NanoBRET assay quantitatively evaluates VEGF binding to the VEGFR2 in real time in living cells

Kilpatrick LE[1], Friedman-Ohana R[2], Alcobia DC[1], Riching K[2], Peach CJ[1], Wheal AJ[1], Briddon SJ[1], Robers MB[2], Zimmerman K[2], Machleidt T[2], Wood KV[2], Woolard J[1], Hill SJ[1]
[1]Division of Physiology, Pharmacology and Neuroscience, University of Nottingham, UK [2]Promega Corporation, Madison, WI, USA

- **Novel labelling technique fluorescently labels VEGF for use in NanoBRET interaction studies**
- **Assay acquires quantitative information on ligand-receptor interaction**
- **PHERAstar® simultaneously detects luciferase and fluorophore signal for accelerated and kinetic reads**

Introduction

Vascular endothelial growth factors (VEGF) are proteins that act as dimers to mediate angiogenesis by binding to their cellular receptors (VEGFR). Upon ligand binding VEGFRs dimerize and initiate a raft of signaling cascades via their intracellular tyrosine kinase domains. VEGFs and their receptors play a role in tumor vascularization and are major targets of anti-cancer therapies.
In order to characterize affinities of VEGF isoforms binding to VEGFR2, assays are required that quantitatively and kinetically report on these interactions.
A novel labeling technique stoichiometrically labels the VEGF family member $VEGF_{165}$a with the fluorophore TMR. The modified ligand was used in conjunction with NanoLuciferase-coupled VEGFR2 (NLuc VEGFR2) in a cellular NanoBRET assay.

Assay Principle

$VEGF_{165}$a was expressed as a HaloTag fusion protein that upon secretion was captured on Halolink resin. The tag was proteolytically removed by HaloTEV and in the presence of 6-TMR-PEG-CBT resulting in the ligand labelled with TMR at a single N-terminal cysteine (Fig. 1A)

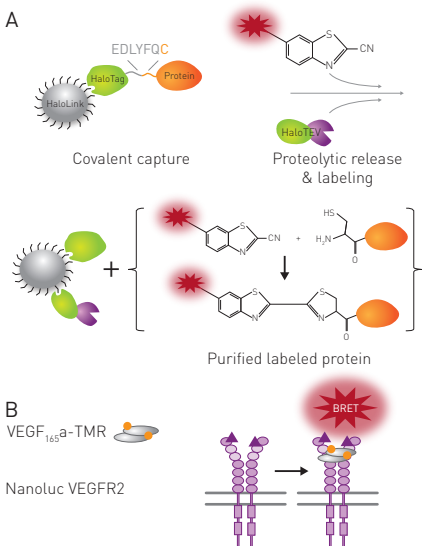

Fig. 1: Synthesis of $VEGF_{165}$a **(A)** and the principle of the NanoBRET assay used to study VEGF-TMR binding to NLuc-VEGFR2 **(B)**. Data taken from reference 1.

The binding of the $VEGF_{165}$a-TMR homodimer to NLuc fused VEGFR2 was detected using BRET. Briefly, oxidation of the NLuc substrate furimazine produces energy which can be transferred from the bioluminescent donor (NLuc) to the acceptor flurophore (TMR) if the two proteins are in sufficiently close proximity (<10nm). Hence, the ratio of fluoro-phore to luciferase emission indicates interaction of fluorescent ligand with its cognate receptor (Fig 1B).

Materials & Methods

- White flat bottomed microplates (Greiner Bio-One #655098)
- PHERAstar® *FS*
- Furimazine (N1130, Promega Corporation)
- HEK293 stably expressing NLuc-VEGFR2

Experimental procedure
HEK293 cells expressing NLuc-VEGFR2 (40,000/well) were seeded 24 h prior to the experiment. Measurements were conducted in HBSS/0.1 % protease free BSA and with a furimazine concentration of 10 µM. Fluorescently labelled $VEGF_{165}$-TMR, unlabelled $VEGF_{165}$a, and Cediranib (an RTK inhibitor, 1 µM) were used at indicated concentrations and measurements were acquired with a PHERAstar *FS* microplate reader using the settings outlined below.

Instrument settings

Optic settings	Luminescence, endpoint or plate mode kinetic, top optic	
	Optic module	NanoBRET; Em 1 460-80 (Nluc) Em 2 LP610 (TMR)
	Gain	2800 (Nluc) 3600 (TMR)
General settings	Settling time	0.0 s
	Measurement interval	1 s
Kinetic settings	Number of cycles	33
	Cycle time	30 s
Incubation	Endpoint	None
	Kinetic	37 °C

Results & Discussion

The TMR-labelled $VEGF_{165}$a was added in concentrations from 1-20 nM to NLuc-VEGFR2 expressing HEK293 cells to monitor ligand-receptor interaction by NanoBRET. The time course of BRET ratios obtained shown in Figure 2 reveals the increase in binding and equilibrium of binding 10 min after the start of the reaction.

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Keywords: **Ligand binding, NanoBRET, Real-time, Receptor, TAMRA, Tyrosine kinase**

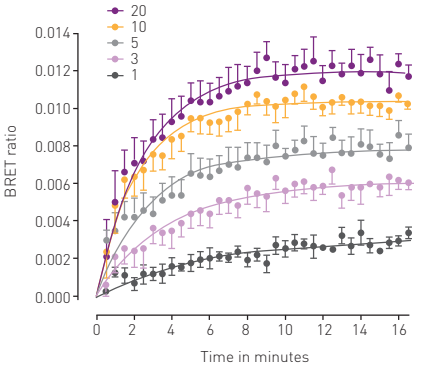


Fig. 2: Ligand binding kinetics of $VEGF_{165}$a-TMR to NLuc-VEGFR2 in HEK293 cells. Furimazine was added to each well and after 5 min of equilibration the reaction was started by adding $VEGF_{165}$a-TMR at the indicated concentration (in nM). Data taken from reference 1.

Next, the ability of the assay to determine ligand binding affinities was tested by displacement of $VEGF_{165}$a-TMR binding to VEGFR2 by unlabeled competing $VEGF_{165}$a. The interaction between $VEGF_{165}$a-TMR and VEGFR2 was impaired by increasing concentrations of the competing isoform as reported by loss of BRET signal (Fig. 3). The pK_i for $VEGF_{165}$a determined by BRET was 9.81.

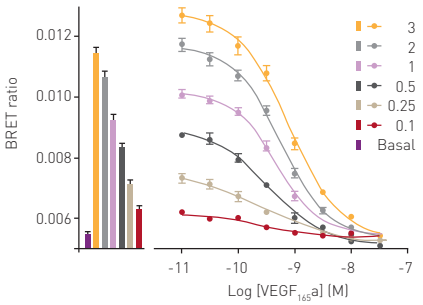


Fig. 3: Inhibition of $VEGF_{165a}$-TMR binding to NLuc-VEGFR2 by $VEGF_{165}$a. Fixed concentrations of $VEGF_{165}$a-TMR (0.1–3 nM) were added simultaneously with increasing concentrations of the unlabeled $VEGF_{165}$a and incubated for 1 h at 37 °C. Data represent mean ± S.E.M of 5 independent experiments, performed in triplicate. Bars represent the binding of $VEGF_{165}$a-TMR obtained at each fluorescent ligand concentration in the absence of competing ligand. Data taken from reference 1.

The effect of the receptor tyrosine kinase inhibitor cediranib on $VEGF_{165}$a-TMR binding to VEGFR2 was monitored for 2 h. In the absence of the inhibitor, ligand bound to VEGFR2 with a peak BRET signal obtained at approximately 15 min after ligand addition (Fig. 4, empty symbols). At later time points, the BRET signal reduced because of receptor internalization and subsequent complex dissociation. In contrast, in the presence of cediranib, high BRET ratios were observed that were sustained for the period of the experiment (2hr). This was due to cediranib preventing VEGFR2 internalisation, which was later confirmed by confocal and super-resolution imaging (Kilpatrick et al., 2017 (1), Figure 7a; data not included in this report).

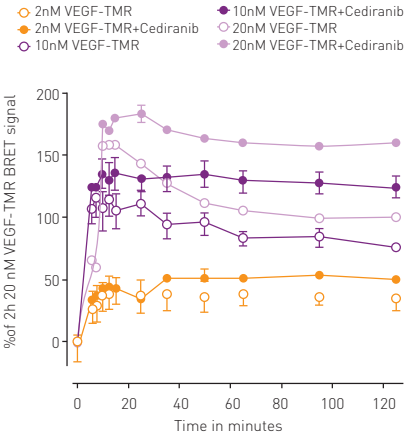


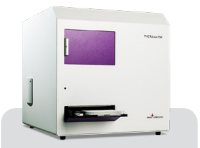
Fig. 4: The effect of the receptor tyrosine kinase inhibitor cediranib on $VEGF_{165}$a-TMR binding to VEGFR2 was monitored for 2 h. In the absence of the inhibitor, the ligand initially binds to VEGFR2 with a peak BRET signal obtained at approximately 15 min after ligand addition (Fig. 4, empty symbols). At later time points, the BRET signal reduces because of receptor internalization and subsequent complex dissociation. In contrast, presence of the RTKI cediranib prevents internalization and stabilizes the interaction for the period of the experiment (2 h). This becomes visible by sustained high BRET ratios (Fig. 4 full symbols). Data taken from reference 1.

Conclusion

The novel method for single site labeling a ligand with a fluorophore has paved the way to use NanoBRET to quantify the molecular pharmacology of the anti-cancer target VEGFR2. The PHERAstar microplate reader reliably detected the luminescent and fluorescent signals produced. Simultaneous acquisition of both signals accelerates the reading of a plate and increases the temporal resolution.The combination of up-to-date assay and detection technology helps to better understand and target cancer-related biological events.

References

1. Kilpatrick LE et al. (2017) *Biochem Pharmacol.* Jul 15; 136:62-75. doi: 10.1016/j.bcp.2017.04.006



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