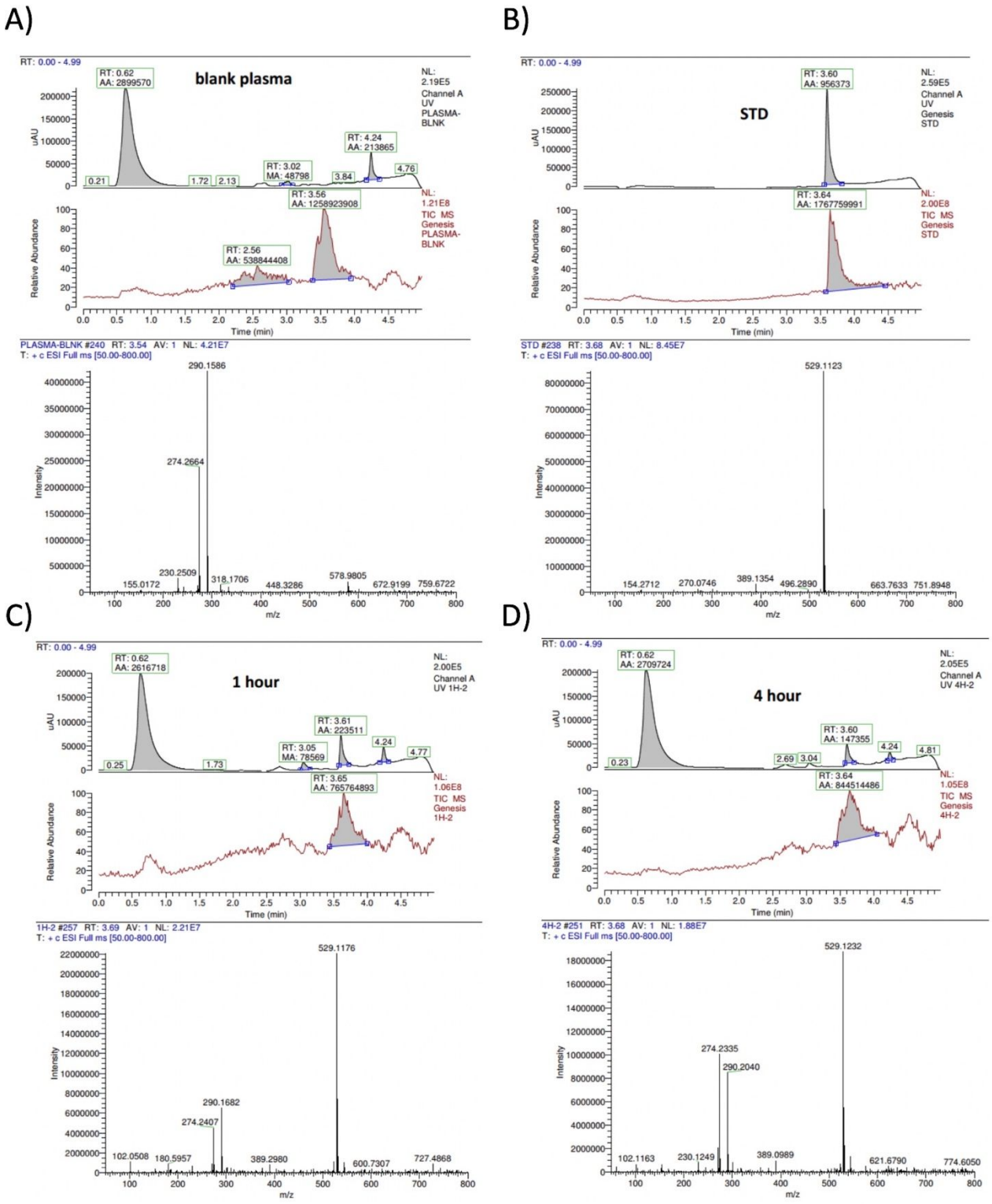

**Supplementary Figure S11:** ***In vivo*** **target inhibition of NPA101.3 in nude mice implanted with cells transformed by RET/C634Y —** NIH3T3 RET/C634Y cells (2 x $10^5$) were inoculated subcutaneously into the right and left dorsal portions of nude mice. When tumours reached approximately ~200 $mm^3$, mice (2 mice/dose) were treated by oral gavage with 3 doses (0, 24, 48 h) of the compound (0.3, 1 and 3 mg/Kg) or left untreated (NT); proteins were harvested 3 h after the last dose. Left) Total lysates (50 μg) from 2 representative NIH3T3 RET/C634Y tumours for each dose were immunoblotted with indicated antibodies. Right) Total lysates (1 mg) from 2 representative NIH3T3 RET/C634Y tumours for each dose were subjected to VEGFR2 immunoprecipitation followed by Western blotting with anti-phospho-VEGFR2 antibody (αpVEGFR2). The blots were normalized using anti-VEGFR2 (αVEGFR2).



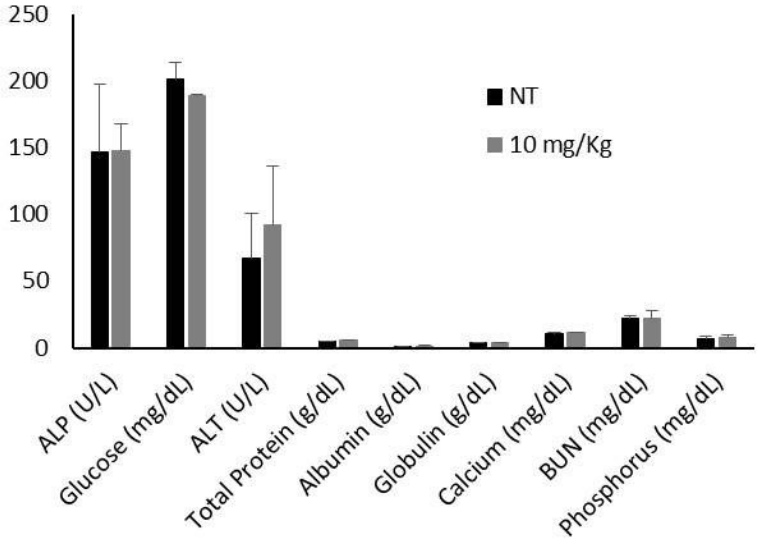

**Supplementary Figure S12: Effects of NPA101.3 on cellular phosphorylation events in nude mice implanted with NIH3T3 cells transformed by RET/C634Y —** Three hours after the last dose, 2 representative tumours for each dose from the experiment reported in Figure 6 were harvested from mice treated with indicated doses of NPA101.3 or left untreated (NT). Proteins were extracted and total protein lysates (50 μg) were immunoblotted with indicated antibodies.




**Supplementary Figure S13: Effects of NPA101.3 on cellular phosphorylation events in nude mice implanted with NIH3T3 cells transformed by HRAS/G12V** —Three hours after the last dose, one representative tumour for each indicated dose from the experiment reported in Figure 6 was harvested and proteins were extracted. Left) total protein lysates (50 μg) were immunoblotted with indicated antibodies. Right) protein extracts (1 mg) were subjected to VEGFR2 immunoprecipitation followed by Western blotting with anti-phospho-VEGFR2 antibody (αpVEGFR2). The blots were normalized using αVEGFR2.




**Supplementary Figure S14**: ***In vivo*** **metabolism study.** NPA101.3 was orally dosed (10 mg/kg) to mice (one animal for each point). Blood plasma samples were collected at 1 h and 4 h after dosing; blood from one untreated mouse (blank) and NPA101.3 solution (STD: standard) were used as controls. NPA101.3 drug peak was detected at 3.61 min (*m/z* = 529) at either time point. No trace of the de-methyl metabolite at *m/z* = 515 was observed. The *m/z* 279 and 290 were not specific peaks identified also in the blank plasma sample.




**Supplementary Figure S15**: **Biochemical analysis.** Plasma concentration of Alkaline Phosphatase (ALP), Glucose (GLU), Alanine Transaminase (AST), Total Protein (T.Prot.), Albumin (ALB), Globulins (Glo), Calcium (Cal), Blood Urea Nitrogen (BUN) and Phosphorus (PHO) were measured upon treating animals with NPA101.3 (10 mg/kg/day) for 7 days or vehicle as indicated (4 animals for each group).