A First Time in Human, Microdose, Positron Emission Tomography Study of the Safety, Immunogenicity, Biodistribution and Radiation Dosimetry of $^{18}$F-FB-A20FMDV2 for Imaging the Integrin αvβ6

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**Running Title**: A first time in human PET study of αvβ6 integrin

**Key words**: FTIH, PET, αvβ6, integrin, A20FMDV2

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Abstract

The αvβ6 integrin is involved in the pathogenesis of cancer and fibrosis. A radio-labelled 20-amino acid αvβ6-binding peptide, derived from the foot and mouth virus (A20FMDV2), has been developed to image αvβ6 levels pre-clinically. This study was designed to translate these findings into a clinical PET imaging protocol to measure the expression of αvβ6 in humans.

Methods

Pre-clinical toxicology was undertaken and a direct immunoassay was developed for FB-A20FMDV2. A first time in human study of 18F-FB-A20FMDV2 was conducted in four healthy subjects (two males and two females). Each subject received a single microdose of 18F-FB-A20FMDV2. Subjects underwent a multibed positron emission tomography (PET) scan of the whole body over 3+ hours.

Results

There were no findings in the pre-clinical toxicology assessments and no anti-A20FMDV2 antibodies were detected before or after dosing with the PET ligand. The mean and standard deviation of the administered mass of 18F-FB-A20FMDV2 was 8.7 ± 4.4 µg (range, 2.7–13.0 mg). The mean administered activity was 124 ± 20 MBq (range, 98–145 MBq). There were no adverse or clinically detectable pharmacologic effects in any of the (4) subjects. No significant changes in vital signs or the results of laboratory studies or electrocardiograms were observed. Uptake of radioactivity was observed in the thyroid, salivary glands, liver, stomach wall, spleen, kidneys, ureters and bladder. Time-activity curves indicated that the highest activity was observed in the bladder content, followed by the kidneys, small intestine, stomach, liver, spleen, thyroid and gallbladder. The largest component of the residence times was the voided urine, followed by muscle, bladder and liver. Using the mean residence time over all subjects as input to Organ Level INternal Dosimetry Assessment/EXponential Modelling software (OLINDA/EXM), the effective dose was determined to be 0.0217 mSv/MBq; using residence times from
single subjects gave a standard deviation of 0.0020 mSv/MBq from the mean. The critical organ was the urinary bladder, with an absorbed dose of 0.18 mGy/MBq.

**Conclusion**

\(^{18}\text{F--FB-A20FMDV2}\) successfully passed toxicology criteria, showed no adverse effects in healthy subjects and has an effective dose that enables multiple scans in a single subject.
Introduction

The αvβ6 integrin is a cell surface adhesion receptor that, in its activated form, interacts with extra-cellular ligands bearing the Arginine-Glycine-Aspartic Acid (RGD) tri-peptide sequence (1). It plays a role in the aetiology and progression of a number of pathological conditions including cancer and fibrosis and as such it is an important prognostic biomarker as well as a potential drug target.

Key ligands for αvβ6 include the latency associated peptides (LAPs) of transforming growth factor beta1 and beta3 (LAPβ1 & LAPβ3) as well as extra-cellular matrix (ECM) ligands such as fibronectin, tenascin and vitronectin (2, 3). Binding of ECM ligands to the integrin receptor can promote cell adhesion, activation of intra-cellular signalling pathways and local release of activated TGFβ from latent complexes in the matrix (1, 4, 5). αvβ6 is up-regulated on many cancers, including pancreatic, breast, ovarian, colon, and over 90% of oral squamous cell carcinoma (6, 7). Furthermore, expression of αvβ6 correlates with development of metastasis in gastric cancer (8), is linked with a dramatic reduction in survival from colon cancer (9), and is reported to promote both the survival and invasive potential of carcinoma cells (10-13). αvβ6-mediated activation of TGFβ promotes myofibroblast differentiation, proliferation and collagen synthesis as part of normal physiological wound healing (14, 15). However, when this process persists and fails to resolve, the result is a pathological elaboration of ECM which results in irreversible organ scarring, ultimately resulting in organ failure and death (16).

The foot and mouth disease virus (FMDV) uses αvβ6 to access the intracellular environment of the host and thus causes the symptoms of foot and mouth disease. In fact, the envelope protein of the virus contains a 20–amino acid peptide sequence, NAVPNLRGDLQVLAQKVART (A20FMDV2) that mediates FMDV infection by binding to αvβ6 (17-19). Phage display analysis identified the DLXXL sequence as a key moiety responsible for αvβ6 specificity while having only minimal interactions with other RGD-integrins (e.g. αvβ3, αvβ5, and αIIbβ3) (20). The high affinity and selectivity (21) as well as the
automated and GMP compatible radiochemistry and pre-clinical radiodosimetry (22) of this A20FMDV2 peptide for αvβ6 have recently been described.

In this manuscript, we describe the safety, tolerability, immunogenicity, biodistribution and radiation dosimetry of $^{18}$F-FB-A20FMDV2 (also known as $^{18}$F-IMAFIB, $^{18}$F-GSK2634673) in preparation of its use as a PET ligand for the delineation of αvβ6 in humans. If successful, this PET ligand may be useful in the clinical management of patients with cancer or fibrosis, since levels of expression of αvβ6 may be of prognostic value (23, 24) and potentially could be used to make treatment decisions in clinical practice. In addition, it may prove to be valuable during development of new therapies that target αvβ6, as an in vivo imaging tool for demonstrating target engagement. Prior to conducting this first time in human study with $^{18}$F-FB-A20FMDV2, various potential risks were identified and strategies to manage these risks were developed. Pre-clinical toxicology was conducted to support microdosing in humans (up to a maximum mass dose of 100 µg, as detailed in the International Committee on Harmonization (ICH) M3 R2). Potential for immunogenic risk was explored through development of an immunogenicity assay to monitor pre-existing as well as induced antibodies as a result of exposure to this FMDV2 peptide within this study.

Methods

Study conduct

Subjects were screened and recruited at Hammersmith Medicines Research (HMR), London, UK and imaging assessments were conducted at the Imanova Centre for Imaging Sciences, London, UK. The study was approved by the London – Brent Research Ethics Committee, UK (reference 13/LO/1792), and permission to administer radioisotopes was obtained from the Administration of Radioactive Substances Advisory Committee (ARSAC) of the UK (Ref: 630/3925/30809). The PETAL (PET study of αvβ6 in lungs)
study is recorded in clinical trials.gov as ‘A Validation and Dosimetry Study of GSK2634673F PET Ligand’ (NCT02052297; RES116235) and the dosimetry data presented here forms part of that study.

Subjects

Four healthy human subjects (two male, two female, ages 48-65) were enrolled in the study after providing written informed consent. The main inclusion criteria were male or female subjects aged 45 years or older and free from any clinically significant illness or disease. The main exclusion criteria included prior radiation exposure to greater than 10 mSv over the past 3 years or greater than 10 mSv in a single year including the proposed study; previous or current exposure to animals that may harbour the foot and mouth disease virus (FMDV2); and previous long term (≥ 3 months) residence in a country where FMDV2 is endemic (such as certain areas of Africa, Asia and South America).

Pre-clinical Toxicology

FB-A20FMDV2 (a non-radioactive homologue of the proposed PET labelled peptide) was screened against a broad range of in vitro pharmacological targets (receptors, ion channels, enzymes and transporters) that were distinct from the intended therapeutic target (αvβ6 integrin receptor) to assess the potential for off-target interactions.

A limited number of nonclinical toxicity studies were performed to evaluate the potential toxicity of the triacetate salt of FB-A20FMDV2, based on the principles of the ICH M3(R2) guideline (ICH 2009) to support the human microdose PET study. The haemolytic potential of FB-A20FMDV2 solutions in 0.9% w/v aqueous sodium chloride (Fresenius Kabi, Bordon, UK) at concentrations of 0.04 and 0.4 mg/mL were assessed in vitro in rat and human blood. These concentrations were selected to match the concentrations used in a subsequent rat toxicity study. The rat was selected as a relevant species as they are known to express αvβ6 integrin receptors (22, 25). In order to examine the toxicity and irritancy potential, groups of 10 male and 10 female Crl:WI (Han) rats (9 to 11 week of age) received a single
intravenous dose of vehicle (0.9% w/v aqueous sodium chloride) or FB-A20FMDV2 at 0.2 mg/kg or 2 mg/kg, at a dose volume of 5mL/kg. These doses provided approximately 100-fold and 1000-fold the maximum permissible human microdose on a mg/kg basis. Animals in this cohort were sacrificed 24 hours after dosing. Additional groups of 5 male and 5 female rats were treated similarly and then remained off-dose for a 14-day period to investigate the regression/progression of any target organ toxicity present at the end of the treatment period or delayed onset of any target organ toxicity. All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals.

Immunogenicity Assay

A direct immunoassay was developed and validated using a pool of A20FMDV2 (GSK3225458) peptide labelled with biotin either at the N- or C- terminus to capture antibodies to the peptide. Captured antibodies were detected using a horseradish peroxidise (HRP) conjugated polyclonal goat anti-human IgG H+L reagent capable of binding to human antibodies regardless of isotype (Supplementary). Negative control (NC) was prepared from pooled normal human serum and was used to define sensitivity and to normalize results. Screening and confirmation cut points were determined during method validation.

Serum samples for immunogenicity screening were obtained from all four subjects prior to administration of ¹⁸F-FB-A20FMDV2 and at follow up (2 – 4 weeks after administration of ¹⁸F-FB-A20FMDV2. Samples were stored at -70°C until assayed. More details are available in the supplementary information.

Imaging

Healthy human subjects were imaged for up to 3.5 hours on a Siemens Biograph 6 TruePoint PET-CT scanner (Siemens Healthcare, Erlangen, Germany) following intravenous administration of ¹⁸F-FB-
A20FMDV2, prepared according to (22). The effective dose for each AC CT scan in this study was estimated to be 2.3 mSv using the ImPACT CTDosimetry spreadsheet system (26).

Prior to injection, each subject received a CT scan for attenuation correction (AC) over 6 to 7 PET bed lengths covering a range from vertex of head to mid-thigh using 130 kV tube potential, and 15 effective mAs exposure with 0.6 second gantry rotation time and spiral pitch of 1.5. CT image data was acquired with 6x3 mm channels, and reconstructed to 5 mm slices at 3 mm intervals and 700 mm field of view.

For each subject, PET scanning was initiated at the time of injection, and a series of 6 whole body scans was performed. Each scan consisted of 6-7 bed positions (depending on subject size), with the duration increasing for subsequent scans, through 1, 2, 5, 5, 7 and 7 minutes per bed position. Between whole body scans 4 and 5, the subject was removed from the scanner bed to void their bladder and move around for around 20 minutes to limit discomfort. After this break, a second AC CT was performed before PET imaging was resumed. PET images were reconstructed on a 256x256 matrix, with a zoom of 1.3 m using ordered subsets with expectation maximisation (OSEM) algorithm employing 3 iterations and 21 subsets and a 5 mm Gaussian image filter. Corrections for scatter, attenuation and decay (to the start of each scan) were applied.

Image Analysis

Image analysis was performed on a Siemens Inveon Research Workplace (IRW; Siemens Healthcare, Erlangen, Germany) workstation, version 4.0.1.14. Regions of Interest (ROIs) were drawn on the PET-CT datasets, delineating as many organs that are relevant to dosimetry as possible, in particular those that are defined by OLINDA/EXM, version 1.1 (Organ Level INternal Dosimetry Assessment/EXponential Modelling software) (27) as source organs. CT anatomy and/or PET uptake was used as a guide, with one set of ROIs being drawn for the first four scans, and another drawn after the bladder void and rest prior to scans 5 and 6. A separate bladder ROI was used for each frame, which took into account the change
in size as the bladder filled and emptied over the course of the study. The activity concentrations in these ROIs were exported from IRW, and imported to Microsoft Excel (Microsoft, Redmond, Washington, US) for further analysis. Organ ROI activity concentrations were tabulated for each frame, to create time activity curves (TAC) which were integrated, with the activity in each organ at the end of the sixth scan being assumed to decay in situ without further redistribution. These values were then multiplied by the organ volume in the Cristy and Eckerman adult phantom according to (28) to calculate the organ residence time (this is the equivalent duration in hours that a single MBq of activity is present in that organ per injected MBq of radioligand, units of MBq.h/MBq).

The bladder was treated separately, with the total activity in the bladder in each frame being input to a model employing an exponential fill and void, fitting parameters for the exponential rate and magnitude as well as the void time and voided fraction. This model was then used with a 2 hour voiding period being fitted to the measured data, and integrated to calculate the bladder residence time.

The total residence times for all organs for an injected radioligand should add up to the mean lifetime for that isotope, given by the half-life divided by the natural logarithm of 2, which for fluorine-18 is 2.64 hours. A ‘remainder’ residence time was assigned to the activity injected which was not measured within ROIs in the image, and is given by the mean lifetime minus the total residence time over all organs and voided urine.

The mean organ residence times over all subjects were used as the input to OLINDA/EXM, which calculated the absorbed dose to each of the relevant target organs (units mGy/MBq), and provided the resulting effective dose per unit injected activity (units mSv/MBq). Organs without specific tissue weighting factors result in an effective dose contribution of 0, as do the testes due to OLINDA/EXM using the higher of the testes and ovaries absorbed doses to characterise the gonad dose.
To provide a measure of the variability of this result, the effective dose per unit injected activity was also calculated using the residence times from each individual subject.

Results

Pre-clinical Toxicology

FB-A20FMDV2 did not demonstrate any off-target activity when screened against a broad range of in vitro pharmacological targets (receptors, ion channels, enzymes and transporters), including a cardiovascular liability panel. Since FB-A20FMDV2 is a large molecular weight peptide, access to the ion channels was limited and it was considered unlikely the peptide would affect the hERG channel. Therefore, taken together, further nonclinical safety pharmacological assessment was not considered necessary.

The haemolytic potential of FB-A20FMDV2 in 0.9% w/v aqueous sodium chloride at concentrations of 0.04 and 0.4 mg/mL was assessed in vitro in rat and human blood. As anticipated, no evidence of haemolysis was observed at either concentration tested, and therefore, this formulation was deemed suitable for intravenous use. Local irritancy and target organ toxicity was investigated in an intravenous extended single dose toxicity study in rats at 0.2 or 2 mg/kg with a 14 day off-dose period. Both doses were well tolerated and no treatment-related clinical signs of toxicity, changes in body weight gain or food consumption were observed. In addition, no treatment related changes in clinical pathology parameters (haematology, clinical chemistry and coagulation) were observed at either dose. At both terminal necropsy and following the 14 day off-dose period, there were no FB-A20FMDV2 -related macroscopic or microscopic observations. Stage-dependent qualitative evaluation of spermatogenesis in the testes was performed on all male rats given vehicle and 2 mg/kg FB-A20FMDV2. The testes revealed normal progression of the spermatogenic cycle and the expected cell associations and proportions in the various stages of spermatogenesis were present.
Thus FB-A20FMDV2 was shown to be non-toxic and supports microdosing in humans up to 100 μg $^{18}$F-FB-A20FMDV2 (equivalent to 2 μg/kg for a 50 kg individual), with anticipated cover (at the human dose) of 1000-fold on a mg/kg basis.

Clinical Safety and tolerability

The PET scans were well-tolerated and all four subjects completed the scans as planned. None of the 4 subjects imaged had any $^{18}$F-FB-A20FMDV2 or PET procedure associated adverse events. Three AEs were reported in this study comprising abdominal cramps, erythema of conjunctiva and heaviness of arms. The three AEs occurred in two subjects. Conjunctival erythema occurred in subject 1 and heaviness of arms and abdominal cramps occurred in subject 4. Subject 4 who had two of the three adverse events had the lowest mass injected (2.7 μg), whereas subject 1 who had the other adverse event had the highest mass administered (13 μg). Therefore, there was no relationship with the mass administered. All the AEs were mild and transient and resolved the same day. Causality was determined by the study physicians based on their combined experience of Phase I trials as well as studies of other novel radiotracers. In the opinion of the investigator none of these AEs was due to the radioligand or the imaging procedure.

Immunogenicity

The screening and confirmation cut points were statistically established during validation and were 2.10 relative O.D. (ROD) and 48.4% inhibition, respectively. To differentiate peptide-boosted true positive from the pre-existing positive responses in healthy subject population, a ratio of post-dose ROD over pre-dose ROD was calculated. Samples with a ratio of ≥2 were reported as peptide-boosted positives. Based on clinical sample testing results, all four human subjects were negative in the anti-peptide antibody assays. Details of the assay validation are available in the supplementary information.
PET imaging

Four subjects were scanned, with demographic and radioligand administration details given in Table 1. PET images, through a single coronal slice, are presented in Figure 1 and show the activity uptake and clearance by tissue organs over the 3+ hour total study time for subject 1. Organs that are clearly visible in this slice through the body include the thyroid, salivary glands, liver, stomach wall, spleen, ureters and bladder. Figure 2 shows coronal and sagittal CT views through subject 1, overlaid with organ ROIs.

Time-activity curves (TACs) from the ROIs through the 13 most active organs are shown in Figure 3 (colours of organ ROIs and TACs are matched. TACs with max value < 2 kBq/mL are not shown and include: muscle, testes, cortical bone, trabecular bone, bone marrow, lungs and brain). Greatest activity concentration was observed for bladder content which, in contrast to the other organs, increases after the first scan, with the interruption of the void at 2 hours. The other notable organs of uptake include the kidneys, small intestine, stomach wall, liver, spleen, thyroid and gallbladder, whose TACs generally have their highest point in scan 1, and decrease broadly linearly over this logarithmic graph, indicating exponential clearance. TACs for all four subjects were broadly similar.

Organ residence times for OLINDA source organs that could be identified from scan images, for each subject, as well as the mean across all subjects, are shown in Table 2. Residence times for breast tissue were assessed for female subjects only, and testes for males only. ‘Organ total’ is the sum of the residence times measured in individual organ ROIs. ‘Voided urine’ is calculated from the quantity of urine voided according to the modelled bladder fit. The largest component of the mean residence time is the voided urine, followed by remainder, muscle, bladder and liver. The residence time is affected not only by the activity concentration but also the organ mass, hence the influence of larger organs like muscle and liver.
The mean residence times were entered into OLINDA/EXM, and the results are shown in Table 3, showing the organ beta, gamma and total absorbed doses (mGy) and effective dose contribution (mSv) from each organ, as well as the total effective dose per injected MBq. The beta dose to the stomach wall was adjusted to correct for OLINDA/EXM’s use of the stomach contents as a source organ, rather than the wall itself.

The critical organ is the urinary bladder, with an absorbed dose of 0.198 mGy/MBq. The effective dose derived from the mean of the organ residence times was 0.0217 mSv/MBq. The effective dose derived by using each individual subject’s residence times range from 0.0217 to 0.0247 mSv/MBq, resulting in a standard deviation of 0.0020 mSv/MBq.

Discussion

This study describes the first-in-human administration of 18F-FB-A20FMDV2 a PET ligand that has been shown pre-clinically to bind with high affinity and specificity to the integrin αvβ6 (21, 22, 29-31). αvβ6 is involved in the aetiology and pathogenesis of cancer and fibrosis and its expression levels have prognostic and theranostic implications for patient management and drug development. Thus, a PET ligand that can be used to non-invasively measure expression levels of this integrin in humans is likely to have broad clinical application in both patient management and the development of new treatments that target αvβ6. The current study provides information about the safety, tolerability and biodistribution of 18F-FB-A20FMDV2 in healthy subjects, as well as the radiation dosimetry of this ligand.

The observed benign profile in the four humans tested in this study supports the lack of findings in the pre-clinical toxicology. In addition, no antibodies to the ligand were detected either before or after administration of the ligand.
Immunohistochemistry in various mouse organs, using a monoclonal antibody specific for αvβ6, revealed that this integrin is expressed constitutively at high or moderate levels in the epithelium of the gall bladder, stomach, duodenum, ileum and colon (32). Low levels of αvβ6 expression were detected in the mouse skin (32). These data were supported by SPECT imaging data using indium-labelled A20FMDV2 but the lung was added to the list of organs expressing αvβ6 (32). PET images from the current human study show focal uptake in the small intestine, stomach, liver, spleen, thyroid and gallbladder, but the majority of the activity was cleared to the bladder by the kidneys. The largest organ doses were found within the abdominal cavity, with the highest value to the bladder wall, then the kidneys, liver, small intestine, uterus, stomach and spleen.

The effective dose, which is 0.0217 mSv/MBq, is lower than the figure from rat data of 0.0335 mSv/MBq (22), largely resulting from the lower residence time in the bladder and small intestine in human, which have longer residence times in rat. The result is fairly typical for a fluorine-18 based ligand, and is close to the value of 0.019 mSv/MBq (33) for the most widely used PET ligand, ¹⁸F-Fludeoxyglucose (¹⁸F-FDG). The critical organ is the urinary bladder, which is common for a ligand that is cleared from the body by a renal route.

In conclusion, ¹⁸F-FB-A20FMDV2 is a safe PET radioligand for future clinical studies to investigate changes in αvβ6 receptor availability as a result of increased expression in disease or following direct competition with a drug candidate. It has the potential to be used as a diagnostic, prognostic and theranostic marker in the clinical management of cancer and fibrosis. In addition, ¹⁸F-FB-A20FMDV2 may be used in the development of novel therapies that target αvβ6.
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Disclosures

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References


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## Tables

### Table 1. Subject demographic and scan details

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<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Scan duration (minutes)</th>
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<th>Specific activity (GBq/µmol)</th>
<th>Injected mass (µg)</th>
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Table 2. Residence times (hours) of 18F-FB-A20FMDV2 for each organ (each individual and mean)

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<tr>
<th>Residence time (MBq.h/MBq)</th>
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<th>Subject 3</th>
<th>Subject 4</th>
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<td>Organ total</td>
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<td>Voided urine</td>
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Table 3. Absorbed and effective dose to each organ

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<tr>
<th>Target Organ</th>
<th>Beta absorbed dose (mGy/MBq)</th>
<th>Gamma absorbed dose (mGy/MBq)</th>
<th>Total absorbed dose (mGy/MBq)</th>
<th>Effective Dose Contribution (mSv/MBq)</th>
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<tr>
<td>Adrenals</td>
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<td>ULI Wall</td>
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<tr>
<td>Pancreas</td>
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<td>Osteogenic Cells</td>
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<td>9.36E-05</td>
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<tr>
<td>Testes</td>
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<td>Urinary Bladder Wall</td>
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<td>6.26E-02</td>
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<td>9.92E-03</td>
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<tr>
<td>Uterus</td>
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<tr>
<td>Total Body</td>
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<td>5.48E-03</td>
<td>8.33E-03</td>
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</table>

**Effective Dose (mSv/MBq)**: 2.17E-02
Figure 1. PET activity though a single coronal slice for subject 1, over the 6 scans (scale 0-10 kBq/mL, mid scan time points shown in h:mm:ss).
Figure 2. Organ Regions of Interest (ROIs) for subject 1, organs are coloured to match those in TACs (Figure 3)
Figure 3. Organ time-activity curves for subject 1
Supplementary information

Immunogenicity assay methodology

Materials used were as follows: A20FMDV2 peptide (GSK2634673E), N-terminal biotin-peptide (GSK3225458A), C-terminal [4-FBA]-peptide-biotin (GSK3228218A), Smart Block (Candor Cat # 113-500), Low cross buffer (Candor Cat # 100-500), normal goat serum (Lampire Cat # 7332500), human healthy donor serum (Bioreclamation), biotinylated human IgG (Jackson Cat # 009-060-003), goat anti-human IgG (H+L) HRP conjugate (Jackson Cat #109-035-088), goat anti-rabbit IgG (H+L) HRP conjugate (Jackson Cat # 111-035-045), Nunc streptavidin plate (Nunc Cat # 436014), 10X PBS, 1% Tween 20 (GSK Media Prep), rabbit anti-GSK2634673E positive control antibody pool (GSK lot #. DIO2.3/197923), human plasma fibronectin (EMD Millipore, lot #. 2361790), EXL-405 plate washer (Biotek) and Spectramax plate reader (Molecular Devices).

The negative control (NC) was prepared by pooling 54 normal human serum (NHS) and positive control (PC) was prepared by spiking rabbit anti-peptide antibody in NC serum at 500 ng/mL for high PC and 25 ng/mL for low PC. Unlabelled peptide and N-/C-terminal biotin-labelled peptides (1:1) were tested for plate coating with different fractions of glycine eluded (PC1A, PC2A) and TEA eluded (high affinity and binding, PC1B, PC2B) from two batches of PCs at 1000, 500, 100 and 10 ng/mL. Assay sensitive, PC specificity and RGD motifs interference were evaluated during method validation.

The anti-peptide antibody (APA) was detected by a validated direct binding ELISA. Briefly, assay controls and samples were diluted at 1:50 in assay buffer containing biotin-peptides (2.5 µg/mL) and incubated for 3 hours at room temperature. The pre-incubated samples were transferred to Nunc streptavidin coated plate for one hour incubation. After a wash step, a cocktail of HRP conjugated goat anti-human IgG (H+L) and anti-rabbit IgG (H+L) was added to the plate and incubated for one hour. After wash away any unbound, TMB substrate was added to the plate. The bound conjugates produced colour reaction and the intensity of the signal was directly proportional to the amount of detected antibodies in the sample. Biotinylated human IgGs were used as a plate positive control (Error! Reference source not found.).

To determine a screening cut point, 76 healthy subjects (38 males and 38 females) were tested 3 times by 2 analysts on 2 different days to determine a potential positive (above the cut point) or negative (below). For confirmation assay, 20 randomly-selected subjects (10 males, 10 females) were spiked with 25 ng/mL of PC and the spiked (positive population) and unspiked (negative population) samples were then incubated in the presence and absence of excess peptide (50 µg/mL).

Clinical serum samples (pre- and week 3) were collected from the 4 healthy subjects enrolled in the current study and tested in this immunogenicity assay.

The A20FMDV2 peptide has 100% homology with foot and mouth disease virus (FMDV), type O, VP1 139-158. The peptide strongly and specifically binds to αVβ6 receptor through its RGD motifs. Compared to integrin receptor ligands in vivo, the sequence 1-6 and 11-20 are non-humanized and may potentially raise Abs (Supplementary Figure 5). The RGD motifs of peptide may also be immunogenic to human. Furthermore, if a polyclonal positive control contains anti-RGD motif Abs, it may also bind to ligands such as fibronectin, tenascin-C, fibronogen, LAP-TGFβ and vitronectin in serum resulting in assay issue. Since fibronectin (FN, MW 460 kDa, dimer) is one of ligands specifically binds to αv and β6 chains
of receptor, it was tested with PC during method development to evaluate PC specificity. Results showed that rabbit polyclonal anti-peptide antibody didn’t bind to FN and other RGD motif proteins in human serum and could be used as a positive control for the APA assays.

Utilization of both N- and C-terminal labelled peptides as the capture reagents can potentially eliminate epitope-masking induced false negatives and enhance the direct ELISA’s sensitivity and drug tolerance. This approach may be applied to the immunogenicity method development for other small peptide biotherapeutics, where there may be a concern of risk of epitope-masking when labelling of the peptide is required or is directly immobilized on solid surface. Furthermore, this direct ELISA format can be used for screening, confirmation, titration and isotyping assays. The APA method has been successfully used for this study of four healthy subjects and for other on-going study (data not shown) and will be further validated in patients with idiopathic pulmonary fibrosis (IPF). The impacts of pre-existing antibodies on efficacy, safety and immunogenicity will be studied.

Immunogenicity assay results
To avoid epitope masking due to plate coating or biotinylation of peptide, a mixture of N- and C-terminal biotinylation peptides was tested on 96-well streptavidin plates compared to unlabeled peptide on regular plates. The results shown that the signals of PC1A were higher than those of PC2A; PC2B and PC2B had very low or negative responses on peptide coated plates. The sensitivity of peptide coated plate assay was poor (≥100n g/mL). However, all PCs had strong and similar responses on biotin-peptide plates (Supplementary Figure 3 and Supplementary Figure 4). Assay positive control didn’t have cross-reactive to endogenous RGD-containing proteins and its signals were not inhibited in the presence of FN at concentrations up to 50µg/mL (Supplementary Figure 4). The %inhibitions of PC at 20, 100 and 500 ng/mL by peptide were 53, 73 and 79%, respectively (Supplementary Figure 5). The sensitivity was demonstrated to be 9.2 ng/mL of peptide at 100 ng/mL of PC. The peptide interference level was 100 µg/mL of peptide at 100 ng/mL of PC. The intra- and inter-run precisions for PC samples were 16.9% and 9.0%, respectively.

The screening and confirmation cut points were statistically established during validation and were 2.10 relative OD (ROD) and 48.41% inhibition, respectively. Among 75 healthy subjects, 17% (13/76) were screened and confirmed positive by peptide inhibition and excluded for screening cut point determination. To differentiate peptide-induced true positive from the prevalence of the positive response in clinical subjects, a ratio of pre-dose ROD over post-doses ROD was calculated for all subjects who had a confirmed pre-dose positive samples. Sample with a ratio of ≥2 was reported as peptide-induced positives. Based on clinical sample testing results, all four human subjects were negative in APA assays.

The A20FMDV2 peptide has 100% homology with foot and mouth disease virus (FMDV), type O, VP1 139-158. The peptide strongly and specifically binds to αVβ6 receptor through its RGD motifs. Compared to integrin receptor ligands in vivo, the sequence 1-6 and 11-20 are non-humanized and may potentially raise Abs (Supplementary Figure 5). The RGD motifs of peptide may also be immunogenic to human. Furthermore, if a polyclonal positive control contains anti-RGD motif Abs, it may also bind to ligands such as fibronectin, tenascin-C, fibronogen, LAP-TGFβ and vitronectin in serum resulting in assay issue. Since fibronectin (FN, MW 460 kDa, dimer) is one of ligands specifically binds to αv and β6 chains of receptor, it was tested with PC during method development to evaluate PC specificity. Results showed that rabbit polyclonal anti-peptide antibody didn’t bind to FN and other RGD motif proteins in human serum and could be used as a positive control for the APA assays.
Utilization of both N- and C-terminal labelled peptides as the capture reagents can potentially eliminate epitope-masking induced false negatives and enhance the direct ELISA’s sensitivity and drug tolerance. This approach may be applied to the immunogenicity method development for other small peptide biotherapeutics, where there may be a concern of risk of epitope-masking when labelling of the peptide is required or is directly immobilized on solid surface. Furthermore, this direct ELISA format can be used for screening, confirmation, titration and isotyping assays. The APA method has been successfully used for this study of four healthy subjects and for other on-going study (data not shown) and will be further validated in patients with idiopathic pulmonary fibrosis (IPF). The impacts of pre-existing antibodies on efficacy, safety and immunogenicity will be studied.

Supplementary Figure 1. Assay Format

![Supplementary Figure 1. Assay Format](image-url)
Supplementary Figure 2. Positive Control Signals in Peptide Coated Plate

![Bar chart showing positive control signals in peptide coated plate](image)

**Note:** this assay format had poor assay sensitivity and highly false-negative risk.

Supplementary Figure 3. Positive Control Signals in Biotin-Peptide Coated Plate

![Bar chart showing positive control signals in biotin-peptide coated plate](image)

**Note:** N-/C-terminal biotin-peptides eliminated false-negative, enhanced sensitivity and reduced peptide interference.
Supplementary Figure 4. PC Ab Specificity Test with Fibronectin

Note: the assay positive control antibody was specific to non-human regions of peptide and didn’t have cross-reactive to RGD motifs on fibronectin.

Supplementary Figure 5. Assay Specificity (Signal %Inhibition)

Note: Positive control signals could be specifically inhibited by peptide.
<table>
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<tr>
<th>Protein</th>
<th>Sequence</th>
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<td>GSK2654673</td>
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<tr>
<td>FMDV type O, VP1</td>
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<td>FMDV type C, VP1</td>
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<td>Fibronectin III (dimer)</td>
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<td>Vitronectin</td>
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**RGD**
- Specifically binding to integrins (αvβ6, β1, β3, β5...integrins)

**DIXL**
- Highly specific inhibition of RGD-dependent ligand binding to αvβ6 integrin

Many other integrin ligands (e.g., collagen, laminin...) and some virus proteins also contain RGD motif

GSK2654673: [18F]-FBA-A25FMDV2 peptide (20 a.a. MW: 2284)

4-Fluorobenzamide (FBA) is a linker