Use of NanoBiT and NanoBRET to monitor fluorescent VEGF-A binding kinetics to VEGFR2/NRP1 heteromeric complexes in living cells

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Background and Purpose: VEGF-A is a key mediator of angiogenesis, primarily signalling via VEGF receptor 2 (VEGFR2). Endothelial cells also express the co-receptor neuropilin-1 (NRP1) that potentiates VEGF-A/VEGFR2 signalling. VEGFR2 and NRP1 had distinct real-time ligand binding kinetics when monitored using BRET. We previously characterised fluorescent VEGF-A isoforms tagged at a single site with tetramethylrhodamine (TMR). Here, we explored differences between VEGF-A isoforms in living cells that co-expressed both receptors.

Experimental Approach: Receptor localisation was monitored in HEK293T cells expressing both VEGFR2 and NRP1 using membrane-impermeant HaloTag and SnapTag technologies. To isolate ligand binding pharmacology at a defined VEGFR2/NRP1 complex, we developed an assay using NanoBiT complementation technology whereby heteromerisation is required for luminescence emissions. Binding affinities and kinetics of VEGFR2-selective VEGF$_{165b}$-TMR and non-selective VEGF$_{165a}$-TMR were monitored using BRET from this defined complex.

Key Results: Cell surface VEGFR2 and NRP1 were co-localised and formed a constitutive heteromeric complex. Despite being selective for VEGFR2, VEGF$_{165b}$-TMR had a distinct kinetic ligand binding profile at the complex that largely remained elevated in cells over 90 min. VEGF$_{165a}$-TMR bound to the VEGFR2/NRP1 complex with kinetics comparable to those of VEGFR2 alone. Using a binding-dead mutant of NRP1 did not affect the binding kinetics or affinity of VEGF$_{165a}$-TMR.

Conclusion and Implications: This NanoBiT approach enabled real-time ligand binding to be quantified in living cells at 37°C from a specified complex between a receptor TK and its co-receptor for the first time.

KEYWORDS
BRET, co-receptor, growth factor, kinetics, NanoBiT, receptor tyrosine kinase
1 | INTRODUCTION

Angiogenesis involves the growth of new blood vessels from existing vascular networks (Carmeliet, 2005). This important physiological process can also be dysregulated in numerous pathologies, such as in tumour development (Chung & Ferrara, 2011). VEGF-A is a key mediator of angiogenesis that primarily signals via its cognate receptor tyrosine kinase (RTK), the VEGF receptor 2 (VEGFR2) (Peach, Mignone, et al., 2018; Simons et al., 2016). VEGF-A binds across immunoglobulin-like domains 2 and 3 of VEGFR2 (Leppanen et al., 2010; Ruch et al., 2007). Agonist binding results in conformational changes throughout the VEGFR2 dimer that lead to auto- and trans-phosphorylation of key intracellular tyrosine residues. This triggers numerous signalling cascades that ultimately initiate endothelial cell proliferation, migration, and survival, as well as increased vascular permeability (Koch et al., 2011).

VEGFR2 is subject to complex trafficking via clathrin-dependent and clathrin-independent endocytosis (Basagianannis & Christoforidis, 2016; Basagiannis et al., 2016; Ewan et al., 2006). It internalises in both the presence and absence of VEGF-A (Ewan et al., 2006; Jopling et al., 2009, 2011). VEGF-A can also bind to the VEGFR2 co-receptor neuropilin-1 (NRP1), a type 1 transmembrane glycoprotein (Soker et al., 1998, 2002). VEGFR2 signalling is up-regulated by NRP1 (Djordjevic & Driscoll, 2013; Fantin et al., 2011; Gelfand et al., 2014). Endothelial cells express both VEGFR2 and NRP1 (Soker et al., 1998; Witmer et al., 2002). NRP1 is also overexpressed in numerous tumour subtypes (Goel & Mercurio, 2013; Jubb et al., 2012; Lee et al., 2014) and immune cells in the tumour micro-environment (Roy et al., 2017). Aberrant VEGFR2 signalling in tumour angiogenesis is therefore up-regulated by NRP1 but existing anti-tumour drugs only target VEGF-A/VEGFR2 signalling. VEGF-A interacts with VEGFR2 via residues encoded at the N-terminus of VEGF-A (Brozzo et al., 2011; Leppanen et al., 2010), while the C-terminus can interact with NRP1 (Mamluk et al., 2002; Parker et al., 2012; Vander Kooi et al., 2007).

VEGF-A is an anti-parallel, disulphide-linked homodimer. Alternative splicing of VEGF-A mRNA leads to a number of distinct VEGF-A isoforms (Peach, Mignone, et al., 2018; Woolard et al., 2009). VEGF-A isoforms have different signalling properties in physiological systems with distinct expression profiles in health and disease (Vempati et al., 2018; Woolard et al., 2004). The VEGF-A isoforms differ in length, such as pro-angiogenic VEGF165a or the shorter VEGF121a isoform. A major site of splicing occurs at exon 8, where proximal splicing results in VEGF165a isoforms that contain exon 8a-encoded residues (CDKPRR) and VEGF121b isoforms that instead contain exon 8b-encoded residues (SLTKDD). While VEGF165a stimulates angiogenesis as a full agonist, VEGF121b is a partial agonist with reported anti-angiogenic activity in vivo (Cébe Suarez et al., 2006; Eswarappa et al., 2014; Woolard et al., 2004). The b1 domain of NRP1 can interact with VEGF165a via an arginine residue encoded by exon 8a (Mamluk et al., 2002; Parker et al., 2012; Vander Kooi et al., 2007). In contrast, “anti-angiogenic” VEGF121b isoforms are unable to interact with NRP1 (Cébe Suarez et al., 2006; Delcombel et al., 2013; Kawamura et al., 2008).

What is already known

- Endothelial cells and tumour cells express both VEGFR2 and its co-receptor NRP1.
- VEGFR2 and NRP1 have distinct ligand binding kinetics and receptor localisation when expressed alone.

What this study adds

- Real-time assay quantifying fluorescent VEGF-A binding at defined heteromeric complexes in living cells at 37°C.

What is the clinical significance

- Aberrant VEGFR2 signalling in cancer is up-regulated by NRP1; however, existing drugs only target VEGF-A/VEGFR2.
- NRP1 is a promising target in oncology due to its high expression localised to tumours.

Fluorescence-based technologies have been used to advance our pharmacological understanding of GPCRs, RTKs, and other classes of membrane protein (Stoddart et al., 2017). For example, bioluminescence resonance energy transfer (BRET) is a proximity-based assay that can quantify real-time binding at 37°C in living cells (Stoddart et al., 2015). A receptor is tagged at the N-terminus with a 19-kDa NanoLuciferase (NanoLuc) such that NanoLuc emits luminescence upon oxidation of the furimazine substrate. This can excite a nearby fluorophore in close proximity (<10 nm), such as a compatible fluorescent ligand bound at the receptor’s orthosteric site. We previously developed fluorescent VEGF-A isoforms that were single site labelled with tetramethylrhodamine (TMR), to monitor ligand binding at full-length VEGF2R or NRP1 tagged with NanoLuc (Kilpatrick et al., 2017; Peach, Kilpatrick, et al., 2018; Peach et al., 2019). Despite having a similar nanomolar binding affinity, VEGF165b-TMR binding kinetics were significantly faster at NRP1 than VEGF2R (Peach, Kilpatrick, et al., 2018). VEGF2R and NRP1 were also subject to distinct subcellular trafficking in the absence or presence of ligand when expressed alone. These techniques were limited to quantifying protein–protein interactions at NanoLuc-tagged VEGF2R or NRP1 expressed in isolation; however, endothelial cells and tumour cells endogenously express both VEGF2R and NRP1 in the same cell (Fantin et al., 2013; Koch et al., 2014; Lee-Montiel et al., 2015; Prahst et al., 2008; Whitaker et al., 2001). As these receptors have distinct ligand binding dynamics and subcellular localisation, approaches are required that isolate the pharmacology of VEGF-A ligand binding to distinct complexes involving both VEGF2R and NRP1.

NanoLuc Binary Technology (NanoBiT) uses a modified NanoLuc split into a large fragment (LgBiT; 156 amino acids) and a small
11-amino-acid tag (HiBiT or SmBiT; Dixon et al., 2016). Complementation of fragments is required for luminescence emission. Numerous variants were developed of the small tag with different intrinsic affinities for complementation with the LgBiT fragment, including the “higher affinity” HiBiT fragment ($K_d \sim 0.7$ nM) and the lower affinity SmBiT fragment ($K_d \sim 190$ μM). Used in combination with a fluorescent ligand, interactions between the ligand and a particular protein pairing can be monitored using NanoBiT and BRET. Here, we have used this technology to investigate the kinetics of ligand binding of VEGF<sub>165a</sub>-TMR (Kilpatrick et al., 2017) and VEGF<sub>165b</sub>-TMR (Peach, Kilpatrick, et al., 2018) to oligomeric complexes containing both VEGFR2 and NRP1.

2 | METHODS

2.1 | Cell culture

HEK293T cells (CCLV Cat# CCLV-RIE 1018, RRID:CVCL_0063) were maintained at 37°C/5% CO<sub>2</sub> in DMEM (Sigma-Aldrich, USA) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich). For a consistent cell background with functional studies performed using a reporter gene assay, all HEK293T cells also expressed a Firefly luciferase reporter gene (RE-Luc2P) that was inserted downstream of the NFAT promoter. Control experiments confirmed that HEK293T-NFAT-ReLuc2P cells did not emit luminescence in response to furimazine alone that interfered with NanoBiT or NanoBRET assays. Cells were passaged at 70-80% confluency using PBS (Lonza, Switzerland) and trypsin (0.25% w/v in versene; Lonza).

2.2 | Generating constructs

N-terminal NanoLuc-tagged VEGFR2 (NM_002253) and NRP1 (NM_003873.5) were cloned in a pFN31K vector encoding the secretory IL-6 signal peptide fused to the N-terminus of NanoLuc, followed by a GSSGAIA linker before the receptor. HaloTag-VEGFR2 and HaloTag-NRP1 were cloned in a pFN21A vector with the IL-6 signal peptide followed by a sequence encoding HaloTag and an EPTTEDLYFQSDNAIA linker at the receptor N-terminus. SnapTag-NRP1 was cloned into a pcDNA3.1 vector encoding a murine 5HT3A EPTTEDLYFQSDNAIA linker at the receptor N-terminus. SnapTag-NRP1 and pcDNA3.1/Neo. Transient transfections used HD at a 3:1 ratio of reagent to cDNA with a total 100-ng cDNA per well. Transfection solutions were made up in serum-free DMEM and added as 11 µl per well. Cells were incubated for a further 24 h at 37°C/5% CO<sub>2</sub>. Receptors were then labelled with a solution of serum-free DMEM/0.1% BSA containing both 0.5-µM membrane-impermeant HaloTag-AlexaFluor488 substrate (G1002; Promega Corporation, USA) and 0.5-µM membrane-impermeant SNAP-Surface AlexaFlouro647 (S9136S; New England BioLabs). These were incubated for 30 min (37°C/5% CO<sub>2</sub>). Cells were washed twice with 200 µl per well HBSS/0.1% BSA and then replaced with a final volume of 225 µl per well. Cells were incubated with vehicle, 10-nM unlabelled VEGF<sub>165b</sub>, or 10-nM unlabelled VEGF<sub>165a</sub> for 60 min at 37°C, adding 25 µl to a total volume of 250 µl. Cells were imaged live using a temperature-controlled LSM710 confocal microscope fitted with a 40x water objective (Pan Apochromat objective, NA 1.2). Wavelengths were imaged simultaneously using the 488/561/633
to allow for substrate oxidation, cells were imaged live at 37°C.

HEK293T-NFAT-ReLuc2P cells were plated in white 96-well plates pre-coated with poly-D-lysine (0.01 mg/ml −1 in PBS) at 25,000 cells per well in DMEM containing 10% FBS. Following 24 h, cells were transiently transfected with a constant amount of pcDNA3.1/Zeo (25-ng cDNA per well). Cells were simultaneously transfected with increasing concentrations of HaloTag-NRP1 or SnapTag-NRP1 (2.5- to 100-ng cDNA per well). Additional wells only contained NanoLuc-VEGFR2. These transfection solutions were made up to equivalent to 125 ng per well using empty pcDNA3.1/Zeo vector in serum-free DMEM. Cells were incubated for another 24 h at 37°C/5% CO2. On the day of the experiment, cells were treated with 0.2-μM membrane-impermeant HaloTag-AlexaFluor488 substrate or 0.2-μM SNAP-Surface AlexaFluor488 substrate in serum-free DMEM/0.1% BSA. Cells were incubated for 30 min at 37°C/5% CO2. They were then washed twice with 100 μl per well HBSS/0.1% BSA and replaced with a final volume of 50 μl per well HBSS/0.1% BSA. At this stage, fluorescence emissions were quantified using the PHERAstar FS platereader using filters for excitation at 485 nm and emission at 520 nm. Cells were then incubated with the NanoLuc substrate furimazine (10 μM) for 5 min. Emissions were recorded using the PHERAstar FS platereader using filters simultaneously measuring NanoLuc emissions at 475 nm (30-nm bandpass) and AlexaFluor488 emissions at 535 nm (30-nm bandpass). BRET ratios were calculated as fluorescence over luminescence emissions from the second of three cycles.

2.5 | Bioluminescence imaging of NanoBiT complexes

HEK293T-ReLuc2P cells were plated in to poly-ω-lysine (0.01 mg/ml −1 in PBS) coated four-chamber 35-mm dishes (10-mm glass coverslip; CellVis Greiner, 627871) at 100,000 cells per quadrant in DMEM/10% FBS. On day 3, medium was replaced with HBSS/0.1% BSA containing furimazine (26 μM). Following incubation for 10 min to allow for substrate oxidation, cells were imaged live at 37°C using the inverted Olympus LV200 Bioluminescence Imaging System, fitted with a 60x oil immersion objective (super Apochromat UPLSAPO 60x/O objective; NA 1.35) with a 0.5x tube lens to focus the image; therefore, images had a final magnification of 30x. Luminescence was collected using a Hamamatsu Image EMx2 Electron Multiplying Charge Coupled Device (EMCCD) camera. Transmitted light images were collected using the camera in conventional CCD mode with a 250-ms exposure time. Luminescence emissions from the full-length NanoLuc or the NanoBIT complex were measured for 10-s exposure with a gain of 15-30. Images were taken as 8-bit images with 512 x 512 pixels per frame.

2.6 | BRET between NanoLuc-VEGFR2 and fluorescent NRP1

HEK293T-NFAT-ReLuc2P cells were plated in white 96-well plates pre-coated with poly-ω-lysine (0.01 mg/ml −1 in PBS) at 25,000 cells per well in DMEM containing 10% FBS. Following 24 h, cells were transiently transfected with a total 125-ng cDNA per well using FuGENE HD at a 3:1 ratio of reagent to cDNA with a gain of 15. Images were taken as 8-bit images with 512 x 512 pixels per frame.

To characterise luminescence emissions from a NanoBIT complex, HEK293T-NFAT-ReLuc2P cells were plated as 25,000 cells per well in white 96-well plates pre-coated with poly-ω-lysine (0.01 mg/ml −1 in PBS) in DMEM containing 10% FBS. Following 24 h, cells were transiently transfected using FuGENE HD at a 3:1 ratio of reagent to cDNA with a total 100-ng cDNA per well. Cells were transfected with a combination of LgBIT-tagged (50-ng cDNA per well) and HiBIT-/SmBIT-tagged receptors (50-ng cDNA per well). Alternatively, cells were transfected with single constructs (50-ng cDNA per well) with empty pcDNA3.1/Zeo vector (50-ng cDNA per well). Transfection mixtures were made up in serum-free DMEM and added as 5 μl per well without replacing DMEM/10% FBS on cells. Cells were incubated at 37°C/5% CO2 for a further 24 h. Medium was replaced with HBSS/0.1% BSA containing 10-μM furimazine, in the absence or presence of purified LgBIT protein (N401B, Promega Corporation) or HiBIT protein (N301A, Promega Corporation). Cells were incubated at 37°C for 10 min to allow NanoBIT complementation and the oxidation of furimazine. To prevent the loss of signal through the bottom of the plate, an adhesive plate BackSeal was added at this point. Luminescence emissions were measured on the PHERAstar platereader using the filter settings measuring emissions between 475 and 505 nm.

Additional experiments aimed to disrupt the recombined NanoBIT complex using increasing concentrations of competing receptor. HEK293T-NFAT-ReLuc2P cells were plated as 25,000 cells per well in white 96-well plates pre-coated with poly-ω-lysine (0.01 mg/ml −1 in PBS) in DMEM containing 10% FBS. Following 24 h, cells were transiently transfected using FuGENE HD at a 3:1 ratio of reagent to cDNA. Cells were transfected with a constant amount of NanoLuc-VEGFR2 (25-ng cDNA per well). Cells were simultaneously transfected with increasing concentrations of HaloTag-NRP1 or SnapTag-NRP1 (2.5- to 100-ng cDNA per well). Additional wells only contained NanoLuc-VEGFR2. These transfection solutions were made up to equivalent to 125 ng per well using empty pcDNA3.1/Zeo vector in serum-free DMEM. Cells were incubated for another 24 h at 37°C/5% CO2. On the day of the experiment, cells were treated with 0.2-μM membrane-impermeant HaloTag-AlexaFluor488 or 0.2-μM SNAP-Surface AlexaFluor488 in serum-free DMEM/0.1% BSA. Cells were incubated for 30 min at 37°C/5% CO2. They were then washed twice with 100 μl per well HBSS/0.1% BSA and replaced with a final volume of 50 μl per well HBSS/0.1% BSA. At this stage, fluorescence emissions were quantified using the PHERAstar FS platereader using filters for excitation at 485 nm and emission at 520 nm. Cells were then incubated with the NanoLuc substrate furimazine (10 μM) for 5 min. Emissions were recorded using the PHERAstar FS platereader using filters simultaneously measuring NanoLuc emissions at 475 nm (30-nm bandpass) and AlexaFluor488 emissions at 535 nm (30-nm bandpass). BRET ratios were calculated as fluorescence over luminescence emissions from the second of three cycles.
of the experiment, cells were treated with 0.2-μM membrane-impermeant HaloTag-AlexaFluor488 substrate in serum-free DMEM/0.1% BSA (30 min, 37°C/5% CO2). They were then washed twice with 100 μl per well HBSS/0.1% BSA and replaced with a final volume of 50 μl per well HBSS/0.1% BSA. Fluorescence emissions were quantified using the PHERAstar FS platteread using filters for excitation at 485 nm and emission at 520 nm. Cells were incubated with 10-μM furimazine for 10 min; then luminescence and fluorescence emissions were recorded using PHERAstar FS platteread. Emissions were simultaneously measured for NanoLuc at 475 nm (30-nm bandpass) and AlexaFluor488 at 535 nm (30-nm bandpass).

2.8 Fluorescent VEGF-A binding at a VEGFR2/NRP1 NanoBiT complex

HEK293T-NFAT-Reluc2P cells were plated in six-well plates at 400,000 cells per well in DMEM containing 10% FBS. On day 2, cells were transfected using FuGENE HD at a 3:1 ratio of reagent to cDNA with a total 1,500-ng cDNA per well made up in serum-free DMEM. Cells were transfected with equal amounts of LgBiT-VEGFR2 (750-ng cDNA per well) and HiBiT-NRP1 WT or Y297A (750-ng cDNA per well) or equal amounts of LgBiT-VEGFR2 (750-ng cDNA per well) with SmBiT-NRP1 WT (750-ng cDNA per well). For experiments monitoring kinetics at HiBiT complexes, matched controls were performed alongside in which cells were transfected with single cDNA per well using FuGENE HD at a 3:1 ratio of reagent to cDNA with a total 1,500-ng cDNA per well made up in serum-free DMEM. Cells were transfected with equal amounts of LgBiT-VEGFR2 (750-ng cDNA per well) and HiBiT-NRP1 WT or Y297A (750-ng cDNA per well) or equal amounts of LgBiT-VEGFR2 (750-ng cDNA per well) with SmBiT-NRP1 WT (750-ng cDNA per well). For experiments monitoring kinetics at HiBiT complexes, matched controls were performed alongside in which cells were transfected with single cDNA per well using FuGENE HD at a 3:1 ratio of reagent to cDNA with a total 1,500-ng cDNA per well made up in serum-free DMEM.

2.9 Data analysis

Data were analysed using GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA, USA; RRID:SCR_002798). Data are presented as mean ± SEM. All experiments were performed in three to six independent experiments with duplicate or triplicate wells (see figure legends for details). Drug additions were randomly allocated to wells within each 96-well plate. Statistical significance was defined as P < .05. Confocal images were collected using Zen 2010 software (Zeiss, Germany). Confocal images were processed and analysed using ImageJ Fiji 1.52 software (National Institutes of Health, USA; RRID:SCR_003070). The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology.

For co-localisation analysis, confocal images were corrected to the background fluorescence intensity from each experimental replicate determined using untransfected cells in each field of view (HaloTag-VEGFR2, 488 nm; SnapTag-NRP1, 647 nm). The mean background intensity was calculated for each experimental replicate (n = 6) and subtracted from each image for manual thresholding. To quantify co-localisation, regions of interest (ROIs) were drawn around each cell that co-expressed HaloTag-VEGFR2 and SnapTag-NRP1. Following subtraction of the region outside the ROI, co-localisation was determined using pixel-based measures between HaloTag-VEGFR2 and SnapTag-NRP1 using the ImageJ plugin Coloc 2. Manders’ overlap coefficients measure co-occurrence as the proportion of SnapTag-NRP1 pixels (red) overlapping with HaloTag-VEGFR2 (green). Pearson’s correlation coefficients measure whether there is a correlation between these channels. Co-localisation parameters were calculated on a per cell basis, with a total number of 119 cells (vehicle), 78 cells (VEGF165α stimulation), and 93 cells (VEGF165b stimulation), pooled from six independent experiments. Blinding was not performed for these imaging experiments because of the complexity of the experimental design. However, Coloc 2 analysis was performed automatically on all cells in every field of view that co-expressed HaloTag-VEGFR2 and SnapTag-NRP1.

Saturation binding curves were fitted simultaneously for total (VEGF165α-TMR or VEGF165b-TMR alone) and non-specific binding (obtained in the presence of 100 nM of unlabelled VEGF-A) using the equation:

\[
\text{Total Binding} = B_{\text{max}} \frac{|L|}{|L| + K_d} + M \cdot |L| + C
\]

describing the nanomolar fluorescent ligand concentration, [L]; maximal specific binding, B_{max}; the equilibrium dissociation constant of the
labelled ligand, $K_d$, in the same units as $[L]$; the slope of the non-specific binding component, $M$; and the y-axis intercept, $c$.

Association kinetic studies were performed with four concentrations of ligand simultaneously for global fitting in order to determine the $k_{on}$ and $k_{off}$. Kinetic studies of fluorescent ligand binding measured over time were fitted to a mono-exponential association function:

$$\text{Binding} = \gamma_{\text{max}} \cdot (1 - e^{-k_{\text{obs}} \cdot t})$$

describing time, $t$, plotted on the x axis; maximum response at infinite time, $\gamma_{\text{max}}$; and the rate constant observed for association, $k_{\text{obs}}$. Additionally, $k_{\text{on}}$ and $k_{\text{off}}$ values were determined by simultaneously fitting association curves at different fluorescent ligand concentrations ([L]). This utilised the following relationship with $k_{\text{obs}}$:

$$k_{\text{obs}} = k_{\text{on}} \cdot [L] + k_{\text{off}}$$

further describing association rate, $k_{\text{on}}$, in units of min$^{-1}$·M$^{-1}$; and dissociation rate, $k_{\text{off}}$, in min$^{-1}$. GraphPad Prism was used to fit each association curve to the above equations with the parameters for $k_{\text{on}}$ and $k_{\text{off}}$ shared between the fits for the four different concentrations of fluorescent ligand used in each experiment. This allowed values for $k_{\text{on}}$ and $k_{\text{off}}$ values to be determined for each experiment. These kinetic data were also used to estimate the binding affinities, due to the relationship between dissociation and association rates within an equilibrium:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}}$$

### 2.10 Materials

Fluorescent VEGF$_{165\alpha}$ and VEGF$_{165\beta}$ were labelled at a single N-terminal cysteine residue with TMR using the HaloTag mammalian protein detection and purification system (G6795; Promega Corporation, USA) as described previously (Kilpatrick et al., 2017; Peach, Kilpatrick et al., 2018). Fluorescent ligands were characterised in terms of labelling efficiency, dimerisation, and function as described in Kilpatrick et al. (2017) and Peach, Kilpatrick et al. (2018). Ligands were stored at −20°C in 2.5 mg·ml$^{-1}$ protease-free BSA (Millipore, USA). Unlabelled recombinant human VEGF isoforms were purchased from R&D Systems (Abingdon, UK). Furimazine and purified NanoBiT fragments were purchased from Promega Corporation (Madison, USA).

### 2.11 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

### 3 RESULTS

#### 3.1 Co-localisation between VEGFR2 and NRP1 co-expressed in living HEK293T cells

To investigate where VEGFR2 and NRP1 were localised when both receptors were expressed together in HEK293T cells at 37°C, we labelled each cell surface receptor with a distinct fluorophore. Receptors were simultaneously labelled using different substrates containing a HaloTag chloroalkane or SnapTag benzylguanine moiety, exploiting the fact that the membrane-impermeant fluorophore-conjugated substrate only labels receptors at the plasma membrane. HaloTag-VEGFR2 and SnapTag-NRP1 were labelled with membrane-impermeant HaloTag-AlexaFluor488 and SnapTag-AlexaFluor647 (Figure 1a). Constitutive internalisation of HaloTag-VEGFR2 was observed (Figure 1a, green regions) whereas SnapTag-NRP1 was largely expressed at the plasma membrane (Figure 1a, magenta regions). Sites of spatial overlap between VEGFR2 and NRP1 were both intracellular and at regions around the plasma membrane (Figure 1a, white). The same cell population was stimulated with a saturating concentration of unlabelled VEGF$_{165\beta}$ (upper panels) or VEGF$_{165\alpha}$ (lower panels) for 60 min (Figure 1a, right panels). Representative images show a large proportion of NRP1 remained at the plasma membrane independent of VEGF-A stimulation. To account for heterogeneity between cells, ROIs were drawn around any cell successfully co-expressing both RTK and co-receptor to quantify co-localisation between HaloTag-VEGFR2 and SnapTag-NRP1. Upon stimulation with VEGFR2-selective VEGF$_{165\beta}$, there was a reduction in the proportion of NRP1 in VEGF$_{165\beta}$-positive regions relative to vehicle (Figure 1b). In contrast, there was a higher correlation between VEGFR2/NRP1 co-localisation upon VEGF$_{165\alpha}$ stimulation compared to vehicle (Figure 1c). Both parameters indicated that VEGFR2 and NRP1 were co-localised in the absence of ligand.

BRET can also be applied to monitor proximity between receptors tagged with a bioluminescent donor (NanoLuc) and fluorescent acceptor (AlexaFluor488). Receptor-receptor BRET was used to monitor whether VEGFR2 and NRP1 were in proximity (<10 nm) when co-expressed in HEK293T cells. This unbiased technique monitors proximity from a whole cell population in 96-well plates. Cells were simultaneously transfected with a constant amount of bioluminescent donor, NanoLuc-VEGFR2, and increasing amounts of cell surface fluorophore-labelled NRP1. In the absence of ligand, there was clear saturation of the BRET signal with increasing amounts of fluorescent NRP1 acceptor (Figure 2a). This was observed for both SnapTag-NRP1 and HaloTag-NRP1, therefore independent of the fluorophore labelling approach. Confirming that increasing amounts of HaloTag-NRP1 and SnapTag-NRP1 were successfully transfected, there was also a saturable BRET signal when plotted against raw fluorescence emissions (Figure 2b). These complementary data were obtained using the same technique to confirm that increased fluorescent protein led to saturation of the BRET signal. Both the confocal imaging studies (Figure 1) and the saturation BRET studies (Figure 2) provided...
evidence for the constitutive formation of heteromeric complexes between VEGFR2 and NRP1 in living cells.

3.2 Complementation of NanoBiT fragments using N-terminal tagged VEGFR2 and NRP1

We then applied a split NanoBiT approach to isolate luminescence emissions from a defined VEGFR2/NRP1 heteromeric complex. Enzymic luciferase activity requires complementation between the large fragment (LgBiT) and the short 11-amino-acid tag (HiBiT or SmBiT). To determine the optimal configuration for luminescence emissions, each NanoBiT fragment was appended to the N-terminus of both full-length VEGFR2 and NRP1. Luminescence emissions were higher for the combination with LgBiT-tagged VEGFR2 and the short fragment attached to NRP1 (Figure 3a). Emissions from the HiBiT complex were approximately 10-fold higher than the SmBiT complex. NanoBiT-tagged receptors expressed independently emitted minimal luminescence in the presence of furimazine relative to the complemented NanoBiT complex (Figure 3b). Addition of purified NanoBiT fragments to exogenously complement the NanoBiT tag confirmed that individual constructs were appropriately expressed despite low luminescence emissions in isolation (Figure 3c). The luminescence signals from both HiBiT and SmBiT complexes were also prevented by competition with increasing amounts of unlabelled HaloTag NRP1 (Figure 3d). Thus, despite the intrinsic affinity between HiBiT and LgBiT (Dixon et al., 2016), luminescence emissions were reduced by increasing amounts of NRP1.

A bioluminescence widefield imaging system was used to visualise where the NanoBiT luminescence signal was localised. To determine the cellular location of the luciferase signal, cells were incubated with membrane-permeable furimazine in the absence of ligand.
NanoLuc-VEGFR2 was largely intracellular, whereas NanoLuc-NRP1 was at the cell surface. The NanoBiT complex between HiBiT-NRP1 and LgBiT-VEGFR2 was localised to both intracellular sites and the plasma membrane. This spatial distribution was comparable to the regions of white overlay between HaloTag-VEGFR2 and SnapTag-NRP1 observed in Figure 1a.

### 3.3 Influence of NanoBit tags on VEGFR2-mediated signalling

We confirmed that the NanoBiT fragments did not interfere with VEGFR2 signalling using an NFAT reporter gene assay (Kilpatrick et al., 2017). Concentration–response curves for VEGF165a were compared between cells stably expressing VEGFR2 tagged at the N-terminus with LgBiT, HiBiT, or SmBiT (Figure 5). Each receptor exhibited a concentration-dependent increase in NFAT gene transcription in response to increasing concentrations of VEGF165a. Each cell line had a similar potency derived for VEGF165a (LgBiT-VEGFR2 pEC50 = 9.95 ± 0.11; HiBiT-VEGFR2 pEC50 = 10.06 ± 0.12; SmBiT-VEGFR2 pEC50 = 10.23 ± 0.23; n = 5 for each). These were comparable to potency values derived for VEGF165a at wild-type VEGFR2 (Kilpatrick et al., 2017).

### 3.4 Nanomolar affinity of fluorescent VEGF-A at a defined VEGFR2/NRP1 complex

Fluorescent VEGF-A ligand binding was monitored at full-length VEGFR2 and NRP1 tagged at their N-terminus with LgBiT and HiBiT, respectively. As the uncomplemented receptors cannot oxidise furimazine, luminescence was confined to proteins where complementation from a defined heteromeric VEGFR2/NRP1 NanoBiT complex had occurred (Figure 6a). BRET therefore only derived from the receptor/co-receptor complex and the fluorescent VEGF-A acceptor. We have previously demonstrated that VEGF165b-TMR selectively binds to NanoLuc-VEGFR2 (and not NRP1), whereas VEGF165a-TMR can bind to both NanoLuc-VEGFR2 and NanoLuc-NRP1 with nanomolar affinity (Peach, Kilpatrick, et al., 2018). At the complemented HiBiT complex, there was saturable binding in the presence of increasing concentrations of VEGF165b-TMR (Figure 6b) or VEGF165a-TMR (Figure 6c). This was displaced by a high concentration of unlabelled ligand, demonstrating low non-specific binding. Both fluorescent ligands had equilibrium dissociation constants (Kd) in the nanomolar range at the VEGFR2/NRP1 complex (VEGF165b-TMR Kd = 16.26 ± 3.81 nM, pKd = 7.82 ± 0.11; VEGF165a-TMR Kd = 2.53 ± 0.49, pKd = 8.61 ± 0.09; n = 3 for both). Estimated ligand binding affinities were similar to those derived at isolated receptors tagged with full-length NanoLuc (Peach, Kilpatrick, et al., 2018).

### 3.5 Real-time kinetics of fluorescent VEGF-A isoforms at a heteromeric VEGFR2/NRP1 NanoBiT complex

Taking advantage of the NanoBiT approach to monitor real-time ligand binding at 37°C to a complex, we compared the kinetics of ligand binding of VEGF165b-TMR with that of VEGF165a-TMR at the VEGFR2/NRP1 NanoBiT complex in living cells. The kinetic binding profile of VEGF165b-TMR (which should only bind to VEGFR2, Peach,
Kilpatrick, et al., 2018) continued to increase over the full 90-min time course in intact cells, producing a classic ligand binding association maintained for each concentration of VEGF165b-TMR (Figure 7a). Fitted to a global association curve (Table 1), VEGF165b-TMR had a slightly slower association rate constant ($k_{on}$) for the VEGFR2/NRP1 complex ($2.29 \times 10^6 \pm 0.30 \times 10^6$ min$^{-1}$ M$^{-1}$) compared to...
NanoLuc-VEGFR2 alone (7.29 × 10^6 min^-1/C1 M^-1; Peach, Kilpatrick, et al., 2018). We then directly compared the real-time binding profile for a saturating concentration of VEGF165b-TMR between the NanoBiT complex and cells expressing NanoLuc-tagged receptors alone in matched time course experiments (Figure 7b). Compared to NanoLuc-VEGFR2, the small decline in BRET signal after a peak at 20 min in intact cells was absent when monitored at the NanoBiT complex for VEGF165b-TMR. There was no BRET detected between VEGF165b-TMR and NanoLuc-NRP1. However, this selective ligand had a distinct long-term kinetic profile at the VEGFR2/NRP1 complex compared to VEGFR2 alone (Figure 7b).

Kinetic experiments were repeated with four concentrations of VEGF165a-TMR (Figure 7c). Unlike VEGF165b-TMR, there was a small decline in BRET ratio between 30 and 60 min for VEGF165a-TMR at the HiBiT complex (Figure 7c). Association binding curves were globally fitted to kinetic data from the initial 20 min due to this decline (Table 1). VEGF165a-TMR had a slower dissociation rate constant (k off) at the HiBiT complex (0.046 ± 0.007 min^-1; Table 1) compared to that previously reported for NanoLuc-NRP1 expressed alone (0.26 min^-1; Peach, Kilpatrick, et al., 2018). As a consequence, the kinetic binding profile for 10-nM VEGF165a-TMR was directly compared between the NanoBiT complex and either NanoLuc-VEGFR2 or NanoLuc-NRP1 (Figure 7d). VEGF165a-TMR association kinetics at the NanoBiT complex in the initial 20 min were more comparable to NanoLuc-VEGFR2 than NanoLuc-NRP1 (NanoBiT k obs = 0.33 ± 0.04 min^-1, NanoLuc-VEGFR2 k obs = 0.31 ± 0.03 min^-1, NanoLuc-NRP1 k obs = 0.93 ± 0.09 min^-1; n = 5 per group). These observed rate constants were significantly slower at the complex than NRP1 alone (repeated-measures ANOVA and Holm–Šidák’s multiple comparisons; P < .05, n = 5 for each). These data suggest that the ligand binding profile for VEGF165a-TMR at the NanoBiT complex reflected VEGFR2 binding kinetics, as opposed to the faster binding observed at NRP1.

### 3.6 Fluorescent VEGF-A kinetics were similar for the SmBiT complex

Considering the distinct kinetic observations at the HiBiT complex, we further probed ligand binding kinetics at the SmBiT complex to explore possible influences of the NanoBit tag characteristics (Dixon et al., 2016). Using four concentrations of VEGF165b-TMR, binding
was monitored over 90 min (Figure 8a). The binding profile remained elevated throughout the time course with similarities to kinetics observed with the HiBiT complex. Kinetic data were globally fitted to a simple exponential association (Table 1). VEGF<sub>165b</sub>-TMR had a slower dissociation rate (k<sub>off</sub>) from the SmBiT complex compared to the HiBiT complex. Plotting the individual observed association rate constants (k<sub>obs</sub>) against VEGF<sub>165b</sub>-TMR concentration, there was a linear relationship observed at both HiBiT and SmBiT complexes (Figure 8b). The interaction between VEGF<sub>165b</sub>-TMR and the NanoBiT complex can therefore be defined as a first-order reaction.

Binding kinetics were also monitored at the SmBiT complex using four concentrations of VEGF<sub>165a</sub>-TMR (Figure 8c). From the fitted data from the initial 20-min period using a global fit, there were no differences between the association kinetic parameters derived for VEGF<sub>165a</sub>-TMR for the HiBiT and SmBiT complexes. There was a linear relationship between the derived observed association rate (k<sub>obs</sub>) constants and VEGF<sub>165a</sub>-TMR concentration (Figure 8d). Despite having the potential to bind to both receptors within the complex, the interaction between VEGF<sub>165a</sub>-TMR and the NanoBiT complex could also be defined by a first-order reaction.
3.7 Similar complex pharmacology using a binding-dead mutant of NRP1

In addition to comparing binding between selective and non-selective fluorescent VEGF-A isoforms, site-directed mutagenesis provided an alternative approach to probe the contribution of NRP1 engagement to the pharmacological characteristics of the VEGFR2/NRP1 complex. Using a previously characterised binding-dead NRP1 mutant (Y297A; Herzog et al., 2011; Fantin et al., 2014; Peach, Kilpatrick, et al., 2018), comparisons were made using the same ligand in the absence of interactions between VEGF₁₆₅b-TMR and NRP1 within the heteromeric NanoBiT complex (Figure 9a). Upon co-expression of LgBiT-VEGFR2 and either HiBiT- or SmBiT-NRP1 (Y297A), there were high luminescence emissions resulting from NanoBiT complementation (Figure 9b). Luminescence emissions from this NanoBiT complex were comparable to wild-type NRP1, implying that this amino acid residue was not required for constitutive VEGFR2/NRP1 complex formation. NanoBiT constructs expressed in isolation from their complementary fragment also had minimal luminescence emissions in the presence of furimazine (Figure 9b). Isolating ligand
binding from this VEGFR2/NRP1 Y297A complex, VEGF165a-TMR exhibited saturable binding at the NanoBiT complex (Figure 9c). This was displaced by a high concentration of unlabelled VEGF165a, confirming that there was low non-specific binding. Derived equilibrium dissociation constants were in the nanomolar range and similar to the wild-type NanoBiT complex (VEGF165a-TMR/NanoBiT Y297A $K_d = 1.55 \pm 0.38$; $p_{K_d} = 8.84 \pm 0.11$; $n = 3$). Binding kinetics at the mutant NanoBiT complex were then monitored using four concentrations of VEGF165a-TMR (Figure 9d). This had a profile identical to that of VEGF165a-TMR at the wild-type HiBiT complex (Figure 7c), in that there was a small decline in BRET ratio following 30–60 min. Association kinetics were derived from the initial 20 min using a global fit ($k_{on} = 3.71 \times 10^7 \pm 0.21 \times 10^7 \text{M}^{-1}\text{s}^{-1}$; $k_{off} = 0.054 \pm 0.008 \text{min}^{-1}$; kinetic $p_{K_d} = 8.85 \pm 0.04$; $n = 5$). These data suggest that VEGF165a-TMR bound the NanoBiT complex with similar kinetics, regardless of the ability to simultaneously engage NRP1.

4 | DISCUSSION

NanoBiT technologies were used to quantify the real-time binding of two fluorescent VEGF-A isofoms at a defined receptor/co-receptor complex between VEGFR2 and NRP1 in living cells at 37°C. Previous work identified differences between VEGFR2 and NRP1 pharmacology in terms of their binding kinetics and localisation when expressed on their own (Peach, Kilpatrick, et al., 2018). VEGFR2 and NRP1 are, however, endogenously co-expressed together in endothelial cells and tumour cells (Fantin et al., 2013; Koch et al., 2014; Lee-Montiel et al., 2015; Prahst et al., 2008; Whitaker et al., 2001). We first demonstrated that full-length VEGFR2 and NRP1 constitutively formed a heteromeric complex in living HEK293T cells. To then probe how this specific receptor/co-receptor heteromer interacted with ligand, we established a novel approach to quantify fluorescent VEGF-A binding at a defined complex using split NanoBiT fragments (Dixon et al., 2016). VEGFR2 and NRP1 tagged at their N-terminus with HiBiT and LgBiT tags led to NanoBiT complementation with minimal luminescence when each was expressed alone. The formation of this NanoBiT complex could be prevented by increasing amounts of an unlabelled version of one of the heteromer components. As such, the BRET signal was specific to interactions between the VEGFR2/NRP1 heteromer (BRET donor) and fluorescent VEGF-A (BRET acceptor). This allowed us to monitor ligand binding to a defined RTK/co-receptor oligomeric complex. Uncomplemented VEGFR2 or NRP1 that still binds to the fluorescent ligand does not, however, contribute to the BRET signal due to the lack of complemented donor luminescence and the requirement for donor and acceptor to be within 10 nm of each other.

Numerous biochemical techniques have suggested that VEGFR2 and NRP1 form heteromeric complexes, including co-immunoprecipitation studies in endothelial cells (Gelfand et al., 2014; Prahst et al., 2008; Whitaker et al., 2001) and proximity ligation assays using antibodies in situ on tumour tissue (Koch et al., 2014). Förster resonance energy transfer (FRET) has also been used to demonstrate complex formation using truncated VEGFR2 and full-length NRP1 tagged with fluorophores at their C-terminus (King et al., 2018). Here, we initially used BRET between full-length VEGFR2 and NRP1 tagged at their N-terminus with NanoLuc or a fluorophore to confirm complex formation in the absence of added VEGF-A. The approach monitored complex formation that originated at the cell membrane, because membrane-impermeant fluorophore-conjugated HaloTag or SnapTag substrates were used. Basal VEGFR2/NRP1 complex formation was also confirmed using both HiBiT-VEGFR2 and LgBiT-NRP1 complementation and the reverse LgBiT-VEGFR2 and HiBiT-NRP1 orientation.

Following the discovery that VEGF165a had faster binding kinetics for binding to NRP1 than to VEGFR2 when expressed on their own (Peach, Kilpatrick, et al., 2018), it was proposed that the presence of NRP1 might enhance VEGF165a binding to the heteromeric complex. The application of both NanoBiT technology and NanoBRET to monitor exclusively VEGF165a-TMR binding to VEGFR2/NRP1 complexes allowed us to test this hypothesis directly. Interestingly, the initial association kinetics (during the first 20 min) for VEGF165a-TMR binding to the VEGFR2/NRP1 heteromeric complex were closer to those observed at NanoLuc-VEGFR2 in isolation than to NanoLuc-NRP1. This was evident from quantification of the observed rate constant

**Table 1** Summary of binding parameters derived at the NanoBiT complex for VEGF165b-TMR and VEGF165a-TMR, compared to published values from receptors expressed alone

|                  | Kinetic $p_K_d$ | $k_{on}$ (min$^{-1}$ M$^{-1}$) | $k_{off}$ (min$^{-1}$) |
|------------------|----------------|-------------------------------|-------------------------|
| VEGF165b-TMR     |                |                               |                         |
| HiBiT complex    | 7.81 ± 0.10    | 2.29 × 10$^3$ ± 0.30 × 10$^3$ | 0.037 ± 0.007           |
| SmBiT complex    | 8.43 ± 0.17    | 2.94 × 10$^6$ ± 0.55 × 10$^6$ | 0.012 ± 0.003           |
| VEGF165a-TMR     |                |                               |                         |
| HiBiT complex    | 8.83 ± 0.12    | 3.12 × 10$^7$ ± 0.43 × 10$^7$ | 0.046 ± 0.007           |
| SmBiT complex    | 8.83 ± 0.31    | 2.83 × 10$^7$ ± 0.69 × 10$^7$ | 0.046 ± 0.020           |

Note: The number of independent experiments are shown in parentheses, each with duplicate wells. Kinetic parameters were derived from a global association fit from the full 90-min time course (VEGF165b-TMR) or the initial 20 min (VEGF165a-TMR). Experiments with LgBiT-VEGFR2 and HiBiT-NRP1 (HiBiT complex) are shown in Figure 7a,c. Kinetic experiments with LgBiT-VEGFR2 and SmBiT-NRP1 (SmBiT complex) are shown in Figure 8a,c.

$^*P < 0.05$, significantly different from SmBiT complex; Kruskal–Wallis test.
from matched experiments at a saturating concentration (10 nM) of fluorescent VEGF165a where $k_{\text{obs}}$ was $0.33 \pm 0.04 \text{ min}^{-1}$ for the VEGFR2/NRP1 NanoBit complex, $0.31 \pm 0.03 \text{ min}^{-1}$ for NanoLuc-VEGFR2, and $0.93 \pm 0.09 \text{ min}^{-1}$ for NanoLuc-NRP1. Furthermore, the removal of the binding site for VEGF165a on NRP1 by site-directed mutagenesis of residue Y297 to alanine did not alter the ability of VEGFR2 and NRP1 to form complexes or the binding of VEGF165a-TMR to the heteromeric complex. It is possible therefore that heteromerisation between VEGFR2 and NRP1 masks the high affinity binding site for VEGF165a on NRP1 and just leaves the VEGFR2 binding site available.

There were some subtle differences between the kinetics of fluorescent VEGF-A isoforms at the VEGFR2/NRP1 heteromeric complex. Pro-angiogenic VEGF165a and anti-angiogenic VEGF165b are functionally distinct VEGF-A isoforms, although these isoforms only differ by six amino acid residues at their C-terminus. Despite observed physiological distinctions between VEGF-A isoforms, there were no differences observed at the level of ligand binding to
NanoLuc-VEGFR2 when it was expressed alone (Peach, Kilpatrick, et al., 2018). VEGF165b is, however, selective for VEGFR2 and unable to interact with NRP1 (Peach, Kilpatrick, et al., 2018). The real-time BRET signal for VEGF165b-TMR remained elevated in intact cells at the NanoBiT complex over the full 90-min time course. This resembled observations made with NanoLuc-VEGFR2 in membrane preparations and was quite different to the decline in BRET signal normally observed in intact HEK293T cells (Peach et al., 2019). In contrast, the profile for VEGF165a-TMR at the HiBiT complex had a small decrease at latter time points, albeit to a lesser extent than at NanoLuc-VEGFR2 in intact cells (Peach et al., 2019). This reduction in BRET signal for NanoLuc-VEGFR2 following
20 min has been linked to VEGF-A/VEGFR2 endocytosis leading to a change in localisation and local pH, as this decline was absent in membrane preparations and not observed for binding to NanoLuc-NRP1 (Peach et al., 2019). These data suggest that the presence of NRP1 in VEGFR2 heteromeric complexes may reduce the extent of VEGFR2 endocytosis normally seen when VEGFR2 is expressed alone.

Imaging studies exploited the compatibility of HaloTag and SnapTag technologies to label distinct receptors co-expressed by the same cell to monitor co-localisation at 37°C. Unlike immunofluorescent antibody labelling, these experiments can be performed in living cells and do not require cell fixation or cell permeabilisation to access internalised receptors. These distinct tags confirmed that VEGFR2 was largely intracellular whereas NRP1 was highly localised around the plasma membrane when they were both co-expressed in the same cell. NRP1 was also localised in filopodia-like projections in HEK293T cells that resembled the filopodia of endothelial tip cells (Fainst et al., 2013, 2015). Although co-localisation studies were limited by the axial resolution limit of basic confocal microscopy, experiments monitoring receptor–receptor BRET confirmed that VEGFR2 and NRP1 were in proximity (<10 nm). Live cell confocal imaging and bioluminescence imaging data both suggested that VEGFR2 and NRP1 were co-localised in both intracellular compartments and at the plasma membrane. VEGFR2 is subject to macropinocytosis in the absence or presence of ligand (Basagiannis & Christoforidis, 2016; Basagiannis et al., 2016). This bulk transport mechanism could therefore non-selectively engulf surrounding NRP1 in living cells. There is evidence in HUVECs for co-localisation between VEGFR2 and NRP1 both at the plasma membrane in the absence of stimulation (Lee-Montiel et al., 2015) or within intracellular sites following 20-min VEGF165α stimulation (Muhl et al., 2017). As the NanoLuc/NanoBiT substrate furimazine is membrane permeable, luminescence could be emitted from complexes anywhere in the cell regardless of subcellular localisation.

NanoBiT technologies take advantage of NanoLuc, a small enzyme engineered from a deep sea shrimp with bright, ATP-independent luminescence emissions (Hall et al., 2012). The small, 11-amino-acid NanoBiT fragment also has mutations that confer differing intrinsic affinities for the LgBiT fragment. For example, HiBiT has a much higher intrinsic affinity for LgBiT than SmBiT (Dixon et al., 2016). Luminescence emissions from HiBiT-containing complexes were higher than for the corresponding SmBiT-containing complex, as observed previously for NanoBiT-tagged GPCRs (Botta et al., 2019). This is likely to be due to differences in the affinity of LgBiT for SmBiT or HiBiT. Dixon et al. (2016) confirmed the rapid kinetics of NanoBiT tag complementation (seconds) and, therefore, this is unlikely to affect the ligand binding kinetics monitored at the VEGFR2/NRP1 complex (minutes). The intrinsic affinity between HiBiT and LgBiT can vary according to the expression system and protein conformation, as observed for chemokine GPCRs using the purified exogenous tag in different assay set-ups (White et al., 2020). While the intrinsic affinity between NanoBiT tags must be taken into consideration, luminescence emissions from both HiBiT and SmBiT complexes were displaceable by increasing amounts of competing NRP1 (Figure 3d). The kinetic parameters derived from HiBiT and SmBiT complexes were also comparable, suggesting that VEGFR2–NRP1 complex formation was not being driven by the affinity of the HiBiT tag for LgBiT.

Despite its ability to up-regulate VEGF-A/VEGFR2 signalling in physiological and pathophysiology, the mechanism by which NRP1 up-regulates VEGFR2 signalling remains largely unknown. NRP1 can interact with a number of other growth factors (Banerjee et al., 2006; Rizzolio et al., 2012; West et al., 2005). Therefore, understanding how NRP1 co-expression influences RTK function has implications for other receptors contributing to cancer drug resistance. Our NanoBiT approach allowed us to isolate VEGF-A ligand binding at a defined complex of VEGFR2 and NRP1 and suggested that NRP1 did not increase the affinity or association binding kinetics of VEGF165α at VEGFR2. While NRP1 appeared to have no direct effect on ligand binding to a VEGFR2/NRP1 complex expressed within the same cell, NRP1 (which is quite often expressed endogenously at higher levels than VEGFR2) could still act as a reservoir for growth factors and create a localised gradient due to its interactions with the extracellular matrix (Shintani et al., 2006; Windwarder et al., 2016).

In summary, we have described here an approach using NanoBiT technology and NanoBRET to monitor in real time the binding of VEGF-A isoforms to defined heteromeric complexes containing both VEGFR2 and NRP1. Understanding the ligand binding properties of a specific heteromeric receptor oligomer is important for studying the molecular pharmacology of individual VEGF-A isoforms in primary cells, such as endothelial and tumour cells, which often endogenously co-express both receptors. This specific technique allowed us to determine for the first time the ligand binding kinetics of VEGF165α–TMR and VEGF165β–TMR to a defined VEGFR2–NRP1 complex. We were able to use bioluminescence imaging and confocal microscopy to determine that VEGFR2–NRP1 complexes are localised in both intracellular compartments and at the plasma membrane. At the plasma membrane, the presence of NRP1 within the heteromeric complex appeared to reduce the extent of agonist-induced VEGFR2 endocytosis normally observed when it is expressed alone. The presence of NRP1 within the VEGFR2–NRP1 heteromeric complexes did not enhance VEGF165α–TMR binding, and a NRP1 binding-dead mutant (Y297A) had no effect on the binding of VEGF165α–TMR, or the formation of VEGFR2–NRP1 complexes, suggesting that the high affinity binding site for VEGF165α on NRP1 might be masked within the heteromeric complexes. In keeping with this conclusion, VEGF165β–TMR, which does not bind to NRP1, had a very similar binding profile to the heteromeric complex to that observed with VEGF165α–TMR. This approach to monitor the binding profile of defined oligomeric complexes should be applicable to a wide range of receptor systems and facilitate drug discovery aimed at a heteromeric complex. This approach could also be developed further to observe ligand interactions with a specified oligomer in vivo due to the small size of the HiBiT/SmBiT tag and bright luminescence emissions, in a similar way to that we have recently reported for a GPCR tagged with the full-length NanoLuc (Alcobia et al., 2018). Given the clinical
importance of therapeutic agents targeting VEGF or VEGFR2 in cancer and other pathologies, understanding the mechanism by which NRPI up-regulates VEGF-A/VEGFR2 signalling is a priority for identifying new targets to improve the long-term efficacy and adverse effects of VEGF-directed therapeutics in cancer.

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AUTHOR CONTRIBUTIONS
S.J.H., J.W., L.E.K., and C.J.P. conceived the study. C.J.P., L.E.K., S.J.H., and J.W. participated in research design. C.J.P. and L.E.K. conducted experiments. C.J.P. and S.J.H. performed data analysis. C.J.P., L.E.K., J.W., and S.J.H. wrote or contributed to the writing of the manuscript.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJPh guidelines for Design & Analysis, and as recommended by funding agencies, publishers, and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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