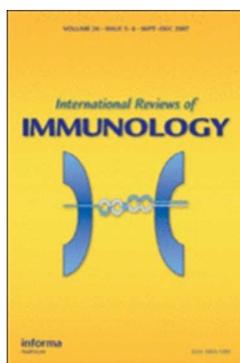# Application of camelid heavy-chain variable domains (VHHs) in prevention and treatment of bacterial and viral infections.

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Application of camelid heavy-chain variable domains (VHHs) in prevention and treatment of bacterial and viral infections

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Manuscripts

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2   **Application of camelid heavy-chain variable domains (VHHs) in**

3   **prevention and treatment of bacterial and viral infections**

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28 ## Abstract

29 Camelid heavy-chain variable domains (VHHs) are the smallest, intact, antigen-

30 binding units to occur in nature. VHHs possess high degrees of solubility and

31 robustness enabling generation of multivalent constructs with increased avidity –

32 characteristics that mark their superiority to other antibody fragments and monoclonal

33 antibodies. Capable of effectively binding to molecular targets inaccessible to classical

34 immunotherapeutic agents and easily produced in microbial culture, VHHs are

35 considered promising tools for pharmaceutical biotechnology. With the aim to

36 demonstrate the perspective and potential of VHHs for the development of

37 prophylactic and therapeutic drugs to target diseases caused by bacterial and viral

38 infections, this review article will initially describe the structural features that underlie

39 the unique properties of VHHs and explain the methods currently used for the

40 selection and recombinant production of pathogen-specific VHHs, and then thoroughly

41 summarise the experimental findings of five distinct studies that employed VHHs as

42 inhibitors of host–pathogen interactions or neutralisers of infectious agents. Past and

43 recent studies suggest the potential of camelid heavy-chain variable domains as a

44 novel modality of immunotherapeutic drugs and a promising alternative to monoclonal

45 antibodies. VHHs demonstrate the ability to interfere with bacterial pathogenesis by

46 preventing adhesion to host tissue and sequestering disease-causing bacterial toxins.

47 To protect from viral infections, VHHs may be employed as inhibitors of viral entry by

48 binding to viral coat proteins or blocking interactions with cell-surface receptors. The

49 implementation of VHHs as immunotherapeutic agents for infectious diseases is of

50 considerable potential and set to contribute to public health in the near future.

51

52 **Key words:** nanobodies, immunotherapy, single-domain antibody, infectious disease

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55 ## Introduction

Structure and properties of VHHs

Immunoglobulin G (IgG) is the most abundant antibody type in serum and constitutes for about 75 % of circulating antibodies. Due to its great availability, IgG is used as the principal antibody in immunological research. It is composed of two identical light chains and two identical heavy chains connected by disulphide bridges [1,2].

Apart from conventional IgG glycoproteins with normal antibody assembly, sera of *Camelidae* were found to contain a high abundance of IgG subclasses, i.e. IgG_2 and IgG_3, devoid of light chains. Moreover, these heavy-chain-only antibodies lack the constant heavy chain 1 domain (C_H1) which causes direct connection of their heavy chain variable domain (V_H) to the hinge. The camelid heavy-chain variable domain exhibits general structural features of a conventional V_H, but is unique in its amino acid sequence, and therefore denoted as VHH [3].

Equivalent to conventional V_Hs, VHHs consist of four framework regions (FRs) separating the three complementarity determining regions (CDRs) (or hypervariable regions) that are involved in antigen binding. VHHs are fully functional for antigen binding and their binding affinities are not affected by the absence of light chains. Moreover, the repertoire of antigen-binding sites is increased due to broadly size-distributed CDRs with diverse amino acid patterns. Particularly, CDR3 of VHHs distinguishes itself from CDR3 of conventional V_Hs by higher variability of its amino acid residues. CDR3 is on average longer and parts of this region that, in conventional IgG, associate with the light chain variable domain (V_L) are available for antigen binding in VHHs. These features improve antigen recognition and binding strength, and thus compensate for the absence of the V_L, which normally accounts for half of the antigen-binding surface. Furthermore, the presence of Cys residues in CDR1 and CDR3 enables the formation of disulphide bridges that stabilise the structure of the antigen-binding site [4−6].

85   VHH domains are the smallest, naturally occurring, antigen-binding units with a

86   molecular weight of 15 kDa [7]. Their small size allows for rapid penetration of tissues

87   and enables construction of engineered multivalent formats with higher avidity than

88   monoclonal antibodies (mAbs) and other antigen-binding units [8]. Moreover,

89   solubility is notably increased in VHHs due to the presence of hydrophilic amino acid

90   residues in FR2. Hence, these antibody fragments are resistant to aggregation and

91   their monomeric nature is preserved in solution [9].

92   Amino acid substitutions cause considerable reshaping of the VHH surface [10]

93   resulting in a large structural diversity of VHH paratopes, e.g. cavity, protruding loop

94   and flat surface. Thereby, the binding of epitopes inaccessible to mAbs, such as those

95   located in the active site of enzymes, is enabled [11].

96   Their small size, variability, stability, avidity and solubility characteristics

97   render VHHs promising tools for immunotherapy.

98

Selection and production of VHHs

100   In order to obtain pathogen-specific VHHs for drug development, their generation

101   must be artificially induced in an experimental host. For this purpose, camelids are

102   immunised with a particular antigen isolated from the infectious organism. Peripheral

103   blood (PB) is collected from which PB lymphocytes are prepared by density gradient

104   centrifugation. The cells are lysed and mRNA is isolated. Extracts of mRNA are used as

105   templates for cDNA synthesis by reverse transcription polymerase chain reaction (RT-

106   PCR). In a "nested" approach, Ig heavy chains are amplified with gene-specific

107   primers. Reamplification of *VHH* genes with specialised primer sets that introduce

108   restriction endonuclease (RE) recognition sites at the 5' and 3' ends of the RT-PCR

109   products enables precise RE digestion of *VHH* cDNAs [12] which are then processed

110   for screening by phage, ribosome or yeast display [13]. For phage display, the most

111   common of display technologies, digested products are cloned into the bacteriophage

112   coat protein gene present in phagemid vectors [14]. The resulting recombinant DNA

113   molecules are transformed into competent bacterial cells for gene expression.

114   Bacterial cells are cultured to high density before infected with a helper phage [12],

115   which provides packaging functions for phagemids, and thus, promotes phage particle

116   formation plus their secretion into culture medium [15]. Thereby, a library of

117   phagemid virions, each expressing a different VHH as fusion with their coat protein, is

118   constructed. In a selection process, i.e. panning, VHH-displaying phage clones are

119   applied to microtitre wells coated with the specific antigen. Adsorption of phage

120   particles on the immobilised antigen, due to antigen–antibody interaction, is revealed

121   by an indirect enzyme-linked immunosorbent assay (ELISA) using horseradish

122   peroxidase-conjugated secondary antibodies directed against the phage coat protein.

123   Vector DNA from positively selected clones is then introduced into *Escherichia coli* for

124   bacterial production, with yields between 3 and 6 mg L^{-1} [12].

125   For increased cost efficiency, VHH fragments may be alternatively generated in

126   lower eukaryotic organisms, esp. yeasts, as these are less fastidious and more

127   productive, with secretion levels above 250 mg L^{-1}. For this purpose, cDNA encoding

128   the selected VHH is ligated into an episomal yeast expression vector. Cellular entities

129   of *Saccharomyces cerevisiae* or *Pichia pastoris* are transformed with the recombinant

130   vector and the pathogen-specific VHH is produced by fermentation [16,17].

131   Subsequently, purified expressed products are subjected to antibody specificity

132   and affinity testing by ELISA methods [12], in order to assess their suitability for

133   further *in vitro* or *in vivo* studies.

134

VHHs directed against bacterial pathogens

Treatment of dental caries

137   Insufficient oral care, in combination with a high-sugar diet, augments the

138   development of diverse biofilm communities on tooth surfaces, i.e. the formation of

139   dental plaque. Acidic conditions and adhesion sites, provided by the dental plaque,

140   favour the growth of the oral pathogen *Streptococcus mutans* on tooth surfaces. *S.*

141   *mutans* further decreases pH by the production of acidic compounds from fermentable

142   carbohydrates. Thereby, severe damage is caused to tooth enamel resulting in tooth

143   decay, which is symptomatic of dental caries [18–20].

144   Adherence of *S. mutans* to the salivary pellicle is mediated by the cell surface

145   streptococcal antigen (SA) I/II [21]. Prevention of adhesin–receptor interactions could

146   therefore assist in the removal of *S. mutans* from tooth surfaces. In 2006, Krüger *et*

147   *al.* developed an immunotherapeutic approach for the treatment of caries in patients

148   with hyposalivation that uses llama VHHs targeted against SA I/II.

149   Krüger *et al.* (2006) constructed a VHH antibody library from PB lymphocytes

150   of llamas immunised with disrupted and intact *S. mutans* HG982. Screening for llama

151   VHH directed against *S. mutans* yielded an antibody specific to SA I/II, denoted S36-

152   VHH [20]. Previous studies had shown that coupling of VHH to enzymes enhances

153   their therapeutic effect [22]. Therefore, by linkage of the selected VHH and *Aspergillus*

154   *niger*-derived glucose oxidase (GOx), a fusion protein (S36-VHH-GOx) was formed to

155   enhance the salivary peroxidase system (SPS) [20]. When glucose and oxygen are

156   available, GOx produces hydrogen peroxide (H_2O_2), which is required by peroxidase to

157   oxidise the salivary components iodide (I^-) and thiocyanate (SCN^-) to the antibacterial

158   compounds hypoiodite (OI^-) and hypothyocyanite ($OSCN^-$) [23]. Genes encoding S36-

159   VHH and S36-VHH-GOx were ligated into *S. cerevisiae* expression vectors and

160   produced as described previously [16]. ELISAs demonstrated that the fusion of GOx to

161   S36-VHH had no negative effect on the binding of SA I/II [20].

162   Therapeutic effects of these VHHs on cariogenesis were further assessed *in*

163   *vivo*. For this purpose, the clinical situation in patients with hyposalivation was

164   simulated by a desalivated rat model. In order to establish a basis for cariogenesis,

165   rats received a diet high in sucrose and were repeatedly infected with *S. mutans* NG8.

166   After three weeks, rats were administered with a single dose of either VHH per day.

167   Both, S36-VHH and S36-VHH-GOx, were found to decrease colonisation with *S.*

168   *mutans* and to exert an anticariogenic effect, however, not to statistically significant

169   degrees. Furthermore, no additional therapeutic effect of GOx in the S36-VHH-GOx

170   fusion protein was observed. Its nonappearance was believed to result from heavy

171 plaque accumulation, partial inhibition of enzyme activity by the prevailing low pH as

172 well as the lack of the electron acceptor oxygen within the plaque [20].

173   Although postulated, the SPS could not be enhanced. Due to virtual absence of

174 saliva, the GOx substrate glucose was scarce, which resulted in minimal H_2O_2

175 generation. Consequently, the amount of oxidisable substrate, i.e. I^- or SCN^-, was

176 limited leading to considerably low antibacterial effects. Krüger *et al.* (2006) suggest

177 administering a mixture of S36-VHH-GOx, lactoperoxidase, and I^- or SCN^-, in case of

178 hyposalivation, to achieve a bactericidal effect of the SPS.

179

180 **Reduction of lipopolysaccharide toxicity**

181 Lipopolysaccharide (LPS), or endotoxin, is a constituent of the outer membranes of

182 gram-negative bacteria. When released into the blood stream after infection, it causes

183 an acute inflammatory response with detrimental effects to organs and tissues, a

184 condition described as sepsis. Sepsis results from the interaction of LPS with high-

185 affinity LPS receptor complexes present on cells of the immune system and in plasma,

186 which induces the release of interleukin 8 (IL-8) and other inflammatory cytokines

187 [24–26].

188   An important cause of sepsis (and meningitis) is the gram-negative pathogen

189 *Neisseria meningitidis* [27]. Meningococcal LPS is classified into twelve distinct

190 immunotypes (L1 to L12) based on structural diversity of the outer core – a result of

191 phase variation [28,29]. Albeit the heterogeneous structures of meningococcal LPS, its

192 lipid A moiety and inner-core regions exhibit considerable conservation among gram-

193 negative bacteria [30].

194   Protection against a variety of gram-negative bacteria could therefore be

195 provided by immunotherapy targeting these conserved LPS components [31].

196 Prerequisites for the efficient use of antibodies in the prevention of sepsis comprise

197 the recognition of an epitope located in the conserved inner core of the LPS molecule

198 and the ability to compete with cell surface LPS receptor complexes. El Khattabi *et al.*

199 explored the potential of anti-LPS VHHs for sepsis therapy in 2006.

200   From PB lymphocytes of llamas, El Khattabi *et al.* (2006) generated a

201 nonimmune, i.e. without immunisation of the animal, VHH phage display library. Four

202 VHH-displaying phages that specifically bound to LPS immunotype L3 from the *N.*

203 *meningitidis* strain H44/76 were selected, and purified anti-LPS VHHs were

204 subsequently produced. All VHHs were found to immunoprecipitate meningococcal LPS

205 forms, while some VHHs additionally reacted with LPS of other gram-negative

206 pathogens (*E. coli* B4:O111*, Salmonella enterica* serovar Typhimurium and *Bordetella*

207 *pertussis*). Epitope mapping was performed to determine the binding sites of the anti-

208 LPS VHHs. It was revealed that one anti-LPS VHH (VHH 5G) bound within the lipid A

209 and KDO regions of an *rfaC* mutant strain, while the other three reacted with an LPS-

210 deficient mutant strain (*lpxA*) for unknown reasons.

211   The effect of anti-LPS VHH on the binding of meningococcal L3 LPS by LPS

212 receptors on innate immune cells was investigated and it was demonstrated that L3

213 LPS–receptor interactions could be efficiently blocked in the presence of VHH 5G.

214 Furthermore, it was studied whether effector cell response to LPS could be prevented

215 by VHH 5G. LPS was effectively sequestered by VHH 5G inhibiting the release of

216 proinflammatory molecules. In addition, LPS depletion due to immunoprecipitation by

217 anti-LPS VHHs was assessed to be sufficient for the detoxification of solutions

218 contaminated with LPS [32].

219   With VHH 5G, an antibody was isolated that recognises LPS from different

220 gram-negative bacteria when present in their outer membranes and in a purified, free

221 form. VHH 5G is able to disturb interactions between LPS and its receptors, disrupt

222 corresponding signalling pathways that normally generate sepsis-related effector

223 molecules, and deplete LPS from aqueous solutions with high efficiency.

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227 **Prevention of enterotoxigenic *E. coli*-induced post-weaning diarrhoea**

228 Enterotoxigenic *E. coli* (ETEC) strains are causative of human and porcine morbidity

229 and mortality [33] as these bacteria express heat-labile and heat-stable enterotoxins

230 that cause ionic imbalance and secretory type diarrhoea in infected subjects [34,35].

231 ETEC virulence is determined, *inter alia*, by F4 fimbriae, which are filamentous protein

232 appendages that interact with F4-specific receptors (F4Rs) present on the epithelium

233 of the small intestine, and thereby mediate the intestinal colonisation by ETEC [36].

234 Intestinal isolates of ETEC from piglets with post-weaning diarrhoea (PWD) – a cause

235 of absent maternal immunity [37] – exhibit prevalence of the serological variant F4ac

236 [38]. In 2005, Harmsen *et al.* aimed to use monoclonal VHHs, raised against ETEC

237 F4ac fimbriae, for PWD immunotherapy.

238   Harmsen *et al.* (2005) isolated F4ac fimbriae from the F4 positive (F4+) ETEC

239 strain CVI-1000, which is devoid of F5, F6, F17, F18 and F41 fimbriae, and used these

240 to immunise a llama and recover its VHH repertoire from PB lymphocytes. Yeast VHH

241 expression libraries were created whence six clones, directed against the F4 fimbriae

242 major subunit, were selected. Two clones did not significantly inhibit bacterial

243 attachment to jejunal brush borders and displayed cross-reactivity with other F4

244 variants, whereas four VHHs that specifically recognised the F4ac fimbrial variant

245 prevented F4+ ETEC attachment. The llama VHH K609 showed the strongest inhibitory

246 activity and was therefore subjected to further studies.

247   First, small intestinal segments of F4R positive (F4R+) piglets, i.e. piglets with

248 brush borders that bound above six ETEC per unit, were perfused with solutions of

249 ETEC and different concentrations of K609. Net fluid absorption was measured to

250 determine the effect of VHHs on ETEC-induced fluid loss. Perfusion with K609 at 4 mg

251 L^{-1} accounted for maximal reduction of ETEC-induced fluid loss, however, only to

252 about 30 %. Second, faecal dry matter content was analysed in two groups of weaned

253 piglets with severe diarrhoea, evoked by the oral challenge with porcine rotavirus

254 strain RV277 and the ETEC strain CVI-1000. Subjects of the experimental group were

255 orally administered daily with either low or high doses of K609, while the control

256 group received no treatment. Faecal dry matter content of the group treated with

257   K609 was higher compared to control piglets, but statistical significance of this

258   difference was merely recorded for piglets administered with high doses. Overall,

259   reduction of diarrhoea was poor and improvement of piglet mortality was insignificant.

260   Possible errors that may have contributed to the limited effectivity of K609

261   immunotherapy have been suggested. These include degradation of K609 by

262   proteases of the gastrointestinal tract and the expression of adhesion factors, other

263   than F4 fimbriae, by the ETEC strain CVI-1000. Furthermore, intestinal colonisation of

264   ETEC expressing other fimbrial types or F4 variants cannot be prevented by K609

265   alone, owing to its specificity. It is rather suggested to produce a mixture of VHHs

266   directed against various ETEC adhesion factors, in order to effectively obviate PWD

267   [39].

268

VHHs directed against viruses

Treatment of human respiratory syncytial virus infection

271   Human respiratory syncytial virus (RSV) is the main contributor to lower respiratory

272   tract infection (LRTI) in infants [40]. Patients who encountered RSV-induced

273   bronchiolitis or pneumonia during infancy are at increased risk of developing asthma

274   and chronic obstructive pulmonary disease in adulthood [41,42]. RSV specifically

275   infects the apical membrane of ciliated respiratory epithelial cells [43] and triggers

276   clinical symptoms of LRTI after a short incubation period [44].

277   RSV pathogenesis is promoted by virulence factors, esp. the envelope

278   glycoproteins G and F, encoded by a linear single-stranded, nonsegmented, negative-

279   sense RNA molecule [45]. The G protein mediates viral attachment to epithelial cell

280   receptors [46], whereas the F protein induces the fusion of viral and epithelial cell

281   membrane which enables the entry of the RSV ribonucleoprotein into host cell

282   cytoplasm [47]. Furthermore, the F protein evokes the fusion of infected cells with

283   adjacent cells to form multinucleated cells (syncytia) [48].

284   RSV is classified into two subgroups, i.e. subgroup A (RSV-A) and subgroup B

285   (RSV-B), based on antigenic variation of the G protein [49]. In contrast, high

286   conservation has been identified in the amino acid sequence of the F protein [50]. The

287   development of therapeutic agents specifically targeting the F protein could therefore

288   be highly valuable in the inhibition of viral entry. A trivalent VHH, specific to the RSV F

289   protein, was recently designed and characterised by Detalle *et al.* (2016) and

290   subjected to a functional comparison with its monovalent form and the prophylactic

291   anti-RSV mAb palivizumab [51].

292   Llama immune libraries were generated by injection with soluble recombinant F

293   protein, inactivated RSV-A or a combination of the two. A monovalent VHH specific to

294   the RSV F protein (Nb017) was identified from the library. Three units of Nb017 were

295   additionally formatted into a trivalent VHH, denoted ALX-0171, using flexible GS

296   linkers. Both RSV-neutralising VHHs were readily produced in a *P. pastoris* strain.

297   Assessment of the binding to the RSV F protein, by surface plasmon resonance

298   analysis, revealed that both ALX-0171 and Nb017 bind its pre-fusion conformation,

299   where the trimeric format exhibited a marked increase in binding affinity.

300   Furthermore, the effect of trimeric formatting on RSV neutralisation capacity was

301   determined by microneutralisation assays. The potency of the trivalent ALX-0171

302   against RSV-A and RSV-B strains was found to be several thousandfold higher than

303   that of the monovalent Nb017. Moreover, a significant increase in potency, as

304   compared to palivizumab, was ascertained.

305   The capabilities of ALX-0171 and palivizumab to completely suppress RSV

306   replication were compared at equivalent concentrations. ALX-0171 caused complete

307   blockage of virus replication in 87 % of viruses tested, whereas palivizumab reduced

308   viral titres by only 18 %. By studying the binding to RSV mutants with alterations in

309   antigenic site II or IV of the RSV F protein, it was demonstrated that ALX-0171

310   specifically targets antigenic site II. Furthermore, it was observed that ALX-0171

311   partially competes with palivizumab for the binding of the RSV F protein which

312   suggests overlap of their epitopes.

313   As Detalle *et al.* (2016) intended to administer ALX-0171 by nebulisation, it

314   was determined whether the nebulisation process causes aggregation, fragmentation

315   or reduced potency of ALX-0171. Higher- and lower-molecular-weight species were

316   detected at minimum levels, but ALX-0171 potency remained unaffected.

317   The *in vivo* efficacy of ALX-0171 against RSV was studied in cotton rats. ALX-

318   0171 was administered at different doses either by nebulisation before or intranasally

319   after RSV challenge. Viral loads in the nose and lungs were significantly reduced for all

320   doses of intranasally administered ALX-0171. Delivered prophylactically via

321   nebulisation, ALX-0171 reduced nasal RSV titres in a dose-dependent matter and in

322   the lungs it completely blocked RSV replication, even at the lowest dose tested (1 mg

323   kg^{-1}). ALX-0171 is therefore superior to palivizumab, which had no effect on viral

324   titres at doses lower than 15 mg kg^{-1}.

325   ALX-0171 is a potential therapeutic VHH that specifically and efficiently binds

326   antigenic site II of the RSV F protein. In comparison to Nb017 and palivizumab, ALX-

327   0171 distinguishes itself by increased neutralisation capacity and inhibition efficiency,

328   which are a result of its trivalency. Moreover, administration by nebulisation enables

329   direct delivery of ALX-0171 to the site of infection and hence a faster exertion of its

330   antiviral effect [52].

331   In May 2016, positive results for ALX-0171 in Phase I/IIa clinical trials were

332   reported by the Belgian biopharmaceutical company Ablynx [53].

333

334   **Eradication of poliovirus-induced infantile paralysis (poliomyelitis)**

335   Poliomyelitis, or infantile paralysis, is a highly contagious disease caused by infection

336   with poliovirus (PV) [54]. Three distinct serotypes, PV1, PV2 and PV3, all of which

337   cause paralytic disease, have been identified based on differences in their antigenic

338   determinants [55]. The single-stranded positive sense RNA genome of PV [56] is

339   enclosed in a non-enveloped capsid composed of 60 monomers of four different

340   polypeptides, i.e. viral protein 1 (VP1), VP2, VP3 and VP4 [57], that are arranged in

341   icosahedral symmetry [58]. PV recognises and binds CD155, a transmembrane

342   glycoprotein of the Ig superfamily [59]. Extracellular domains of CD155 interact with a

343   conserved narrow surface depression in the PV capsid, termed canyon [60], inducing a

344 conformational change of the virion that initiates cell entry and uncoating [61,62]. In

345 rare cases, PV reaches the central nervous system where it replicates in motor

346 neurons of the spinal cord and thereby causes muscle paralysis [63].

347   Prophylactic treatment by inactivated polio vaccine (IPV) [64] and oral polio

348 vaccine [65] led to near eradication of poliomyelitis [66]. Other treatment options

349 such as mAb 35-1f4, which neutralises PV1 by virion cross-linking [67], and the

350 capsid-binding pyridazinamine analogue R75761 [68] have been developed. In 2010,

351 Thys *et al.* isolated and characterised VHHs specific to PV1 and assessed their antiviral

352 activity *in vitro*.

353   By repeated infection of a dromedary with PV1 Sabin strain and isolation of its

354 VHH repertoire, an immune library was created. Fifteen VHH clones that positively

355 reacted with PV1 Sabin strain in an ELISA were further analysed. In a standard

356 neutralisation assay their abilities to block infectivity of PV1 (vaccine and wild-type

357 strain), PV2 and PV3 were determined. Neutralising activity against PV2 and PV3 was

358 non-existent, whereas both the vaccine and wild-type strain of PV1 were neutralised

359 by five VHHs (PVSP6A, PVSS8A, PVSP19B, PVSS21E, and PVSP29F). HeLa cell cultures

360 were incubated with dilutions of these VHHs and cell viability was monitored, in order

361 to reveal possible cytotoxic effects. Cytotoxicity was, however, not demonstrated for

362 any of the tested VHHs.

363   Antiviral activities of the neutralising VHHs at different concentrations were

364 assessed by examination of their cytopathic reduction effect in HeLa cells infected with

365 PV1, and were compared with that of mAb 35-1f4 and R75761. Full protection from a

366 PV1-induced cytopathic effect was provided by PVSP6A and PVSP29F at a

367 concentration below that of R75761. Moreover, these VHHs exhibited protective

368 activities comparable to mAb 35-1f4. Among the VHHs tested, the lowest half maximal

369 effective concentration (EC_{50}) values were obtained for PVSP6A and PVSP29F.

370   In order to further define antiviral activities, abilities of the VHHs to reduce

371 infectious virus yields were investigated in PV1-infected HeLa cells. Certain

372 concentrations of PVSP6A and PVSP29F completely abolished virus replication. In

373 contrast, cells treated with equal concentrations of mAb 35-1f4 showed residual virus

374 titres.

375 For the generation of PV1 neutralisation escape mutants, mixtures of PV1 and

376 VHH were created. Isolation of resistant viruses failed for PVSP6A and PVSP29F due to

377 complete inhibition of plaque formation. The other three VHHs were unable to

378 neutralise about 100 plaque forming units (PFU), which was consistent with the

379 number of PFU expected for a neutralising mAb [69].

380 Thys *et al.* (2010) aimed to isolate a VHH that could inhibit cellular attachment

381 and cell entry by binding within the canyon region. Recent studies have demonstrated

382 that all of the PV1-neutralising VHHs recognise epitopes in the canyon region that

383 overlap with the binding site for CD155 and thereby block ligand–receptor interactions

384 [70,71]. Furthermore, PV1 neutralisation escape mutants have been found to bear

385 amino acid substitutions in capsid VPs closely located to the VHH binding sites [72].

386 PVSP6A and PVSP29F were the most potent inhibitors of PV1, among the VHHs

387 tested. Moreover, their efficacies were superior to R75761 and mAb 35-1f4 at

388 equivalent concentrations. These VHHs could therefore be used to develop advanced

389 antiviral drugs that prevent or treat PV1-induced poliomyelitis. Additionally, Thys *et al.*

390 (2010) plan to adapt their immunisation protocols to generate a VHH with protective

391 activity against all of the three PV types, with the aim to contribute to worldwide

392 eradication of poliomyelitis.

393

Conclusions

395 VHH antibody fragments compare to conventional mAbs in their selectivity, antigen

396 specificity and binding affinity, but excel in terms of their small size, robustness and

397 increased solubility, which allow for construction of potent multivalent molecules with

398 high avidity, and thus are advantageous for the development of therapeutic drugs.

399 VHHs were found to exceed the *in vivo* efficacies of certain marketed drugs, especially

400 when assembled into multimeric formats or linked to enzymes. Microbial production of

401 VHHs is furthermore a feasible alternative to the generation of mAbs by mammalian

402 cell culture [73] and the chemical synthesis of immunological agents. Albeit the great

403 pharmaceutical potential, no VHH-based agents have been approved for therapy thus

404 far. However, some VHHs, such as ALX-0171, are already in advanced clinical phases.

405 　　Besides their application in prevention and treatment of viral and bacterial

406 infections, VHHs have been used to combat pathologies such as arthritis [74], cancer

407 [75] and thrombosis [76], to trace antigens and biomarkers for *in vivo* diagnostic

408 imaging [77,78], and to protect against lethal scorpion and snake envenoming

409 [79,80].

410 　　Based on their versatile applicability in the biomedical field, VHHs are predicted

411 as major contributors to the solving of public health problems of the future.

412

413

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416

417

Declaration of interest

419 None to declare.

420

421

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425

426

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