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Short communication

Evaluation of a synthetic peptide as a replacement for the recombinant fusion protein of respiratory syncytial virus in a potency ELISA

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ABSTRACT

This report describes the development of a potency ELISA using a peptide derived from the motavizumab binding epitope of respiratory syncytial virus (RSV) F-protein. Motavizumab is an antibody therapeutic studied for the prevention of RSV disease. It binds to the RSV glycoprotein F (F-protein), blocking the ability of RSV to fuse with target cells. This binding is the basis for a potency ELISA, however, due to inefficient F-protein production, development of an alternative ligand for the potency ELISA was investigated. A series of synthetic peptides spanning the motavizumab epitope on F-protein were evaluated for motavizumab binding activity. A 26-mer peptide was identified with desirable motavizumab binding kinetics, as shown by ELISA and surface plasmon resonance. The peptide corresponds to a portion of the motavizumab binding domain on the F-protein, and is referred to as F-peptide. The binding of motavizumab to the F-peptide is used in a new motavizumab potency ELISA, which was shown to be robust and statistically comparable to the F-protein ELISA. In addition, based on a qualitative observation, this new ELISA may be able to detect motavizumab degradation with greater sensitivity compared to the F-protein ELISA.

1. Introduction

Respiratory syncytial virus (RSV) causes acute respiratory tract infections and is one of the most common childhood diseases. Infants are particularly prone to RSV infection; virtually all infants are infected with RSV prior to their second birthday. Furthermore, re-infection with RSV is common, potentially leading to serious upper and lower respiratory tract disease, especially in the elderly, immunocompromised, or in patients with cardiopulmonary disease [1,2]. RSV infectivity is facilitated, in part, by two viral coat glycoproteins, F and G. RSV G-protein binds to a specific receptor on the cell surface while the F-protein initiates fusion of the virus with the cell. In addition, F-protein is expressed on the surface of infected cells and precipitates cell–cell fusion of RSV infected cells, leading to the formation of syncytia [3].

RSV F-protein is a prime target for RSV neutralizing antibodies [4–6] and is an inviting target for development of RSV vaccines and small molecule anti-virals [7,8]. Motavizumab is a humanized monoclonal antibody therapeutic that binds to the RSV F-protein and subsequently blocks the ability of RSV to enter host cells [5,9]. The interaction between RSV F-protein and motavizumab serves as the basis for an ELISA that measures motavizumab potency. Therefore, a continuous supply of purified F-protein is required. However, recombinant F-protein production and purification have proven to be difficult due to inefficient expression of recombinant F-protein [10].

Due to the difficulties in getting a consistent F-protein reagent for the motavizumab potency assay, the use of a fragment of the full-length F-protein was explored. Because of the linear nature of the highly conserved binding epitope for motavizumab [11,12], a synthetic peptide, representing the motavizumab binding region on the antigenic site A of the full-length F protein, was selected to provide an alternative to the F-protein in the potency ELISA. This report describes the selection and suitability of a synthetic peptide ligand to be used in place of RSV F-protein in motavizumab potency ELISA.

2. Materials and methods

2.1. Materials

2.1.1. F-protein

Recombinant RSV F-protein was produced in-house from baculovirus-Sf9 insect cells and purified by affinity chromatography as described [5,13].

Abbreviations: ELISA, enzyme-linked immunosorbant assay; IgG, immunoglobulin G.

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Table 1
Binding of motavizumab to F-protein derived peptides.

Peptide	Amino acid sequence	EC_{50} of motavizumab binding measured by potency ELISA (M)
Wild-type F-protein sequence (residues 248–283)	STYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQ	N/A[a]
F-peptide-10	NSELLSLINDMPITNDQKKLMSNNOC	$8.5 \times e^{-10}$
F-peptide-11	NSELLSLINDMPITNDQKKLMSNNC	$1.1 \times e^{-9}$
F-peptide-12	NSELLSLINDMPITNDQKKLMSNN	$4.6 \times e^{-9}$
F-peptide-30C	NSELLSLIHDMPITNDQKKLMSNNVQIVRQ	$7.1 \times e^{-10}$
F-peptide-30N	STYMLTNSELLSLIHDMPITNDQKKLMSNN	$8.1 \times e^{-10}$

[a] N/A: not applicable.

2.1.2. Peptide synthesis

The peptides used in this study were synthesized by AnaSpec (Fremont, CA). The peptides were synthesized under GMP/GLP guidelines and supplied with a certificate of analysis.

2.2. Methods

2.2.1. Potency ELISA

Microtiter plates (Corning, Lowell, MA) were coated with 4 μg/ml of either recombinant F-protein or F-peptide in phosphate buffered saline (PBS) overnight at 4 °C. The plates were washed three times with PBS + 0.05% Tween-20 (PBST) and then blocked for 1 h with 250 μl/well of PBST + 0.5% bovine serum albumin (BSA; Sigma–Aldrich, Saint Louis, MO). After washing, the standard curve, controls and motavizumab sample dilutions were loaded onto each plate and incubated for 1 h at room temperature. The plates were washed three times with PBST and then goat anti-human IgG H + L (KPL, Gaithersburg, MD) was added to each well. Following a 30 min incubation and a final wash step, the plates were developed with TMB (3,3′,5,5′-tetramethylbenzidine; KPL), stopped after 10 min with 2N H_2SO_4 (VWR, West Chester, PA) and read at a wavelength of 450 nm on a spectrophotometer.

The motavizumab standard curve was optimized to include the upper and lower asymptotes of the curve to ensure an accurate measurement of the half-maximal effective dose (ED_{50}) value. This was achieved by extending the curve range for both standards and sample from 1000 to 0.46 ng/ml using 3-fold serial dilutions.

2.2.2. Surface plasmon resonance (SPR) analysis

All SPR equipment and reagents were purchased from GE Healthcare Biosciences (Uppsala, Sweden), unless otherwise noted. All SPR analysis was performed at 25 °C on a Biacore 3000 instrument. Goat anti-human IgG was immobilized on two flow cells of a research grade Sensor Chip CM5 using standard amine coupling chemistry. HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% P-20, pH 7.4) was used as the continuous flow buffer. Motavizumab was diluted to 10.9 μg/ml and injected for 1 min at a flow rate of 5 μl/min across one goat anti-human IgG flow cell. The other goat anti-human IgG flow cell served as a reference and was not injected with motavizumab. For the kinetic ranking of peptides and F protein binding to motavizumab, each F-protein or candidate peptide sample was diluted to 100 nM and injected for 2 min at a flow rate of 20 μl/min across the motavizumab-containing flow cell and the reference flow cell. Dissociation of each sample was monitored for 2 min. After dissociation, the surface was regenerated with 10 mM glycine, pH 1.7. BIAevaluation software version 4.1 was used for data processing and evaluation. Sensorgrams for all samples were fit simultaneously to a 1:1 interaction model.

Table 2
SPR determination of the motavizumab kinetic binding constants.

Sample	k_a (s^{-1})	k_d ($M\,s^{-1}$)	K_D (M)
F-peptide-10	$1.53 \times e^5$	$7.87 \times e^{-4}$	$5.20 \times e^{-9}$
F-peptide-11	$2.13 \times e^5$	$1.02 \times e^{-2}$	$4.90 \times e^{-8}$
F-peptide-30C	$2.49 \times e^5$	$7.60 \times e^{-3}$	$3.10 \times e^{-8}$
F-peptide-30N	$2.52 \times e^5$	$2.97 \times e^{-3}$	$1.19 \times e^{-8}$
F-protein[a]	N/A[b]	N/A	$3.46 \times e^{-11}$ [9]

[a] The K_D of motavizumab binding to F-protein is provided for reference.
[b] N/A: not applicable.

2.2.3. RSV microneutralization assay

The RSV microneutralization assay measures the ability of motavizumab to prevent RSV from infecting Hep-2 target cells and was performed as described [4].

2.2.4. High performance size exclusion chromatography

High performance size exclusion chromatography was used to monitor motavizumab stability in terms of time-dependent fragmentation and aggregation. To determine the chromatographic profile of motavizumab, sample was injected onto a TosoHaas G3000SWXL column (7.8 mm × 30 cm). The sample was eluted isocratically with 0.1 M disodium phosphate containing 0.1 M sodium sulfate and 0.05% sodium azide, pH 6.8, at a flow rate of 1.0 ml/min. Eluted protein was detected using UV absorbance at 280 nm. The results were reported as the area percent of the product monomer peak compared to all other peaks, excluding the buffer-related peak observed at approximately 12 min.

3. Results

3.1. Functional screen of candidate peptides

The linear epitope for motavizumab binding extends from residue Asn-254 to Asn-277 of the RSV F-protein sequence, corresponding to the antigenic site A binding domain [4,14]. To select an optimized peptide in terms of motavizumab binding affinity and specificity, a series of synthetic peptides spanning the motavizumab binding epitope were synthesized and character-

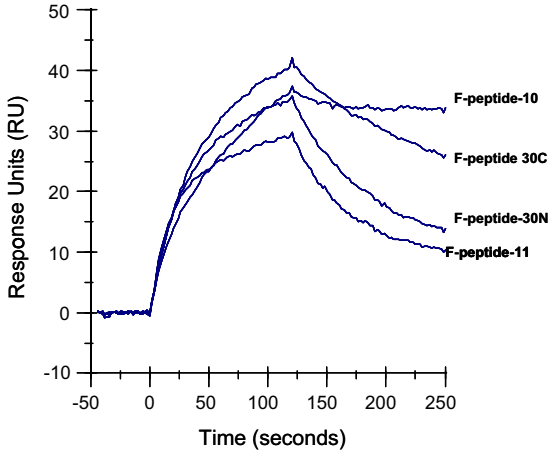

Fig. 1. SPR analysis of F-protein peptide mimetics.

Table 3
Plackett–Burman analysis of candidate peptides.

Parameter	Range	Low (−)	High (+)
Plate coating time	N/A[a]	Overnight @ 4 °C	2 h @ 37 °C
Plate blocking time	1–4 h	1 h	4 h
Sample incubation time	1 h ± 10 min	50 min	70 min
Detection antibody incubation time	1 h ± 10 min	50 min	70 min
Coating molecule	N/A[a]	F-protein	F-peptide

[a] N/A: not applicable.

ized. Table 1 illustrates five peptides that were initially evaluated in this study. Two peptides, F-peptide-30C and F-peptide-30N, were extended by six amino acid residues on either side of the motavizumab binding epitope to determine if the additional sequence could enhance binding. Peptides F-peptide-10 and F-peptide-11 were prepared with a terminal cysteine to facilitate coupling to a carrier protein in the event that the peptide could not be coated directly onto an ELISA plate in a manner similar to F protein.

Motavizumab binding to the candidate peptides was determined by potency ELISA. Duplicate columns of a microtiter plate were coated with each candidate peptide and with the F-protein. The plates were processed as described in Section 2, and binding to motavizumab was determined for each peptide relative to F-protein. Four of the peptides had favorable motavizumab binding relative to F-protein in terms of EC_{50} values, and were selected for further evaluation. The four selected peptides are F-peptide-10, F-peptide-11, F-peptide-30C and F-peptide-30N (Table 1).

3.2. Evaluation of kinetic binding parameters by SPR

Kinetic parameters were determined for each of the four candidate peptides (F-peptide-10, F-peptide-11, F-peptide-30C and F-peptide-30N) using SPR on a Biacore 3000 instrument. Motavizumab was captured on a sensor chip surface as described in Section 2. Each sample was passed over the motavizumab surface, allowed to disassociate, and the surface was regenerated following completion of data capture. Qualitative inspection of the resulting sensograms demonstrates that F-peptide-10 had the slowest apparent off-rate (Fig. 1) compared to F-peptide-11, F-peptide-30C and F-peptide-30N. Peptides F-peptide-10 and F-peptide-30C bound to motavizumab with binding constants (K_D) of 5.2 and 11.9 nM, respectively (Table 2). Peptides F-peptide-11 and F-peptide-30N bound to motavizumab with a K_D at least 3.8-fold lower. For reference, F protein binds to motavizumab with a K_D of 34.6 pM [9]. The difference in the motavizumab kinetic binding parameters between F-protein and the candidate F-peptides is discussed in Section 4. Based on these SPR results, the two peptides with the most favorable kinetic profiles, F-peptide-30C and F-peptide-10, were selected for further testing to determine the best candidate peptide to replace F-protein.

3.3. Robustness of ELISA with candidate peptides

A Plackett–Burman (PB) experimental design [15] was used to evaluate the robustness of the F-peptide binding ELISA for the remaining two candidate F-peptides (F-peptide-10 and F-peptide-30C). This design allows the capacity of an assay to remain unaffected by small but deliberate variations in method parameters to be measured, including microtiter plate coating incubation time, blocking incubation time, sample incubation time, detection antibody incubation time and coating molecule concentration. For each candidate peptide, eight runs of the experiment were conducted, with assay conditions randomly assigned to each run, to minimize any systematic bias. The range of experimental parameters and the 8-run PB design are displayed in Tables 3 and 4, respectively. Since this was not a full-factorial PB design (8 runs but only 5 factors evaluated), two "dummy" placeholder factors were added to estimate the error of each ELISA method.

The results of each experiment were analyzed. The effect of each of the 5 parameters on the assay was estimated as the mean difference between the ELISA titer at the low and high levels. The five effects were ranked and compared to their theoretical expected values based on a linear regression analysis. If the assay is robust and insensitive to the changes within the operation ranges of the parameters, the plot of the estimated effects versus their theoretical values should form a straight line. The robustness criterion is defined as $R^2 \geq 0.90$. The F-peptide-10 ELISA was shown to be robust ($R^2 = 0.931$), while F-peptide-30C ELISA was not robust ($R^2 = 0.739$). Due to the lack of robustness of the F-peptide-30C peptide, as well as its faster dissociation kinetics as detected in the Biacore assay (Table 2), the F-peptide-30C peptide was eliminated as a candidate peptide.

3.4. Evaluation of F-peptide-10 as a suitable replacement for F-protein

A series of studies were performed to evaluate if F-peptide-10, hereafter referred to as "F-peptide", was a suitable replacement for F-protein as the coating antigen in a motavizumab potency ELISA.

Table 4
Plackett–Burman analysis experimental design.[a]

Run #	Plate coating time	Plate blocking time	Sample incubation time	Detection antibody incubation time	Coating molecule concentration	"Dummy" 1	"Dummy" 2
1	+	+	+	−	+	−	−
2	−	+	+	+	−	+	−
3	−	−	+	+	+	−	+
4	+	−	−	+	+	+	−
5	−	+	−	−	+	+	+
6	+	−	+	−	−	+	+
7	+	+	−	+	−	−	+
8	−	−	−	−	−	−	−

[a] + and − signs refer to the high and low of each factor, respectively. Refer to Table 3 for the + and − value of each factor.

Table 5
F-protein and F-peptide ELISA potency of photo-degraded motavizumab samples.

Day	UV?	% relative potency		Microneutralization (% relative potency)	HPSEC (% purity)
		F-protein	F-peptide		
0	–	95	100	95	99.5
1	–	89	106	NP[a]	99.5
	+	87	103	NP	96.1
2	–	111	112	NP	99.5
	+	103	100	NP	92.1
3	–	119	120	NP	99.5
	+	94	82	NP	89.1
7	–	110	108	91	99.5
	+	119	85	70	75.9
14	–	104	101	94	99.4
	+	61	37	44	62.2

[a] NP: not performed.

3.4.1. Statistical equivalence of the F-protein and F-peptide potency ELISAs

To determine if the F-protein and F-peptide potency ELISAs were statistically equivalent, an analysis of covariance (ANCOVA) was performed to compute the difference in the mean potency between the two ligands. The data for this analysis was generated from motavizumab samples that were stored under four temperature conditions (−70 to −90 °C, −40 °C, 2–8 °C, and 23–27 °C) for a duration of up to 36 months. The ANCOVA was adjusted for storage temperature and duration. The variance component analysis was used to estimate the intra-assay variability for the F-protein binding ELISA using a mixed model, with storage temperature and duration as random effects. The acceptance criterion for equivalence was the 90% confidence interval (90% CI) of the mean difference that was fully contained within the F-protein binding ELISA intra-assay variability (standard deviation; SD).

The ANCOVA results showed that the mean difference between the potency values obtained from the F-protein and F-peptide ELISAs, when adjusted for storage temperature and duration, was −2.22% with a 90% CI of (−4.43%; −0.02%). These values were fully contained within one SD of the F-protein binding ELISA potency (−10.60%; 10.60%). These results suggest that the expected difference between the F-protein and F-peptide assays would be smaller than the assay variability of the F-protein binding ELISA. Therefore, the potency values obtained for both the F-protein and F-peptide binding ELISAs were statistically equivalent.

3.4.2. Evaluation of forced motavizumab degradation studies

Motavizumab samples were placed under conditions that are known to induce degradation and then tested in the F-protein and F-peptide ELISA to compare the stability indicating potential of each method. Aliquots of motavizumab were exposed to ultraviolet (UV) and white light in a room temperature incubator for 14 days. Samples were removed at various time points and analyzed using the F-protein and F-peptide ELISAs. In addition, two orthogonal methods were used to determine sample integrity: a cell-based bioassay (RSV microneutralization) and high performance size exclusion chromatography (HPSEC). For the F-protein and F-peptide ELISAs and the RSV microneutralization assay, the relative potency of the UV and white light treated samples was determined in comparison to an untreated motavizumab reference standard sample and expressed as a relative potency value.

The binding activity of motavizumab to F-protein has been previously shown to be resistant to UV light-induced degradation for up to 7 days [16]. However, a decrease in motavizumab binding for F-protein can be observed in samples that have been incubated in the presence of UV light for 14 days (see below). After 2 days of light exposure, the UV-irradiated motavizumab sample had similar potency values in the F-protein and F-peptide ELISAs and in the RSV microneutralization assay (Table 5). In contrast, there was a decrease in the intact antibody purity for the UV-exposed motavizumab sample as determined by HPSEC, with a percent purity of 92.1%, demonstrating physical degradation of motavizumab. There was no decrease in potency or purity for the samples exposed to white light for the same amount of time.

After 14 days, the UV-treated motavizumab sample had a relative potency of 37% in the F-peptide ELISA, a value comparable to the relative potency of the same sample in the RSV microneutralization assay (44%). Yet the same UV-irradiated sample had a potency of 61%, in the F-protein ELISA, suggesting that the F-peptide ELISA may be more sensitive as an indicator of motavizumab stability under these study conditions.

4. Conclusions

These studies demonstrate that a synthetic peptide representing the linear epitope of the A binding site of F-protein, "F-peptide", is a suitable replacement for F-protein as a coating antigen reagent in the motavizumab potency ELISA. Early data trends suggest that the use of an ELISA that measures motavizumab binding to F-peptide may be a better indicator for monitoring product stability. The F-peptide ELISA was able to detect motavizumab degradation earlier than the F-protein ELISA, closely resembling the stability-indicating capacity of a cell-based assay (RSV microneutralization). The enhanced stability indicating property of F-peptide may be due to the lower affinity of motavizumab for F-peptide compared to F-protein.

The molecular basis for the interaction between the antigen binding fragment of motavizumab and a peptide representing the A binding domain of RSV F-protein has been determined by X-ray crystallography [17; Protein Data Bank ID: *3IXT*]. The crystal structure of motavizumab and its target epitope may provide clues as to why the F-peptide ELISA may potentially be more sensitive to motavizumab degradation than the F-protein ELISA. There are 4 primary interactions between the motavizumab Fab fragment and F-peptide: 3 hydrogen bonds and a salt-bridge. Because there are only four interactions between motavizumab and F-peptide, disruption of even one of these interactions would significantly decrease the affinity of motavizumab for F-peptide, leading to a greater sensitivity of the assay in response to time and temperature-dependent degradation of motavizumab. In contrast, there are additional interactions between motavizumab and F-protein, including a number of residues that are critical for motavizumab binding activity but lie outside the primary binding epitope. Therefore, disruption of one of the primary interactions between motavizumab and its epitope on F-protein may not lead to a significant decrease in binding affinity.

This study demonstrates that a synthetic peptide "F-peptide" mimicking the naturally occurring motavizumab epitope of F-protein can be used in the potency ELISA for measuring motavizumab potency and for stability monitoring.

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