CHAPTER 5

CONCLUSION

The first objective of the study was to identify the alternative signaling cascades and multiple check points in allergy signals as potential pharmacological targets. With this in view, three known anti-allergic molecules namely, Dexamethasone, a commercial anti-allergic steroid, Cyclosporin A, fungal polysaccharide with documented anti-allergic properties and Wortmannin, a documented PI3K inhibitor were comparatively analyzed at the level of their effect on molecular signal transduction. Based on this analysis, Cyclosporin A was determined to be the most potent of the three anti-allergic molecules, in terms of degranulation inhibition, with an IC50 of 40 µM. Its molecular mechanism of anti-allergic action was found to be stabilization of the mast cell membrane via the stabilization of the membrane actin dynamics observed at 30s of FcεRI activation. This activation of actin polymerization was determined to be mediated by the up regulation in the phosphorylation levels of WASP Ser^{483/484} residues. Wortmannin was found to selectively sequester membrane cholesterol, comparable to the commercial positive control, Methyl β- Cyclodextrin. While all three molecules inhibited total cellular phosphorylation levels, Wortmannin exhibited better sustenance of the effect. This is predicted to be on account of its effect both on the direct inhibition of cellular phosphorylation (similar to PI3K inhibition), as well as, due to inhibition of FcεRI activation, through disruption of receptor cross-linking. Dexamethasone was found to exert its effect on inhibition of the activation of cellular mediators resulting in an inhibition of the late phase mediators of allergy, such as the transcription
factor NFkB and secreted cytokine, TNF-α. Interestingly, Cyclosporin A was the only molecule inhibiting AKT Ser\(^{473}\) phosphorylation; its inhibition of NFkB (p65) nuclear translocation is predicted to be mediated via its inhibition of Akt phosphorylation. Thus by comparatively analyzing the anti-allergic potential of the three anti-allergic molecules, and their check points in allergic signaling, alternative allergic signals have been identified; these include the WASP-Arp 2/3 complex-actin pathway, the PI3K- PKC- Calcium-actin pathway, the PI3K-MAPK-NFkB pathway, the Akt-NFkB pathway, the TNF-α- Akt/Cot- NFkB pathway and the NFkB-TNF-α pathways.

Another major aim of this study was to isolate potential anti-allergic drug candidates from ethano-botanically classified medicinal plants with documented anti-allergic properties. Towards this end, two plants Solanum xanthocarpum and Clerodendron serratum, classified ethano-botanically as mast cell stabilizers were screened for their in vitro anti-allergic efficacy. Based on the in vitro bio-screen for degranulation inhibition, four anti-allergic molecules were isolated as potential drug candidates. Of these, SXM 2 was determined using spectroscopic studies to be Solasodine.

Of the four molecules, SXM 3, exhibited the least IC50 value of 0.004 mM, followed by Solasodine (0.009 mM), SXM 4 (0.07 mM) and SXM 1 (98.6 mM). The results clearly depict that, the pharmacophore responsible for the exhibited bio-activity is Solasodine, the C27 steroidal alkaloid (Cholestane) skeleton. This conclusion is derived from the comparative analysis of the IC50s for degranulation inhibition, between the commercial anti-allergic steroid derived from Diosgenin, Dexamethasone, and Solasodine. Solasodine exhibited a 10 fold enrichment in IC50 at 0.009 mM (~0.01 mM), compared to the 125 µM (~0.1 mM) exhibited by Dexamethasone. No cytotoxicity of the isolates was observed in RBL-2H3 in vitro allergy model, as well as on the normal human PBMCs.
The mechanism of anti-allergic action of SXM and the isolated drug candidate Solasodine was then studied in the RBL-2H3 in vitro allergy model. The stabilization of the characteristic 15% fall in actin content at 30s of FcεRI activation, is predicted to be due to inhibition of actin depolymerization, rather than due to an induction of actin polymerization. This conclusion is based on the fact that inhibition of WASP phosphorylation is observed at 30s of FcεRI activation. Simultaneous inhibition of N-WASP phosphorylation is predicted, which is thought to contribute towards the actin stabilization observed at 30s. The inhibition in WASP phosphorylation observed at 10min is thought to be responsible for the inhibition in actin polymerization, observed by 20min of FcεRI activation. This stabilization in membrane actin dynamics is reflected in the inhibition of CCE, despite the activation of store operated calcium channels seen upon SXM treatment. The reduced calcium levels are reflected in the inhibition of Akt phosphorylation and consequent NFκB activation. The inhibition in the activation of ITAM exhibited by SXM is a true effect of inhibition of phosphorylation/activation, rather than through disruption of receptor cross-linking and activation.

Solasodine exhibits an inhibition of actin phosphorylation at later time points, reflected in its WASP phosphorylation status. This is reflected in both the membrane actin levels, as well as, in the inhibition of actin “comet-tail” formation. The pharmacophore solasodine is more effective at inhibiting calcium fluxes, than SXM. Solasodine in addition exhibited inhibition in the cPLA2 activity levels. The failure of the molecule to inhibit COX 2 activity despite significant down regulation of cellular calcium fluxes, indicates that the inhibition of COX 2 activity is independent of calcium responses. In addition, Solasodine did not inhibit COX 1 enzyme activity, thus negating any possible adverse side-effects. The inhibition of ITAM phosphorylation/activation, despite Solasodine not inhibiting membrane cholesterol sequestration, indicated that the observed down regulation in cellular
mediators’ activation was a true effect on inhibition of phosphorylation rather than through disruption of FceRI cross-linking. The inability of Solasodine to sustain the inhibition of cellular mediators’ phosphorylation/activation, despite membrane cholesterol sequestration (0.8 fold), maybe explained by the large increases in the cytosolic cholesterol concentration, exhibited by the cells, treated with the pharmacophore.

Thus, it was seen that, the steroidal pharmacophore Solasodine, had greater effects on the inhibition of calcium flux, and consequently the late phase mediators, Akt and NFkB; while the glycosyl moiety, conferred potential for greater sequestration of membrane cholesterol, and consequent disruption of FceRI cross-linking. The effect on membrane and cytosolic actin polymerization and depolymerization, for the most part was seen to rest upon the effect of the molecule on WASP and N-WASP phosphorylation/activation.

Finally, the effect of the molecule on degranulation inhibition, in combinations of two and three were studied, to determine the existence of possible synergism or antagonism between the molecules, in terms of their anti-allergic activity. It was found that the molecules in all their combinations exhibited antagonistic effects on degranulation inhibition. This is in keeping with the observations from previous studies, which have shown that, steroidal glycoalkaloids in combination exhibit synergism for cholesterol sequestration and membrane disruption, and this is reflected as antagonism in their degranulation inhibition potential.

Thus in summary, the comparative analysis between the commercially available anti-allergic molecules, identified, multiple and alternative check points in the allergic signal transduction, as potential pharmacological targets. In particular, the PI3K-PKC and the Akt pathways emerged as alternative signaling mediators and as potent anti-allergic targets.
The pharmacophore Solasodine exhibited the maximum sustained inhibition of Akt, comparable to Cyclosporin A, which was found to be the most potent of the three tested anti-allergics in its bio-activity. Solasodine, typical to its steroidal structure, exerted more potent effects on late phase allergy mediators, the Akt and NFkB, while only slightly sequestering membrane cholesterol; Solasodine also exhibited inhibitory effects on receptor activation. Finally, reflective of earlier studies, the isolated drug candidates in combination, true to their steroidal glycoalkaloid structure-activity relationship, exhibited antagonistic effects in combination on degranulation inhibition, on account of their synergism in membrane disruption.

In conclusion, the study strived to provide a comprehensive anti-allergic drug discovery chart, replete with identification of potential molecular targets, isolation of anti-allergic molecules, studying the molecular targets and mechanism of action, testing their true potential for allergy therapy, and finally, studying their in vitro pharmaco-dynamics in terms of drug interactions and combinatorial effects. The potential way to take this research work forward and a step closer to realizing the potential of the molecules as commercial anti-allergics would be to, perform in vivo studies on mice and rat models of airway hypersensitivity, patch test and rat paw edema. Optimizing their formulation, dosage and routes of entry is essential for maximizing the drug efficacy. Performance of pharmaco-kinetic and metabolism studies and in vivo toxicity testing are the next step in the evaluation of the isolates as potential drug candidates. Finally, testing of serum IgE levels in allergic patients and patch tests, after proper FDA approval, would represent the final step in the clinical evaluation of the isolates, before they can become commercially available anti-allergic medications.

The study has for the first time documented the invitro anti-allergic properties of Solanum xanthocarpum. The study has also for the first time
identified the pharmacophore responsible for its observed anti-allergic potential. The molecular mechanism of action of the pharmacophore has been deciphered. The study tested the individual drug efficacy on identified pharmacological targets and has also recorded their potential drug interactions in terms of degranulation inhibition.