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Research paper

Structure-activity relationship study of the tumour-targeting peptide A20FMDV2 via modification of Lys16, Leu13, and N- and/or C-terminal functionality

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ABSTRACT

The 20-residue linear peptide A20FMDV2 has been shown to exhibit high selectivity and affinity for the tumour-related αvβ6 integrin and has potential as a vector for therapeutic drugs. However, it exhibits poor half-life in plasma in part due to its high susceptibility to serum proteases. In this study fourteen A20FMDV2 analogues incorporating non-proteinogenic substitutes of the native Lys16 and Leu13 residues and six A20FMDV2 analogues containing modified N- and C-termini were synthesised to increase the half-life and activity of A20FMDV2. The analogues incorporating modified terminal motifs of A20FMDV2 were found to strongly bind to the αvβ6 integrin and were subsequently functionalized with the diethylenetriaminepentaacetic acid chelating agent to facilitate coupling with radioactive indium-111 for human plasma stability and *in vivo* biodistribution studies. A20FMDV2 peptide variants incorporating an N-terminal D-Asn and C-terminal D-Thr exhibited improved relative activity in vitro and were less susceptible to plasma degradation.

1. Introduction

Integrins are αβ heterodimeric molecules that encompass a large family of cell surface receptors involved in several key processes including cell adhesion, invasion, proliferation and apoptosis [1]. In humans there are 24 members of which 8 classes recognize substrates via a highly conserved tripeptide motif Arg-Gly-Asp, present on extracellular ligands such as fibronectin and vitronectin [2]. Integrin αvβ6 is expressed in high levels on numerous cancers [3–8] such as oral, head and neck, pancreatic, ovarian and breast and increased expression level of integrin αvβ6 has been correlated with tumour progression. Integrin αvβ6 also can promote fibrosis [9] and some viral infections including foot and mouth disease [10]. As such, integrin αvβ6 is a major target [11,12] for imaging, diagnostics and therapy in the field of oncology and beyond.

A20FMDV2 (H-N^{1}AVPNLRGDLQVLAQKVART^{20}-OH) is a 20-residue linear peptide derived from the viral protein of foot-and-mouth disease virus [13–15]. This peptide has been shown to exhibit high selectively and affinity for αvβ6, that is highly expressed on cancer cells. A20FMDV2 binds to αvβ6 through the Arg-Gly-Asp tripeptide of the Arg^{7}-Gly-Asp-Leu-Gln-Val-Leu^{13} fragment, and the two leucine residues present in the RGDLQVL fragment enhance the binding of the peptide to the receptor *via* hydrophobic interactions [15]. The peptide is also stabilised by an α-helix at the C-terminal region, LQVLAQKVART [13].

A20FMDV2 has been used in positron emission tomography (PET) for diagnostic imaging applications [16] by conjugating to 4-[^{18}F]fluorobenzoic acid (FBA) (or derivatives) [17–19], and ^{64}Cu labelling using A20FMDV2 peptide that incorporates a metal

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chelator such as DOTA [20–22]. Recently, [^{18}F]-FBA-A20FMDV2 has progressed to a clinical setting [23] in the treatment regime of idiopathic pulmonary fibrosis [24] while an ^{111}In labelled A20FMDV2 derivative has been shown to be highly specific for imaging of breast cancer expressing the αvβ6 integrin using single-photon emission computed tomography (SPECT) [25]. These studies outline the importance of A20FMDV2 to ongoing diagnostic tools targeting the αvβ6 integrin and underpins αvβ6 as a valid therapeutic target.

The therapeutic value of A20FMDV2 is limited by its short half-life in blood caused, in part, by its high susceptibility to serum proteases such as endo- and exopeptidases. Hausner et al. [17] reported that 4-[^{18}F]fluorobenzoyl-A20FMDV2-amide was rapidly degraded by plasma proteases into three distinct metabolites, although the peptide sequences were unable to be identified, although it was postulated that a possible cleavage site was at ^{10}Leu-^{11}Gln. Similarly, ^{111}In-DTPA A20FMDV2-acid (DTPA = diethylenetriaminepentaacetic acid), when exposed to mouse serum exhibited a half-life of 4 h [25]. This data suggests that improving the half-life is therefore a key step in the development of A20FMDV2 as a clinically viable cancer-targeting drug.

One proven method of improving half-lives of susceptible peptides (and proteins) is the introduction of polyethyleneglycol (PEG) [26]. Hausner et al. prepared PEGlyated versions of 4-[^{18}F]-FBA-A20FMDV2, by placement of one or two PEG_{28} moieties at the N terminus [27] or a single PEG_{28} at both the N and C termini [28] and concluded that a bi-terminally PEGylation A20FMDV2 derivative, [^{18}F]-FBA-PEG_{28}-A20FMDV2-PEG_{28} exhibited the most favorable pharmacokinetics. An increase in affinity for cells displaying the αvβ6 integrin was also noted. To the best of our knowledge, these findings are the sole reports on the modification of the parent A20FMDV2 peptide and to date a systematic evaluation has not been undertaken.

There are other approaches that can be used to minimise enzymatic peptide degradation [29]. To evade endopeptidases which cleave internal peptide bonds, pseudopeptides such as azapeptides and peptoids are used to either protect or replace the target peptide bonds. Alternatively, incorporation of non-proteinogenic amino acids such as D-amino acids and *N*-alkylated amino acids can offer a more economical option to increase plasma stability against endopeptidases. To minimise degradation by exopeptidases which cleave amino acid residues from peptide termini, the use of terminal D-amino acids or simple modification of the *N*-amino and *C*-carboxyl groups can prevent site recognition by specific peptidases [30]. Head-to-tail cyclisation of linear peptides is also a well-established method to minimise degradation by exopeptidases [31]; however, careful experimentation is required to prevent undesired side reactions such as polymerisation or racemisation.

In this study the amino acid residues of A20FMDV2 prone to enzymatic recognition have been structurally modified to enable investigation of the pharmacological effects of non-proteinogenic amino acids on A20FMDV2 activity and stability in human plasma. The native Lys16 and Leu13 amino acids were selected to undergo structural modification as these amino acids are readily cleaved by endopeptidases such as trypsin (Lys), chymotrypsin (Leu) and pepsin (Leu) [32]. As the Leu13 residue is involved in the specific binding of A20FMDV2 to the αvβ6 integrin via hydrophobic interactions, it was envisaged that the use of appropriate non-proteinogenic hydrophobic substitutes of Leu might also enhance the binding activity of A20FMDV2. Five A20FMDV2 analogues incorporating non-proteinogenic substitutes of Lys16 and nine analogues containing non-proteinogenic and hydrophobic substitutes of Leu13 were synthesised by Fmoc solid-phase peptide synthesis (SPPS). To investigate the impact of exopeptidases on peptide activity and degradation, six A20FMDV2 analogues incorporating modified N- and C-termini and D amino acids at the N- and C-termini were also synthesised by Fmoc SPPS.

To enable binding activity to the αvβ6 integrin by flow cytometry to be measured, all peptides were synthesised incorporating a Lys2 (D-biotin) residue instead of native Ala [24,25]. Following activity studies, biotinylated peptides exhibiting potent binding activity were then selected and the metal chelator diethylenetriaminepentaacetic acid (DTPA) [33] was incorporated to facilitate coupling of ^{111}In for plasma stability studies and *in vivo* biodistribution studies. The synthesis, binding studies and human plasma stability of biotinylated A20FMDV2 (**1**), its non-proteinogenic derivatives **2**–**15**, N-terminally acetylated or/and C-terminally amidated mimics **16**–**18,** D amino acids variants **19**–**21** and biotinylated and DPTA-incorporated **22**–**26** analogues, are described in detail below.

2. Results and discussion

2.1. Synthesis of biotinylated A20FMDV2 ***1****, Lys16 or Leu13-modified and biotinylated peptides* ***2****–****15****, N- and/or C-terminus-modified and biotinylated peptides* ***16****–****21*** *and (DTPA)-containing peptides* ***22****–****26***

To enable investigation of introducing non-proteinogenic amino acids (listed in Fig. 1) on A20FMDV2 binding activity, biotinylated peptides **1**–**15** (see Table 1), except for peptide **6**, were synthesised by standard Fmoc SPPS on the acid liable hydroxymethylphenoxypropionic acid linker (HMPP) which delivers a C-terminal carboxylic acid using to the conditions depicted in Scheme 1. The desired peptide sequences were assembled using 20% piperidine/DMF to remove the Fmoc protecting group and *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)/DIPEA as coupling reagents.

Since specific binding to the αvβ6 integrin was to be studied by flow cytometry, the native alanine at the second residue in A20FMDV2 (**1**) and all analogues thereof, were substituted with a biotinylated lysine residue. This substitution has previously been shown to be well tolerated [24,25]. We chose to install the D -biotin moiety by selective deprotection of a 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) [34] group on the side chain *N*ε-amino group followed by condensation with D-biotin using HBTU/DIPEA. Trifluoroacetic acid (TFA)/H_2O/3,6-dioxa-1,8-octanedithiol (DODT)/triisopropylsilane (TIPS) (94:2.5:2.5:1.0, v/v/v/v) effected cleavage of the synthesised peptides from the corresponding peptidyl-resins. Peptides **1**–**15** were obtained in good yields ranging from 2% to 50% and purity exceeding 99% (see peptide characterization data, SI).

For the synthesis of peptide **6** containing an *N*-L-methyllysine modification we employed an on-resin N-methylation protocol [36] which furnished peptide **6** in good yield (30%) following TFA-mediated peptide cleavage and RP-HPLC purification.

The lead peptide, A20FMDV2, which contains all naturally-occurring amino acids would be susceptible to degradation by exopeptidases which act on the amino- and carboxy terminuses. To mitigate this, six N- and/or C-terminus-modified and biotinylated A20FDMV2 mimics were prepared wherein we systematically modified the amino and carboxy ends (peptides **16**–**18**) and the N-terminal and C-terminal amino acids (Asn1 and Thr20, respectively, peptides **19**–**21**). N-terminal/C-terminal modified peptides **16**–**18** were obtained by capping of the N-terminus with acetic anhydride (**16**) or by employing the Rink amide linker to afford the C-terminal carboxamide (**17**) or a combination of both (peptide **18**).

Peptide **19**, bearing the unnatural D-Asn1 in place of the native Asn1 at the N-terminus of biotinylated A20FMDV2 (**1**) was obtained