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Regular Article

Differences in the inhibition of coagulation factor XIII-A from animal species revealed by Michael Acceptor– and thioimidazol based blockers

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ABSTRACT

Introduction: The A-subunit of blood coagulation factor XIII is a pro-transglutaminase, which cross-links α- and γ-fibrin-chains in its activated form. Selective inhibitors against FXIII-A may be desirable drugs to prevent the development of thromboses. Animal models are generally used for proof of principle and for toxicological studies in drug development. The aim of the study was to investigate the specificity of a set of FXIII-A-blockers against FXIII-A from different species, i.e. human, dog, mouse, rat and pig. Thus the usefulness of different animal species for FXIII-A-blocker drug development should be evaluated.
Materials and Methods: FXIII-A proteins were recombinantly produced in insect cells and purified to homogeneity. They were characterized by SDS- and native PAGE, a transamidase assay and isopeptidase assay. The inhibition second-order rate constants of different irreversible inhibitors were determined using the isopeptidase assay.
Results: All FXIII-A species were able to assemble with recombinant human FXIII-B into a heterotetrameric complex. Kinetic parameters of FXIII-A species were determined. Second-order rate constants for FXIII-A inhibition by two irreversible inhibitors were determined and differed considerably. FXIII-A species of dog, mouse and rat were inhibited in a manner similar to human FXIII-A. Pig FXIII-A however was resistant to a previously described non-peptidic inhibitor. Furthermore, the results showed considerably better inhibition with the novel peptide-based inhibitor compared to the non-peptidic compound.
Conclusions: Our data shows that biochemical interspecies comparison studies are a prerequisite for animal studies. Peptide-derived inhibitors carrying a Michael Acceptor Pharmacophore (MAP) are a promising new class of FXIII-A-inhibitors.

Introduction

Blood coagulation factor XIII is a heterotetrameric pro-transglutaminase (FXIII-A_2B_2)[2], consisting of two catalytic A subunits (FXIII-A) and two carrier B subunits (FXIII-B). FXIII-A comprises cross-linking activity and is normally present in an inactive form in plasma. Upon cleavage between Arg37 and Gly38 by thrombin, the N-terminal activation peptide is removed. The heterotetrameric complex subsequently dissociates in the presence of Ca^{2+}, allowing the truncated FXIII-A to obtain its active conformation to catalyze the formation of inter- and intramolecular ε-(γ-glutamyl)-lysine bonds [2]. The carrier subunit FXIII-B binds to FXIII-A, preventing spontaneous activation [3]. In addition, it prevents proteolytic degradation of FXIII-A [4]. Under normal conditions, only tetrameric FXIII is found in plasma, with an excess of about 50% free FXIII-B [5–7].

The plasmatic blood coagulation process includes a cascade of enzymatic steps leading to the activation of pro-thrombin to thrombin (factor IIa). Thrombin then cleaves off the fibrinopeptides from fibrinogen. The resulting fibrin molecules assemble non-covalently resulting in a soft, urea-soluble fibrin clot [8]. Finally thrombin activates FXIII to FXIIIa, which by its transglutaminase activity cross-links fibrin and covalently attaches anti-fibrinolytic factors like α_2-antiplasmin [2]. Since FXIII-A is the essential enzyme in clot stabilization and maturation, selective inhibitors may provide a new therapeutic option for anticoagulation prophylaxis. Primary plug formation by platelet aggregation and non-stabilized fibrin clot formation should not be affected. Lorand and coworkers [9] showed that by inhibiting fibrin-crosslinking with an excess of competing substrate for FXIII-A, the resulting soft clot was much more susceptible to fibrinolysis compared to control.

Abbreviations[2]: Abz, ortho-aminobenzoic acid; CAD, cadaverine; DNP, 2,4-dinitrophenyl; tBu, tert-butyl ester; Trt, trityl; Fmoc, Fluorenylmethyloxycarbonyl; DMF, Dimethylformamide; TBTU, O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate; HOBt, Hydroxybenzotriazole; DIPEA, N,N-Diisopropylethylamine; ClTrt, Chlorotrityl; HATU, 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Boc, tert-Butyloxycarbonyl; TFA, Trifluoro acetic acid; MAP, Michael Acceptor Pharmacophore.

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[2] with regards to "Factor XIII: terms and abbreviations" recommended by Muszbek and coworkers [1].

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In early phases of the drug development process, animal studies generally have to be performed for proof of principle, pharmacological and toxicological studies. Transferability of results from animals to humans requires comparable activities of drug candidates on the animal and human targets. Interspecies differences in the activity of anticoagulant drugs are especially described for coagulation factor Xa (FXa), where substantial differences were observed using a variety of different inhibitors and animal models [10–13]. This illustrates the potential hazard of interspecies differences which may lead to inappropriate extrapolation of experimental results. In order to provide highly active but isotype-selective transglutaminase inhibitors, we developed a novel group of peptide-based transglutaminase blockers called TIMAs: Transglutaminase Inhibiting Michael Acceptors. TIMAs generally are composed of a peptidic or peptidomimetic backbone and a Michael acceptor as pharmacophore (Fig. 1A). The Michael acceptor, an electrophilic α,β-unsaturated carbonyl compound, is attacked by the active site cysteine of the transglutaminase, which then forms an irreversible complex (Fig. 1B) and leads to enzyme inactivation. Michael acceptors have already been used for cysteine protease inhibitors [14]. In cysteine protease blockers, Michael acceptors are linked to the C-terminus of the peptide or peptidomimetic backbone. In contrast, TIMAs contain the Michael acceptor in the side chain, thus mimicking substrate glutamine. To mention an example, ZED754 targets tissue transglutaminase. Structure analysis of TG2-ZED754 conjugate co-crystals confirmed the open conformation of inhibitor bound TG2 as described [15,16].

We recently developed potent blockers of human FXIII-A based on peptides carrying a Michael acceptor warhead. The first main part of the study was to express and purify the recombinant FXIII-A from animals commonly used as preclinical models and human FXIII-B, followed by their characterization. The second main part was to investigate whether there are differences in the inactivation of human FXIII-A compared to aminal FXIII-A. Examined inhibitors were a TIMA inhibitor and a previously described non-peptidic inhibitor, 1,3-dimethyl-2[(2-oxopropyl)thio]imidazolium chloride (D003) [17]. Moreover, the influence of Val34Leu-polymorphism of recombinant human FXIII-A and complex-formation with recombinant human FXIII-B (rFXIII-A_2B_2) in respect of inhibition were analysed. In the following text, the term "species" relates to recombinant FXIII-A from human, dog, pig, rat and mouse.

Methods

cDNA-cloning

The cDNAs encoding FXIII-A from different species and human FXIII-B were obtained from the following sources: *Canis lupus familiaris* F13A1-cDNA (NCBI reference sequence XM_535876.3) was isolated from a kidney tissue-derived cDNA library (Biocat, Heidelberg, Germany) by PCR. *Mus musculus* F13A1-cDNA (NCBI reference sequence NM_028784.3) and human F13B-cDNA (NCBI reference sequence NM_001994.2, containing R115H exchange) were isolated by PCR from IMAGE clones (IMAGENES, Berlin, Germany). F13A1-cDNAs of *Rattus norvegicus* and *Sus scrofa* were synthesized (GeneArt, Regensburg, Germany) according to NCBI reference sequences NM_021698.2 and XM_001927630.4, respectively. Human F13A1-cDNA (NCBI reference sequence NM_000129.3) was isolated from a cDNA-clone (Open Biosystems, Lafayette, USA). For cloning into vector pFastBac1 (Invitrogen, Karlsruhe, Germany), all cDNAs were combined with compatible restriction sites for cloning. Furthermore, a sequence encoding a hexahistidine-tag was introduced at the 5'end of F13A1-cDNAs. The pFastBac1-vector containing human F13A1-cDNA served as template for generating human F13A1-Val34Leu-cDNA using site directed PCR mutagenesis. All constructs have been checked for accuracy by DNA sequencing.

Insect Cell Expression

Recombinant baculoviruses were generated using the Bac-to-Bac Expression system (Invitrogen, Karlsruhe, Germany). Virus titers were determined using BacPak Baculovirus Rapid Titer Kit (Clonetech, Saint-Germain-en-Laye, France) as described by the manufacturer. Sf9 insect cells were cultured in serum free medium (Sf 900 II SFM, Invitrogen)

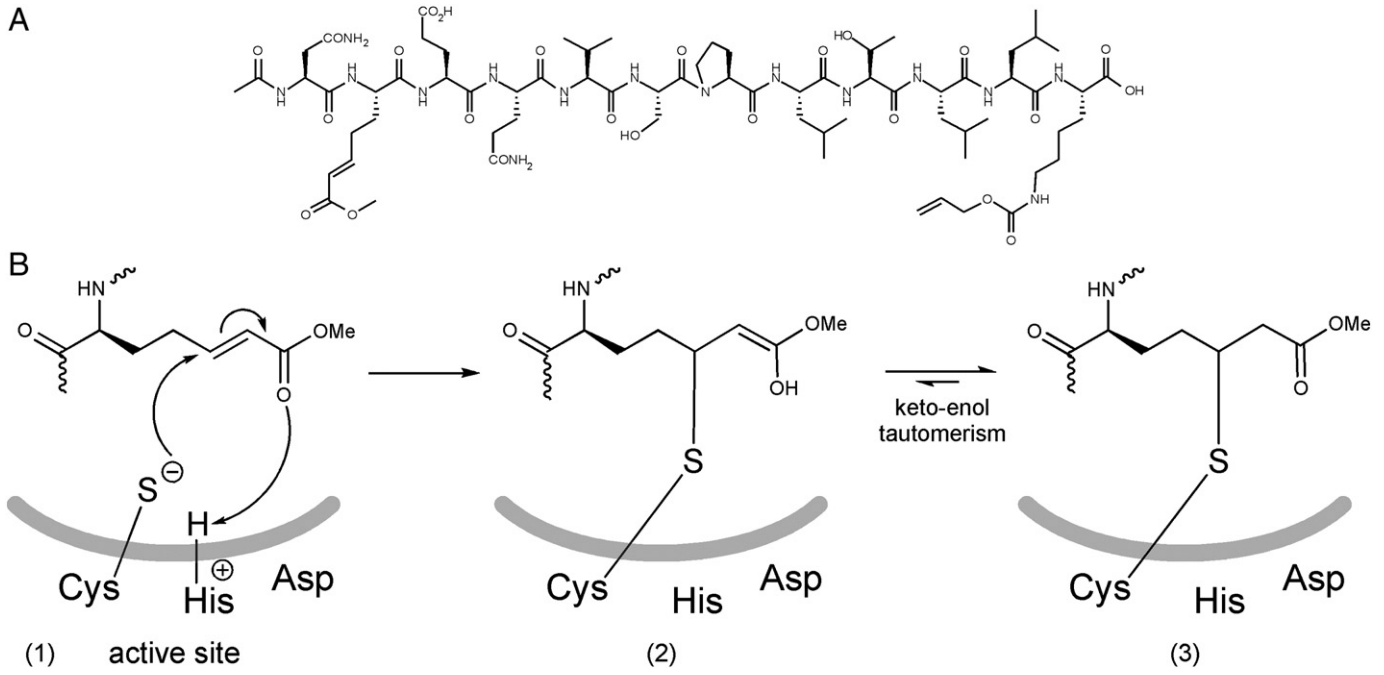

Fig. 1. Michael-acceptor based peptidic inhibitor ZED1251; **A**: Structure of ZED1251. **B**: The electrophilic α,β-unsaturated carbonyl compounds are attacked by the active site cysteine residue (1) to form an irreversible complex (2,3).