Supplementary Material to

Vinorelbine Delivery and Efficacy in the MDA-MB-231BR Preclinical Model of Brain Metastases of Breast Cancer

A. Vinorelbine Quantitation and Integrity Confirmation by Chromatography

LC-MS/MS Analysis

Integrity of vinorelbine was evaluated in brain, liver and kidney after 0.5, 2, and 8 h of circulation *in vivo* in healthy female NuNu mice by LC-MS/MS (Figure S1). The following m/z ion pairs were monitored: 779/122 for vinorelbine, 737/122 for deacetylvinorelbine, and 811/224 for the internal standard vinblastine. In addition to diacetylvinorelbine, transitions for the reported vinorelbine metabolites vinorelbine 3,6 ether (777/122), hydroxyl vinorelbine isomers (795/122), and desmethylvinorelbine (765/122) were also monitored (1). Deacetylvinorelbine, an active metabolite of vinorelbine, was <20% of the total vinorelbine concentration in brain at all time points. No additional metabolites other than deacetylvinorelbine were observed in brain.

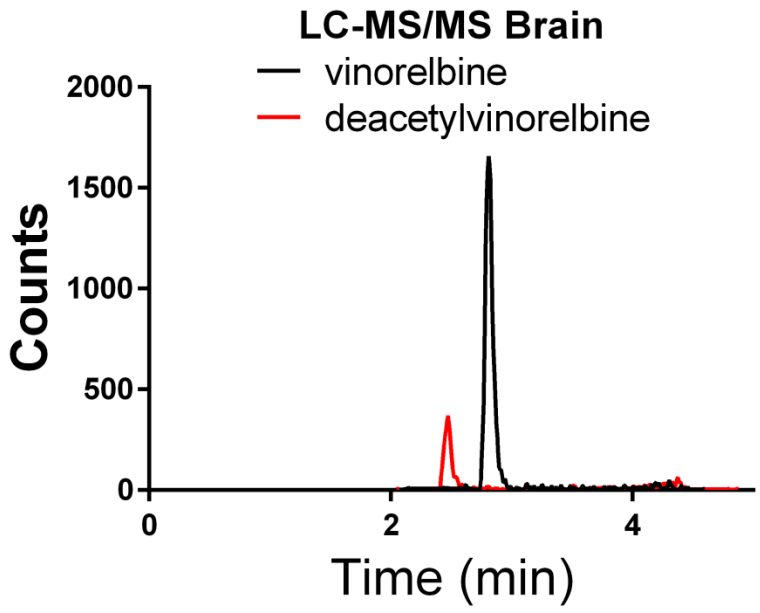

Figure S1 LC-MS/MS chromatogram of brain extract from mouse administered vinorelbine for 2 h. No additional metabolites were detected.

Radiotracer HPLC Analyses

Quantification and integrity of ^{3}H-vinorelbine was evaluated

in plasma, lung, and kidney after 2 and 8 h of circulation *in vivo* using HPLC coupled to a flow scintillation analyzer (FSA) for inline radiotracer detection. Chromatographic conditions were set so that deacetylvinorelbine eluted as a shoulder before vinorelbine with FSA detection, because of the large path length necessary for sufficient FSA sensitivity, whereas it was a separate peak with UV detection prior to flowing into the FSA (Figure S2A). With this method, the single FSA peak at the time of vinorelbine elution represented all active radiolabeled vinorelbine-related species. As illustrated in Figure S2 for 8 h samples, no other metabolites were observed in injectate (B), plasma (C), or kidney (D).

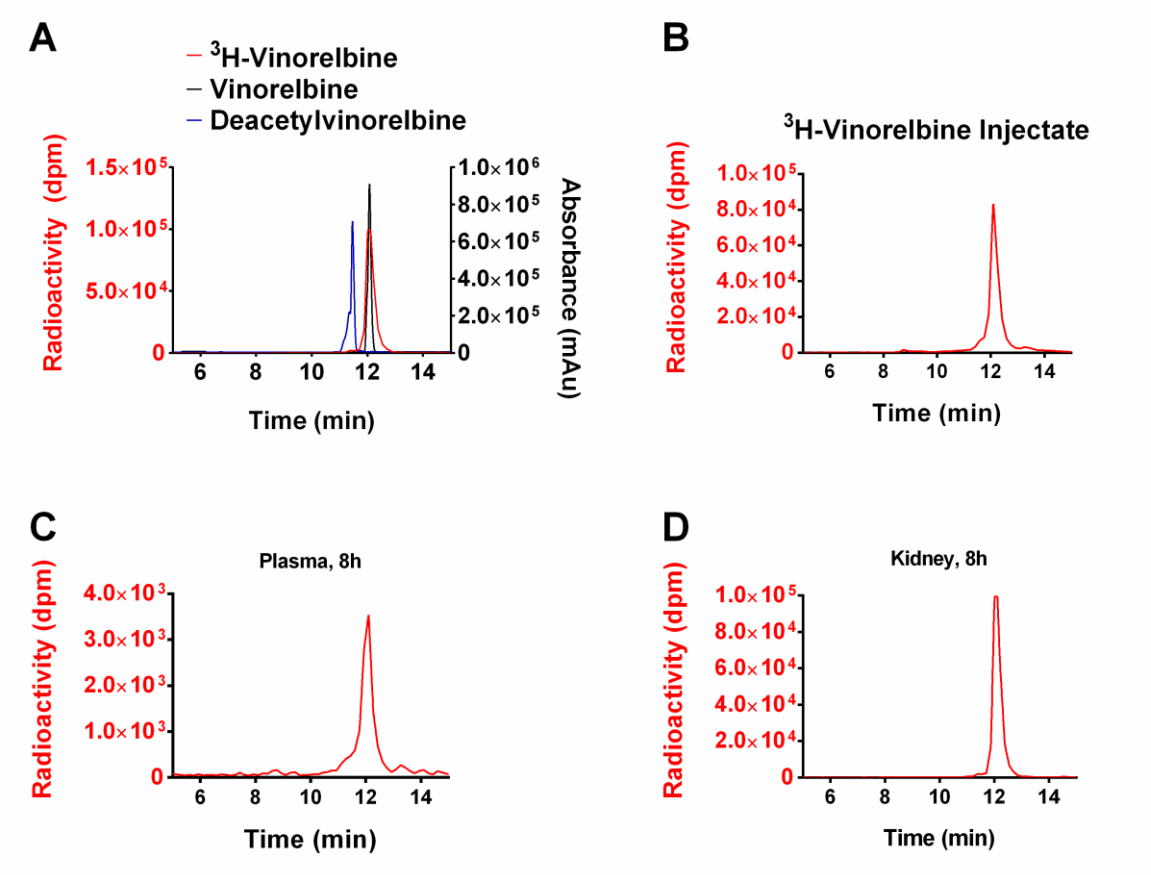

Figure S2 Tracer Purity and Integrity as Determined by HPLC

A Superposition of radiochromatogram of [^{3}H] vinorelbine, cold vinorelbine and deacetylvinorelbine, tracer purity was found to be >99%. Radiochromatogram of **B** injectate, **C** plasma, and **D** kidney extracts after 8 h circulation.

Volatile tritiated species have been reported in prior radiotracer studies (2), so especial care was taken to minimize and monitor for this problem. We administered only highly pure vinorelbine (>99% pure, confirmed by HPLC prior to use) and evaporated samples prior to analysis to remove any potential volatile species present. Samples were spot-checked by HPLC for confirmation that no volatile species were present. The stability of ^{3}H-vinorelbine was assessed and was found to be >99% intact after 24 h. Hence, ^{3}H-vinorelbine was not spontaneously breaking down at 37°C and pH 7.4. In addition, we evaluated the level of volatile species present in the original specimens (without evaporation) and only observed volatile ^{3}H in plasma at later (8h) time point, which was calculated to represent <1% of injected tracer, based upon tracer distribution in animal water space. Therefore, we determined that volatile ^{3}H was not a confounding contributor to radiotracer signal in this work.

Sample Preparation and Chromatography Conditions

For LC/MS-MS analysis, tissue samples were homogenized with 4 volumes (w/v) of PBS using a mechanical homogenizer. Drug was extracted from tissue homogenate by protein precipitation with three volumes of ice cold acetonitrile containing 0.3% formic acid to suppress vinorelbine decomposition, followed by centrifugation at 14,000***g*** for 15 minutes at 4°C. Chromatography was performed on a Shimadzu NEXERA UHPLC system coupled to an ABSciex QTRAP 5500 MS/MS with electrospray interface operating in positive mode. Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 rapid resolution column (2.1x 50 mm 3.5 µm) which was protected by a Zorbax Extend C18 guard column. Mobile phase was 10 mM ammonium acetate (pH 3.2 adjusted with formic acid) (A) and methanol (B) using gradient elution from 20% to 75% B over 4 minutes, followed by 2 minute re-equilibration. Vinblastine was used as

internal standard for both vinorelbine and deacetylvinorelbine. Instrument control, data acquisition, and quantitation were performed using Analyst 1.5.2 software.

For HPLC analysis, lung and kidney tissue samples were homogenized with 3 volumes (w/v) of 100 mM phosphate buffer. Drug was extracted from tissue homogenate or plasma by protein precipitation with two volumes of ice cold acetonitrile followed by centrifugation at 20,000***g*** for 15 minutes at 4°C. Chromatography was performed on a Varian HPLC system (Varian Chromatographic Systems), coupled with a Flow Scintillation Analyzer (Radiomatic 150 TR, Perkin Elmer). Chromatographic separation was achieved on a Luna 5µ C18 (2) (250×4.6 mm) (Phenomenex) reversed phase column, with gradient elution of 0.1% TFA in water (A) and acetonitrile (B) as the mobile phase. Total HPLC run time was 20 minutes, with a ramp from 90% A at 0 min to 90% B at 20 min followed by a 5 minute equilibrium time. The HPLC outlet from the column directly fed into the flow scintillation analyzer and the radio-flow chromatographic data was acquired with Galaxie Workstation software (Varian). The same methods were used for analysis of plasma and buffer samples from dialysis experiments.

B. Equilibrium Dialysis for Brain Slice Binding Method Validation

Equilibrium dialysis has been used to determine the unbound (free) fraction of a large number of central nervous system and anticancer drugs in plasma and brain tissue. To establish the validity of our brain slice binding method, we compared results from equilibrium dialysis of paclitaxel (a drug that also works by binding to tubulin) and doxorubicin (a commonly-used anticancer drug that has been well characterized in our laboratory) to literature values (3, 4) and then to results from our brain slice binding technique (data not shown). The conditions necessary for successful equilibrium dialysis

(e.g., pH and choice of ions and ionic strength in buffer solutions) were carefully evaluated and subsequently used with the brain slice binding method. After establishing

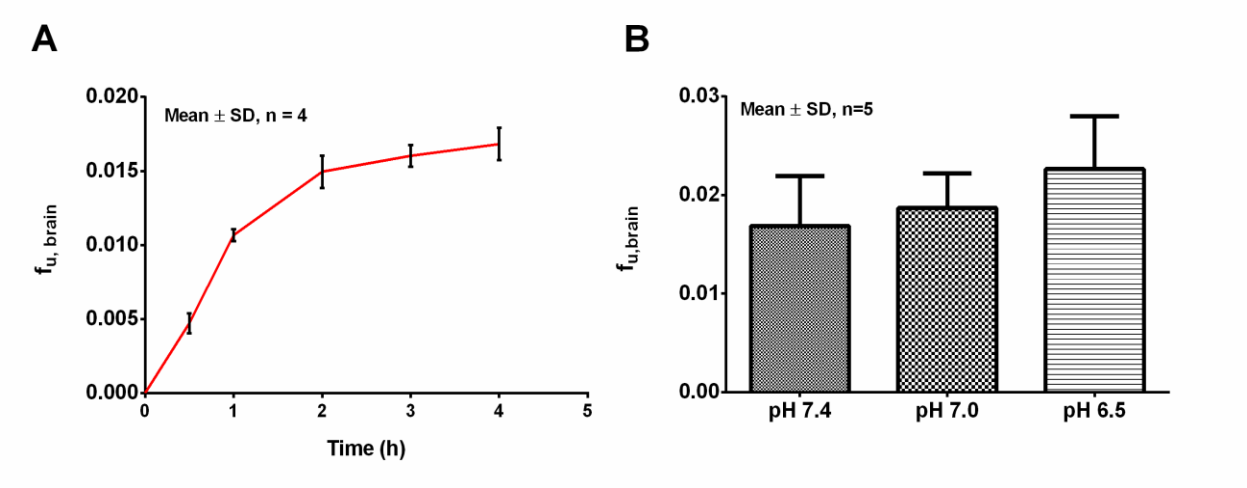

Figure S3 Dependence of $f_{u, brain}$ on equilibration time and pH by equilibrium dialysis. **A** Vinorelbine equilibrium dialysis with brain homogenate reached equilibrium by 4 hours (n=4 per time point). **B** Unbound fraction of vinorelbine in brain did not differ significantly with pH between pH 6.5 and 7.4.

the validity of the method, vinorelbine unbound fraction was similarly evaluated by both methods. Equilibration time was found to be 4 h for vinorelbine (Fig. S3A) and the pH did not have a significant effect on $f_{u,brain}$ (Fig. S3B) by the equilibrium dialysis method.

Equilibrium Dialysis Method

Control brain tissues were homogenized with 9 volumes (w/v) of 100 mM phosphate buffer at pH 7.4 (dilution factor 10) using an ultrasound probe sonicator (Fisher Scientific, Sonic dismember, Model 500) for 3 seconds at 10% amplitude. Plasma or brain homogenate were spiked with ^{3}H-vinorelbine to achieve a final concentration of 2 µCi/mL and dialyzed against an equal volume of 100 mM phosphate buffer. Dialysis was performed using micro-equilibrium dialyzers (Harvard Apparatus) in an orbital shaker at 100 rpm, 37°C for 5 hours. Micro-equilibrium dialyzers consist of two chambers of 250 µL volume capacities, separated by Spectra/por2 semi permeable membrane with molecular weight cut off of 12-14 kDa (Spectrum

Laboratories, Inc). The membranes were preconditioned in 50% alcohol overnight and in 100 mM phosphate buffer for one hour before dialysis. At the end of equilibrium, 25 µL samples from donor and receiver chambers were mixed with 5 mL of ScintiSafe scintillation fluid and radioactivity was determined using LS 6500 multi-purpose scintillation counter (Beckman Coulter). Representative samples were also collected and % purity of the sample was determined using HPLC; radioactivity in each sample was corrected for purity.

Brain Slice Binding Method

A hydrophobic well was drawn around the brain sections, using a Liquid Blocker Super Pap Pen (Daido Sangyo Co. Ltd). Sections then were incubated at 37°C for 45 minutes with 100 mM phosphate buffer at pH 7.4 containing 1 µCi/ml of ^{3}H-vinorelbine. The incubation process was performed in a humidified chamber in order to avoid evaporation of the PBS. At the end of the incubation period, PBS was collected and slides were washed with cold PBS. The ^{3}H-vinorelbine concentration in the collected PBS is called the "*in vitro* unbound concentration". Slides were allowed to dry at room temperature, placed in cassettes along with tissue calibrated standards for QAR analysis. Total *in vitro* concentration in the brain slice was calculated using MCID by interpolating the measured radioactivity from the radioactive standards co-exposed with brain

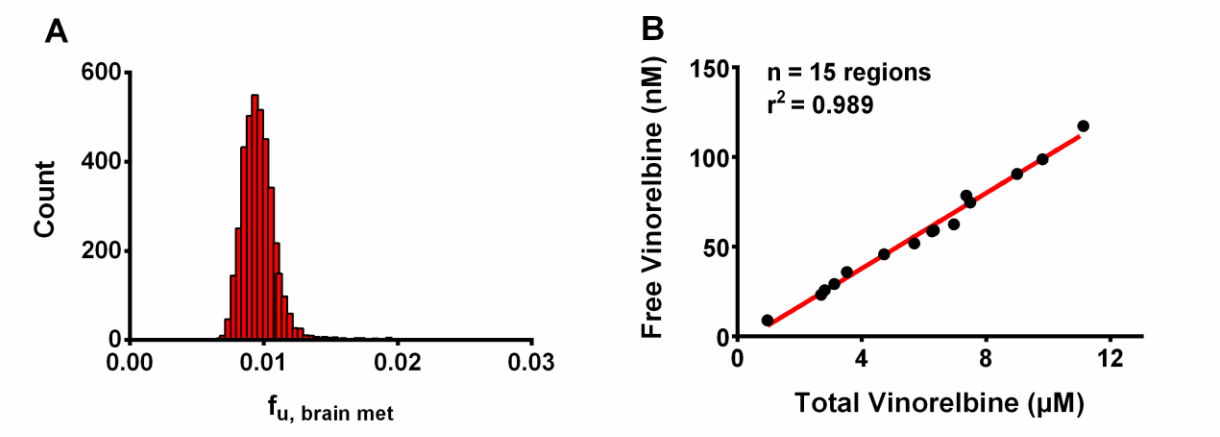

Figure S4 Representative f_u distribution within a metastasis, and correlation between free and total vinorelbine concentrations. f_u exhibits a narrow, Gaussian distribution (**A**), resulting in a strong correlation between free and total vinorelbine (**B**) (n=15 regions, r^2=0.989

sections. The ratio of free drug concentration in buffer to total *in vitro* concentration in the brain section, calculated with MCID, provides the *in vitro* free fraction of drug in brain. To obtain the free *in vivo* vinorelbine concentrations in the brain section, the MCID transformation function was used to multiply the image of the *in vivo* total drug concentration with the image of the *in vitro* unbound fraction obtained from its adjacent section on the other slide. This transformation provides a color-coded, digital image with location-specific *in vivo* free drug concentrations. Similarly the product of the images of *in vivo* total drug concentration with (1- *in vitro* unbound fraction) provides an image of the *in vivo* bound vinorelbine concentrations. The distribution of f_u within a metastasis is essentially Gaussian and exhibits little heterogeneity, resulting in a linear correlation between free and total vinorelbine concentrations.

C. TUNEL Staining for Apoptosis Quantitation

To observe and quantify early apoptosis, DNA breaks in the nuclei were visualized using the terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) assay. The basic principle of the TUNEL assay is the introduction of modified deoxyuridine triphosphates (dUTPs) by the enzyme terminal deoxynucleotide transferases (TdT) at the 3`-OH ends of fragmented DNA. The Click-iT® TUNEL Alexa Fluor® imaging assay (Invitrogen) that we used utilizes a dUTP modified with an alkyne, a small bio-orthogonal functional group that enables the nucleotide to be easily incorporated by TdT. Upon incorporation of alkyne modified dUTP, Alexa Fluor azide is added to alkyne group of dUTP, with the reaction being catalyzed by copper (I). The small molecular size of Alexa fluor azide (MW ~1,000) in this TUNEL method provides easy penetration into samples, which in turn permits detection of a higher number of apoptotic cells. Apoptosis in brain metastases is heterogeneous and shows substantial early

apoptosis in some regions of the metastasis. In addition, apoptosis up to 100% was observed in *in vitro* cell culture for the *in vitro* IC_{50} determination, as shown in Figure 11B of the manuscript.

TUNEL Staining Methodology

In Vivo Brain Sections

Slides with thaw mounted brain sections were allowed to equilibrate to room temperature for 1 hour, hydrated in PBS for 10 minutes and fixed with 4% PFA for 30 minutes at room temperature. Sections were washed 3 times in PBS for 5 minutes, then brain sections were permeabilized with 1% SDS for 5 minutes, followed by washing in PBS and distilled water. Sections were then incubated for 10 minutes with 100 µL of reagent buffer, followed by washing with 3% BSA. Slides were incubated with 100 µL of Alexa Flour cocktail for 30 minutes. DNA staining was performed by incubating sections with Hoescht 33342 dye for 15 minutes, followed by washing in PBS. Finally, the sections were covered with Fluorosave mounting media and a glass cover slip, followed by fluorescence imaging using IX81 (Olympus) fluorescence microscope at 40X zoom. Excitation and emission wavelengths were 650 and 670 nm for Alexa Fluor 647, and 350 and 460 nm for Hoechst 33342 bound to DNA. Fluorescence data was acquired using Slidebook 5.0 software (Olympus), after exposing the sections for 300-500 milliseconds.

In Vitro Cells Grown on Cover Glass

In a 12-well plate, MDA-MB-231BR cells were plated on a glycine-coated cover glass at 10,000 cells in 200 µL media. Cells were grown at 37°C in 5% CO_2-95% air environment in DMEM containing 10% FBS. After 24 hours, media was removed and 1 mL of fresh media with different concentrations of vinorelbine was added and incubated for 2 hours. At the end of incubation,

media was removed carefully and TUNEL staining, imaging, and analyses were performed following the same procedures used for brain sections above. Equilibrium dialysis of vinorelbine (1 μCi/mL) in cell culture media was performed in separate experiments to determine $f_{u,\ in\ vitro}$ (0.54 ± 0.04), which was used to calculate *in vitro* unbound vinorelbine concentration.

D. Brain Section Image Analyses Methodologies

Brain sections were removed from the -80°C freezer, and were allowed to equilibrate to room temperature in a desiccator for one hour.

Fluorescence Analysis

Spatial distribution and quantification of Texas red in entire brain sections were performed using MVX10 microscope (Olympus) with a 2X objective (NA, 0.5) and an optical zoom of 0.63X. Excitation and emission wavelengths for various fluorescent dyes were 560 ± 55 and 645 ± 75 nm for Texas red, and 740 ± 35 and 780 longpass filter for near-infrared ICG. Fluorescence data was acquired using Slidebook 5.0 software (Olympus), after exposing the sections for 300 milliseconds. Sum intensity of Texas red per gram of tissue was calculated in tumor and corresponding normal regions, using a brain section thickness of 0.02 mm and 1.04 g/cm^3 as the density of brain tissue (29). Fluorescence imaging of TUNEL stained sections was performed using an A1R confocal microscope (Nikon Instruments, Inc) at 40X zoom. Excitation and emission wavelengths were 650 and 670 nm for Alexa Fluor 647, and 350 and 460 nm for Hoechst 33342 bound to DNA. Fluorescence data acquisition and analysis was performed using Nikon A1R Elements software.

QAR Analysis

QAR was employed to study the spatial distribution and quantification of ^{3}H-vinorelbine in brain, brain metastases, peripheral soft tissue tumors and subcutaneous tumors. Phosphor images were developed by exposing 20 µm tissue sections along with tissue-calibrated tritium standards for 30 days to tritium-sensitive phosphor screens (Fujifilm Corporation), which were then digitized using a Fuji phosphoimager (Fujifilm Life sciences) and converted to color-coded vinorelbine concentrations using MCID Analysis 7.0 software (Research Inc). The limit of detection (1 month exposure time) of ^{3}H-radiotracer was 0.23 ± 0.04 nCi/mg of tissue.

Cresyl Violet Staining

After QAR exposure, slides were submerged in ice cold PBS for 5 minutes, and then were fixed in 4 % PFA for 10 minutes, followed by washing in ice-cold PBS for 5 minutes. Then brain sections were stained for 10 minutes with 0.1% cresyl violet acetate aqueous solution (Electron Microscopy Sciences), followed by washing in water for 5 minutes, in 70% alcohol for 15 seconds, and finally in 100% alcohol for 2 minutes. Slides were air-dried and a coverslip was placed on brain sections using tissue mountant for histology (Sigma). Bright field images (taken with 2X objective) for tumor localization, were imported into Slidebook and MCID software for correlation with permeability by fluorescence and drug uptake by QAR analyses respectively.

Immunofluorescence staining for ABCB1 and CD31

At necropsy, the mouse brains are dissected and immediately frozen in OCT, in a dry ice and isopentane bath. Eight micron sections were performed on a cryostat. Brain sections were washed in 4°C PBS for 5 minutes to remove excess OCT prior to fixation. Slides were fixed with -20°C methanol (5 minutes), washed in PBS 3 times for 5 min, and blocked in PBS, 5% goat serum (Dako). ABCB1 (Santa Cruz Biotechnology, sc-55510, dilution 1/100) and CD31

(Millipore cat# 550274. Dilution 1/500) antibodies were incubated overnight at 4°C. Secondary antibodies (Alexafluor, from Molecular Probes) and DAPI were incubated 1:500 in blocking buffer for 1 hour at room temperature in the absence of light. Nuclei were visualized using DAPI. Brain sections were then mounted using Fluorescence Mounting Medium (Dako). Pictures were taken with Zeiss fluorescent microscope and ZEN software.

References

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