SUMMARY AND CONCLUSIONS

Invasive fungal infections are rising worldwide as the number of immunocompromised patients increases. Unfortunately, our armamentarium of antifungal drugs is limited. Although current therapies are effective in treating some of the most prevalent infections, the development of novel treatments is vital because of emerging drug-resistant strains and the toxicity of certain current therapies. The systematic study of products from actinomycetes and fungi has led to the development of immunosuppressive drugs such as cyclosporin A (CsA), FK506 (tacrolimus) and rapamycin (sirolimus). These drugs exert potent antifungal effects against a variety of pathogenic fungi. In fact, these were initially discovered as antifungal agents and later their potential as immunosuppressants was realized. By taking into consideration this point we started our study for screening of bioactive compounds with antifungal activities by isolating various microbes from soil and water samples from the cold Himalayan region of Kaza and Spiti in Himachal Pradesh.

This resulted in the isolation of two new species of Actinomycetes that produced bioactive compounds having widespread antifungal spectrum. These two strains were designated as RMV-1378 and RMV-A16.

Polyphasic characterization of the strain RMV-1378^T, isolated from cold desert of the Himalayas, India, clearly confirmed that the strain belong to the genus *Actinoalloteichus.* Physiological and biochemical tests allowed genotypic and phenotypic differentiation of the strain RMV-1378^T from its closest phylogenetic relative. Analysis of 16S rDNA sequence revealed that the isolate is very closely related to *Actinoalloteichus cyanogriseus* with similarity of 99 %. However, results of DNA-DNA hybridization, showed low genomic relatedness with *Actinoalloteichus cyanogriseus* (51 %). Therefore, we proposed that the isolate be classified as a new species of *Actinoalloteichus*, for which we proposed the name *Actinoalloteichus spitiensis* sp. nov.

The taxonomic position of the strain RMV-A16^T, isolated from cold desert of Himalayas, India, was also examined by a polyphasic approach. The sequence of almost complete 16S rDNA and comparison with those previously studied streptomycetes confirmed that the strain belongs to the genus *Streptomyces.* On the basis of biochemical and chemotaxonomic data, as well as 16S rDNA gene sequence analysis the isolate appears to be a novel species of the

genus *Streptomyces*. Therefore, we have characterized this isolate as a new actinobacterium species, for which we have proposed the name *Streptomyces himgiriensis* sp. nov.

Though both these isolates showed antifungal activity. Strain RMV-1378 showed greater activity against the MDR *Candida* strains and was hence pursued for further studies. It was found to produce Caerulomycin A (CaeA). CaeA inhibits the growth of pathogenic fungi such as *Aspergillus fumigatus*, *Fusarium oxysporum*, *Trichophyton mentagrophytes* and *Microsporum gypseum* etc. CaeA has also categorically inhibited the growth of fluconazole resistant clinical strains of *Candida albicans*. We used clinical isolates of *Candida albicans* named as F2/F5 and Gu4/Gu5 for this study. In which isolate F5 over expresses the MDR1 gene and Gu5 over expresses the CDR1 and/or CDR2 gene and are both fluconazole resistant whereas F2 and Gu4 are their wild type strains and are fluconazole susceptible. Interestingly, CaeA significantly decreased the viability of these strains in a dose dependent manner at very low MICs as compared to the well-known antifungal agent Fluconazole. Thereby suggesting that CaeA could be tried as a lead molecule in the treatment of fungal infections. Since the well known immunosuppressive drugs [Cyclosporin A (CsA), FK506 (tacrolimus), and Rapamycin] used now days were initially discovered as antifungal agents, we become interested to study its effect on the immune system.

Immune dysregulation plays a vital role in a wide spectrum of inflammatory diseases, including hypersensitivity responses to environmental Ag (allergic disorders), false recognition of self-Ag (autoimmune diseases), and robust immune attack against allo-Ag (graft rejection and graft-vs-host disease). Existing treatments for unwanted immune responses, such as allergic reactions, autoimmunity, and graft rejection, are limited and often toxic.

We have isolated and characterized a drug named as Caerulomycin A (CaeA). Interestingly, till now nothing is known about its action on the immune system. We therefore, did extensive experiments to categorically demonstrate its role on the cells of the immune system and by using a mechanistic approach to track its mode of action. We initiated experiments using splenocytes isolated from the BALB/c mice stimulated with T cell (ConA) and B cell (LPS) mitogens. It was quite remarkable to observe that CaeA significantly inhibited the proliferation of mitogen stimulated T and B cells.

The role of $CD4^+$ T cells is well established in many autoimmune diseases including experimental autoimmune encephalitis (EAE) and multiple sclerosis (MS) etc. We therefore became interested to evaluate the role of CaeA on $CD4^+$ T cells. It was very interesting to observe that CaeA suppressed the proliferation of antigen specific $CD4^+$ T cells. Since $CD4^+$

T cell plays a crucial role in the initiation and regulation of immune responses, inhibition of their activation therefore, provides a powerful approach for immunosuppressor therapy. Next we did experiments using antigen specific D10G4.1 Th2 clones stimulated either with anti-CD3 Ab or antigen-pulsed splenocytes. As Th2 cells are responsible for allergic reaction. Sensitization with a foreign antigen mimicking self can induce an allergic immune response of Th2 cells and is associated with autoreactivity. This experiment gives evidence that CaeA is capable of acting at the level of clonal population. We also evaluated the role of CaeA on the mixed lymphocyte reaction (MLR) and compared its immunosuppressive activity with a well-known immunosuppressive drug cyclosporin A (CsA). We observed a dose dependent inhibition in MLR by both the drugs. Surprisingly, CaeA was found to be more potent than CsA in inhibiting MLR. CaeA inhibited maximum proliferation using a 10 fold lesser dose than CsA. $CD4^+$ T cells can be sub-divided on the basis of cytokines profile. Th1 (T helper 1) cells mainly produce IL-2, IFN-γ and lymphotoxin. Th2 (T helper II) cells chiefly secrete IL-4, IL-5, IL-9, IL-10 and IL-13. Th1 cells are responsible for cell-mediated immunity or delayed type hypersensitivity (DTH), whereas Th2 cells are essential for humoral immunity. We therefore monitored the action of CaeA on both Th1-like and Th-2-like cytokines. CaeA drastically inhibited the production of IFN-γ and IL-4. We could not observe any impact on the secretion of IL-10. We also substantiated our finding that CaeA affects the activity of Th1 cells by utilizing 3DO.54.8 Th1 hybridoma. CaeA affected the growth of Th1 hybridoma and showed significant synergy with anti-CD3 Ab in inhibiting proliferation.

We also wanted to see that at what stage of T cells proliferation CaeA induces its suppressive activity. We therefore, stimulated D10G4.1 Th2 clones with anti-CD3 Ab and added CaeA into the cultures at different time intervals. Interestingly, CaeA not only acts on the early stages of activation but also at later phases of division like the already known immunosuppressive drug Rapamycin.

We have positively established very categorically using a variety of cells and repeating the experiments several times that CaeA inhibited *in vitro* proliferation of mixed lymphocytes cultures, T cells, B cells, $CD4^+$ T effector cells, Th2 clones and MLR reaction. We therefore, did additional experiments to demonstrate whether CaeA retains immunosuppressive property *in vivo* also. Therefore, OVA primed mice were administered different doses of CaeA for 7 days. Later, the animals were sacrificed and splenocytes were cultured *in vitro* with antigen and different doses of CaeA. As compared to the mice that were not administered CaeA, the animals inoculated with CaeA showed significant level of retardation

in the proliferation. It may be conjectured from these experiments that CaeA also has immunosuppressive activity *in vivo*. These results are quite convincing and further give a signal that CaeA may work effectively as an immunosuppressive drug in transplantation and autoimmune diseases. It is worth mentioning here that we also did toxicity assays in BALB/c mice after administration of different doses of CaeA (25-100 μg/100 μl/mice/day) for 7-9 days. No mortality rate or change in the behavior of animals was observed. Thus indicating that the doses used in our experiments are safe to use and do not induce any toxic effects in the animals.

This raises a question that is the inhibition of proliferation due to over proliferation and death of the cells? To address this issue, experiments were carried by stimulating lymphocytes with ConA. The culturing of lymphocytes with CaeA did not effect their viability, as evidenced by propidium iodide and annexin V staining. This event specifies that suppression in proliferation was not due to the death of lymphocytes rather the drug was delivering inhibitory signals without altering viability of the lymphocytes. To affirm these results, we went on to elucidating the mode of action of CaeA in inhibiting the proliferation of lymphocytes. It is very well known that CTLA-4, which is expressed at a later stage on T cells activation, delivers inhibitory signals to restrict their proliferation. In contrast, CD28 is constitutively expressed on T cells and is known to deliver stimulatory signals. We therefore, monitored the effect of CaeA in modulating the expression of CD28 and CTLA-4 on $CD4^+$ T cells. It was quite noteworthy to observe that CaeA significantly upregulated the expression of CTLA-4. In contrast, it substantially down-regulated the exhibition of CD28. We also noticed that CaeA increased the quantity of $CD4^+$ T cells expressing CTLA-4 and decreased the number of CD28 positive cells. Similar results were observed in the case of non-$CD4^+$ T cells. Thus it is reasonable to conclude that immunosuppression induced by CaeA was a consequence of upregulation of CTLA-4 and downregulation of CD28 expression on $CD4^+$ and non-$CD4^+$ T cells. Leukocyte function-associated antigen-1 (LFA-1) is expressed on T-cells, neutrophils and eosinophils, is a ligand for Intracellular adhesion molecule-1 (ICAM-1 or CD54), which is expressed on antigen presenting cells (APCs) and activated endothelial cells. LFA-1 interaction is essential for T-cell activation as well as for migration of T-cells to target tissues. Therefore we become interested to know the effect of CaeA on LFA-1, no significant change in percentage or in the expression was observed. We also looked into the impact of CaeA in regulating the expression of activation marker CD69 on thymocytes. It is

well-established fact that CD69 is an early activation marker on T cells. Incorporation of CaeA into the cultures exhibited no change in the CD69 expression.

Many costimulatory molecules (i.e. CD40, B7-1, B7-2, M150, ICAM-1 etc.) are expressed on the surface of APC`s (dendritic cells, macrophages/monocytes, B-cell). But B7-1 and B7-2 are the most potent costimulatory molecules known to date. Their interactions with CD28/CTLA-4 receptors expressed on T cell surfaces are crucial for the proper regulation of T cell activity. Since we observed the enhancement in CTLA-4 and decrease in CD28 expression on T cells induced by CaeA, we therefore also monitored the expression of their ligands B7-1 and B7-2. Interestingly, CaeA upregulated B7-1 but downregulated B7-2 expression on thioglycolate elicited peritoneal macrophages. Like CTLA-4, B7-1 is not expressed constitutively on the cell surface of APC. But is upregulated at a later stage of action. Further, B7-1 binds to both CTLA-4 and CD28 more strongly than does B7-2. However, when the relative interactions are directly compared, B7-1 favors binding to CTLA-4 over CD28 by 20-fold, whereas B7-2 only favours CTLA-4 over CD28 by about 8-fold. In view of the above-mentioned findings, the potent role of CaeA as an imunosupppression agent can be viewed by a mechanism of enhanment of the expression of CTLA-4 on T cells and B7-1 on APC. The role of CTLA-4 and B7-1 in delivering inhibitory signals is very well documented in literature (Hirokawa et al. 1996, Jeannin et al. 1997, Suvas et al., 2002, David et al. 2003). Interestingly, we observed augmentation in the expression of B7-2 in J774 cancer cells but no detectable change was noticed in the case of B7-1. Recent data shows that the B7-2 expression above a threshold elicits anti-tumour immunity (Pizzoferrato 2004).

MHC-peptide is known to deliver first signal to T cell, which is cardinal in the initiation of cascade of events necessary for the activation of T cells. Inhibition in the expression of MHC molecules may render APC incapable of presenting peptides to T cells. Thus blocking their activation. It was of interest to also monitor the expression of MHC molecules on the surface of APC. CaeA very significantly inhibited the expression of IA^d. This indicates that CaeA not only downregulates the expression of costimulatory molecules that delivers positive signals for the proliferation and differentiation of T cells but also inhibits the expression of MHC molecules.

Biochemical analysis had revealed that cyclophilin-CsA and FKBP-FK506 complexes bind to and inactivate Ca^{2+}-dependent serine/threonine phosphatase calcineurin. Since it has been believed that inhibition of calcineurin is a molecular basis of the immunosuppressive properties of CsA and FK506. We also observed that CaeA acts through Ca^{2+} dependent

pathways, as evidenced by the inhibition in the proliferation of lymphocytes stimulated with PMA and ionomycin.

After establishing the immunosuppression activity of CaeA on the lymphocytes, we became curious to test whether it could also inhibit the growth of cancer cells *in vitro*. Therefore, we evaluated its impact on mouse T cell thymoma (EL-4 cells) and B cell lymphomas (WEHI-279 and A20), on human lung cancer cell lines H226, A549 (NSCLC) and human breast cancer cell line MCF-7. Remarkably, CaeA was not only potent in inhibiting the growth of mouse cancer cells but human cancer cell lines as well. Interestingly, unlike normal mouse cells, CaeA significantly reduced the viability of cancer cell lines. We also established that the suppression in proliferation of cancer cells was due to apoptosis. H226 (NSCLC) cells were treated with CaeA and analyzed for the activation of caspase 3 (a downstream caspase) and cleavage of PARP, a well-known substrate for caspase 3, 6 and 7. We observed a time-dependent activation of downstream caspase 3 and cleavage of a 118 kDa PARP protein into an 87-kDa fragment, another characteristic feature of cells undergoing apoptosis. This evidence indicates that CaeA induced apoptosis in NSCLC cells. Next, we argued that induction of apoptosis in cancer cells might be mediated through cell cycle perturbations resulting in cell growth arrest. The cell growth and viability assays have shown that CaeA results in an arrest of cell growth and an increase in the loss of viability in a time-dependent fashion that could be related to apoptosis. To further prove the point we conducted DNA cell cycle analysis that showed a progressive arrest of the cells in G_0-G_1 phase, in fact within 24 h of CaeA treatment a G1/S arrest was also observed in treated cells.

Finally, we tested the role of CaeA in a mouse model in which a tumor was induced using J774 cancer cells. As compared to the control groups, CaeA was able to suppress the *in vivo* initiation of tumor formation and could also regress the growth of already developed tumour. This result shows that the drug could be tried as a therapeutic agent in the treatment of cancer. It also could be used as a lead molecule as an immunosuppressive agent in the treatment of autoimmune diseases and transplantation.