Summary

cently, our group at CSIR-IMTECH has described the use of caerulomycin A (CaeA) an immunosuppressive agent (Singla et al., 2012). CaeA exhibited better munosuppression than the clinically used drug cyclosporin A. CaeA suppresses both ll-mediated immunity as well as humoral immunity. In addition, CaeA suppresses cretion of proinflammatory cytokines IFN-γ and IL-4 that play deleterious role in ıtoimmune diseases and transplantation. At molecular level, CaeA possesses a much mpler chemical structure compared to cyclosporin A. The molecular mass of CaeA is 29.23, whereas molecular mass of cyclosporin A is 1202.61. *In vitro* studies have shown ıat CaeA requires 10-fold lesser amount by weight compared to cyclosporin A for chieving similar level of inhibition of lymphocytes (Singla, 2004; Singla et al., 2012). hus, if successfully developed as drug, CaeA is expected to lead to a significant decrease ı cost of immunosuppressive therapy.

rior to this work, CaeA has been described as a bioactive molecule possessing ntifungal, antibacterial and antiamoebic properties. Orignally, CaeA was isolated from *Streptomyces caeruleus* (Chandran et al., 1968; Funk and Divekar, 1959). However, in our group CaeA was isolated from a novel species of actinomycetes, *Actinoalloteichus spitiensis* (Singla et al., 2005).

The objective of the present study was to find the target of CaeA and elucidate its mechanism of immusuppressive activity.

Most common targets of organic small molecule drugs are proteins and nucleic acids (Hughes et al., 2011; Triggle, 2007). However, analyzing the structure of CaeA, we quickly realized that the primary target of this molecule may not be a macromolecule. The core structure of CaeA is 2,2'-bipyridine. 2,2'-Bipyridine. is known to be a strong chelator of metal ions, such as Mo, Ru, Fe, etc. (Kaes et al., 2000; Liu and Hider, 2002). The ability of bipyridine to chelate iron ions appears noteworthy and relevant to immunosuppression property of CaeA. Iron being a redox active, plays a crucial role in metabolic processes. Iron is a cofactor for several enzymes (Le and Richardson, 2003; Papanikolaou and Pantopoulos, 2005). The function of these proteins would be impaired if the supply of iron is inadequate, which in turn would affect the proliferability of cells.

The immune system consists of a complex array of cells and molecules, whose activities are regulated in highly specific way following the antigenic challenge. Thus, the abnormal status of iron can lead to impaired immune functions (Brock and Mulero, 2000; Weiss, 2005).

From the forgoing discussion, it may be inferred that immunosuppressive property of CaeA may be related to its ability to chelate iron. If such a process occurs intracellularly, it will cause iron deficiency and impaired function of several enzymes, which will have determinant effect on all the proliferating cells. Therefore, our approach at unraveling target of CaeA involved, studying the effect of CaeA on intracellular iron pool and consequently its effect on key molecules involved in cell proliferation and cell cycle. We termed this approach as 'Rational Approach'. Jurkat cell line was used in this study. The known iron chelator DFO was used for comparative analysis of results obtained with CaeA.

As a first step, we demonstrated depletion of intracellular iron pool on treatment of Jurkat cells with CaeA using atomic absorption spectrometry (AAS). 2.5 μM CaeA caused 90% reduction in intracellular iron pool after 24 h treatment. In comparison, 100 μM DFO caused only 26% reduction in iron pool under similar conditions.

The transferrin mediated iron uptake and mobilization from cells are important indices for determining the ability of chelator to induce iron depletion in the cells (Le and Richardson, 2003). Using radiolabelled transferrin-^{55}Fe, we demonstrated that CaeA caused concentration and time dependent reduction in iron uptake by Jurkat cells. Prior to estimation of radio counts, the cells were treated with pronase to remove any surface-bound ^{55}Fe. CaeA required about 40-fold lesser concentration to achieve similar reduction in iron uptake by Jurkat cells after 3 h.

In iron efflux study, Jurkat cells prelabelled with radioactive ^{55}Fe-transferrin were incubated with CaeA or DFO. The amount of ^{55}Fe released in the media was estimated by radioactive count after removing the cells by centrifugation compared to untreated cells, CaeA resulted in concentration dependent as well as time dependent increase in release of iron from the cells. Compared to DFO, CaeA at 40-fold lower concentration caused 1.78-fold higher release of iron from the ^{55}Fe-prelabelled cells.

nsferrin receptors (TfRs) are transmembrane proteins that allow the controlled access ron to cells (Aisen et al., 2001). Tf-TfR mediated pathway for uptake of iron is central ıll iron requiring cells (Chua et al., 2007). Ferritins are ubiquitously expressed proteins all the species from bacteria to plants and man. They are involved in the sequestration l storage of iron (Han et al., 2011). Intracellular iron level regulates the abundance and ıilability of TfR and the intensity of ferritin protein post transcriptionally through iron gulatory proteins. Iron deplete conditions cause increased TfR translation, decreased R degradation and translation block of ferritin (Ganz and Nemeth, 2012).

nce we have shown that CaeA causes iron-deplete condition in cells, we studied the fect of CaeA on the expression levels of TfR1 and ferritin-H. 2.5 μM CaeA caused 72-fold increased expression of TfR1 after 24 h and approximately 9-fold decreased :pression of ferritin-H. In comparison, 100 μM DFO caused 1.57-fold increased :pression of TfR1 and about 2.6-fold decreased expression of ferritin-H.

. major consequence of intracellular depletion of iron would be on the function of nzymes that require iron as cofactor. Ribonucleotide reductase (RNR) is one such nzyme, whose activity is essential for the proliferation of cells. Ribonucleotide reductase r 2'-deoxyribonucleoside-diphosphate:thioredoxin-disulfide-2'-oxidoreductase is a biquitous radical-containing enzyme, involved in the rate limiting step, in the synthesis ıf DNA. Inactivation of RNR stops DNA synthesis, which in turn inhibits cell ıroliferation (Cerqueira et al., 2007). Using ^{3}H-cytidine incorporation assay, we lemonstrated that CaeA caused concentration dependent decreased synthesis of DNA. The decrease was in the range of 21-55% at CaeA concentration of 0.3-2.5 μM. Significantly, no effect on the synthesis of DNA was observed when Jurkat cells were :reated with CaeA in presence of excess of ferrous ions.

To confirm that the depletion in DNA is due to the effect of CaeA on RNR, we estimated intracellular pool of deoxyribonucleotide phosphates (dNTPs). Treatment of Jurkat cells with 2.5 μM CaeA resulted in significant decreased intracellular levels of dUTP, dGTP and dATP. The effect on dTTP levels was insignificant, possibly due to the compensatory deoxyribonucleotide salvage pathway (Koc et al., 2004). These results confirmed that decreased synthesis of DNA is due to the impaired function of RNR.

RNR is composed of two subunits R1 and R2. R1 subunit is constitutively expressed throughout the cell cycle, whereas R2 subunit is expressed in S-phase of cell cycle where large amounts of deoxyribonucleotides are required in proliferating cells (Ahmad and Dealwis, 2013; Shao et al., 2006). However, we found that treatment of Jurkat cells with 2.5 μM CaeA caused 2.24-fold increased expression of R2 subunit. We speculate that the increased level of R2 subunit may be due to its decreased degradation. We have also shown that treatment of Jurkat cells with 2.5 μM CaeA resulted in significant decrease in % of cells in G1-phase and significant increase in % of cells in S-phase, which indicated that the cell cycle arrest has occurred in S-phase. These findings are consistent with previous reports, which showed the arrest of lymphocytes in S-phase by DFO (Lederman et al., 1984). The increased concentration of R2 may help cells to overcome the replication stress induced by CaeA through depletion of dNTPs in the cells as described above.

Next, we studied the effect of CaeA on MAPK signaling transduction pathways. MAP kinase pathway occupies a central position in signal transduction machinery, which links external signal to the varied cellular processes (Kim et al., 2002; Le and Richardson, 2002; Noulsri et al., 2009). The c-Jun NH_2-terminal protein kinase (JNK) and p38 MAP kinase are known to have anti-proliferative and pro-apoptotic function, while ERK1/2 MAP kinase is primarily known for proliferative role (Dhillon et al., 2007; Raman et al., 2007; Yu and Richardson, 2011). The p38 and JNK are known for cell cycle arrest by phosphorylating p53 and increasing the stabilization of cdk inhibitor $p21^{WAF1/CIP1}$ (Wilkinson and Millar, 2000; Yu and Richardson, 2011).

It is known in literature that iron chelation significantly reduce ASK1-thioredoxin complex formation. Reduced iron levels promote disulfide formation and consequently dissociation of thioredoxin from the complex that leads to increased phosphorylation of ASK1, which activates the JNK and p38 MAPKs and their downstream target molecules p53 and ATF2 (Yu and Richardson, 2011).

We studied the effect of CaeA on phosphorylation of JNK and p38 MAPKs at various time points. The phosphorylation for JNK initiated at 3h of treatment and maximum phosphorylation with respect to total protein was observed at 12 h of treatment with 2.5 μM CaeA. However, we did not find a significant increase in phosphorylation of JNK

h 100 μM DFO. A significant increase in phosphorylation of p38 was observed with h 2.5 μM CaeA and 100 μM DFO.

other member of MAPKs is ERK1/2, which is primarily known for proliferative role. early stages 2.5 μM CaeA caused increased phosphorylation of ERK1/2. Thus, the el of phospho-ERK1/2 increased up to 1.63-fold in 6 h; after which the level started :reasing and reached close to normal level after 24 h. These results are similar to those served earlier for DFO, which demonstrated increased phospho-ERK1/2 levels after ubation of up to 12 h. This intial increase in ERK1/2 phosphorylation may be related an early response against stress (Yu and Richardson, 2011). In contrast CaeA caused nificant increase in phospho-JNK and phospho-p38 levels at 24 h, where anti-)liferative effects of these pathways may be important in the immunosuppressive tivity of CaeA.

e also studied the effect of CaeA on cell-cycle molecules, cyclin D1, cdk4 and $1^{CIP1/WAF1}$. Cell cycle progression depends upon the sequential activation and bsequent inactivation of cdks. Cdks rely on cyclins to modulate their phosphorylation tivity. The activity of cyclin-cdk complexes are modulated by cyclin-dependent kinase hibitors (CKI). One of the inhibitors, known as $p21^{CIP1/WAF1}$ is a universal cdk inhibitor .e and Richardson, 2003). It has been demonstrated that p38 MAPK activates $!1^{CIP1/WAF1}$ on Fe chelation and results in decreased expression of cyclin D1, which leads cell cycle arrest, JNK is also known to cause cell cycle arrest by stabilizing $p21^{CIP1/WAF1}$:pression (Yu and Richardson, 2011).

hese studies prompted us to determine the effect of CaeA on the expression of cyclin 1, its partner cdk4 and on the mRNA expression of $p21^{CIP1/WAF1}$. CaeA caused)ncentration dependent decrease in cyclin D1 and cdk4 expression level. Compared to)ntrol (untreated cells), 2.5 μM CaeA caused 10-fold higher decrease in expression of dk4, whereas 100 μM DFO caused about 2.5-fold higher decrease in expression of cdk4. .5 μM CaeA caused 3.4-fold higher expression of $p21^{CIP1/WAF1}$ mRNA compared to)ntrol, whereas 100 μM DFO increased the expression of $p21^{CIP1/WAF1}$ mRNA by 2-fold.

.eactive oxygen species (ROS) have the ability to commence sequence of chemical :actions with many biomolecules, leading to DNA oxidation, mitochondrial damage, and

peroxidation of membrane lipids that result in cell death or apoptosis. Iron has ability to generate ROS through Fenton's reaction (Kalinowski and Richardson, 2005). CaeA being chelator of iron may decrease ROS levels in the cells and prevent apoptosis. Compared to untreated cells, 2.5 μM CaeA caused 2.1-fold decrease in ROS level in Jurkat cells, under similar conditions 100 μM DFO caused 1.7-fold reduction in ROS level in Jurkat cells. Significantly, 70-80% cells were found to be viable even after 24 h treatment with 2.5 μM CaeA determined by viability assays.

In brief, the results from 'Rational approach' may be summarized as given below.

1. We have shown that CaeA causes (i) decreased intracellular iron concentration, (ii) reduced uptake of iron by the cells, and (iii) increased iron release from cells.

2. Following consequences resulting from intracellular iron depletion by CaeA were observed (i) increased expression of TfR1, (ii) decreased synthesis of DNA by cells, (iii) increased phosphorylation of ERK, JNK and p38 MAP kinases and (iv) decreased expression of cell cycle progression molecules cyclin D1 and cdk4.

3. CaeA inhibits synthesis of DNA, probably through inhibition of RNR, which catalyze rate limiting step in the synthesis of DNA. Inhibition of RNR activity was confirmed by measuring dNTPs pool in the cells. dNTPs pool was depleted on treatment of cells with CaeA.

4. CaeA caused significant decrease in ROS levels in the cells.

At the beginning of the project on identification of target of CaeA, we also started activity based protein profiling (ABPP) approach in parallel with the rational approach described above. In literature, this chemical proteomic approach has proved very useful in identification of protein targets of small organic molecules (Berger et al., 2004; Cravatt et al., 2008). In this dissertation, we have recorded our plans and initial experiments done in pursuit of ABPP approach. However, we did not continue to follow this approach in view of the initial results obtained from rational approach, which implicated iron as the primary target of CaeA.

To achieve more comprehensive understanding of the biological effect of CaeA in Jurkat cells, we also carried out the gene expression profiling studies using DNA microarray

chnique. Jurkat cells were treated with 2.5 µM CaeA and 100 µM DFO for 24 h at 37 and microarray data obtained as described in the materials and methods section. The ta was analyzed by GeneSpring GX and Genotypic Biointerpreter-Biological Analysis ftware. Pathway maps of the affected pathways were collected from Kyoto cyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2010). We focused our tention on the effect of CaeA on some of the pathways that are important for generation immune response. We found significant decrease in the mRNA expression of SPA1A, HSPA1B, HSPA6, HSPA8, CD28, CD40LG, ZAP70, C1s, C1QA, C9, CD2, ELL, ITGA4, CD40LG and CD226 on treatment of Jurkat cells with 2.5 µM CaeA.

ISPA1A, HSPA1B, HSPA6 and HSPA8 encode for heat shock protein HSP70 family nd HSP90AA1 encodes for a member of HSP90 family. The endogenous antigenic eptides are presented by MHC I complex, whereas exogenous determinants are isplayed by MHC II complex molecules. It is known that the exogenous antigen haperoned by heat shock proteins (HSPs) can be channeled into the conventional ndogenous pathway presented by MHC I complex to CD8+ T cells (Suto and Srivastava, 995). Among HSPs, heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) re known for cross antigen presentation (Murshid et al., 2012; Yang et al., 2009).

D28/B7 is primary costimulatory signal. Its downregulation may result in immune suppression or antigen specific tolerance. Zeta-associated protein-70 (ZAP70) activation transduces NF-κB signaling pathway, which can induce expression of CD40 ligand on T cells. The CD40-CD40LG interaction stimulates B cells and they undergo clonal expansion and proliferation and produce antibody for secretion on interaction with cytokines secreted by the T cells (Rainiene, 2005). CaeA reduces the expression of CD40LG remarkably at transcriptional level, which indicates that CaeA also affects the activity of B cells.

An important component of immune system is the complement system. The complement cascade gets activated by three pathways: the classical, alternative and mannose lectin pathways. Complement system can sense DAMPs that activate various components of complement pathways and mediate inflammation, antibody-dependent cytotoxicity, and also potentiates T-cell activation (Oberbarnscheidt et al., 2011; Ponticelli, 2012). Through

microarray it was deciphered that CaeA was able to downregulate C1s and C1QA the components of C1 complement protein, which initiates the classic biochemical pathway of complement system and also decreases the C9 component of the complement system.

The immunosuppression can be attained by lymphocytes depletion, diversion of traffic of lymphocytes or by jamming the reaction pathway of lymphocytes (de la Cruz-Merino et al., 2012; Duncan and Wilkes, 2005; Rainiene, 2005). The molecules CD2, CD28, SELL, ITGA4, CD226 and CD40LG play a role in lymphocyte trafficking and stimulation of lymphocytes.

CaeA also upregulated genes, which play a role in enhancing the effector response of lymphocytes like GZMB, JUN, FOS, AP1, TNFRSF10A, IL23A, CXCL16, and ICOSLG. Therefore, CaeA could maintain balance between immunosuppression and its undesirable effects, important for preventing increased risk of infection, malignancy and drug related toxicities. Thus, CaeA is an excellent candidate molecule, which could be evaluated for inclusion in modern-day immunosuppression regimen.