6 Summary and Outlook

The aim of this work was the development and the implementation of a peptide-based screening system for the systematic investigation of the molecular interactions of fluoroalkyl-substituted amino acids in protein environments. A *coiled coil*-based model polypeptide had to be designed and applied in order to evaluate the properties, fluorinated amino acid side chains exert in specific native protein interfaces and in fluorous interactions with other fluorine-substituted side chains. Furthermore, this *coiled coil*-based screening system had to be adapted for the screening of peptide libraries by applying phage display technology to find the preferred interaction partners for the fluoalkyl-substituted amino acid side chains out of the pool of the twenty native residues. The studies using this newly developed screening system should help clarifying controversies about the behavior of organic fluorine in protein environments such as space filling of fluoroalkyl groups, the impact of fluorination on the polarity of alkyl moieties, the nature of the stabilizing effect of perfluorination on hydrophobic protein cores (increasing lipophilicity versus fluorous interactions), and the ability of fluorine to participate in hydrogen bonding. The new information obtained from these studies can now be applied in the design of peptide based drugs with improved phamacological properties and binding activities.

An α-helical peptide dimer with an antiparallel orientation of both helix strands was designed to act as the *coiled coil*-based polypeptide environment. Two amino acid positions, one in the hydrophobic core and another in the polar interaction domain, served as substitution positions for the fluorinated building blocks to be investigated. Two screening methods were established for the evaluation of the interaction properties of non-natural amino acids with native polypeptides. One measures the thermostability of the substituted *coiled coil* dimers, and the other determines the rates of their self-replication via native ligation.
Both screening methods could be successfully optimized in terms of reproducibility of the obtained results. A very high sensitivity towards single amino acid substitutions has been demonstrated. The thermostability screen and the self-replication technique were able to detect even differences of one single fluorine atom between amino acid side chains in a 82mer polypeptide. In order to prove the principle concept of the screening system, molecular modeling studies and alanine scans in both substitution positions were perfomed. The results proved the contribution of both substitution positions to structural stability of the *coiled coil* model polypeptide.

Alkyl- and fluoroalkyl-substituted peptide building blocks with systematically altered side chains in terms of size of the alkyl moiety and content of fluorine atoms were incorporated in

both substitution positions in *coiled coil* screening peptides. Thereby, the fluorinated and alkyl-substituted analogues of *(S)*-ethylglycine 4,4-difluoroethylglycine, 4,4,4-trifluoroethylglycine, and 4,4-difluoropropylglycine have been used. New information could be won about the properties of fluoroalkyl groups in protein environments by screening these peptides, applying the described methods: Although it is often stated in literature that CF_3 is a good mimic of the isopropyl group in hydrophobic environments, it was found here that either space filling or lipophilicity of trifluoromethyl groups or even both fall behind that of isopropyl.
The results obtained from this work suggest in general that fluorination of alkyl groups has opponent electrostatic and steric consequences on hydrophobic protein interfaces. While the enhancement of the side chain volume by alkyl fluorination stabilizes hydrophobic cores, the polarization of alkyl hydrogen atoms in proximity to the fluorination site strongly disturbs the hydrophobic environment. The statement often found in literature that fluorination increases the lipophilicity of alkyl groups cannot be supported by these results. The stabilization of hydrophobic protein cores by perfluorination has rather to be attributed to specific fluorous interactions between fluoroalkyl-substituted amino acid side chains. The studies performed in this work proved that such fluorine-fluorine interactions can significantly affect protein folding, which supports the theory of the "fluorous effect". In the future, more fluoroalkyl-substituted amino acids with systematic side chain alterations should be screened in order to obtain more and detailed information about the properties of organic fluorine. The implementation of improved techniques such as calorimetry and surface plasmon resonance (SPR) would provide exact thermodynamic and kinetic data for the quantification of the results that were obtained from both screening methods.

To combine the unique properties of alkyl fluorination with the resistance towards proteolysis that is provided by $C^{\alpha,\alpha}$-dialkylated amino acids, both enantiomers of trifluoromethylalanine (TfmAla), its non-fluorinated analogue aminoisobutyric acid (Aib), and α-methylleucine (MeLeu) have been evaluated with the *coiled coil*-screening system. The results demonstrate that $C^{\alpha,\alpha}$-dialkylated amino acids strongly affect conformation and structural stability of the α-helical folding motif. While both of the trifluoromethylalanine enantiomers destabilize the helix dimer in both protein interaction domains, it was found that Aib leads to enhanced dimer stability when introduced into the polar interface. Thereby, the stabilizing influence on the helix backbone overcompensated the loss of polar interaction, caused by the amino acid substitution. Furthermore, the insertion of α-methylleucine into the hydrophobic core did not destabilize the helical dimer.
These results suggest that peptide-based drugs can be designed, which reveal strongly improved metabolic stabilities *in vivo* and are able to form *coiled coil* structures with target proteins. This approach appears to be powerful in the design of *coiled coil*-based inhibitors

for HIV-1 infection, a pathological process that was introduced in Chapter 1. Furthermore, the implementation of perfluorinated $C^{\alpha,\alpha}$-dialkylated derivatives of hydrophobic amino acid residues, such as valine, isoleucine, and leucine, could help to create *coiled coil* proteins with both enhanced structural stability, introduced by fluorous interactions, and increased resistance towards *in vivo* metabolism caused by $C^{\alpha,\alpha}$-dialkylation. However, the conclusions drawn from investigations on this kind of non-natural peptide building blocks have to be proven in further studies with *coiled coil* models, varying in the extent of amino acid substitution and structure of introduced non-natural amio acids.

Based on the model polypeptide that has been successfully used for the evaluation of the interaction properties of fluoroalkyl-substituted amino acids, a *coiled coil* protein was designed for being displayed on phage surface. The substituted *coiled coil* fragments that have already been applied for the thermostability and self-replication screens, should be used for screening phage-displayed libraries for best interaction partners of the fluorinated amino acids. A library could be successfully constructed and its size was optimized. The binding and native ligation of both *coiled coil* fragments, one to be displayed on phage surface and one for library screening, have been demonstrated on the surface of the soluble maltose binding protein (MBP). However, the incorporation of the library protein, possessing a free N-terminal cysteine residue for native ligation into the phage coat, could not be accomplished, although several attempts of redesigning the protein have been made and different strategies for its presentation on the surface of the virion were tried. Since the reason for the failure has been discovered, screening for interaction partners of fluoroalkyl-substituted amino acids from phage-displayed *coiled coil*-based libraries can be successfully accomplished by applying a completely redesigned protein model system lacking the N-terminal cysteine residue.

The screening system that has been developed in this work and implemented for the investigations on fluoralkyl-substituted amino acids can be applied for any kind of non-natural peptide and protein building blocks.