Overview of the work carried out:

- Cloning, expression and purification of the rHuEpo from *E. coli*
- Identification of the protease sensitive peptide bonds on the unglycosylated Epo molecule
- Introduction of mutations at the susceptible site(s) and analysis of mutants for improved protease resistance leading to enhanced *in vivo* half-life.
- *In vivo* plasma half-life comparison of the mutants and the wild-type.

The cloning, expression and purification of Epo from *E. coli* has been done before. Nahri et *al.,* (1991) expressed it in *E. coli*, refolded it from the IBs using $CuSO_4$ and purified it by ion-exchange and hydroxyl-apatite chromatography. They obtained protein which was homogeneous as analyzed by SDS–PAGE. Boissel *et al.,* (1993) cloned the *epo* gene in pET16b plasmid and expressed it BL21 (DE3) cells. They too resorted to refolding the IBs dissolved in 6 M guanidine HCl. In the same study it was also determined that C29Y and C33Y mutants are fully active. However, in our study when we made three constructs (C29S, C33S and double mutant C29/33S), all of them expressed in *E. coli* upon induction by IPTG but none of the molecule was recoverable after the final step of refolding. We had already observed that incubation of multimeric species with 10 mM DTT causes all of the bands to converge to a single band (Figure 2m), implying that the multimers were aggregates formed by disulfide linkages. Interestingly these three cysteine mutants showed up as a clear single band in SDS–PAGE when their IBs were solubilized in 8 M urea, unlike other mutants and wild-type Epo which always showed multiple bands, at this particular stage of the protein prep.

As mentioned in Chapter 3, we identified various sites on unglycosylated Epo that are susceptible to protease cleavage. With the aim to bring about a minimal change in the molecule, we set out to mutate only the sites that appeared on the loop region of the molecule. We identified various other protease sensitive sites on Epo that appeared on its helices (details in chapter 3) but did not pursue them as we wanted to carry out minimal changes in the molecule avoiding any possible structural perturbation. Therefore we chose to carry out the mutations on the crossover loops' residues rather than those on helices.

Although we started our work by carrying out limited proteolysis of the Epo molecule with proteases like trypsin and pepsin, we fine-tuned our strategy to concentrate on more relevant proteases, that is, the ones present in the lysosome. Recently there have been reports on the role of Epo in preventing cathepsin-mediated cell death by preventing the release of lysosomal proteases (Ozden *et al.*, 2011, Dev *et al.*, 2013). Although these reports talk about cathepsins and Epo on the same platform, they do not, in any way, link cathepsins with *in vivo* Epo clearance; a connection which according to us appears quite plausible.

In our study, *in vivo* mouse-model study is yet to confirm that the mutants designed to be resistant against cathepsin L have an increased half-life compared to the wild-type molecule. Epo-dependent cell proliferation assay has confirmed the all the molecules are as bioactive as the commercial, glycosylated, wild-type molecule. Therefore these mutations may be particularly advantageous in that they may confer resistance to proteolytic cleavage to the molecule, which is possibly one of the major routes of clearance of the molecule in the body.

This work may give some insights into the likely trail of degradation of Epo in the body. If the results do finally show the mutants to have a half-life greater than that of the wild-type molecule, it would further support the study of Epo degradation in cell lines which ascribed it to the lysosomal pathway. Further experiments can be conducted by expressing the mutants in a glycosylated form and checking the degree to which they persist in the plasma compared to the wild-type molecule. Studies can also be conducted to test the tissue protective efficacy of the mutants, which may further add to the knowledge on this topic.