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Target-mediated clearance and bio-distribution of a monoclonal antibody against the Kunitz–type protease inhibitor 2 domain of Tissue Factor Pathway Inhibitor

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ABSTRACT

Introduction: A humanised monoclonal antibody, concizumab, that binds with high affinity to the Kunitz-type protease inhibitor (KPI) 2 domain of human tissue factor pathway inhibitor (TFPI) is in clinical development. It promotes coagulation by neutralising the inhibitory function of TFPI and may provide a subcutaneous prophylaxis option for patients with haemophilia. We aimed to study biodistribution and pharmacokinetics (PK) of concizumab.

Materials and Methods: Blockage of cellular TFPI by concizumab was measured by tissue factor/Factor VIIa-mediated Factor X activation on human EA.hy926 cells. Biodistribution of concizumab was analysed in rabbits by immunohistology, and the PK was measured in rabbits and rats.

Results and Conclusions: Concizumab bound to cell surface TFPI on EA.hy926 cells and neutralised TFPI inhibition of Factor X activation. The antibody cross-reacted with rabbit TFPI, but not with rat TFPI, allowing for comparative PK studies. PK data in rats described a log-linear profile typical for a non-binding antibody, whereas PK data in rabbits revealed a non-linear, dose-dependent profile, consistent with a target-mediated clearance mechanism. Immunohistology in rabbits during target-saturation showed localisation of the antibody on the endothelium of the microvasculature in several organs. We observed a marked co-localisation with endogenous rabbit TFPI, but a negligible sub-endothelial build-up. Concizumab binds and neutralises the inhibitory effect of cell surface-bound TFPI. The PK profile observed in rabbits is consistent with a TFPI-mediated drug disposition. Double immunofluorescence shows co-localisation of the antibody with TFPI on the endothelium of the microvasculature and points to this TFPI as a putative target involved in the clearance mechanism.

Introduction

Current treatment for bleeding in haemophilia A and B patients involves intravenous (i.v.) replacement therapy with the missing coagulation factor, Factor VIII (FVIII) or Factor IX (FIX) administered as a drug of either plasma or recombinant origin. As an adverse complication many haemophilia patients develop inhibitory antibodies against the replenished coagulation factor. These patients are then treated with bypassing agents, i.e. plasma-derived activated prothrombin complex concentrate (pd-aPCC) or recombinant Factor VIIa (rFVIIa). Prophylactic therapy with either replacement or bypassing agents requires frequent i.v. injections with negative implications for adherence to therapy and the general quality of life for patients [1]. Subcutaneous injections and reduced dosing frequency are thus highly desired options for haemophilia treatment, which may be attainable with a therapy that involves an antibody having high bioavailability and long half-life.

Tissue factor pathway inhibitor (TFPI) is an endogenous inhibitor of the extrinsic coagulation initiation pathway. TFPI is made in at least two alternatively spliced isoforms, TFPIα and TFPIβ [2–4]. TFPIα consists of three Kunitz–type protease inhibitor (KPI) domains with short stretches of amino acids between the domains. The KPI-1 of TFPI binds the tissue factor (TF)/FVIIa complex and KPI-2 binds Factor Xa (FXa) resulting in the formation of a quaternary complex of TF/FVIIa/FXa/TFPI [5]. KPI-3 binds to protein S, which enhances the inhibitory activity of soluble TFPIα towards FXa [6–8]. Following KPI-3, a highly basic C-terminal

Abbreviations: AUC, area under the curve; Cl, clearance; DAB, 3,3′-diaminobenzidine; dIF, double immunofluorescence; EGFR, epidermal growth factor receptor; GAG, glycosaminoglycans; GPI, glycosylphosphatidylinositol; FVIII, Factor VIII; FIX, Factor IX; FXa, Factor Xa; FCS, fetal calf serum; T½, half-life; HRP, horseradish peroxidase; ABC-HRP, HRP-coupled avidin-biotin complex; Hz, humanised; IHC, immunohistochemistry; i.v, intravenous; KPI, Kunitz-type protease inhibitor; C_{max}, maximum concentration; mAb, monoclonal antibody; NCA, non-compartmental analysis; AUC_{extrap}, percentage of AUC that is extrapolated; PK, pharmacokinetics; PBS, phosphate buffered saline; pd-aPCC, plasma-derived activated prothrombin complex concentrate; rFVIIa, recombinant Factor VIIa; SPR, Surface plasmon resonance; TMDD, target-mediated drug disposition; TMB, tetramethylbenzidine; TF, tissue factor; TFPI, Tissue factor pathway inhibitor.

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tail allows binding of TFPIα to the endothelial surface glycosaminoglycans (GAG). TFPIβ is a truncated form of TFPI that contains KPI-1 and KPI-2 followed by a unique 12 amino acid sequence at its C-terminus that attaches it to the cell surface via a glycosylphosphatidylinositol (GPI)-anchor [2,4].

In humans, the majority of TFPI is found in and on endothelial cells, while small but significant amounts are found in plasma and in platelets (reviewed in [4,9]). Endothelial cells contain several pools of TFPI: i) a GPI-anchored pool on the surface, which was recently identified as TFPIβ [10], ii) a heparin-releasable pool that is presumed to be bound to GAGs on the cell surface, and iii) an intracellular pool subject to release by certain stimuli eg. heparin. The latter two TFPI pools were identified as mainly TFPIα, and their amount was estimated to be ~65% of the TFPIβ pool [10]. The mRNA for TFPIα in endothelial cells is, however, 5-10-fold more abundant than the TFPIβ mRNA [11], and endothelial cells secrete almost exclusively the TFPIα isoform [10]. Plasma is thus the second significant reservoir of TFPI. Approximately 20% of plasma TFPI is full length TFPIα, while 80% is a truncated form of TFPI that lacks a KPI-3 domain and is bound primarily to lipoprotein particles [4]. The third significant source of TFPI is located inside resting platelets. Platelet TFPI is found to be TFPIα and accounts for 7-10% of the total TFPI found within whole blood [9]. The total amount of plasma TFPI in the human body is around 4.8 nmol in a blood volume of 3 L, compared with 170 nmol of just the TFPIβ isoform on the surface of endothelial cells [10]. TFPI bound to endothelial cell surfaces, including GPI-anchored TFPIβ and GAG-bound TFPIα, thus constitute the majority of TFPI that is exposed to the bloodstream in a normal human body.

Concizumab (previously known as mAb 2021) is a high affinity monoclonal antibody, specific for the KPI-2 domain of TFPI [12]. Binding of the antibody to KPI-2 prevents TFPI from inhibiting FXa and abolishes the negative feed-back loop that limits initiation of the coagulation process via the TF/FVIIa complex. Blockage of the TFPI inhibition of TF/FVIIa-mediated FXa generation will result in a coagulation process driven by TF/FVIIa tenase activity. Under haemophilic conditions this would compensate for a defective FIXa/FVIIIa-mediated tenase activity as previously demonstrated in both in-vitro and in-vivo experiments [13]. These findings have recently been further substantiated by studies where rabbits made temporarily haemophilic were treated with the KPI-2 binding antibody by either i.v. or subcutaneous administration [12].

The present study aims at describing the specific interaction of the monoclonal KPI-2 binding antibody, concizumab, with the endothelium and the consequence of this interaction for its bio-distribution and clearance from the circulation. This was studied *in vitro* by cell binding studies with human endothelial cell lines and analysis of the functional effect of the antibody on the inhibition of cell surface TFPI. The data demonstrated that the antibody bound to TFPI on endothelial cells and neutralised TFPI inhibition. The pharmacokinetic profile of the antibody in rabbits and rats was studied, taking advantage of the fact that the KPI-2 antibody cross-reacts with rabbit TFPI but not with rat TFPI. Pharmacokinetic analysis showed that the specific interaction of the antibody with rabbit TFPI resulted in a non-log-linear profile consistent with a target-mediated drug disposition (TMDD) divergent from the log-linear clearance profile in rats. Finally, the bio-distribution of the antibody in rabbits was studied by immunohistology. Double immunofluorescence data pointed towards a potential clearance pathway by demonstrating co-localisation of the injected antibody and endogenous TFPI in a pattern consistent with the capillary endothelium.

Materials & Methods

Antibodies

A murine monoclonal antibody 4F36 (mAb 4F36), IgG1 isotype, directed against the KPI-2 domain of human TFPI was raised in RBF mice, and humanised in an IgG4 isotype version (concizumab, previously known as mAb 2021) [12]. An irrelevant isotype control monoclonal antibody directed against a trinitrophenyl moiety [14] was raised in a mouse IgG1 version (murine isotype control mAb) and humanised as an IgG4 version (Hz isotype control mAb). All antibodies were formulated in phosphate buffered saline (PBS), pH 7.2. Endotoxin levels were below 1 EU/mg IgG. Surface plasmon resonance (SPR) data showed a strong binding of concizumab to human and rabbit TFPI (K_D = 0.03 nM and 0.05 nM respectively), whereas binding to rat TFPI was below the detection limit.

Endothelial Cell Studies

EA.hy926 and ECV304 cells

The human endothelial cell-like immortalised cell line EA.hy926 WT, derived from umbilical vein cells is positive for TFPI. This cell line was used as a positive TFPI control for the binding of concizumab. As negative control we used the aerolysin-resistant EA.hy926 AR cells that do not express GPI-anchored proteins on their surface, and thus lack GPI-anchored TFPI [15]. Both EA.hy926 WT and AR cell lines were tested for binding by staining for concizumab and matching isotype mAb. For labelling, 50 μl of cell preparations were added to 96 well plates (Greiner, cat no. 65021) together with 50 μl of diluted mAbs (6–300 μg/ml) and incubated at 4 °C for 1 hour. After incubation and wash (PBS with 5% fetal calf serum (FCS), centrifuged for 5 minutes at 200 g) the secondary PE-conjugated goat anti-human IgG (Jackson Immunoresearch #109-116-170, West Grove, PA) (2.5 μg/ml in PBS) was added and incubated for 1 hour at 4 °C. Finally, cells were washed and fixed with paraformaldehyde (1% w/v in PBS). Samples were analysed on a BD FACS-array flow cytometer. Signals were collected in the PE-channel. Analysis of results was done using FSC-Express Version 3 (Nr 300.0707) and GraphPad Prism Software (version 5).

EA.hy926 WT and the human immortalized endothelial cell-like cell line ECV304 were grown to 100% confluence in 96 well plates in DMEM (Gibco) and in Medium 199 (Gibco), respectively, both supplied with 10% FCS and 1% penicillin/streptomycin. EA.hy926 WT, EA.hy926 AR and ECV304 cell lines were used for testing of neutralisation of cell surface TF-dependent FX activation. Cells were washed twice with 25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, pH 7.4 before FX activation was measured in 25 mM HEPES, 137 mM NaCl, 3.5 mM KCl, 5 mM $CaCl_2$, 1 mg/ml BSA pH 7.4. After incubation for 15 min at 37 °C, neutralisation of TFPI was induced by addition of concizumab (0 – 9 μg/ml; 0 – 60 nM) for 120 min. This was followed by incubation with 50 pM rFVIIa (Novo Nordisk A/S) for 15 min. Generation of FXa was then initiated by addition of 50 nM FX for 40 min at 37 °C. The reaction was stopped by mixing 40 μl supernatant with 10 μl (75 mM) EDTA and FXa activity was finally measured with 0.6 mM chromogenic substrate S-2765 (Chromogenix). FXa concentration was calibrated towards a FXa standard curve and the specificity of cell surface TF/FVIIa activity was verified in control experiments with addition of 0.5 mg/ml neutralising goat anti human TF polyclonal antibody (Novo Nordisk A/S). In addition a possible unspecific effect of the humanized IgG1 was tested within the relevant concentration range in control experiments with a humanized isotype control monoclonal IgG4 antibody. Data were analysed with GraphPad Prism Software (version 5).

Animals

This study conformed to the Danish legislation governing animal experiments, and was carried out under a permission granted from the Danish Animal Experiments Inspectorate. Female New Zealand White rabbits (Charles River Laboratories) weighing 2.9 ± 0.6 kg, (n = 27) were included. All rabbits were allowed at least 5 weeks of acclimatisation before inclusion in the study. The rabbits were housed in colonies of 8 to 10 animals with controlled room temperature and humidity. Male Sprague–Dawley rats (Charles River Laboratories)