Proof of Efficacy Protocol

In order to further characterize the Aducanumab’s behavior, we employed bioinformatic modeling to investigate the impact of non-cleavable linkers, such as (G4S)n, on antibody binding. Our examination, centered on steric hindrance, sought to understand the effects of the length of the linker and its influence on brain penetration. The complex formed between Aducanumab and Aβ has already been documented in the Protein Data Bank (PDB ID: 6c03) (22). Leveraging this information, we conducted modeling experiments focusing on Aducanumab, an Alzheimer's disease treatment that was previously withdrawn from the market. Our methodology involved pre-processing and standardizing the available antibody structure using UCSF Chimera and structure precition of transferrin attached to the light chain of Aducanumab via linker, by AlphaFold2. Amyloid-beta was then docked onto the constructs from AF2 using HADDOCK, both before and after linkage with transferrin via the (G4S)n linker. Subsequently, we evaluated binding affinity and interaction patterns through the PRODIGY server, with particular attention to the linker's length (Figure 1-5).

The results of our protein-protein complex analysis reveal significant differences in binding affinity and interaction characteristics (Table 1). For the Aducanumab-Abeta complex, the ΔG value was calculated at -18.1 kcal mol-1, with a corresponding Kd of 5.10E-14 M at ℃. The interface analysis demonstrated varying degrees of interactions, with notable contributions from charged-charged and apolar-apolar interactions. Upon conjugation with transferrin using different linkers, we observed enhanced binding affinity and altered interaction profiles. Specifically, the Transferrin-(G4S)3-Aducanumab-Abeta complex exhibited the highest ΔG value (-29.2 kcal mol-1) and the lowest Kd (4.10E-22 M at ℃), indicative of stronger binding. Interface analysis further revealed increased interactions across all categories, particularly in polar-apolar and apolar-apolar interactions. These findings underscore the importance of linker length in modulating protein binding and highlight the potential of our approach for optimizing drug-protein interactions. These findings underscore the critical role of linker length in modulating binding properties. Comparative analysis using (G4S) or (G4S)3 linker has revealed superior flexibility with the latter, facilitating unimpeded binding to proteins with minimal steric hindrance around the amyloid-binding pocket. Furthermore, these findings indicate that linking the antibody's light chain with transferrin does not compromise antibody binding to brain proteins nor impede amyloid's accessibility to its binding domain.

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Figure 1. Amyloid-beta (blue) bound to Aducanumab (tan)

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Figure 2. Amyloid-beta (blue) bound to Transferrin-Aducanumab complex (tan) bound by long-linker (G4S)3 (green)

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Figure 3. Amyloid-beta (blue) interacting residues with Transferrin-Aducanumab complex (tan) bound by long-linker (G4S)3

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Figure 4. Amyloid-beta (blue) is bound to the Transferrin-Aducanumab complex (tan) and is bound by a short-linker (G4S) (green)

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Figure 5. Amyloid-beta (blue) interacting residues with Transferrin-Aducanumab complex (tan) bound by long-linker (G4S)

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| **Protein-protein complex** | **ΔG (kcal mol-1)** | **Kd (M) at ℃** | **ICs charged-charged** | **ICs charged-polar** | **ICs charged-apolar** | **ICs polar-polar** | **ICs polar-apolar** | **ICs apolar-apolar** | **NIS charged** | **NIS apolar** |
| Aducanumab-Abeta | -18.1 | 5.10E-14 | 22 | 20 | 64 | 1 | 19 | 29 | 21.2 | 40.1 |
| Transferrin-(G4S)-Aducanumab-Abeta | -21.3 | 2.60E-16 | 39 | 27 | 83 | 1 | 18 | 43 | 28.5 | 35.5 |
| Transferrin-(G4S)3-Aducanumab-Abeta | -29.2 | 4.10E-22 | 32 | 33 | 136 | 7 | 38 | 79 | 28.9 | 35.7 |

Table 1. The binding properties of various conjugates.