Structure-guided residence time optimization of a dabigatran reversal agent

Felix Schiele1,*, Joanne van Ryn2, Tobias Litzenburger1, Michael Ritter1, Daniel Seeliger3, and Herbert Nar3

1New Biological Entities Discovery; Boehringer Ingelheim GmbH & Co. KG; Biberach, Germany; 2CardioMetabolic Diseases Research; Boehringer Ingelheim GmbH & Co. KG; Biberach, Germany; 3Lead Identification and Optimization Support; Boehringer Ingelheim GmbH & Co. KG; Biberach, Germany

Keywords: antidote, thrombin, Pradaxa, rational protein design, residence time, idarucizumab

Abbreviations: CDR, complementarity determining region; Fab, fragment antigen binding; RMSD, root mean square deviation; VKA, vitamin K antagonist

Novel oral anticoagulants are effective and safe alternatives to vitamin-K antagonists for anticoagulation therapy. However, anticoagulation therapy in general is associated with an elevated risk of bleeding. Idarucizumab is a reversal agent for the direct thrombin inhibitor, dabigatran etexilate (Pradaxa®) and is currently in Phase 3 studies. Here, we report data on the antibody fragment aDabi-Fab2, a putative backup molecule for idarucizumab. Although aDabi-Fab2 completely reversed effects of dabigatran in a rat model in vivo, we observed significantly reduced duration of action compared to idarucizumab. Rational protein engineering, based on the X-ray structure of aDabi-Fab2, led to the identification of mutant Y103W. The mutant had optimized shape complementarity to dabigatran while maintaining an energetically favored hydrogen bond. It displayed increased affinity for dabigatran, mainly driven by a slower off-rate. Interestingly, the increased residence time translated into longer duration of action in vivo. It was thus possible to further enhance the efficacy of aDabi-Fab2 based on rational design, giving it the potential to serve as a back-up candidate for idarucizumab.

Introduction

Thromboembolic disorders such as myocardial infarction, stroke and venous thromboembolism are the most common cause of mortality and morbidity in Western societies. These thromboembolic events can be triggered by an excessive activation of coagulation, which involves multiple factors, with thrombin being one of the critical components.

Orally available vitamin K antagonists (VKAs) such as warfarin have been used for decades for long-term anticoagulation. VKAs are cumbersome to use as they display multiple interactions with other drugs and food, which result in the need for regular patient monitoring. Thus, these agents have a narrow therapeutic window between the desired anticoagulant effect and potential adverse hemorrhagic effects. As a consequence many patients do not receive adequate anticoagulation therapy or they do not receive any anticoagulation therapy.

Newer oral anticoagulants, such as dabigatran, were designed to circumvent these disadvantages of warfarin therapy and thereby increase appropriate use of anticoagulation to prevent thromboembolic events resulting in thrombosis and stroke. Dabigatran (Fig. 1A) is a potent, non-peptidic direct thrombin inhibitor. The orally administered double prodrug, dabigatran etexilate, is hydrolyzed in vivo by esterases into the active form, dabigatran. Dabigatran has a half-life of 11–13 hours and is renally cleared. Dabigatran etexilate is approved for the treatment and prevention of venous thromboembolism and the prevention of stroke in patients with atrial fibrillation. In all indications, a fixed-dose regimen has provided effective anticoagulation with a favorable bleeding profile compared to warfarin, without regular monitoring or dose adjustment.

In general, anticoagulation therapy is associated with an elevated risk of bleeding. Outcomes after 30 days in patients receiving dabigatran and warfarin who had major or life-threatening bleeding were compared in all dabigatran Phase 3 clinical trials and found to be similar or slightly improved in patients with dabigatran. This may be due to its shorter duration of action because of its shorter half-life as compared to warfarin. Despite all these data, there is a perception that the lack of a specific reversal agent will prevent adequate treatment of these infrequent events, resulting again in patients not receiving adequate anticoagulation therapy.

We previously reported that the antibody fragment idarucizumab (referred to here as aDabi-Fab1 for consistency) binds dabigatran and immediately neutralizes its anticoagulant effect. Similar results have also been obtained in healthy volunteers, where dabigatran anticoagulation could immediately be reversed after idarucizumab administration. We report here a distinct, novel antibody fragment against dabigatran (aDabi-Fab2) that was identified in a search for backup clinical candidates to
Figure 1. Structures of aDabi-Fab2 in complex with dabigatran. (A) Chemical structure of dabigatran. (B) Representative electron density (2Fo-Fc) for dabigatran bound to aDabi-Fab2. The benzamidine group is completely defined whereas the electron density becomes weaker toward the carboxamide moiety. (C) The benzamidine group of dabigatran (purple colored and represented in sticks and balls) is buried in a pocket formed at the interface of the Fab’s heavy chain (orange) and light chain (light yellow) whereas the benzimidazole and pyridine moieties are partially exposed to the solvent. (D) Four crystallographically independent structures reveal distinct conformations of dabigatran and H:Tyr103. In 3 of the 4 structure, H:Tyr103 forms an H-bond to L:Asp33. (E) Unliganded aDabi-Fab2 (green) superimposed onto dabigatran bound aDabi-Fab2 (silver). The distal part of CDR:H3 from H:Ser101 – H:Phe106 blocks the binding pocket in the unbound form. (F) Representative complex structure of aDabi-Fab2. Amino acids that were manually selected for mutations are indicated in red.
Table 1. Crystallographic data and refinement statistics

|                  | aDabi-Fab2a apo (PDB-ID: 4YGV) | aDabi-Fab2a: Dabigatran 1 (PDB-ID: 4YHI) | aDabi-Fab2a: Dabigatran 2 (PDB-ID: 4YHK) | aDabi-Fab2b: Dabigatran (PDB-ID: 4YHL) | aDabi-Fab2b: Dabigatran apo (PDB-ID: 4YHM) | aDabi-Fab3 apo (PDB-ID: 4YHN) | aDabi-Fab3: Dabigatran (PDB-ID: 4YHO) |
|------------------|-------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| **Data collection** |                               |                                        |                                        |                                        |                                        |                                        |                                        |
| Space group      | P1                            | P2,1                                   | P2,2,2,1                               | P6,1                                   | P2,2,2,1                               | P1                                     | P2,2,2,1                               |
| Cell parameters  |                               |                                        |                                        |                                        |                                        |                                        |                                        |
| a (Å)            | 55.9                          | 51.8                                   | 48.2                                   | 103.7                                  | 60.0                                   | 55.6                                   | 60.3                                   |
| b (Å)            | 56.2                          | 128.9                                  | 59.7                                   | 103.7                                  | 78.4                                   | 56.1                                   | 78.1                                   |
| c (Å)            | 81.5                          | 60.3                                   | 127.7                                  | 84.9                                   | 87.7                                   | 78.5                                   | 93.4                                   |
| α (deg)          | 87.4                          | 90.0                                   | 90.0                                   | 90.0                                   | 90.0                                   | 88.9                                   | 90.0                                   |
| β (deg)          | 83.0                          | 92.3                                   | 90.0                                   | 90.0                                   | 90.0                                   | 84.9                                   | 90.0                                   |
| γ (deg)          | 65.6                          | 90.0                                   | 90.0                                   | 120.0                                  | 90.0                                   | 65.7                                   | 90.0                                   |
| Resolution range (Å) | 51.13 – 1.76                 | 128.92 – 1.90                          | 127.69 – 2.21                          | 51.85 – 2.09                           | 87.67 – 2.16                           | 29.98 – 2.31                           | 42.59 – 1.82                           |
| No. observations | 154351 (22607)                | 202218 (29154)                         | 119943 (17227)                         | 309519 (46252)                         | 144508 (17047)                         | 56747 (11255)                          | 259467 (63260)                         |
| No. unique observations | 85149 (12315)            | 61726 (9015)                           | 19263 (2767)                           | 30655 (4455)                           | 22495 (3019)                           | 33587 (6981)                           | 40152 (9420)                           |
| R_{free} (%)     | 10.7 (44.5)                   | 12.1 (60.1)                            | 15.8 (63.8)                            | 10.6 (62.7)                            | 14.0 (59.4)                            | 8.3 (38.5)                             | 12.1 (49.7)                            |
| Average I/σ(I)  | 7.5 (2.3)                     | 9.6 (2.4)                              | 9.8 (3.2)                              | 15.8 (3.4)                             | 11.0 (3.2)                             | 10.0 (2.7)                             | 11.6 (4.1)                             |
| Completeness (%) | 96.2 (95.0)                   | 99.4 (99.8)                            | 99.9 (99.8)                            | 100 (100)                              | 98.7 (93.5)                            | 89.4 (77.6)                            | 99.7 (97.9)                            |
| Multiplicity     | 1.8 (1.8)                     | 3.3 (3.3)                              | 6.2 (6.2)                              | 10.1 (10.4)                            | 6.4 (5.6)                               | 1.7 (1.6)                              | 6.5 (6.7)                              |
| **Refinement statistics** |                       |                                        |                                        |                                        |                                        |                                        |                                        |
| Molecules in ASU | 2                             | 2                                      | 1                                      | 1                                      | 1                                      | 2                                      | 1                                      |
| No. reflections  | 85139                         | 61647                                  | 19095                                  | 30575                                  | 22428                                  | 33555                                  | 40033                                  |
| R_{work} (%)     | 17.8                          | 18.1                                   | 17.4                                   | 18.1                                   | 17.0                                   | 18.9                                   | 18.0                                   |
| R_{free} (%)     | 19.8                          | 21.5                                   | 23.4                                   | 22.1                                   | 22.2                                   | 22.0                                   | 21.0                                   |
| No. protein atoms | 607                           | 6733                                   | 3356                                   | 3330                                   | 3354                                   | 6708                                   | 3376                                   |
| No. dabigatran atoms | 70                             | 35                                     | —                                     | 35                                     | —                                     | 35                                     | —                                      |
| No. water molecules | 827                         | 855                                    | 250                                    | 326                                    | 321                                    | 325                                    | 439                                    |
| RMSD from ideality |                      |                                        |                                        |                                        |                                        |                                        |                                        |
| Bond lengths (Å) | 0.008                         | 0.008                                  | 0.008                                  | 0.008                                  | 0.008                                  | 0.008                                  | 0.008                                  |
| Bond angles (deg) | 1.03                          | 1.07                                   | 1.10                                   | 1.07                                   | 1.08                                   | 1.11                                   | 1.04                                   |

Values in parentheses correspond to the highest resolution shell.

aDabi-Fab1. By structure-guided protein design, we were able to improve the affinity and residence time (defined as the inverse of the binding dissociation rate constant, k_d) of the original aDabi-Fab2 significantly. The optimized mutant aDabi-Fab3 exhibits prolonged and more effective neutralization of dabigatran’s anticoagulant effects in vivo compared to the parental antibody fragment.

### Results

**Antibody generation**

aDabi-Fab2 was generated by immunizing mice with dabigatran-derived hapten and subsequent screening of hybridoma clones for binding to dabigatran as described previously.11 aDabi-Fab2 had a K_d of ~180 pM, and thus a ~90-fold weaker affinity than aDabi-Fab1 (K_d ~ 2 pM). Structural studies were performed to understand the difference in affinities at a molecular level.

**Overall structure of Fabs**

We chose recombinant expression of Fab to allow for a more homogenous product with defined chain length; cleavage of IgG with papain would have resulted in a more heterogeneous C-terminal end of the H-chain, which could have affected the immunogenic and pharmacokinetic properties of the molecule. Purification over a benzamidine matrix facilitates the enrichment of Fabs with functional binding activity and eliminates non-functional molecules such as L-chain homodimers.

Two variants of aDabi-Fab2 that differ only at 2 positions in the framework regions 3 and 4 of the light chain were crystallographically characterized. In total, 5 distinct crystal structures were determined, yielding structural information on 4 crystallographically independent aDabi-Fab2-dabigatran complex structures and 3 unbound forms (detailed crystallographic information shown in Table 1) at resolutions of 2.3 Å or better. Both Fabs show no major structural differences in both liganded and unliganded states, respectively. Therefore, for convenience, the further structural description will refer to a representative single Fab structure. If the respective features differ significantly between protomers in the different crystal systems, they are mentioned explicitly. The aDabi-Fab2 is composed of 2 variable domains (VL and VH) and 2 constant domains (CL and CH3). These domains show the typical immunoglobulin fold.14 The antigen binding site of the antibodies is formed by the complementarity-determining regions (CDRs) on the surface of the variable domains, which are named from L1 to L3 and H1 to H3. The heavy and light chain are covalently linked via a disulfide bond formed by their C-terminal cysteines.
Table 2. Characterization of generated mutants

| aDabiFab          | Expression / Purification | Binding in ELISA | Octet | Kinexa $K_a$ [pM] | Kinexa $K_d$ [s$^{-1}$] | Kinexa $k_1$ [M$^{-1}$ s$^{-1}$] |
|-------------------|---------------------------|------------------|-------|------------------|--------------------------|----------------------------------|
| Dabi-Fab1         | +                         | +                | 2     | 7.3 x 10$^{-7}$  | 3.4 x 10$^4$              |                                  |
| aDabi-Fab2        | +                         | +                | 173   | 2.3 x 10$^{-4}$  | 1.3 x 10$^6$              |                                  |
| H: T30E D31E Y33H E50R N52D N55T Y103M F106H L: Y37H D75G F94H A96G Y101E Q105G | No | –                | –     | –                | –                         |                                  |
| H: T30E D31E Y33H E50R N52D N55T Y103M F106H L: Y37H D75G F94H A96S Y101E Q105G | No | –                | –     | –                | –                         |                                  |
| H: T30E D31E Y33H E50R N52D N55T Y103E F106H L: Y37H D75G F94H A96S Y101E Q105G | No | –                | –     | –                | –                         |                                  |
| H: Y33W, Y103W, N52H | No | –                | –     | –                | –                         |                                  |
| L:Y33H            |                           | +                | No    | –                | –                         |                                  |
| L:Y33L            |                           | +                | No    | –                | –                         |                                  |
| L:Y37L            |                           | +                | No    | –                | –                         |                                  |
| H:N52H            |                           | +                | No    | –                | –                         |                                  |
| H: Y33W, Y103W, N52H | No | –                | –     | –                | –                         |                                  |
| L:Y33H            |                           | +                | No    | –                | –                         |                                  |
| L:Y33L            |                           | +                | No    | –                | –                         |                                  |
| L:Y37L            |                           | +                | No    | –                | –                         |                                  |
| H:N52H            |                           | +                | No    | –                | –                         |                                  |

**Binding mode of aDabi-Fab2 to dabigatran**

aDabi-Fab2 binds dabigatran in a different conformation and with different molecular interactions compared to aDabi-Fab1 (Supplementary Data S1) or thrombin. All CDR loops except L2 are involved in dabigatran binding. The binding is mediated by hydrophobic interactions, H-bonds and a salt bridge. The benzamidine moiety of dabigatran inserts into a cavity formed by heavy and light chain, whereas the benzimidazole, carboxamide and pyridine moieties are partially exposed to solvent (Fig. 1B, C). It is noteworthy that the benzamidine moiety of dabigatran is bound in an identical fashion in all complex structures with a rmsd (root mean square deviation) of 0.03 Å, whereas the rest of the ligand adopts slightly different conformations with a maximum rmsd of 1.58 Å over all atoms and also displays higher B-factors, which indicates higher flexibility for this part of the bound dabigatran molecule (Fig. 1D). As a consequence, the contact area (defined as the difference in total accessible surface area divided by 2) between dabigatran and the Fab for the various aDabi-Fab2-dabigatran complexes varies between 468 Å$^2$ and 492 Å$^2$. This variation is almost exclusively caused by contacts formed to the heavy chain of the aDabi-Fab2 as it ranges from 370 Å$^2$ to 392 Å$^2$, whereas the contact areas for the light chain remain constant at about 100 Å$^2$.

The most important interaction in the binding of dabigatran to aDabi-Fab2 is a bidentate salt bridge formed within a deep hydrophobic pocket in the interface region of heavy and light chain. The benzamidine moiety of dabigatran inserts into this cavity and interacts with L:Glu39 in CDR:L1 with nitrogen-oxygen distances of 2.7 Å. This interaction is stabilized as one of the amidine nitrogens forms a hydrogen bond to the carbonyl oxygen of H:Tyr103 (2.6 Å) in CDR-H3. The amine nitrogen in the biatomic bridge between the benzamidine and the benzimidazole and the amidine nitrogen of the benzimidazole moiety of dabigatran both participate in a H-bonding network, which is mediated by 2 water molecules. These waters bind to H:Tyr33 and H:Gly99, but, as they are partially solvent exposed, these H-bonds are not thought to contribute significantly to binding affinity. In three of the 4 protomers of the complex structures, the carboxyl group of dabigatran forms a fully solvent-exposed H-bond with H:Arg54.

Interactions of aryl rings are ubiquitously found in protein structures as well as in protein ligand complexes. Two π-systems interact predominantly via T-shaped edge-to-face geometry or in a parallel-displaced stacking arrangement. Both orientations are observed in the costructures of dabigatran and its neutralizing aDabi-Fab2. In the binding pocket for the benzamidine moiety, H:Phe106 displays a N-H/π interaction with an amidine nitrogen (3.4 Å distance of nitrogen to ring centroid). In this nonclassical hydrogen bond, the π-electrons of the phenyl ring act as H-bond acceptor. H:Tyr33 is involved in displaced π-stacking with the benzimidazole moiety (3.3–3.4 Å), and interacts in an edge-to-face fashion with the pyridine ring of the ligand (3.5–3.8 Å). This pyridine group forms an additional cation-π interaction with the guanidinium group of H:Arg53 in CDR-H2 with a distance of 3.8–4.4 Å. Arginine is the predominant amino acid providing a cation in cation-π interactions. The H:Tyr103 side chain forms one edge of the dabigatran binding pocket of the antibody fragment. Its position is stabilized by...
a hydrogen bond of its OH group to the carboxylate group of L: Asp33. While the H:Tyr103 side chain is thus in a conserved conformation for 3 of the 4 crystallographically independent structures, we observe a series of distinct and ordered conformations of the benzimidazole pyridine carboxamide part of the dabigatran molecule, which results in a variation of the distance of the closest atoms of the H:Tyr103 phenol and benzimidazole ring systems between 3.8 and 4.4Å. In the fourth structure, the H:Tyr103 OH – L:Asp33 carboxylate hydrogen bond is not seen. Instead, the benzimidazole pyridine carboxamide part of dabigatran is positioned very close to the H:Tyr33/H:Arg53 side of the binding pocket and H:Tyr103 is in edge-to-face π-stacking distance (Fig. 1D). The above observations suggest that the portion of the free energy associated with the edge-to-face π-stacking and the H:Tyr103 OH – L:Asp33 carboxylate hydrogen bond are roughly equivalent. The crystallographic data further suggest that H:Tyr103 exists in 2 defined low energy conformations in the dabigatran bound state of aDabi-Fab2.

Structures of the unbound Fabs
The distal part of CDR-H3 adopts a significantly different conformation between free and complexed form (Fig. 1E). A change in the backbone and side chain conformations of the H: Ser101 – H:Tyr105 region results in a movement of H:Asp104 Cα by 5.2 Å and a shift of 10.2 Å for its carboxylate moiety. This loop and side chain reorientation results in a closed conformation of the antibody fragment that blocks access to L:Glu39, which is the receptor of the dabigatran amidine moiety in the ligand bound conformation. The above residues are involved in a tight crystal contact, which may contribute to induction of the particular loop structure in 2 of the 3 crystallographically independent unbound aDabi-Fab2 structures. However, in the third structure, a very similar loop structure is observed although no crystal contact is present. Here, additional structural changes relative to the bound state are observed in the region H:Phe29 – H: Tyr33 of CDR-H1 with the H:Tyr33 side chain being positioned within the benzimidazole site of the bound form, and thus further closing down the dabigatran binding site.

Functional properties of aDabi-Fab2
aDabi-Fab2 shows tight binding to dabigatran (K_D = 180 pM) (Fig. 2A), with an off-rate of 2.3 × 10^{-4} s^{-1} and neutralizing effects on dabigatran activity in vivo. This also translated into similar inhibition of the anticoagulant effect of dabigatran in vitro in human plasma compared to aDabi-Fab1 (Fig. 2B). However, compared to aDabi-Fab1, there was a significantly reduced duration of action in vivo (Fig. 2C). While aDabi-Fab1 completely reversed the anticoagulant effects of dabigatran for

Figure 2. Characterization of aDabi-Fabs. (A) Constant concentrations of Fab were incubated with increasing concentrations of dabigatran. The concentration of free Fab was then determined by capturing the unbound Fab on a dabigatran-biotin conjugate coupled to Neutravidin beads. (B) Comparison of the effects of aDabi-Fab1, 2 and 3 in inhibiting the anticoagulant effect of 7nM dabigatran in vitro in human plasma. IC_{50} shown in nM, n = 4 determinations. (C) Neutralization of dabigatran anticoagulant activity by aDabi-Fab 1, 2 and 3 after intravenous administration in rats that were pre-treated with a continuous infusion of dabigatran. Anticoagulant activity was measured as the thrombin time using 3U/ml thrombin. Data shown as mean ± SE, n = 6–8.
Design of mutants
Optimization was based on a structural analysis of the bound versus unbound states of aDabi-Fab2, comparison of the dabigatran binding sites between aDabi-Fab1 and aDabi-Fab2 and computational protein design using the enzyme design protocol of Rosetta. We selected a set of paratope residues for mutagenesis (Fig. 1F) and designed 20 mutants of aDabi-Fab2 that were expected to exhibit improved binding affinities, residence times and dabigatran neutralization properties relative to the parental aDabi-Fab2 antibody fragment (Table 2). For this purpose, we considered 2 possible, general approaches: 1) Destabilizing the closed, unbound form of aDabi-Fab2 and driving the conformational equilibrium toward the open, ligand bound state, which could be facilitated by introduction of mutations that would interfere with the conformation observed in the unbound structures; and 2) Stabilizing the complex by enhancing the potential for molecular interactions between protein and ligand. We thus aimed to introduce additional interaction opportunities in the benzamidine pocket, e.g., by replacing H:Phe106 with histidine so that an additional H-bond flanking the salt bridge between dabigatran and L:Glu39 could form. This was seen in the complex structures of dabigatran with thrombin and dabigatran with aDabi-Fab1 (both complexes have sub-nanomolar binding affinities). In addition, we tried to optimize shape complementarity by mutating selected residues that contact dabigatran, e.g., by enlarging amino acid side chains involved in hydrophobic or π-stacking interactions with dabigatran such as H:Tyr33 and H:Tyr103.

Affinity screening of mutated Fabs
Twenty mutated Fabs were transiently expressed in HEK293 cells and purified on a benzamidine matrix, thus enabling selection of Fabs with functional binding domains. Eight Fabs that showed binding to immobilized dabigatran in ELISA were ranked in a kinetic binding assay according to their off-rate (Fig. 1F). Other Fabs showed too low expression for further analysis, and no detectable binding to dabigatran in ELISA. Fab mutant H:Y103W, then designated aDabi-Fab3, which had a single replacement of tyrosine by tryptophan at position 103 of the heavy chain, displayed the lowest off-rate (8.1 × 10^{-5} s^{-1}) in this ranking. Two other Fabs, mutants H:Y103M and H:Y33W, Y103W, where position 103 was mutated to tryptophan or to a methionine, also showed improvement of off-rates compared to the parent aDabi-Fab2. The kinetic assay enabled off-rate ranking, but did not allow for a reliable determination of dissociation constants (Kₐ) due to its limited sensitivity. Therefore, we applied Kinexa technology to determine the Kᵦ in solution of aDabi-Fab3 as was previously done for aDabi-Fab1 and aDabi-Fab2. The data show a Kᵦ of 18 pM for aDabi-Fab3, and thus a 10-fold improvement in affinity over the parental Fab2 (Kᵦ ~180 pM). The on-rate of aDabi-Fab3 was increased ~2-fold compared to aDabi-Fab2; however, the improved affinity of aDabi-Fab3 was mainly the result of a ~5-fold lower off-rate (Table 2).

The attempt to introduce an additional H-bond to tighten interactions in the benzamidine pocket, thereby stabilizing the key interaction, e.g., the salt bridge to L:Glu39, completely impaired binding of the aDabi-Fab2 mutants containing the F106H exchange. This mutant was not able to bind to benzamidine sepharose, a standard step in the purification procedure for dabigatran-binding antibody fragments. As this was one of 5 variants that could not be purified in this way, we assume that the mutation distorts the geometry of the binding pocket. The strongest effect on residence time and Kᵦ was observed for the Y103W mutant, which showed a 10-fold increase in affinity and a 5-fold slower off-rate.

Structure of mutant Y103W (aDabi-Fab3)
In order to elucidate the structural basis for the enhanced binding properties of the Y103W mutant (aDabi-Fab3), we determined the crystal structures of both dabigatran bound and unbound forms. As expected for this surface mutation, we did not observe any structural changes in the unbound form relative to the parental aDabi-Fab2 structure (Supplementary Data S2). However, in the dabigatran complex the tryptophan side chain in aDabi-Fab3 adopts a similar conformation compared to the aDabi-Fab2 tyrosine side chain, preserving the hydrogen bonding to L:Asp33 with its indole nitrogen. The larger size of the tryptophan side chain in turn leads to a much tighter fit of dabigatran in the binding site with the formation of aromatic edge-to-face (Trp103) and face-to-face (Tyr33) interactions of the dabigatran benzimidazole pyridine carboxamide moiety on both of its sides. Indeed, dabigatran is now in a position that closely resembles one of the conformers observed in the aDabi-Fab2 co-structures with Tyr103 in a different position in the binding site having lost the hydrogen bond to L:Asp33 and pushing dabigatran toward the Tyr33/Arg53 side of the binding pocket forming a π-stacking interaction (Fig. 3).

This structural analysis provides a qualitative explanation for the improved affinity of the mutant Fab relative to the parental Fab. Introduction of tryptophan at position 103 of the heavy chain optimizes the steric fit of dabigatran in the paratope while retaining the favorable intramolecular hydrogen bonding that stabilizes the bound Fab conformation.

Functional properties of aDabi-Fab3
aDabi-Fab3 was then tested in vitro in human plasma (Fig. 2B) and in vivo in a rat model of dabigatran anticoagulation (Fig. 2C). As seen with the comparison of aDabi-Fab2 and aDabi-Fab1, there was no difference in the potency of the compounds in reversing the anticoagulant activity of dabigatran in vitro. However, the duration of the aDabi-Fab3 effect on complete reversal of dabigatran anticoagulant activity was prolonged to 15 minutes, which is 3 times longer than the 5 minutes of the parent compound, aDabi-Fab2.
Discussion

We previously reported an antibody fragment (aDabi-Fab1) that binds dabigatran with picomolar affinity and has an extremely slow dissociation rate of $7.3 \times 10^{-7}$ s$^{-1}$.\textsuperscript{11} Compared to aDabi-Fab1, aDabi-Fab2 exhibits a significantly lower binding affinity and also shorter drug residence time in vitro and in vivo. The importance of residence times for the efficacy of drugs has gained increased attention in recent years.\textsuperscript{19,20} We reasoned that increasing affinity and residence time through structure-guided protein design may ultimately translate into prolonged dabigatran neutralization effects in vivo.

In the complex structures of aDabi-Fab2, the benzamidine moiety of dabigatran resides in a pocket that is formed by the hydrophobic side chains of H:His35, H:Tyr103, H:Phe106, L:Tyr37, L:Phe94, L:Ala96 and L:Tyr101. This hydrophobic environment allows for efficient binding of the dabigatran benzamidine moiety and energetically favors the formation of a salt bridge between the amidine group and L:Glu39 at the bottom of the pocket.

For antibody fragments where the unbound and complex structures have been solved, a variety of conformational changes has been reported ranging from side chain movements,\textsuperscript{21} small shifts of CDRs\textsuperscript{22,23} and domain motions\textsuperscript{24} to more pronounced effects in the backbone conformations of the CDRs.\textsuperscript{25,26} CDR-H3 of aDabi-Fab2 undergoes major conformational rearrangements upon ligand binding. The same backbone conformations in different crystallographic environments were observed; thus, we expect these conformations to be the energetic minimum in the presence and absence of dabigatran, since CDR:H3 in the unbound form shields the largely hydrophobic benzamidine pocket from solvent. Both “induced-fit”\textsuperscript{27} mechanisms and “population-shift” models of binding\textsuperscript{28} have previously been reported for antibody-antigen complexes.\textsuperscript{29,30} Computational simulations suggest that in case of antibody-hapten binding, the “population-shift” pathway, where binding of the hapten leads to a redistribution of already pre-existing conformational substrates, is favored over the “induced-fit” mechanism.\textsuperscript{31} On the basis of the structural information, we would suggest that, because the formation of a salt bridge between L:Glu39 and the benzamidine moiety of dabigatran seems to be the major force of binding and as the side chain of L:Glu39 is inaccessible in all the unbound crystal structures of aDabi-Fab2, the “population-shift” mechanism is dominant in this case.

Our rational protein engineering approach involved the mutagenesis of the paratope of aDabi-Fab2 in order to optimize affinity and off-rate. Any mutation that would either shift the conformational preference of aDabi-Fab2 to the bound state by destabilizing the unbound state or that would stabilize the protein-ligand interactions in the bound state or a combination of both effects should result in the desired property optimization. Interestingly, the unbound form of aDabi-Fab3 adopts the same backbone conformation observed in the wild type, excluding dominating effects of the mutation on the stability of the unbound form. The positive effects of this mutation are thus likely to be caused by a stabilization of the complex. The structure of this complex offers a possible qualitative explanation for increased affinity and residence time. The
larger size of the side chain of H:Trp103 compared to H:Tyr103 increases the shape complementarity between dabigatran and the binding pocket. As a consequence, the benzimidazole and carboxamide moieties of dabigatran acquire a more defined orientation compared to the aDabi-Fab2 co-structures. In the aDabi-Fab2 structures, H:Tyr103 adopts 2 distinct positions, with one forming a hydrogen bond to L:Asp33. Mutant H:Y103W satisfies this H-bond formed by the indole nitrogen atom of H:Tyr103. We thus conclude that the 10-fold improved affinity to dabigatran and the 5-fold improved residence time of the mutant is mostly due to a stabilization of the complex. The increased shape complementarity and the maintained H-bond to L:Asp33 lower the free energy of this state compared to the parental aDabi-Fab2. This finding is in line with other reports emphasizing the role of shape complementarity and reduced protein flexibility for residence time.32-34

We describe here the application of a structure-guided approach to optimize affinity and residence time of an anti-drug antibody. By increasing shape complementarity for dabigatran while retaining intramolecular hydrogen bonds that stabilize bound conformations, we successfully increased the affinity and residence time by a factor of 10 and 5, respectively. Most notably, the tighter binding on the molecular level translated into prolonged neutralization of dabigatran’s anticoagulant effects in vivo. This example of structure-based protein design shows the potential of protein engineering approaches for the optimization of other therapeutic proteins in the future.

Materials and Methods

Engineering, expression and purification of Fabs

Based on the aDabi-Fab2 sequence, 20 mutated variants were generated by gene synthesis (Invitrogen). Synthesized V-gene sequences were cloned into a pcDNA3.1-based expression. H-chain and L-chain constructs were combined for transient expression in HEK293E cells, using 293fectin (Cat. No. 12347-019, Life Technologies, NY, USA). Fabs were harvested 5 days after transfection. The pH of the medium containing the secreted Fabs was adjusted to 8.0. 2.5 ml Benzamidine-Sepharose (GE Healthcare, Life Sciences, NY, USA) were mixed with 0.5 l/well 0.1 M citric acid, pH 3.0. The elution fraction was neutralized with 3 M Tris-HCl, pH 9.3 and tested in an Octet QK (Pall ForteBio Corp., CA 94025 USA), using Streptavidin-Biosensors (Part No: 18-5020). After a baseline was established, biotinylated dabigatran at 10 µg/ml was loaded onto the biosensors for 900 s at 1000 rpm. Undiluted Fabs were presented for 900 s at 1000 rpm, leading to a concentration-dependent association, followed by dissociation in PBS for 1800 s at 1000 rpm. The dissociation constants were calculated using a one-phase exponential decay model for each curve individually, leading to a concentration-independent off-rate-ranking.

Affinity determination

The affinities of selected Fabs were determined using Kinexa technology (Sapidyne Instruments, Boise ID). A constant concentration of Fab or antibody was incubated with various concentrations of dabigatran until equilibrium was reached. After the incubation the concentration of free Fab was determined by capturing it on Neutravidin beads coupled with a biotin-conjugated dabigatran analog. The captured humanized Fab was detected with an anti-human IgG conjugated with Cy5. The dissociation constants were determined using a 1:1 binding model.

Binding kinetics determination

Prior to k(on) determination, a Kinexa affinity determination was needed to identify the concentration of dabigatran for 80% inhibition. This concentration of dabigatran was mixed with Fab at the same concentration as in the Kinexa affinity measurement immediately before the start of the k(on) determination. While equilibrating, samples were drawn repeatedly from this mixture and concentration of free Fab was determined by capturing it on Neutravidin beads coupled with a biotin-conjugated dabigatran analog. The captured humanized Fab was detected with an anti-human IgG conjugated with Cy5. k(on) was determined by using a 1:1 binding model. k(off) was calculated from k(on) and K_D according to the law of mass action.

Complex formation

Anti-dabigatran Fabs were further purified using size-exclusion chromatography on a Superdex 75 column equilibrated and run with a buffer containing 50 mM TRIS (Ph 7.5) and 100 mM NaCl. Purified Fabs were concentrated to 10 mg/ml using an Amicon 10 kDa cutoff concentrator. During concentration, the buffer was replaced with bidest water. Dabigatran was dissolved to a 10 mM stock in DMSO with 50 mM HCl. From this stock solution dabigatran was mixed with the Fab at a 2.5:1 molar ratio and incubated on ice for 2 h.
Crystallization of apo Fabs and the Fab-dabigatran complexes

Crystallization trials were set up using vapor diffusion in sitting drops. 200 nl of (free or complexed) protein was mixed with an equal volume of screening solution and equilibrated against 50 μl of the screening solution. aDabi-Fab2a in complex with dabigatran crystallized in 100 mM SPG buffer (pH 6) and 25% PEG 1500. Crystals appeared after 8 days of incubation at 20°C. aDabi-Fab2b in complex with dabigatran crystallized in 100 mM SPG buffer (pH 7) and 25% PEG 1500. Crystals appeared after 8 days of incubation at 20°C. Both uncomplexed Fabs crystallized at 100 mM PCB buffer (pH 6) and 25% PEG 1500. Crystals appeared after 1 day of incubation at 20°C. Crystals were cryoprotected by soaking them in the crystallization solution supplemented with 15% glycerol and flash frozen in liquid nitrogen at 100 K.

Data collection, processing and refinement

Native data were collected at the beamline PXI – X06SA of the Swiss Light Source (Paul Scherrer Institut, Villigen, Switzerland) on a PILATUS 6M pixel detector. Data were collected at 100 K and a wavelength of 0.9100 Å. Data processing and reduction were performed using autoPROC. Molecular replacement was performed with PHASER as implemented in CCP4. A search model was assembled using CHAINSAW to prune the light and heavy chain of a Fab (PDB code 1CIE) to its CB-atom. As the elbow angles between variable and constant domains vary between crystal structures, the variable and constant domains of each chain were used as separate search models during molecular replacement. It was first searched for the constant domains of heavy and light chain followed by their respective variable domains. Model building and refinement were performed using Coot and autoBUSTER. During the first 3 rounds of iterative refinement and model building, dabigatran was omitted from the model of the complex structures. Positive electron density became clearly visible at the interface of heavy and light chain and dabigatran was built into this density. The models were refined against high resolution datasets with NCS restraints as implemented in autoBUSTER. Model quality was checked using Molprobity. Model figures and superimpositions were prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC). The contact areas were calculated with PISA package. Atomic distances were measured using Coot and PyMOL. Root mean square deviations (rmsd) were calculated using SUPEROSE.

ACCESSION NUMBERS: Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 4YGV, 4YHI, 4YHK, 4YHL, 4YHM, 4YHN and 4YHO.

Modified thrombin time

aDabi-Fab 1, 2 and 3 were serially diluted in pooled human plasma. Dabigatran was added at a final concentration of 7 nM and incubated with Fab sample for 10 min at 37°C. Thrombin time was measured in a CL4 coagulometer (Behnk Elektronik, Norderstedt, Germany) using bovine thrombin reagent from Siemens Diagnostics (Marburg, Germany). Thrombin reagent was dissolved to 0.4 U/mL with HEPES and stored on ice until use. Plasma samples were prewarmed (37°C) for 1 minute, and then an aliquot of thrombin solution was added to initiate coagulation. The time in seconds to the onset of plasma clotting was determined. IC50 was calculated using GraphPad Prism v6.01 (GraphPad Software, Inc., San Diego, CA).

Thrombin time ex vivo in the rat

All experiments were performed with ethical approval from Animal Use Ethics Committee, Tübingen, Germany. Wistar rats were anesthetized and placed on a 37°C heating pad. The carotid artery was cannulated for blood sampling and the jugular veins for dabigatran infusion and Fab or vehicle administration, respectively. Dabigatran (or saline in control group) was administered as a bolus (0.30 μmol/kg) followed by an infusion (0.10 μmol/kg/hr) for 20 minutes prior to Fab injection to achieve steady state plasma levels. Dabigatran infusion was also continued for the duration of the 30 min study. Blood samples (0.4 mL each) were collected into 3.13% sodium citrate prior to and 1, 3, 5, 10, 15, 20, 25, and 30 minutes post Fab injection to measure thrombin time. Whole blood thrombin time was measured in a CL4 coagulometer (Behnk Elektronik, Norderstedt, Germany) using bovine Thrombin Reagent from Siemens Diagnostics (3 U/mL, Marburg, Germany). Blood samples (0.40 mL) were prewarmed to 37°C. Thrombin Reagent (0.10 mL) was added to initiate coagulation and the time in seconds to the onset of blood clotting was determined.

Disclosure of Potential Conflicts of Interest

All authors are employees of Boehringer Ingelheim Pharma GmbH & Co. KG.

Acknowledgments

The authors would like to thank Johanna Schurer for her excellent technical assistance in performing the functional tests. All experiments were performed with ethical approval from Animal Use Ethics Committee, Tübingen, Germany.

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website

References

1. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006; 3:e442; PMID:17132052; http://dx.doi.org/10.1371/journal.pmed.0030442

2. Hirsh J, Dalen JE, Anderson DR, Poller L, Bussey H, Ansell J, Deykin D. Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. Chest 2001; 119:8S-21S; PMID:11157640; http://dx.doi.org/10.1378/chest.119.1_suppl.85

3. Ansell J, Hirsh J, Poller L, Bussey H, Jacobson A, Hylek E. The pharmacology and management of the vitamin K antagonists The Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest 2000; 126:2045-33S; PMID:15588473; http://dx.doi.org/10.1378/chest.126.3_suppl.204S
1. Schleier F, van Ryn J, Newsome C, Sepulveda E, Park J, Blech S, Ebner T, Ludwig-Schwellinger E, Stangier J, Hauel NH, Nar H, Priepke H, Ries U, Stassen J-M, Darkow T, Vanderplas AM, Lew KH, Kim J, Hauch Jones M, McEwan P, Morgan CL, Peters J, Goodfellow structural biology. Proc Natl Acad Sci U S A 1999; 96:9459-64; PMID:10494714; http://dx.doi.org/10.1073/pnas.96.17.9495
2. Richter F, Leaver-Fay A, Khare SD, Bjelle S, Baker D, De novo enzyme design using Rosetta3. PLoS One 2011; 6:e19230; PMID:21610366; http://dx.doi.org/10.1371/journal.pone.0019230
3. Copeland RA, Pompoliano DL, Mek TD. Drug-target residence time and its implications for lead optimization. Nat Rev Drug Discov 2006; 5:736-9; PMID:16739813; http://dx.doi.org/10.1038/nrd2082
4. Swinney DC. Biochemical mechanisms of new molecular entities (NMEs) approved by United States FDA during 2001-2004: mechanisms leading to optimal efficacy and safety. Curr Top Med Chem 2006; 6:641-78; PMID:1680266776744093
5. Niemi MH, Turunen L, Pulli T, Nevanen TK, Hoytyna M, Siderlund H, Rovinen J, Takkinen K. A Structural Insight into the Molecular Recognition of a (−)-di-sugars-9-Tert-Ethylpyrrolidine. J Mol Biol 2010; 400:803-14; PMID:20630472; http://dx.doi.org/10.1016/j.jmb.2010.05.048
6. Loydah CE, Monzongo AF, Maynard JA, Barnett J, Georgiou G, Iverson BL, Robertus JD. Crystal structure of the engineered neutralizing antibody M18 complexed to domain 4 of the anthrax protective antigen. J Mol Biol 2008; 387:680-93; PMID:19394245; http://dx.doi.org/10.1016/j.jmb.2008.02.003
7. Stanfield RL, Fieser TM, Lerner RA, Wilson IA. Crystal structures of an antibody to a peptide and its complex with peptide antigen at 2.8 A. Science 1990; 248:712-8; PMID:16256877; http://dx.doi.org/10.1126/science.1095030
8. Majeed A, Hwang H-G, Connolly SJ, Eikelboom JW, Ezekowitz MD, Wallentin L, Brueckmann M, Fraessdorf M, van Ryn J, Lang B, Ramael S, Reilly P. A randomized, double-blind, placebo-controlled study of idarucizumab, a specific antidote to dabigatran. Thromb Haemost 2015; 113:931-42; PMID:25832311; http://dx.doi.org/10.1160/TH14-11-0982
9. Bork P, Holm L, Sander C. The immunoglobulin fold: structural classification, sequence patterns and common core. J Mol Biol 1994; 242:309-20; PMID:7932691
10. Burley S, Petsko G. Aromatic-aromatic interaction: a mechanism of protein structure stabilization. Science 1995; 229:23-8; PMID:8902666; http://dx.doi.org/10.1126/science.8902666
11. Meyer EA, Castellano RK, Diederich F. Interactions with aromatic rings in chemical and biological recognition. Angew Chem Int Ed 2003; 42:1210-50; http://dx.doi.org/10.1002/a00399319
12. Gallivan JP, Dougherty DA. Carboxyl interactions in structural biology. Proc Natl Acad Sci U S A 1999;