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Title: Synthesis of novel PET tracer ^{124}I-trametinib for MAPK/ERK kinase distribution and resistance monitoring

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Radioiodinated Trametinib

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Abstract:

Trametinib (MEKINIST™) is an extremely potent allosteric inhibitor of MEK1/2 that has been approved for treatment of metastatic melanoma and anaplastic thyroid cancer in patients with confirmed BRAFV600E/K mutations. Though highly efficacious, adverse side effects including skin, gastrointestinal and hepatic toxicity, are dose limiting and can lead to treatment termination. Development of a non-invasive tool to visualize and quantify the delivery and distribution of trametinib (either as single agent or in combination with other therapeutics) to tumors and organs would be very helpful in assessing therapeutic index, personalizing individual dose and potentially predict resistance to therapy. To address these issues, we have developed a radiolabeled trametinib and evaluated the *in vitro* and *in vivo* properties. ^{123}I-, ^{124}I- and ^{131}I-trametinib, pure tracer analogs to trametinib, were synthesized in >95% purity with average yield of 69.7% and >100GBq/μmol specific activity. Overall, ^{124}I-trametinib uptake in a panel of cancer cell lines can be blocked with cold trametinib confirming specificity of the radiotracer *in vitro* and *in vivo*. ^{124}I-Trametinib was taken up at higher rates in KRAS and BRAF mutant cell lines compared to wild type KRAS cancer cell lines. *In vivo*, biodistribution revealed high uptake in the liver 2 hours post injection followed by clearance through the gastrointestinal tract over 4 days. Importantly, higher than expected uptake was observed in the lung and heart for up to 24 hours. Peak uptake in the skin and gastrointestinal tract was observed between 6 and 24 hours while in B16F10 melanoma bearing mice peak tumor concentrations were achieved between 24 to 48 hours. Tumor uptake relative to muscle and skin was relatively low, peaking at 3.4- to 8.1-fold by 72 hours, respectively. Biodistribution of ^{124}I-trametinib was significantly reduced in mice on trametinib therapy

providing a quantitative method to observe MEK inhibition *in vivo*. ^{124}I-trametinib serves as a tool to personalize *in vivo* the dose instead of using the current single fixed dose scheme and when combined with radiomic data monitor emergence of therapy resistance. In addition, the production of iodinated trametinib affords researchers the ability to measure drug distribution for improved drug delivery studies.

Introduction:

Targeted small molecule therapy is based on identifying and inhibiting an overactive signaling protein downstream in a pathway such as in the tyrosine receptor kinase (TRK). The mitogen activated protein kinase (MAPK) pathway contains kinase kinases (MAPKKK) which phosphorylate another MAPK Kinase (MAPKK) which ultimately activates another MAPK such as an extracellular-signal-regulated kinase (ERK)(*1*). In the TRK pathway, Ras is a G-protein, Raf as a MAPKKK, MAPK/ERK kinase (MEK) as MAPKK and ERK as MAPK. As this pathway is not unique to cancer cells, side effects can arise in healthy tissue upon inhibition. Many cancers possess BRaf and Ras mutations, resulting in hyperactive signaling through MEK and ERK, ultimately turning on proliferative genes (**Figure 1**) (*2*). Trametinib, (Mekinist®) is an FDA approved MEK inhibitor for use in metastatic melanoma containing a BRAF mutation. As a type-III MEK inhibitor, trametinib binds MEK1/2 with an IC_{50} of 0.9nM as an allosteric inhibitor outside the ATP binding pocket, preventing Raf phosphorylation of MEK on S217 (pMEK) and thus ERK (pERK) while overall MEK levels are unaffected (*3*). The use of trametinib as a MEK inhibitor has allowed improvement in survival of patients with melanoma, and new strategies to treat mutant KRAS lung cancers (*4*). More recently activation of Natural Killer cells when treated in combination with cyclin dependent kinase (CDK4/6) inhibitors (*5*) provides even newer uses for MEK inhibitors.

Despite the high potency of trametinib, toxicity in the skin, gastrointestinal tract, and liver reduces compliance of continued use (*6*). This toxicity is “on target / off tumor” as healthy tissue also uses the TRK pathway, so quantification of drug distribution is important for patient dosing, understanding target tissue and organs, as well as

monitoring potential resistance to therapy. We aimed to build a radioactive version of trametinib, allowing for positron emission tomography (PET) or single photon emission computed tomography (SPECT) imaging. According to the FDA toxicology package, trametinib is not highly metabolized by cytochrome P450 enzymes and is primarily metabolized by deacetylation, demethylation, ketone formation, mono-oxygenation and glucuronidation(*7*). The para positioned iodine on the 2,4-fluoro-iodoanilino ring of trametinib would be an ideal site for radiotracer placement as the iodine ring directly engages the MEK sub-pocket (*8,9*). The para positioned iodine also remains intact in the major metabolite, known as M5, which is two-fold less potent for MEK1 and phospho-MEK1 compared to the parent trametinib allowing tracking of the metabolite in addition to the parent. Radioiodine is also ideal as a tracer given the numerous forms readily available for PET- or SPECT- imaging, and radioiodinated trametinib would be identical to trametinib given therapeutically.

To allow facile radioiodination, the 4-iodo group was replaced with a boronato-pinacol prosthetic group on trametinib. Previous work with (hetero)aryl boron reagents identified radiosynthesis via copper mediated iodination with high selectivity in a mild one step procedure(*10-12*). Production of ^{123}I, ^{124}I, and ^{131}I-trametinib from a boronato-pinacol could be initiated and purified within 1.5 hours from the boronato-pinacol-trametinib precursor with approximately 70% relative radiochemical yield. Iodine is not cleaved during metabolism of trametinib, and thus radioiodine will allow tracking *in vivo* trametinib in addition to the relevant deacylated and oxidized metabolites, which are still highly potent inhibitors of MEK1 (2-10 fold loss in potency from parent) is possible(*7*).

Radiolabeled trametinib is taken up with higher avidity in known BRAF and or KRAS mutant cell lines.

Here, we provide a robust synthesis of radioiodinated trametinib, providing a tool for patient level pharmacokinetic modeling, patient specific dosing, and resistance estimation. Radiolabeled trametinib was readily taken up into KRAS and BRAF mutant cell lines and significantly blocked when co-administered with cold trametinib. The distribution of radiolabeled trametinib *in vivo* follows target organs known for toxicity such as the skin and gastrointestinal tract but also reveals distribution in the heart and lung at levels not consistent with distribution by the blood. Accumulation in trametinib naïve tumors peaked at 48 hours. Future work could utilize radiolabeled trametinib as a tool for developing new drug delivery systems, focusing on the distribution of the drug and metabolites. Radioiodinated trametinib can monitor MEK1/2 levels, such as in KRAS and BRAF mutant cancers, thus personalizing dosages needed per patient during therapy and a diagnostic for MEK resistance.

Materials and Methods:

Trametinib Synthesis:

Trametinib was modified using diborylpinacol under palladium catalysis at 130^{O}C with microwave to contain a bora-pinacol group in lieu of natural iodine making the trametinib-boronato-precursor. Radioiodination was attempted via two methods: Chloramine-T as well as a copper mediated insertion. Briefly, the copper mediated insertion uses >37MBq of [^{123}I, ^{124}I, or ^{131}I]-NaI in 20-100 µL added to an Eppendorf tube

containing the trametinib-boronato precursor (200 µg, 200 µL ethyl acetate), copper acetate (0.16 mg,73 µL) and 1,10-phenanthroline (0.20 mg, 200 µL) in methanol:water mixture (4:1). The reaction mixture was heated at 80^{O}C for 35 min and allowed to cool to RT. The progress of the reaction was monitored on a HPLC equipped with a Bioscan radiodetector (Phenomenex Luna C-18 column, 250 X 4.6 mm, 5 µm, 100 Å,) using a gradient of 5-95% CH_3CN in 0.1% TFA in water (2 – 15 min). Iodinated trametinib was isolated by HPLC from reagents in the 95% CH_3CN fraction, diluted to 5% with phosphate buffered saline and captured onto a Waters Sep-Pak Light C18 cartridge. Iodinated trametinib was recovered using either EtOH and rotary evaporated to near dryness, or directly with DMSO.

Radiotracer production:

Iodine-124 (^{124}I) was obtained with gratitude from the Memorial Sloan Kettering Cancer Center Radiochemistry and Molecular Imaging Probe facility after production and purification into 0.05M Na^{124}I. Iodine-131 and Iodine-123 were purchased from National Diagnostic Products and provided as NaI in 0.05N NaOH or sulfate buffer.

Cell uptake studies:

Uptake kinetic studies of ^{124}I-trametinib were done in 24 well plates with cell lines seeded at 1.3 x 10^5 cells per well with standard culture media. Cell lines were administered 1.85 kBq of ^{124}I-Trametinib per well and incubated at 37^{O}C for up to 72 hours. Blocking experiments were done with 1 µg of cold trametinib, and a titration curve of cold trametinib was performed with the B16F10 cell line for two hours before 0.05% trypsin addition for gamma counting of ^{124}I-trametinib in cells. Activity present in each vial was determined

alongside a standard curve by gamma counting on a Perkin Elmer 2480 Wizard 3 with a count time of 60 seconds per sample. Murine cell lines tested: AKP, B16F10, KP1, Raw264.7. Human cell lines tested: 22Rv1, ASPC1, Du145, LNCAP, MDA-MB-231, MiaPaca2, PC3, SW1222.

Animal studies:

All experiments were done in compliance and approval with the Memorial Sloan Kettering Cancer Center research animal resource center and the institutional animal care and use committee. Healthy C57BL/6-J mice were purchased from Taconic. B16F10 melanoma cell line was purchased and cultured from American Type Culture Collection in Dulbecco's modified essential medium with 10% fetal bovine serum and 1% penicillin streptomycin. $3x10^5$ cells were implanted into the right dorsal foot pad or right flank of mice. After 2- or 5-weeks flank or foot pad melanoma bearing mice, respectively were injected with ^{124}I-trametinib in 10% DMSO in saline intravenously and imaged or euthanized at the given timepoint for biodistribution studies. A cold iodine block was administered intraperitoneally up to one hour prior to radiotracer injection. For therapy monitoring, cohort of mice were administered 6 mg kg^{-1} per mouse per day in 10% DMSO, 90% saline intraperitoneally for three days. Daily dosing intraperitoneally was done to mimic daily oral dosing of trametinib, as well as preserve tail veins for ^{124}I-trametinib administration. No further therapy was administered after injection of ^{124}I-trametinib. For biodistribution studies, mice were euthanized in accordance with the research animal resource center and the institutional animal care and use committee. Tissues collected during biodistribution were placed into pre-weighed tubes for determination of tissue mass. Tissues collected: blood,

heart, lungs, liver, spleen, kidneys, complete gastrointestinal tract (stomach through lower intestine including pancreas), thyroid, thigh muscle, femur bone, ear, lymph nodes (popliteal, inguinal, and or mesenteric), and tumor. Activity present in each vial was determined alongside a standard curve by automatic gamma counting on a Perkin Elmer 2480 Wizard 3. Metabolite analysis with ^{131}I-trametinib was performed with a Miltenyi gentle Macs C-dissociation cup and metabolites extracted with acetonitrile for analysis by HPLC using the same conditions as for trametinib purification.

Imaging studies:

Mice injected with ^{124}I-trametinib were imaged at 0, 1, 3, 6, 12, 24, and 96 hours post injection using a Siemens Inveon PET/CT scanner. A fixed 30-minute acquisition was performed for all images. Images represented as percent injected dose per gram overlaid onto the CT for anatomical reference.

Results:

Trametinib **(1)** is not immediately available for direct radioiodination in high yield, requiring a facile precursor for rapid iodination. By using palladium catalysis under elevated temperature and microwave conditions, the stable para-iodine could be removed and exchanged with a boronato-pinacol group. The precursor boronato-trametinib **(2)** was achieved in a 9:1 ratio with a boronato hydroxide impurity, which could also be used for radioiodination and attempted via two methods. Chloramine-T oxidation was unsuccessful, but the copper (II) acetate mediated 1-10-phenanthroline iodination **(Figure 2A)** was successful after heating 80^{O}C for 30 minutes. Iodination was achieved

(3) as seen by the HPLC chromatogram with minimal unreacted radioiodine **(Figure 2B)**. Synthesis with additional radiotracers ^{123}I and ^{124}I yielded high reaction selectivity as well allowing facile iodination across the three iodine sources used. The reaction product was purified by HPLC and collected onto a Sep-Pak Light C18 cartridge for elution into either EtOH or DMSO. Overall iodination and purification were completed in less than 1.5 hours with an overall radiochemical yield of 69.7% and specific activity of > 100 Gbq/μmol for ^{124}I-trametinib. As radioiodine replaces cold iodine on the 2,4-fluoro-iodo-analine, radioiodinated trametinib is a pure tracer without perturbation of the structure. With an available method to produce radioiodinated trametinib from a wide variety of radioiodine sources, the focus of the remaining work used ^{124}I-trametinib for facile PET imaging. ^{124}I is a PET tracer with a 23% β^+ yield and 4.18d $t_{1/2}$ allowing imaging out to 96 hours and beyond for long term biodistribution.

With ^{124}I-trametinib, radiotracer specificity was determined in human and murine cancer cell lines. Addition of ^{124}I-trametinib at 2 hours **(Figure 2C)** was readily blocked by the addition of 1μg of cold, commercially available trametinib. This block persisted through 72 hours in cell lines tested showing the selectivity of the iodinated trametinib produced and the duration of inhibition *in vitro* **(Supplementary Figure 1)**. Internalization of ^{124}I-trametinib at 2 hours varied almost ten-fold, with KRAS mutants having higher internalization percentages. The highest uptake was observed with the MDA-MB-231 human cell line, which has both KRAS and BRAF activating mutations. Murine cell lines were similarly avid for ^{124}I-trametinib as KRAS mutants and the KRAS BRAF wild type cell line Raw264.7 had the lowest uptake **(Figure 2C)**. A titration of cold trametinib with 13.4 kBq of ^{124}I-trametinib revealed the dose dependent inhibition with cold trametinib

and a 50% block by 0.03μM (**Supplementary Figure 2**). Selectivity of trametinib blocking was confirmed with other allosteric MEK inhibitors and partial inhibition with BRAF and pan TRK inhibitors (**Supplementary Figure 3**). KRAS and or BRAF mutant lines overall had higher internalization rates than wild type cancer cell lines providing a nearly ten-fold range between lines tested. Du145 has a UBE2L3-KRAS fusion gene despite having a normal KRAS variant(*13*) though uptake studies suggest the fusion gene does not have an effect on ^{124}I-trametinib uptake. The murine melanoma line B16F10 while not a KRAS or BRAF mutant, contains a INK4a/ARF deletion, allowing oncogenes to drive proliferation(*14,15*). Here the use of iodinated trametinib can separate KRAS and BRAF mutants from wild type in naïvely treated cancer cell lines while blocking studies show ^{124}I-trametinib uptake can determine the degree of MEK inhibition.

Trametinib distribution and associated organ toxicity has been reported in clinical trials(*2,6,16*) as well in pre-clinical toxicology findings(*7*). Despite recorded adverse events, the majority of studies published refer to on-target/off-tumor toxicity of MEK inhibition in tissues but do not correlate amount of drug delivered to each organ and the toxicity observed. According to the trametinib prescribing information(*17*), adverse events include: hemorrhage, venous thromboembolism, cardiomyopathy, ocular toxicity, interstitial lung disease, serious febrile reactions, serious skin reactions, hyperglycemia, and embryofetal toxicity. Most common reactions include rash, diarrhea and lymphedema. Metabolism of trametinib is mainly hepatic, with 70-93% excreted via feces when given orally(*7*). Knowing drug concentration over time in each tissue and the reported adverse interactions establishes a baseline for trametinib biodistribution and a tool to show how new formulations and delivery vehicles could alter and prevent organ

toxicity. Healthy C57BL/6-J mice were administered approximately 1.5 MBq ^{124}I-trametinib for biodistribution studies or 1.85 GBq for imaging studies via tail vein and euthanized after 1, 2, 6, 12, and 24 hours (**Figure 3**). Organs collected were blood, heart, lung, liver, spleen, kidneys, entire gastrointestinal tract (stomach, pancreas, small intestine, large intestine, rectum), thyroid, muscle, bone, bladder, skin and lymph nodes. Imaging studies show rapid clearance of the drug from the blood within two hours, with the majority of activity in the liver (**Figure 3A**). Subsequent images show gastrointestinal clearance and fecal excretion. Image series identify low uptake of ^{124}I-trametinib across the blood brain barrier and into the brain(*7*). Biodistribution studies show highest uptake in liver and kidneys between 1-6 hours with 18% and 11% injected dose per gram (ID/g) respectively, with the gastrointestinal tract and spleen representing 11% and 8% ID/g (**Figure 3B**). ^{124}I-trametinib was rapidly cleared from the blood yet 5% ID/g was observed in the heart and lung that did not appreciably clear until 24 hours after administration. Skin, the area most notable for toxicity observed in patients, was found in mice to have an overall low uptake of 1-3% ID/g, highlighting the sensitivity of the organ to trametinib. Other notable organs include the muscle with 2-3 % ID/g at each timepoint observed.

Next biodistribution studies were completed in mice bearing B16F10 melanomas on their right dorsal foot pad. This melanoma model is highly metastatic yielding metastases in the ascending lymph nodes. Primary tumor uptake was visible as early as 1 hour (**Figure 4A**) after injection and increasing through 24 hours and peaking at 4% ID/g at 48 hours, while all other organs appreciably cleared between 24-72 hours. The tumor accumulation over 48 hours presents a possibility of imaging melanomas after 24 hours with a nearly 6-fold ratio over skin by 48 hours and 8-fold over skin by 72 hours

though the greatest benefit is to identify the MEK avidity of naïve tissue, and inhibition during trametinib therapy. No significant difference in organ uptake was observed compared to healthy mice **(Supplementary Figure 4)**. Uptake in sentinel draining nodes taken from the foot pad primary tumor were elevated at early time points. Numerous studies have shown previously the success of ^{18}F-FDG in mapping melanoma(*18*), thus ^{124}I-trametinib is best suited to other cancers with mutant KRAS and BRAF genotypes that are not ^{18}F-FDG avid.

Mice with flank B16F10 melanomas were divided into a naïve or trametinib treatment groups. Mice under trametinib therapy received trametinib at 6 mg kg^{-1} once daily for three days by intraperitoneal injection. Both cohorts of mice were administered ~15MBq ^{124}I-trametinib intravenously and imaged through 48 hours post injection. PET images show significantly lower ^{124}I-trametinib distribution in trametinib treated mice at 24 hours (**Figure 5A**), and nearly absent from mice by 48 hours (**Figure 5B**). Terminal biodistribution confirmed trametinib treated mice have lower systemic MEK expression with significant reductions in the liver, gastrointestinal tract, bladder, flank tumor, and inguinal lymph nodes (**Figure 5C**). Here, ^{124}I-trametinib can confirm blocking of MEK during trametinib therapy, and when blocked, ^{124}I-trametinib is rapidly cleared.

In another murine model, nude mice with MDA-MB-231 tumors were administered ^{124}I-trametinib. MDA-MB-231 cell lines contain both KRAS and BRAF activating mutations, suggesting higher uptake as seen *in vitro*. Coronal image slices at 24 hours post injection revealed higher uptake at 5 % ID/g which began clearing by 48 hours post injection (**Supplementary Figure 5**). To confirm the uptake observed was due to the parent molecule and not a metabolite, nude mice bearing MDA-MB-231 tumors were

administered ^{131}I-trametinib for analysis. Radio-HPLC traces show trametinib as the parent species extracted from tumor, liver and intestine (including feces), though minor metabolites were observed in the intestine and at 6h post injection in the liver (**Supplementary Figure 6**).

Discussion:

Trametinib is widely used as a therapeutic agent for tumors bearing BRAF V600E/K mutations. Combinatorial success of trametinib with BRAF inhibitors highlights the importance of a targeted blockade in the overactive RTK pathway. The importance of trametinib for KRAS mutant cancers has also opened new therapy opportunities for patients with lung cancers(*4*). Overactive signaling cascades from KRAS and or BRAF mutations use MEK to keep downstream proliferation genes on. By administering trametinib and inhibiting MEK, the overactive signaling cascades can be attenuated (**Figure 1**). Trametinib alone has been shown to block phosphorylation of MEK and downstream ERK but does not affect total MEK amounts. Dimerization of KRAS with mutant and wild type copies present different degrees of MEK phosphorylation impacting MEK inhibitor sensitivity(*19*) and MEK monotherapy can lead to allelic mutants resistant to MEK inhibitors(*20*). In addition, administration of trametinib has presented several on target toxicities while administration in the clinic remains a fixed dose per patient. By utilizing radiolabeled trametinib, concentration-based information is now possible for each organ allowing therapy and resistance surveillance.

Design of radioiodinated trametinib allowed for the radiotracer to replace an existing stable iodine isotope. Synthesis of iodinated trametinib using copper mediated 1-

10-phenanthroline provided high selectivity for ^{123}I, ^{124}I and ^{131}I over chloramine-T oxidation (**Figures 2A, 2B**). Purification and solvent exchange for injection provided nearly 70% radiochemical yield and a total synthesis time of about 90 minutes. This synthesis efficiently uses a variety of iodine isotopes such that trametinib could be imaged on a variety of imaging systems.

Selectivity *in vitro* of ^{124}I-Trametinib across a panel of human and murine cell lines was determined by a reduction in cell uptake upon blocking with 1μg cold trametinib (**Figure 2C**). Cell lines with the highest ^{124}I-trametinib uptake after 2 hours were lines known to have aberrant KRAS and or BRAF mutations, while lines lowest in uptake had wild type alleles per milligram of protein with a ten-fold change between extremes. Uptake in several cell lines through 24 hours are rapidly internalizing ^{124}I-trametinib, while persistent blocking with cold trametinib dramatically limits internalization (**Supplementary Figures 1-3**). Radiolabeled trametinib can be used to determine the avidity of trametinib naïve tumors as a surrogate measurement for MEK signaling avidity. Furthermore, during trametinib therapy, radiolabeled trametinib can measure inhibition of MEK in the patient's tumor, allowing dose finding and therapy personalization with new treatment combinations.

Healthy mice showed rapid metabolism of ^{124}I-trametinib in the liver by 2 hours with subsequent gastrointestinal clearance though 24 hours (**Figure 3A**). Trametinib is known to clear 20% renally and 80% by fecal excretion(*17*), agreeing with observed imaging and biodistribution (**Figure 3B**) and metabolite analysis (**Supplementary Figure 6**). Uptake in skin was not as extensive as liver and spleen, however uptake in the lungs

and heart was observed, that could not be explained by activity in the blood. Adverse event reporting(*17*) mentions pulmonary and cardiac events, though it is unclear if uptake of ^{124}I-trametinib would correlate with susceptible patients. Mice bearing B16F10 melanomas showed slow uptake in the primary tumor through 48 hours (**Figure 4A**), with non-significant differences in organ uptake compared to healthy mice (**Supplementary Figure 4**). Overall, clearance of tissue over tumor by 48 hours allowed ^{124}I-trametinib to serve as a tool compound to identify MEK *in viv*o and thus serve to quantitate avidity of lesions for trametinib therapy. Lastly, ^{124}I-trametinib administered during traditional trametinib therapy was blocked (**Figure 5A**) providing meaningful feedback on systemic therapy for personalized dosing (**Figure 5B**) as well as therapy resistance. Preclinically, development of radioiodinated trametinib can serve as a tool compound for researchers.

Conclusion:

Trametinib is widely used for treatment of metastatic melanoma in combination with other BRAF inhibitors though therapy relies on fixed dosages and suffers from numerous limiting side effects. Here development of radioiodine trametinib (^{123}I-, ^{124}I-, and ^{131}I-) provides three radiotracers with identical properties to cold, naive trametinib allowing visualization of KRAS mutant cell lines not done previously. ^{124}I-trametinib *in vitro* was found to be taken up in KRAS and BRAF mutant lines with greater avidity than wild type cancer lines with overall uptake significantly blocked by cold trametinib. ^{124}I-trametinib was widely distributed to skin, liver, and the gastrointestinal tract but also revealed higher than expected uptake in the heart and lungs. Mice bearing B16F10 melanomas showed highest uptake in tumors by 48 hours post injection. Here, radioiodinated trametinib can

be used to determine trametinib avidity in trametinib naïve tumors, as well as measure inhibition during trametinib therapy, allowing dose personalization, and assessment of potential drug resistance.

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Author contributions: E.C.P., N.P., and J.G. devised and conducted experiments, and wrote this manuscript. O.O. provided synthetic expertise and G.Y provided the trametinib precursor. E.I. and E.S. performed animal experiments.

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Data availability: The data that support the plots within this paper and other findings of this study are available from the corresponding author upon reasonable request.

KEY POINTS:

QUESTION: Can a radioiodinated trametinib serve as a tool radiotracer for identifying levels of MEK *in vivo*, potentially identifying malignant tissue, and MEK resistant tissue.

PERTINENT FINDINGS: Radioiodinated trametinib was made with a variety of iodine sources in high yield and was taken up most avidly in cell lines expressing KRAS and or

BRAF mutations. Uptake of radioiodinated trametinib in melanoma tumors increased through 48 hours and could be blocked when traditional trametinib is administered prior radiotracer administration.

IMPLICATIONS FOR PATIENT CARE: ^{124}I-trametinib, a radiotracer specific to MEK and identical to FDA approved trametinib, can measure the avidity of malignant tissue by PET and determine the degree of tumor inhibition for patients receiving BRAF and MEK inhibitors, identifying any emergence of resistance.

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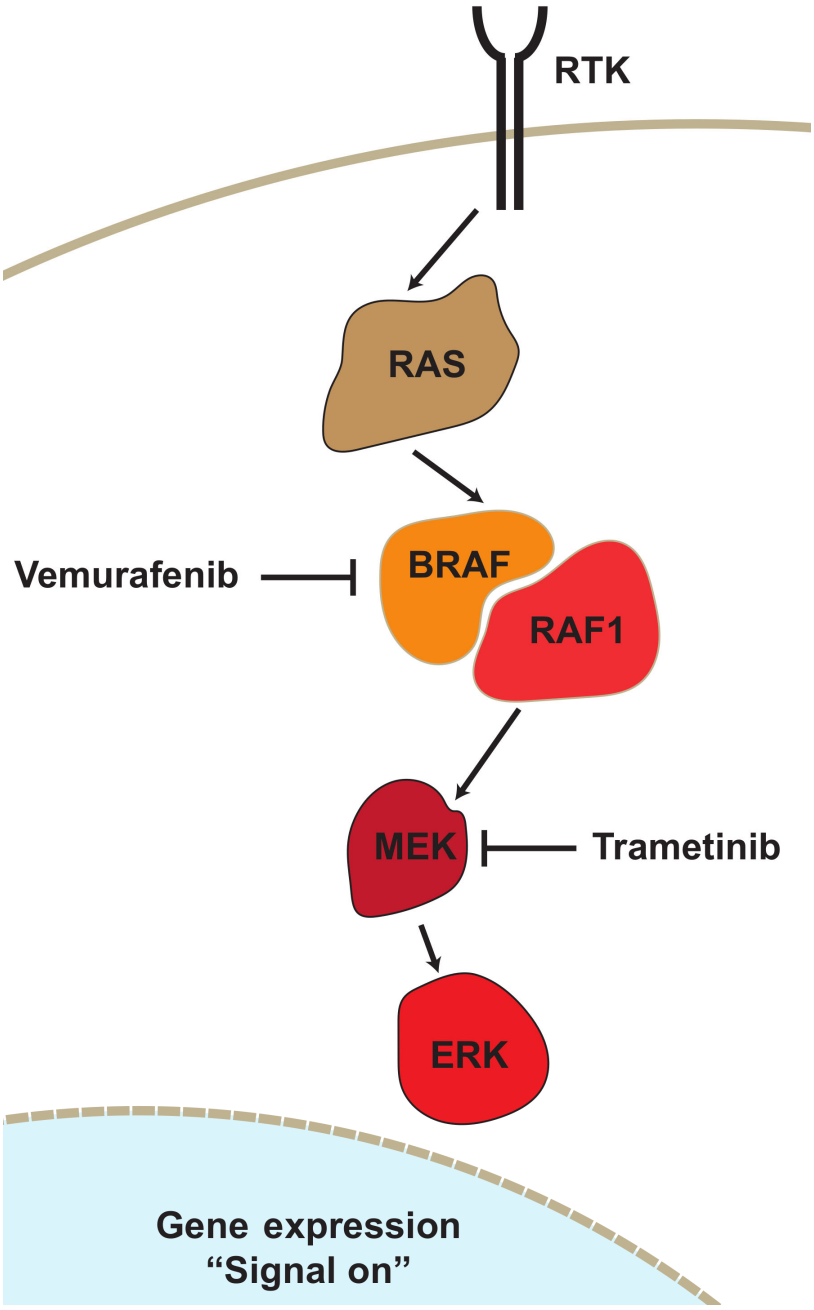


Figure 1. Receptor Tyrosine Kinase pathway containing RAS and BRAF. MEK is found in the Receptor Tyrosine Kinase pathway downstream of both KRAS and BRAF. Trametinib is a MEK 1/2 inhibitor and FDA approved for $BRAF^{V600E}$ mutant cancers. By inhibiting MEK activity trametinib can reduce hyperproliferative signaling from KRAS or BRAF mutants, though on target toxicity is observed even with wild type KRAS and BRAF cells.

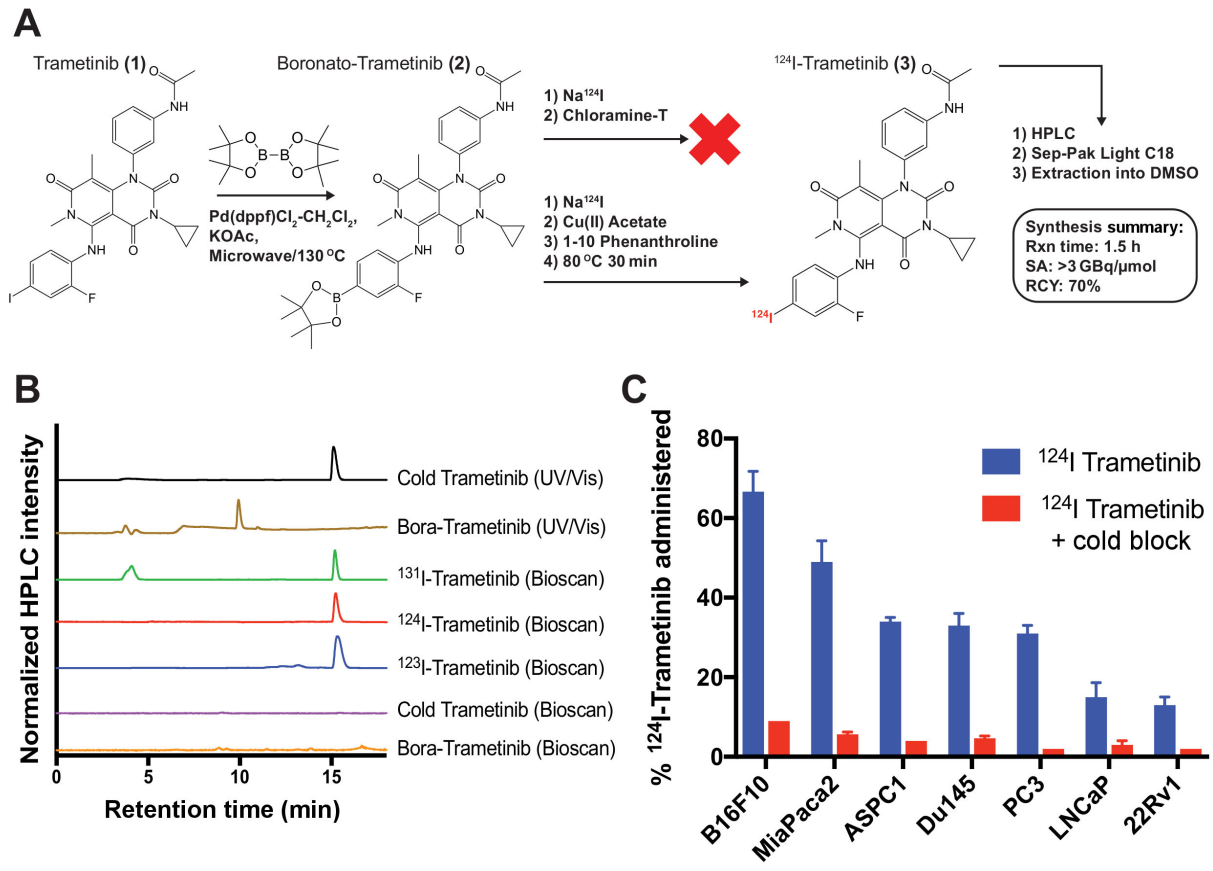


Figure 2. Synthesis and cell line specificity of radioiodinated trametinib. A) Synthesis scheme started from trametinib into a boronato-pinacol precursor readily available for iodination. Radiosynthesis and purification was complete within 1.5 hours with a radiochemical yield (RCY) of 70% for ^{124}I-trametinib with a specific activity >100GBq/μmol. B) HPLC purification shows majority of radioiodinated product is distinct from boronato-precursor and identical to cold trametinib. C) Trametinib is a type-III MEK allosteric inhibitor designed to reversibly inhibit ATP from phosphorylating MEK1/2. ^{124}I-trametinib addition to cell lines expressing reveal a nearly ten-fold range of uptake after 2h, all of which are blocked with 1μg cold trametinib. Human KRAS mutant (G12V, G12C or G12D) or BRAF^{V600E} cell lines retained a higher percentage of ^{124}I-trametinib with the double KRAS BRAF mutant having the highest uptake differentiating KRAS and BRAF mutant tissue from normal. Murine cancer cell lines were all similarly avid for ^{124}I-trametinib except Raw264.7 representing normal KRAS and BRAF and lower uptake.

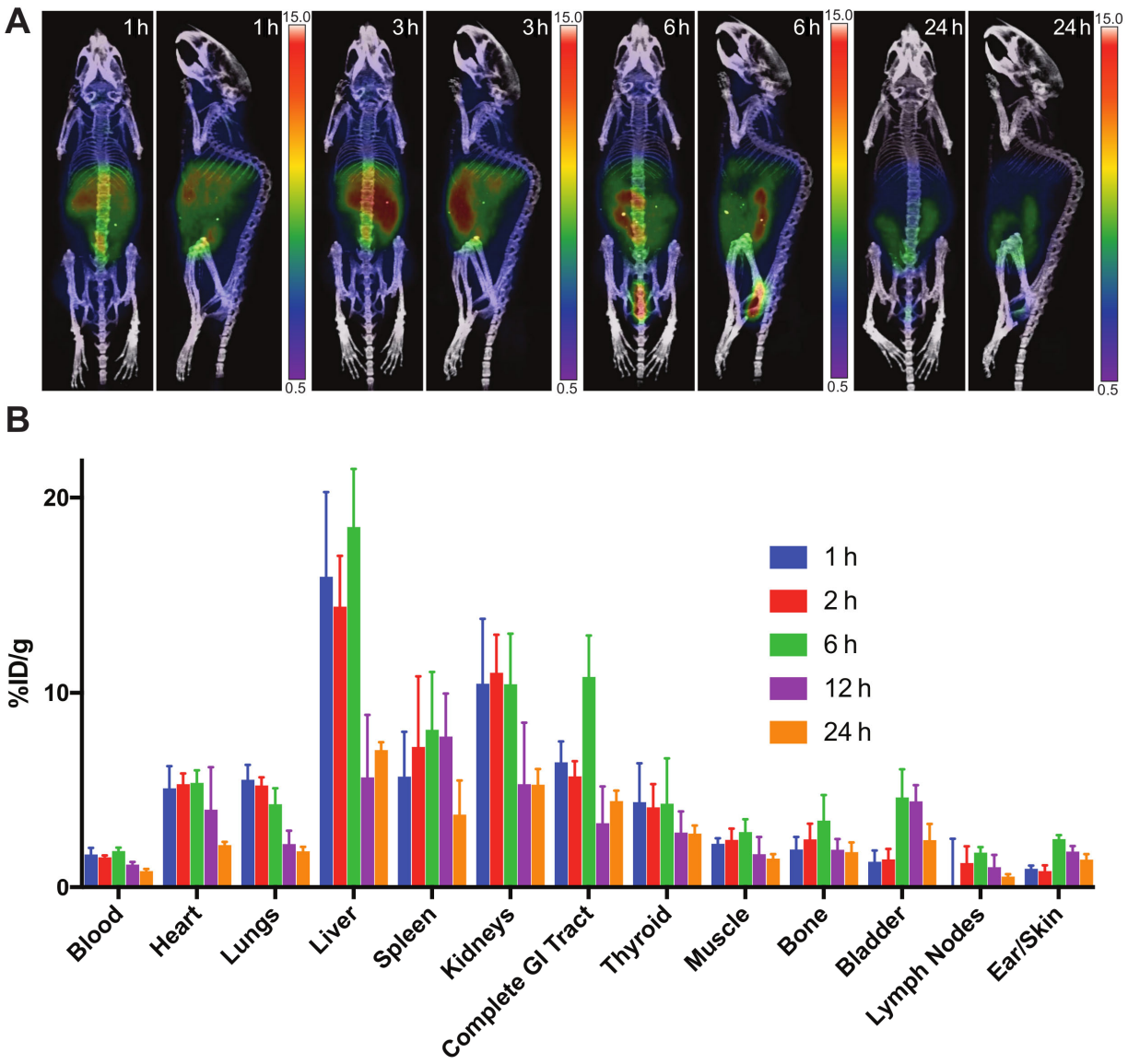


Figure 3. *In vivo* distribution of ^{124}I-Trametinib in healthy mice primarily in the liver with subsequent elimination through the gastrointestinal (GI) tract. A) PET imaging at 1, 3, 6, and 24 hours show broad distribution in tissues with the highest uptake in liver, spleen and kidneys and later GI clearance. B) Terminal biodistributions at up to 12 hours show high heart and lung uptake (5% ID/g) compared to blood (1% ID/g) or the skin (1-2% ID/g). Peak uptake in skin was observed at 6h post injection.

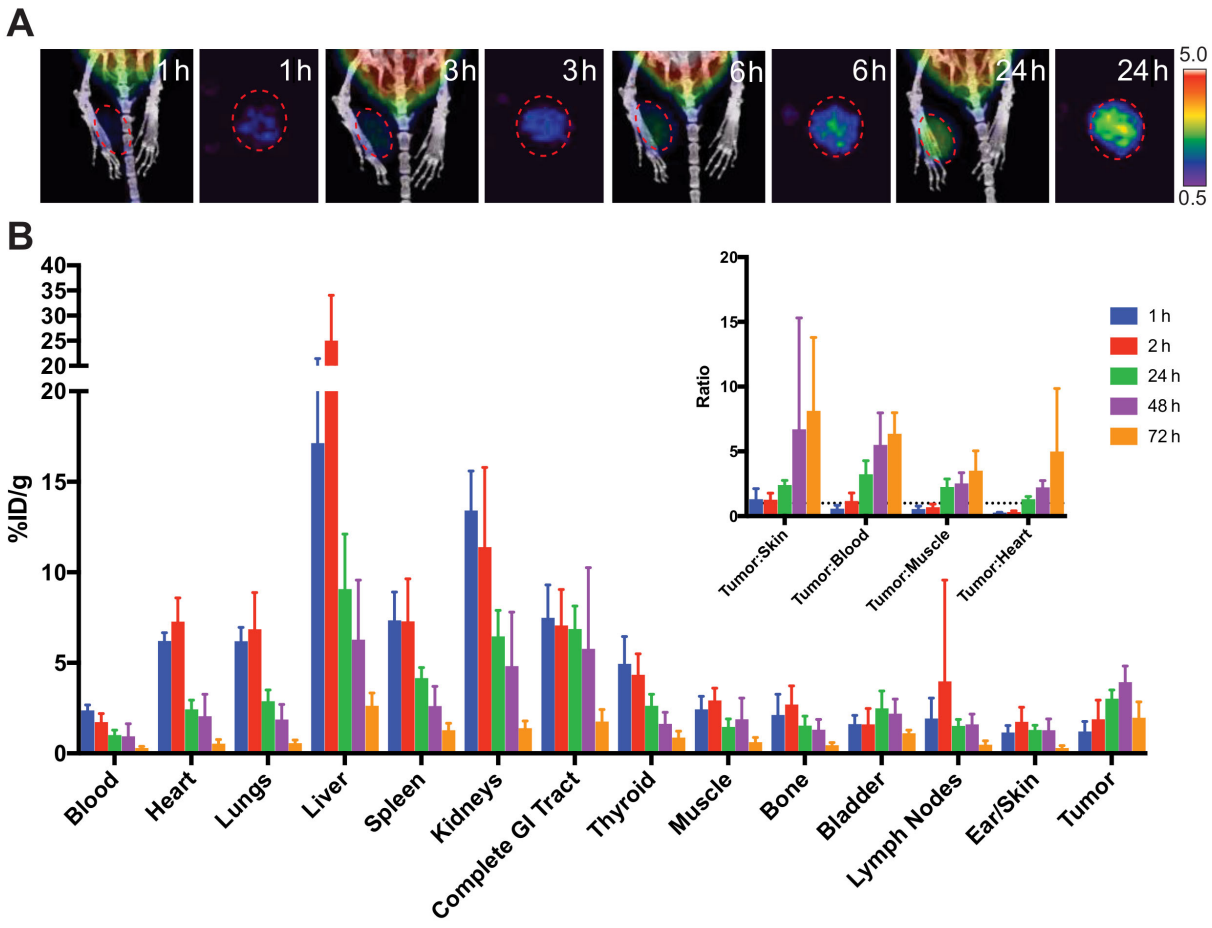


Figure 4. Imaging and Biodistribution of ^{124}I-Trametinib in mice bearing B16F10 melanomas through 72h. A) PET imaging of B16F10 melanoma bearing mice have slow tumor uptake through 72 hours. B) Terminal biodistribution reveals maximal uptake of ^{124}I-trametinib in the B16F10 tumor footpad was observed between 48- and 72-hours post injection. (inset) Ratio of percent injected dose per gram in tumor to skin, blood, muscle, and heart increased after 24 hours showing retention of ^{124}I-trametinib in the tumor relative to other tissues. Tumor uptake after 24 hours would allow imaging of naïve tumors in addition to monitoring tumor resistance during conventional trametinib therapy.

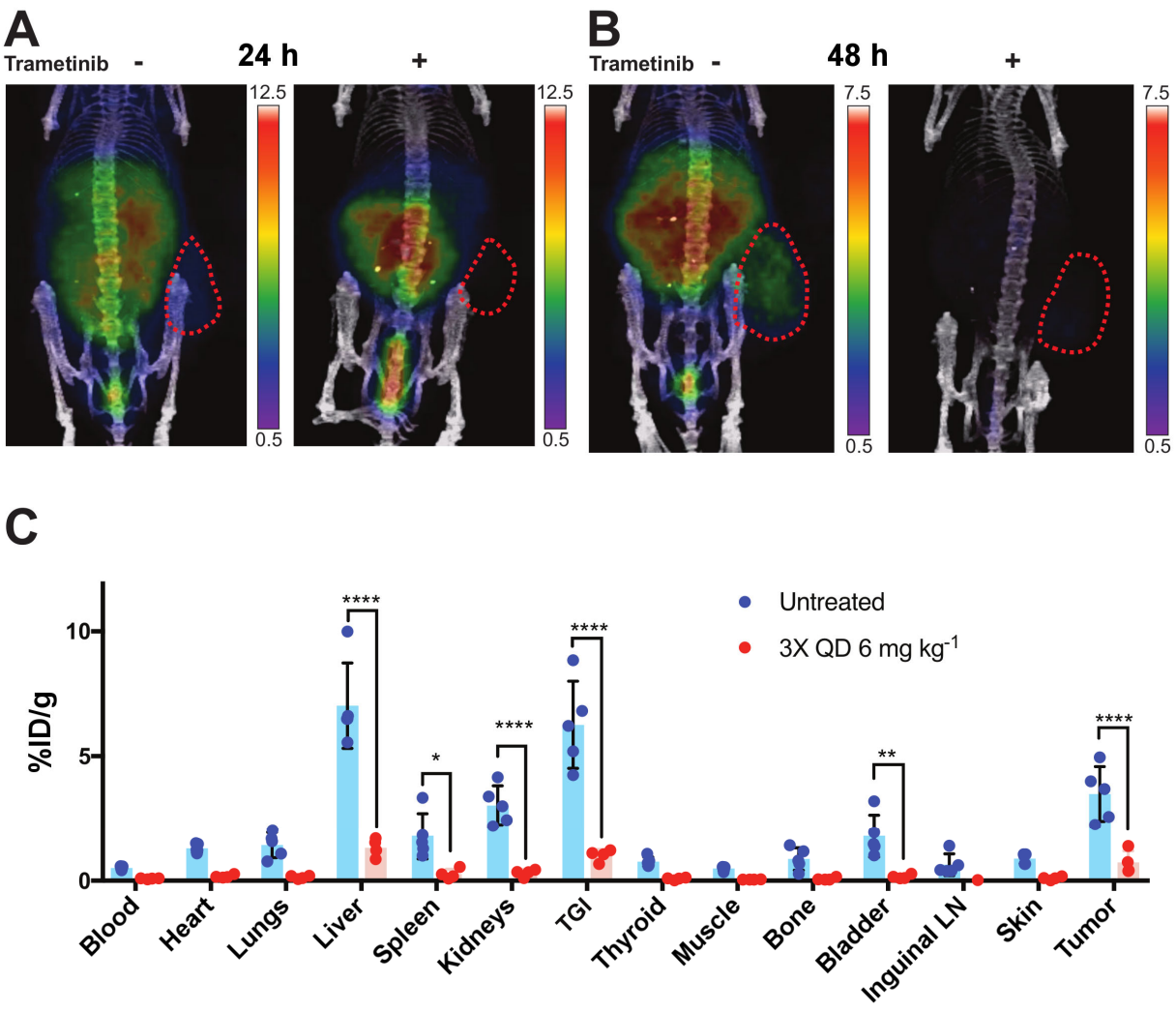
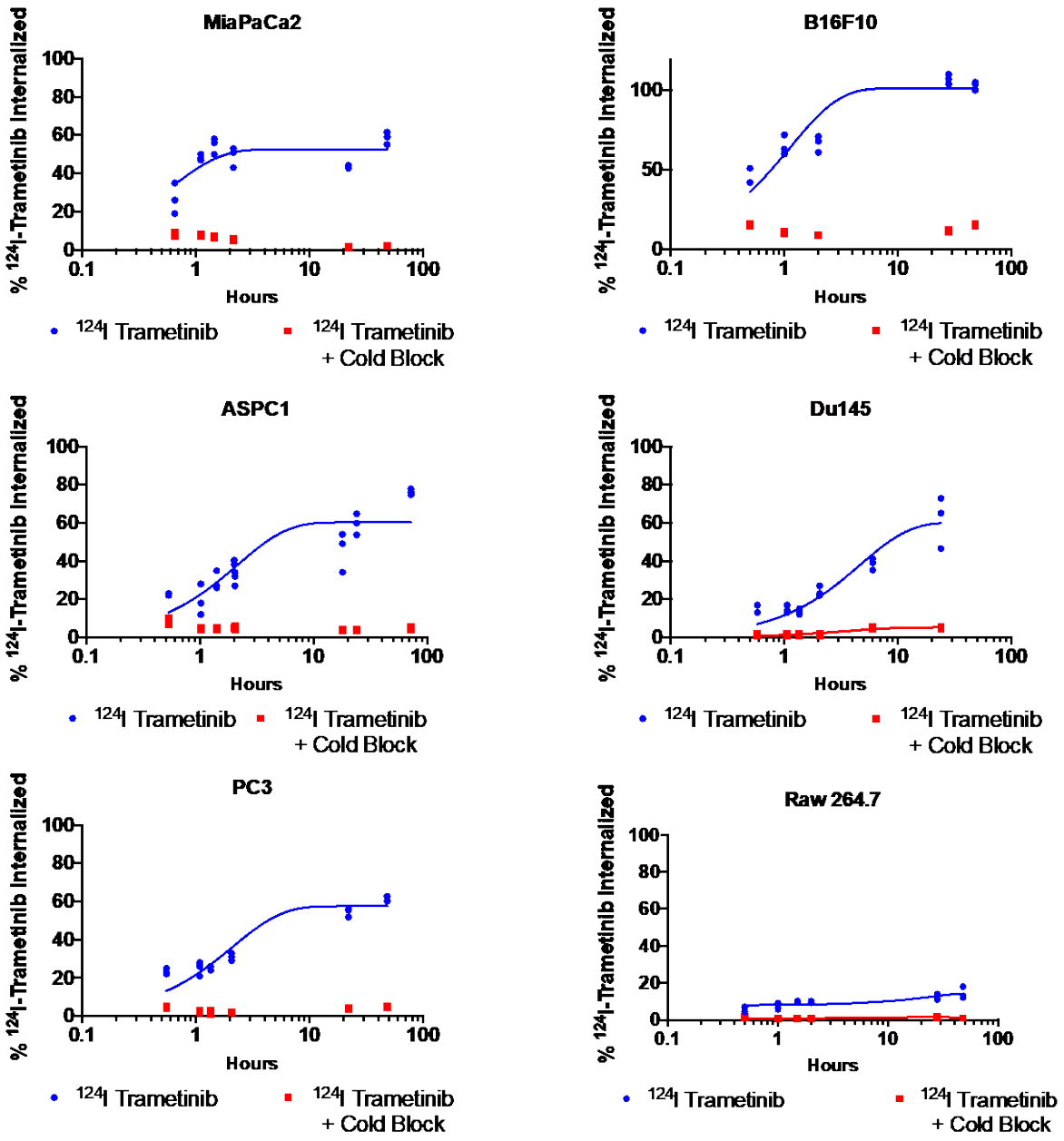
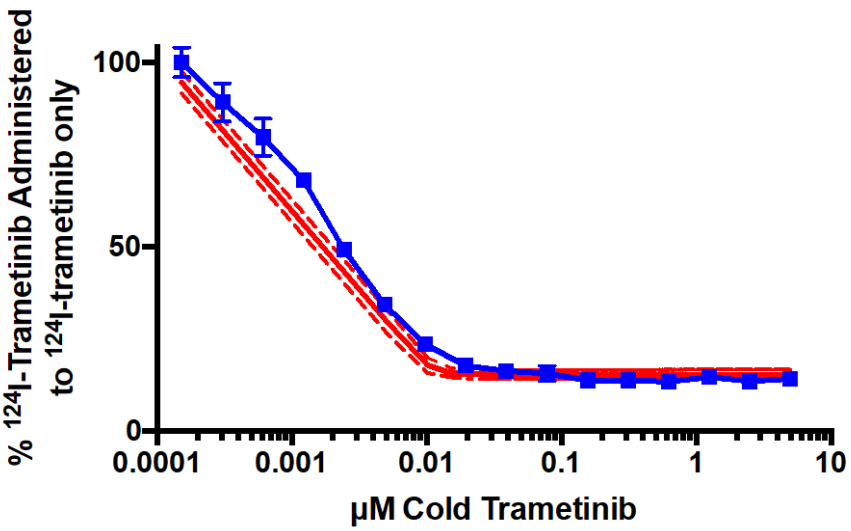


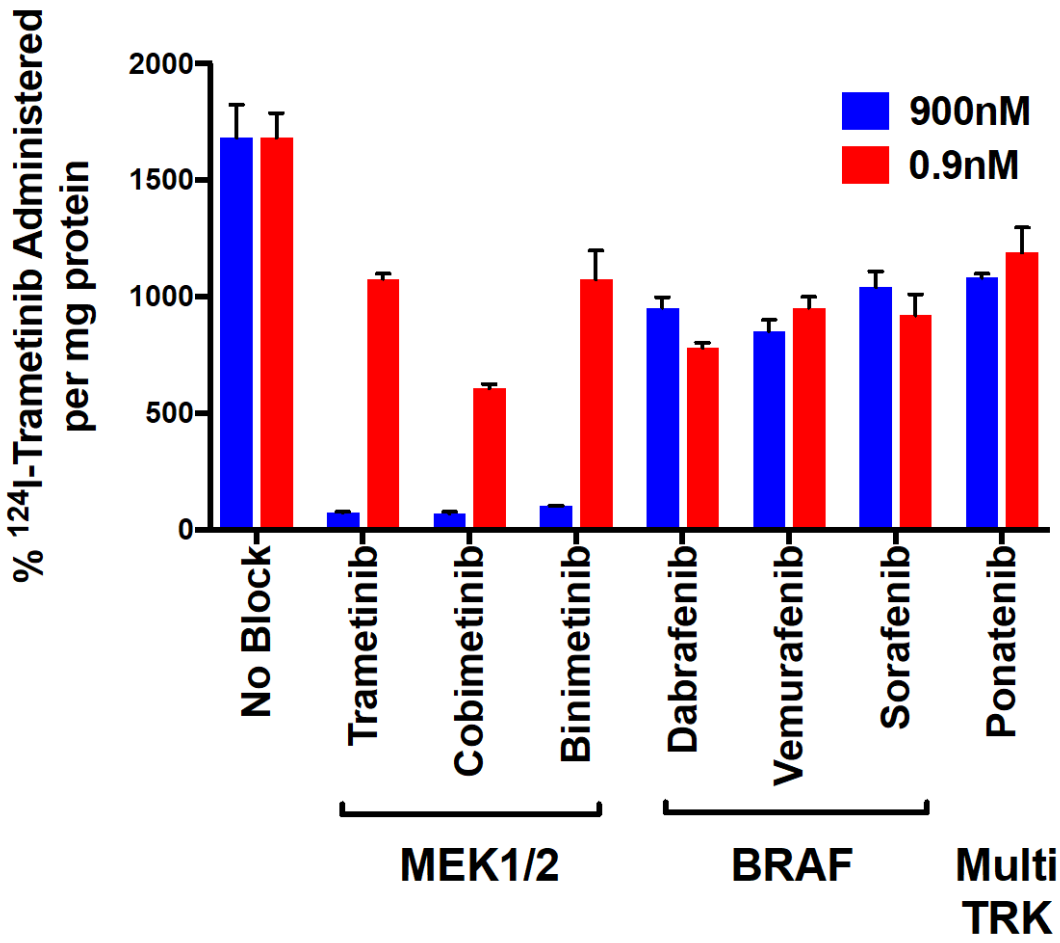
Figure 5. Trametinib therapy reduces uptake of ^{124}I-trametinib systemically. Prior administration of 6 mg kg^{-1} trametinib intraperitoneally once a day (QD) for three days shows reduced uptake of ^{124}I-trametinib at A) 24 hours and B) 48 hours post administration by PET. C) Terminal biodistribution post 48-hour PET scan confirms significant decreases in ^{124}I-trametinib uptake in the liver, spleen, kidneys, total gastrointestinal tract, bladder, and right flank B16F10 tumor. Two-way Anova used for biodistribution analysis, n=4 mice received 3x trametinib treatment, n=5 naïve mice before ^{124}I-trametinib imaging. ****= p<0.001, **=p<0.01, *= p<0.05.



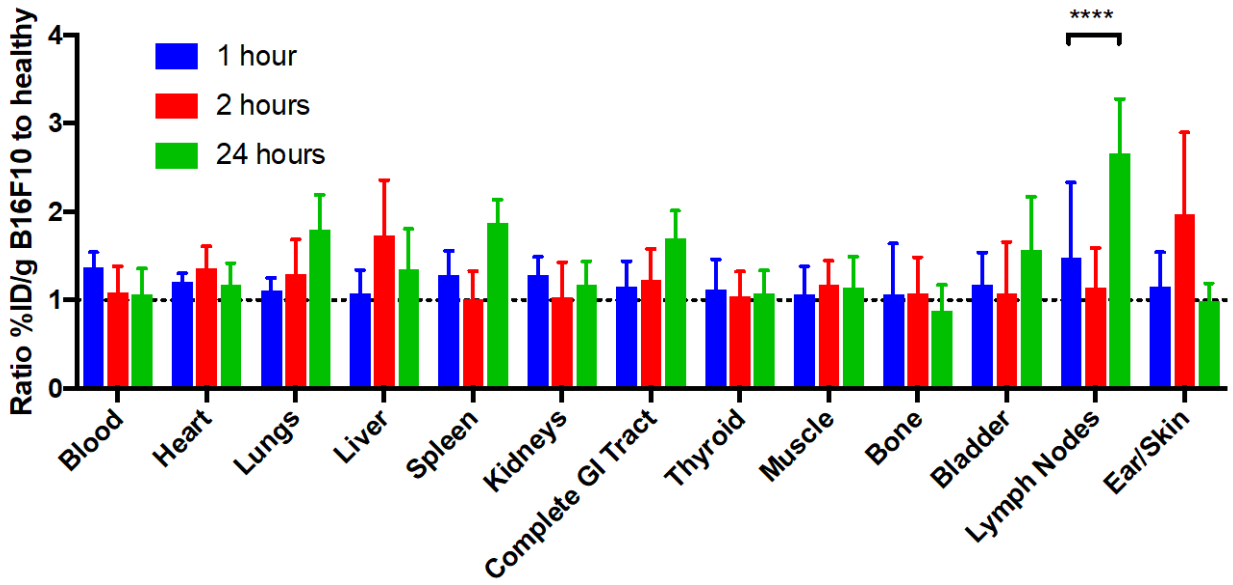
Supplementary Figure 1. Kinetic uptake study of ^{124}I-trametinib over 72 hours across a panel of KRAS mutant and wild type cancer cell lines show a high degree of blocking with cold trametinib, with higher uptake rates in KRAS mutant cell lines MiaPaCa2, B16F10, ASPC1 and Du145 then in KRAS wild type lines PC3, LNCaP, 22Rv1 and Raw264.7.



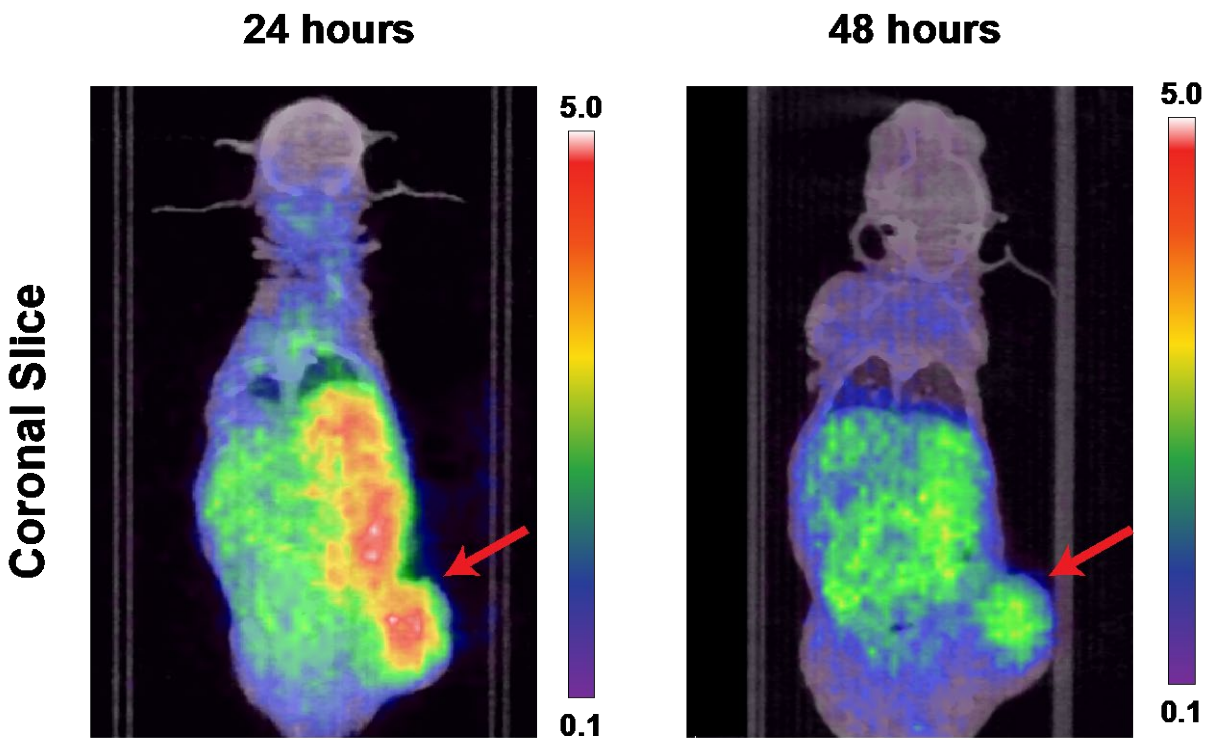
Supplementary Figure 2. Titration of cold trametinib competes with ^{124}I-trametinib in B16F10 melanoma cells. ^{124}I-trametinib uptake decreased (blue line) with increasing amounts of cold trametinib providing direct dose response to estimate inhibition of tumor lines. 50% uptake inhibition was achieved at 0.03µM for B16F10 cell lines after 2 hours incubation (red line = sigmoidal inhibition extrapolation).



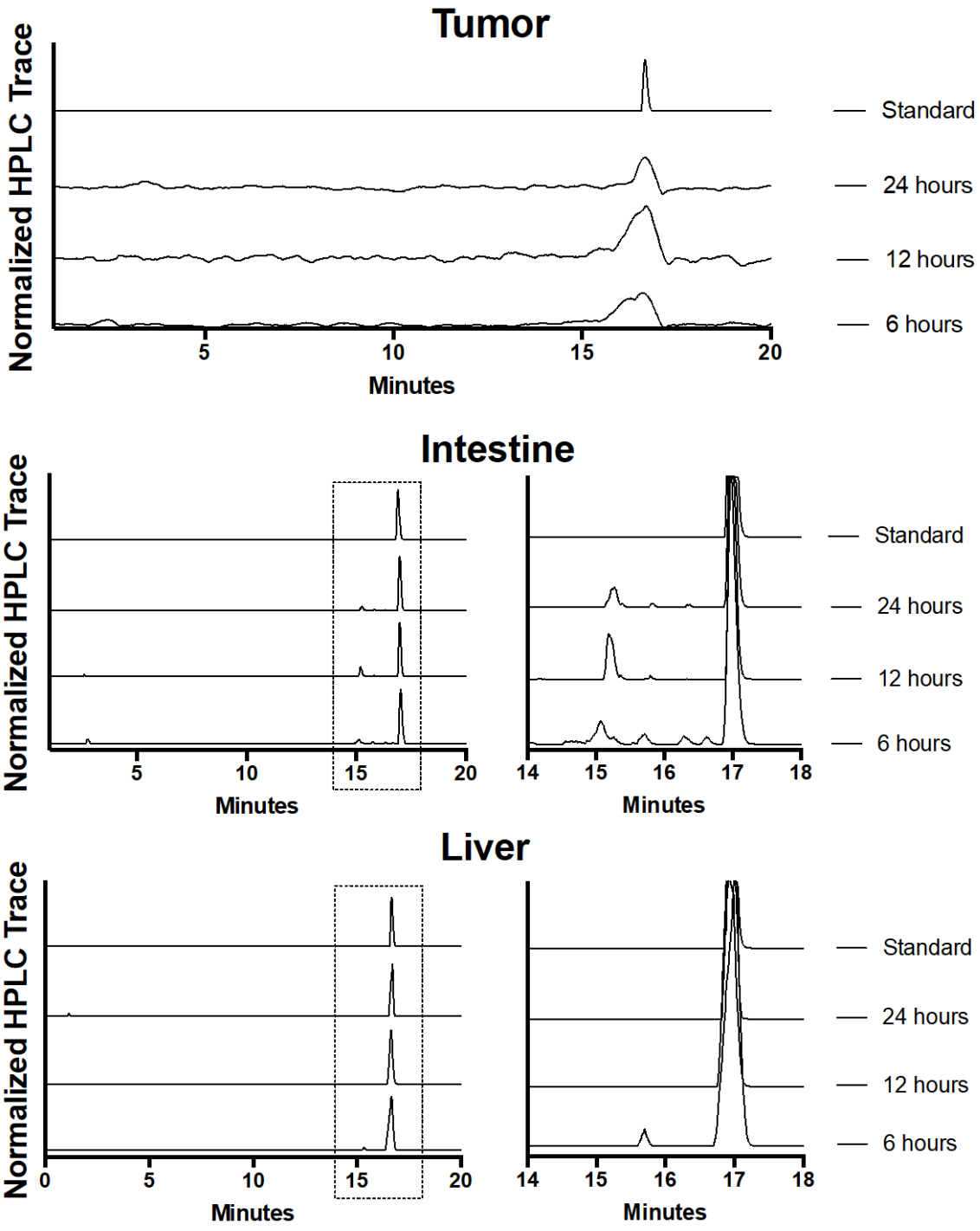
Supplementary Figure 3. Specificity of ^{124}I-trametinib with blocking doses of BRAF and MEK inhibitors. Type 3 MEK inhibitors used include trametinib, cobimetinib, and binimetinib. BRAF inhibitors used included dabrafenib vemurafenib, and sorafenib. Lastly ponatinib was used as a pan TRK inhibitor. Blocking doses were added 1000x and approximately the individual drug's IC_{50} alongside ^{124}I-trametinib for two hours to triple negative breast cancer line MDA-MB-231. Potent blocking was seen with MEK inhibitors, while only partial blocking was observed for BRAF and pan TRK inhibitors.



Supplementary Figure 4. Ratio of biodistribution of ^{124}I-trametinib B16F10 bearing mice compared to healthy C57BL/6-J mice. Slightly higher uptake in lungs and spleen are seen after 24 hours, though statistically not significant, while larger and significant increases in lymph nodes are observed. The increased lymph node uptake in melanoma bearing mice suggests ^{124}I-trametinib can detect abnormalities in draining lymph nodes and could be used for lymph node mapping from systemic delivery.



Supplementary Figure 5. Coronal slices of a nude mouse bearing MDA-MB-231 tumor on their right flank (red arrow). Uptake at 24 hours was observed to be ~5 % ID/g while at 48 hours lower tumor uptake of ~2% ID/g was seen.



Supplementary Figure 6. Metabolite analysis of ^{131}I-trametinib in tumors, liver, and intestinal tract (including feces). Organs were harvested at 6, 12, and 24 hours post injection, extracted with acetonitrile for HPLC analysis. HPLC profile shows the major extracted peak was still parent ^{131}I-Trametinib. Some free radioiodine (2.5 minutes) with four minor metabolites/degradates (between 15 and 17 minutes) were also seen in the radiodetector HPLC trace.