Investigation of homodimeric structure of a fluorescently labelled $VEGF_{165a}$ ligand in solution using Fluorescence Correlation Spectroscopy

Background and Purpose: The Vascular Endothelial Growth Factor $_{165}$a ($VEGF_{165}a$) is a splice variant of the VEGF family of proteins, which activate VEGF Receptor-2 (VEGFR-2), an important drug target for cancer therapy [1]. VEGF ligands are thought to be formed as antiparallel dimers linked by disulfide bonds, which bind to VEGFR2 causing its dimerization and activation [1].

Recently, we have generated a tetramethylrhodamine labelled form of $VEGF_{165}a$ ($VEGF_{165}a$-TMR), where each protomer of the proposed dimeric ligand has been labelled at a single N-terminal cysteine residue. Here we have investigated the homodimeric nature of $VEGF_{165a}$-TMR in physiological buffer, using the technique of Fluorescence Correlation Spectroscopy (FCS), which allows the concentration and diffusion coefficient of fluorescently labelled peptides to be determined [2].

Methodologies: FCS was used to determine the concentration and diffusion coefficient of $VEGF_{165a}$-TMR molecules over a time-course of 20min. $VEGF_{165}a$-TMR and 5-carboxy-tetramethylrhodamine N-succinimidyl ester (TAMRA) (0.25-10nM) were prepared in Hank's buffered salt solution (HBSS, [3]) in the presence or absence of 0.1% protease-free bovine serum albumin (BSA), and placed in Labtek 8-well coverglasses in a final volume of 200µl. Repeated FCS measurements were taken for 1x20s over 0-20 min using a Zeiss LSM510 Confocor 3 (561nm excitation, emitted light collected through an LP580 filter). FCS data were analysed using autocorrelation analysis and fitted to a single component 3D diffusion model, as previously described [3]. $VEGF_{165}a$-TMR was also treated with the reducing agent dithiothreitol (DTT, 10mM, 30min, 37^{o}C) prior to FCS measurements. Data are expressed as mean ± SEM of 3 independent experiments.

Results: In the absence of BSA, the detected concentration of a 10nM $VEGF_{165a}$-TMR solution (theoretical concentration assuming dimeric species) was 3.6 ± 0.2 nM dropping to 2.3 ±0.2 nM (n=3), after 20min. In the presence of BSA, the measured concentration of ligand in solution was 8.9 ± 0.8 nM (n=3) and remained constant for 20min. Similar results were observed for the range of $VEGF_{165a}$-TMR concentrations (0.25-10nM). The linear relationship between the theoretical added concentration and the FCS detected concentration showed a slope of 0.81 ± 0.05, in the presence of 0.1% BSA, and 0.29 ± 0.02 in its absence; linear regression R^2 = 0.8 for both conditions. Under reducing conditions in the presence of BSA, the slope of the line for detected vs. nominal concentration of $VEGF_{165a}$-TMR showed increased to 1.62 ± 0.04, n=3, R^2 = 0.98, suggesting a near doubling of $VEGF_{165a}$-TMR concentration from 0.25-10nM.

Conclusions and Applications: In this study we were able to quantify the concentration of $VEGF_{165a}$-TMR in buffer in the presence or absence of 0.1% BSA. Our results showed that BSA reduces the non-specific binding of $VEGF_{165a}$-TMR ligand to the plastic surfaces and coverglass of the multiwell plates. $VEGF_{165a}$-TMR in buffer under reducing conditions showed a two-fold increase in FCS detected concentration, confirming the homodimeric nature of this fluorescent ligand.

[1] Woolard J *et al.* (2009) *Microcirculation* **16**(7): 572–92.
[2] Briddon SJ and Hill SJ (2007) *Trends in pharmacological sciences* **28**(12): 637–645.
[3] Ayling LJ *et al.* (2012) *Jounal of Cell Science* **125**: 869–886.