7. Summary and conclusions

In case of end state organ failure, especially in case of liver and heart failure, organ transplantation is typically the only remedy available. Kidney failure is another example where organ transplantation is not only a cost effective treatment but also provides better quality for life to patients. Successful organ transplantation requires effective suppression of the immune system of an organ recipient by the use of immunosuppressive drugs (ISDs). ISDs are also prescribed for the treatment of autoimmune diseases, inflammatory disorders, hypersensitivity to allergens, etc. Autoimmune disorders include systemic autoimmune diseases, autoimmune endocrine disorders, and organ-specific autoimmune diseases; such as lupus erythematosus, psoriasis, multiple sclerosis, rheumatoid arthritis, thyroiditis, hemolytic or pernicious anemia, insulin-dependent diabetes mellitus, Crohn's disease, autoimmune hepatitis, and autoimmune pneumonitis.

Although, currently used drugs, such as cyclosporine, FK506, azathioprine, cyclophosphamide, methotrexate, prednisone, prednisolone, etc. have provided significant relief to patients in reducing organ rejection in transplantation, these suffer from one or another drawback, such as poor oral bioavailability, side effects like nephrotoxicity and malignancy, non-specific mode of action, incomplete suppression of belligerent immune cells, unaffordable cost, etc. Cyclosporine A is a widely used drug and has provided significant benefits in organ transplantation, but it suffers from disadvantages such as tolerated dosages do not provide complete suppression of rejection response, thus requiring concomitant treatment with other immunosuppression agents. Significant adverse side effects of cyclosporine A include damage to the kidney and liver, hyperplasia of gum tissue, refractory hypertension, increased incidence of infections and malignancy.

Therefore, there is a continuous need for discovering efficacious, selective and safer new immunosuppressive drugs for improved sustenance of organ transplants and treatment of autoimmune disorders. In particular, there is a need to provide immunosuppressive treatment to patients at significantly decreased cost.

In a program directed at exploitation of microbial wealth of India, our group in collaboration with other groups in IMTECH has recently described the isolation of caerulomycin A (CaeA) from *Actinoallotheicus spitiensis* and its use as immunosuppressive agent. CaeA induces generation of regulatory T cells, significantly

suppresses T cell response and prolongs the survival of allogeneic skin graft. CaeA exhibited better immunosuppression than clinically used drug cyclosporine A. Whereas, CaeA suppresses both the cell-mediated immunity as well as the humoral immunity, cyclosporine A (CsA) suppresses only the cell mediated immunity. At molecular level, CaeA possesses a much simpler chemical structure compared to cyclosporine A or FK506. The molecular mass of CaeA is 229.23, whereas molecular mass of cyclosporin A is 1202.61. *In vitro* studies have shown that CaeA requires 10-fold lesser amount by weight compared to cyclosporine A for achieving similar level of inhibition of lymphocytes.

Having described the activity of CaeA as immunosuppressive agent, the next step was to find its intracellular target and attempt to decipher its mechanism of action. The target deconvolution is essential in order to overcome problems like toxicity, to enhance specificity, efficacy and elucidation of mechanism of action of the drug molecule. Most common targets of organic small molecule drugs are proteins and nucleic acids. 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. Iron is central regulator of immune cell proliferation and function. Lymphocytes are key molecules for specific immunity. Iron is a limiting factor for the proliferation and differentiation of lymphocytes. Accordingly, our group in IMTECH has recently demonstrated that CaeA exerts its immunosuppressive effect by depleting intracellular iron concentration. It has multiple cellular targets, viz., iron containing ribonucleotide reductase enzyme, which is crucial for DNA synthesis and cell cycle control molecules cyclin D1, $p21^{CIP1/WAF1}$ and cdk4, which are important for normal cell cycle progression. Thus, for the first time we reported that that iron chelation is a viable rationale approach to selectively suppress the immune system; because compared to normal cells, rapidly proliferating cells require higher utilization of iron, which is a central regulator for proliferation and function of immune cells. Previously, iron chelation has been used to target cancer cells and also provide relief to patients suffering from secondary iron overdose.

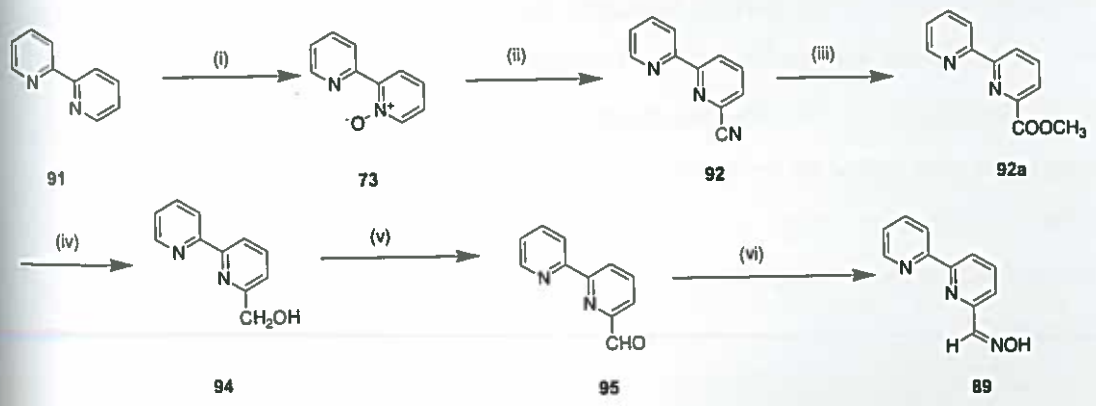
From the above discussion it becomes clear that CaeA offers several advantages over CsA, which is the most commonly used drug in clinic at present. Compared to CsA, CaeA possesses much simpler structure, has a different mechanism of action and requires

lower dose by weight for achieving similar level of immunosuppression. CaeA is therefore an excellent lead molecule for development of new class of immunosuppressive drugs. The next obvious step in the drug development process was to modify structure of CaeA with the aim of obtaining more efficacious molecule/s. Therefore, we initiated this study with following objectives:

1. Synthesize a library of molecules based on Caerulomycin A (CaeA) scaffold.
2. Test the synthesised molecules for immunosuppression using *in vitro* assay methods
3. Test the selected molecules for their ability to prolong survival following allo-skin graft in mouse model.

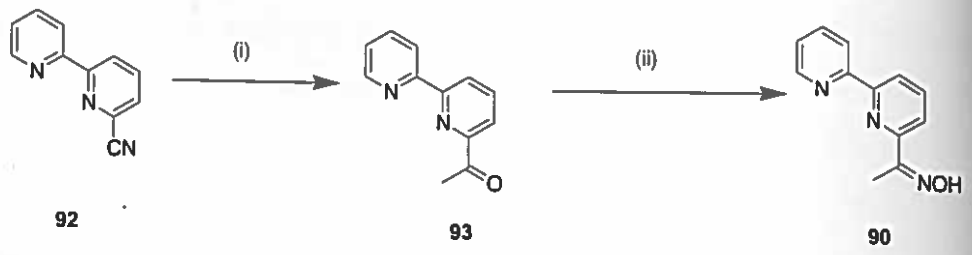
To begin with, we established the synthesis of desmethoxy analog of CeaA, *N*-[([2,2'-bipyridin]-6-yl)methylidene]hydroxylamine (**89**, Scheme 1) and its corresponding ketoxime *N*-[1-([2,2'-bipyridin]-6-yl)ethylidene]hydroxylamine (**90**, Scheme 2). The structures of synthesized compounds **89** and **90** were confirmed by NMR and Mass data.

Scheme 1



Reagents: (i) mCPBA, $CHCl_3$, 25 °C; (ii) $(CH_3)_3SiCN$, $(CH_3)_3NCOCl$, CH_2Cl_2, 25 °C; (iii) HCl, MeOH, 80 °C; (iv) $NaBH_4$, THF, 80 °C; (v) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C; (vi) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

Scheme 2



92 93 90

Reagents: (i) MeMgBr, anhydrous THF, 25 °C; (ii) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

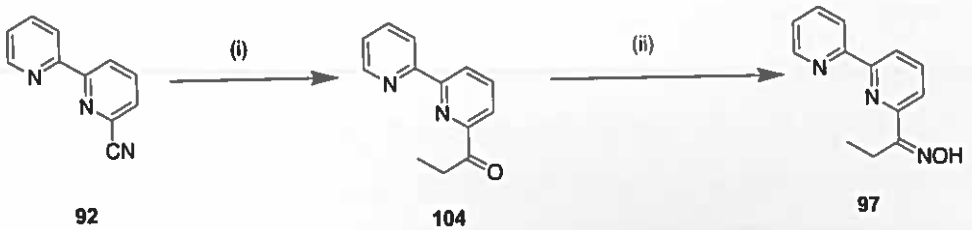
The next step was to compare the activity of **89** and **90** with CaeA by an *in vitro* assay. T cells are known to play an important role in the immune system. Thus, assay of a chemical entity for suppression of the proliferation of T cells *in vitro* is expected to provide reasonable idea about its immunosuppressive potential. Initially we used Jukat T-lymphocytes, a human lymphoblastoid T cell line for assay of anti-proliferative potential of **89** and **90**. As expected, CaeA exhibited 50-60% inhibition of proliferation of Jurkat cells after 48 hrs in concentration range of 0.3 to 1.2 μM in consonance with doubling time of Jurkat cells and our previous report, which showed that at these concentrations CaeA has insignificant effect on viability of cells. We were pleased to note that both the compounds **89** and **90** having simpler structure then parent molecule CaeA were found to be active in inhibiting the proliferation of Jurkat cells. **89** exhibited 20-25% inhibition activity as compared to 50-60% observed for CaeA at 0.3-1.25 μM concentration. Interestingly **90** was found to be more active than CaeA exhibiting 55 to 60% inhibition activity in concentration range of 0.15 to 0.60 μM. Thus, **90** required about two fold lesser concentration than CaeA to achieve similar level of inhibition of the proliferation of Jurkat cells.

We also performed assay using mitogen, concanavaline A (Con A) stimulated T-lymphocyte proliferation assay to ensure that the results obtained with Jurkat cell line would provide reliable results in measuring the immunosuppressive potential of analogs, in comparison to CaeA. Both compound **89** and **90** exhibited concentration dependent inhibition of Con A-stimulated T-cell lymphocytes. Compound **89** required about 4-fold higher concentration than CaeA and compound **90** required about 2-fold lesser concentration than CaeA to achieve similar level of suppression of mitogen activated T-lymphocytes.

The following conclusion could be drawn from the above, (i) methoxyl group of CaeA is not essential for its immunosuppressive activity; it can be replaced with H, (ii) conversion of aldoxime to ketoxime resulted in improved activity, (iii) Jurkat cells based assay method and splenocyte based assay method leads to similar results and conclusions and (iv) Jurkat T-cell lymphocytes based assay method can initially be used for comparison of activity of analogs of CaeA for short-listing of active compounds for the following reasons: (a) theses do not require activation by mitogen and (b) animals need not be sacrificed to obtain splenoctytes. The splenocyte based assay can be performed only on a few selected compounds for the conformation of results obtained with Jurkat cell based assay

Next, we prepared a series of oximes **97-103** in which methyl group of **90** was replaced with normal or branched chain alkyl, cycloalkyl or alkynyl group. The oximes **97** to **103** were prepared from their corresponding ketones **104** to **110** by reaction with hydroxylamine hydrochloride. Ethyl ketone 1-([2,2'-bipyridin]-6-yl)propan-1-one **(104)** was synthesised starting from nitrile **92** by Grignard reaction with CH_3CH_2MgBr in anhydrous THF (Scheme 3).[163] The yield of ketone **104** was poor compared to that obtained with methyl ketone **93**.The structure of ketone **104** and corresponding oxime **97** were in agreement with their NMR spectral data

Scheme 3



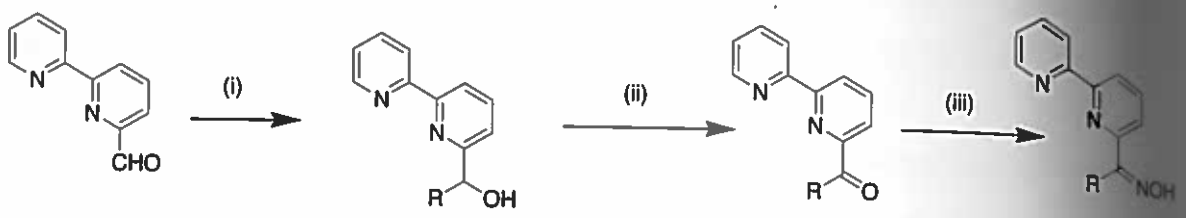
92 104 97

Reagents: (i) EtMgBr, anhydrous THF, 25 °C; (ii) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

Attempted preparation of ketone **105** to **110** by direct Grignard reaction with nitrile **92** failed in our hands. In all examples, starting material was recovered unchanged from the reaction mixture. Therefore, we decided to prepare these ketones starting from aldehyde **95**, synthesis of which has been described above in Scheme 1. Grignard reaction of aldehyde **95** with corresponding Grignard reagent yielded secondary alcohols **111** to **116**

which without any further purification were converted to desired ketones **105** to **110** by Swern oxidation (Scheme 4). The structure of oximes and their intermediate ketones were in agreement with their spectral data.

Scheme 4



95

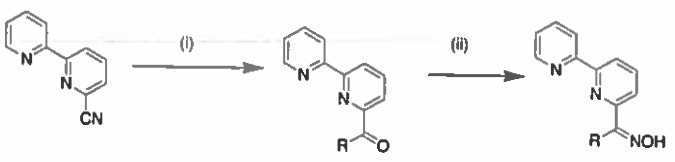
111-116*

105, **98**, R = $CH(CH_3)_2$
106, **99**, R = CH_2-$CH(CH_3)_2$
107, **100**, R = CH_2-CH_2-$CH(CH_3)_2$
108, **101**, R = n-C_8H_{17}
109, **102**, R = cyclopentane
110, **103**, R = CCH_3

Reagents: (i) RMgBr, anhydrous THF, 25 °C; (iii) $(COCl)_2$, DMSO, CH_2Cl_2, -78 °C; (iii) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C. *crude subjected to next reaction without purification

Next, we synthesized a series of 2,2′-bipyridine aryl oximes **117** to **130** from their corresponding ketones **131** to **144**. Ketones **131** to **144** were prepared directly from nitrile **92** by reaction with corresponding aryl Grignard reagent in anhydrous THF (Scheme 5). The structure of ketones and oximes were in agreement with their spectral data.

Scheme 5



92

131-144

117-130

131, **117** R = C_6H_5
132, **118** R = 2-MeO-C_6H_4
133, **119**, R = 3-MeO-C_6H_4
134, **120**, R = 4-MeO-C_6H_4
135, **121**, R = 4-Me-C_6H_4
136, **122**, R = 4-F-C_6H_4
137, **123**, R = 4-OCF_3-C_6H_4
138, **124**, R = 4-$N(CH_3)_2$-C_6H_4
139, **125**, R^3 = 2, 4-DiMeO-C_6H_4
140, **126**, R^3 = 2, 5-DiMeO-C_6H_4
141, **127**, R^3 = 3,4,5-TriMeO-C_6H_4
142, **128**, R = $C_{10}H_7$
143, **129**, R^3 = 6-MeO-$C_{10}H_7$
144, **130**, R^3 = $C_{14}H_9$

Reagents: (i) RMgBr, anhydrous THF, 25 °C; (ii) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

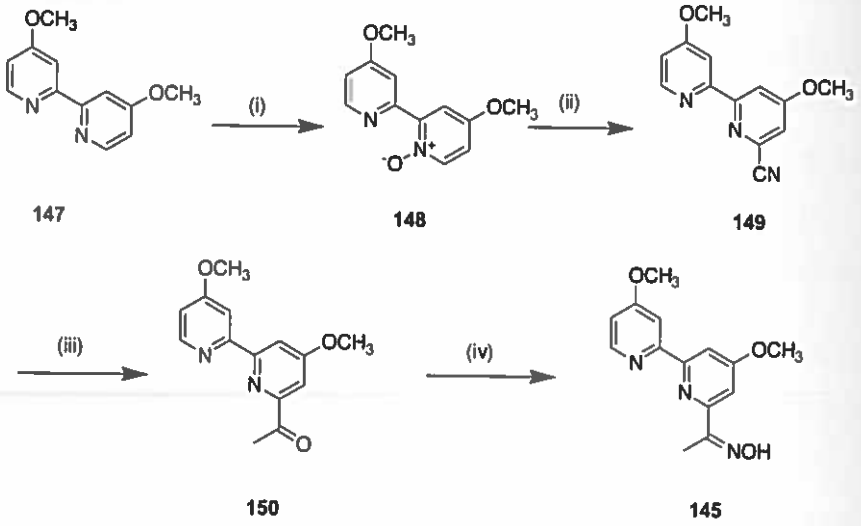
T cell anti-proliferation activity of alkyl, alkenyl and aryl oximes, **97-103** and **117-130** was assayed using Jurkat T-lymphocytes. The results were compared with CaeA and compound **90**. Based on these results, (i) the series for aliphatic and oximes **97-103, 90** and CaeA was arranged as **97** (Ethyl) > **90** (Methyl) > **99** (Isobuyl) > **100** (Isopentyl) > **101** (Octyl) > CaeA > **102** (Cyclopentyl) > **103** (Propynyl) > **98** (Isopropyl) and (ii) the series for aryl oximes was arranged as **125** (2,4 diOMephenyl) > **130** (Phenanthryl) > **90** (Methyl) > **118** (2-OMephenyl) > **123** (4-OCF_3phenyl) > **128** (Naphthyl) > **122** (4-Fphenyl) > **117** (Phenyl) > **129** (6-OMenaphthyl) > **124** (4-$N(CH_3)_2$phenyl) > CaeA > **127** (3,4,5-triOMephenyl) > **120** (4-OMephenyl) > **119** (3-OMephenyl) > **121** (4-Mephenyl) > **126** (2,5 diOMephenyl).

In the aliphatic series only ethyl homologue **97** showed higher activity than parent methyl oxime **90**. The longer chain or branched chain alkyl derivatives, such as isobutyl (**99**) and octyl (**101**) exhibited similar activity as compared to methyl derivative **90**. Cyclic alkyl derivative (**102**) exhibited activity similar to CaeA, however both were less active than methyl derivative **90.** Triple bond substitution (**103**) resulted in further decrease in activity. Isopropyl compound **98** was found to be least active. Thus it may be concluded that in aliphatic series, small chain alkyl groups, methyl and ethyl are ideal for substitution at the oxime carbon. Increasing the hydrophobicity by incorporation of longer alkyl chain or cyclic alkyl group does not improve the activity and sterically hindered group such as isopropyl and cyclopentyl resulted in decrease in the activity.

In the aromatic series, phenyl derivative **117** showed slightly less activity than corresponding methyl derivative **90**. Addition of +I group methoxyl at 2-position (**118**) increased the activity, which was further enhanced by addition of methoxyl group at 2 and 4-position of phenyl ring (**125**). Substitution by OCF_3 (**123**) and F (**122**) at 4-position of phenyl ring resulted in improved activity as compared to phenyl substituted oxime (**117**). Changing phenyl group to more hydrophobic naphthyl (**128**) or phenanrthyl group (**130**) improved the activity. However **130** was found to be toxic to Jurkat cells at higher concentration as cell viability decrease to about 20%. The substitution by methoxyl group at 3 or 5 –position of phenyl ring (**119**, **127** and **126**) resulted in decrease in activity of oxime. Addition of methyl group at 4-position of phenyl ring (**121**) did not improve the activity.

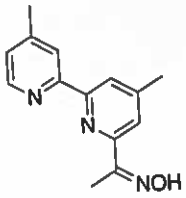
The next step was to vary the substitution in aryl rings of 2,2′-bipyridine system. Initially we selected methyl group on the oxime moiety **90** as it gave better activity than CaeA (1). Although ethyl group **97** gave somewhat better activity and branched chain isobutyl group **99** gave almost similar results as compared to methyl group **90** in Jurkat T-cell proliferation assay, we preferred methyl group because of the ease of its synthesis in high yield compared to other alkyl derivatives. Given the fact that desmethoxy CaeA **89** was less active than CaeA, we decided to add OCH_3 substituent at 4-position in the ring. However preparation of non-symmetrical 2,2′-bipyridine rings is tedious, therefore we decided to synthesis symmetrical, 4,4′-dimethoxy derivative **145** instead of 4-methoxy derivatives (Scheme 6). For comparison, we also prepared corresponding 4,4′-dimethyl derivative *N*-[1-(4,4'-dimethyl[2,2'-bipyridin]-6-yl)ethylidene]hydroxylamine (**146**) by following similar steps of reactions, but starting from 4,4′-dimethyl-2,2′-bipyridine.

Scheme 6

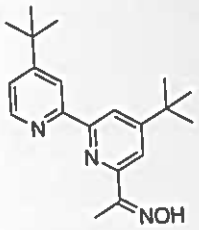


Reagents: (i) mCPBA, $CHCl_3$, 25 °C; (ii) $(CH_3)_3SiCN$, $(CH_3)_3NCOCl$, CH_2Cl_2, 25 °C; (iii) MeMgBr, anhydrous THF, 25 °C; (vi) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

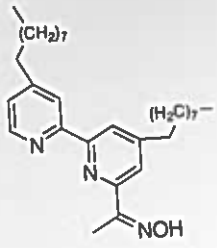
4,4′ dimethyl derivative, **146** was found to be more active than 4,4′-dimethoxy **145** in Jurkat T-lymphocyte proliferation assay. Therefore, we decided to replace methyl group with branched chain and longer chain alkyl group. Thus, **155** and **156** were prepared starting from appropriate bipyridine derivative and following steps similar to those described in Scheme 6 for synthesis of oxime **145**.



146



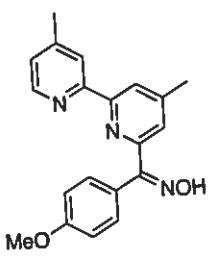
155



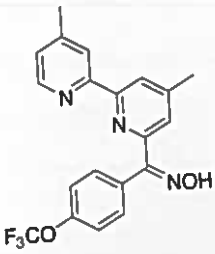
156

T-cell anti-proliferation activity of oximes **145, 146, 155** and **156** was performed using Jurkat T-cell lymphocytes. Amongst all these derivatives, all methyl compound **146** was found to be the most active compound. Substitution with methoxyl and *t*-butyl resulted in derivatives, which were better than CaeA, but similar to parent methyl derivative **90**. Substitution with long chain hydrophobic alkyl group **156** resulted in almost complete loss of activity. The series may be arranged as **146** (4,4′-dimethyl/methyl) >**145** (4,4′-dimethoxy/methyl) >**155** (4,4′-di-*t*-butyl/methyl) >**90** (methyl) >**156** (4,4′-dinonyl/methyl)

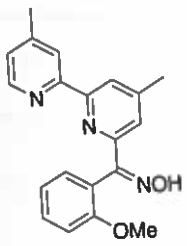
A set aryl oximes, **165, 166, 173** and **174,** were then synthesized for comparison of activity with oxime **146**. The method for synthesis of **165, 166** and **174** was similar to that described in Scheme 6 for oxme **145**. Oxime **173** was synthesised as shown in Scheme 8. T-cell anti-proliferation activity of oximes **165, 166, 173 and 174** was performed using Jurkat T-cell lymphocytes. None of the oxime **165, 166, 173** and **174** exhibited better activity than all methyl oximes **146**. Surprisingly, substitution with aryl group did not improve activity; most of these oximes were slightly less active than simple methyl derivative **90**. Isobutyl derivative **173** showed activity at only higher concentrations.



165

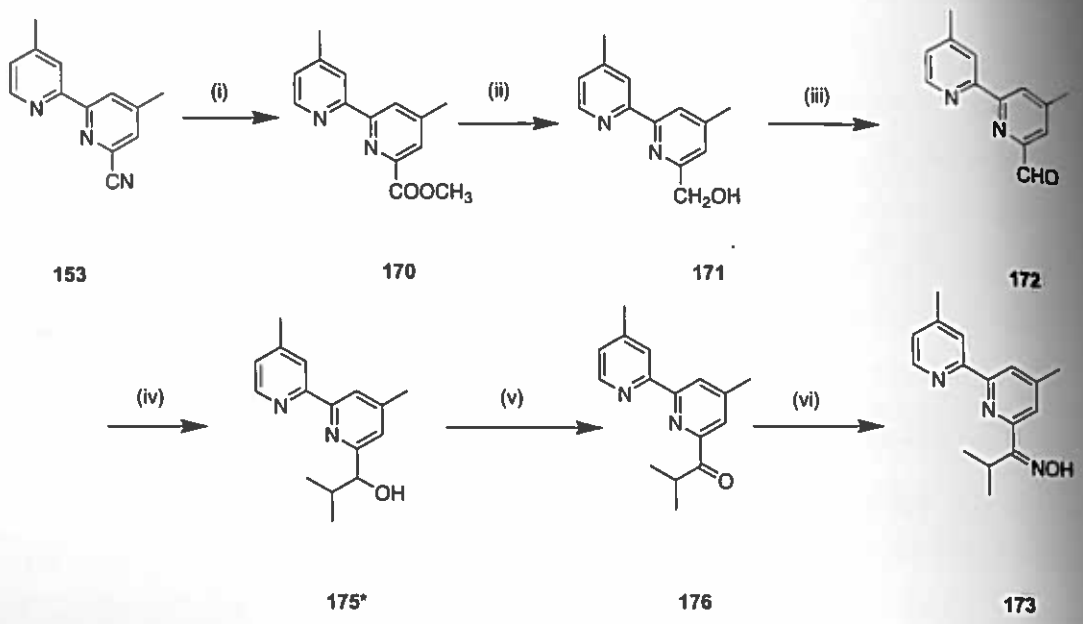


166



174

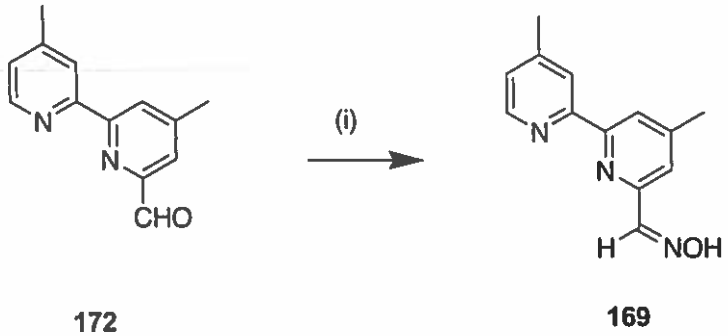
Scheme 8



Reagents: (i) HCl, MeOH, 80 °C; (ii) $NaBH_4$, THF, 80 °C; (iii) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C; (iv) $(CH_3)_2CHMgBr$, anhydrous THF, 25 °C; (v) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, -78 °C; (vi) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.
*crude subjected to next reaction without purification

Selection of oxime for use in animal model based on Jurkat T-lympocytes proliferation assay: All methyl oxime **146** was found to be the best amongst all the compounds tested. However the parent molecule CaeA is an aldoxime, whereas **146** is a keoxime. Therefore, it was decided to add corresponding aldoxime **169** in the test regimen for animal experiments. Oxime **169** was synthesized from aldehyde **172** as described in Scheme 9. The structures of aldehyde **172** and oxime **169** were in agreement with their spectral data.

Scheme 9



Reagents: (i) $NH_2OH.HCl$, C_5H_5N, EtOH, 80 °C.

Although all methyl oxime **146** was found to be the most active, we considered it important to add on aryl derivative (i.e. methyl group at oxime moiety replaced with an aryl group) in the regimen for animal experiments for comparative purpose. The oximes **165**, **166**, and **174** were less active than **146**. However, their activity was more or less similar to methyl derivative **90**. Trifluoromethoxyphenyl derivative were considered better than methoxyphenyl derivative because it is metabolically more stable to demethylation reaction by enzymes. 4,4′-dimethy/4-OCF_3-Ph compound **166** was no better than corresponding 4,4′-H/4-OCF3-Ph **123.** Therefore, **123** was selected for animal studied.

Suppression of mitogen induced mouse T-lymphocytes by selected oxime 123, 146 and 169: So far, the oximes **123**, **146** and **169** were short-listed based on the suppression of proliferation of Jurkat T-cell lymphocytes. However, before proceeding with animal studies, we considered it necessary to confirm the activity of selected oximes by more direct method, which employs T-lymphocytes isolated from mouse spleen. Thus, molecule **123**, **146**, and **169** along with CaeA were assayed for their ability to suppress mitogen (Con A) induced proliferation of mouse T-lymphocytes.

The results for oximes **146** and **169** were similar to those obtained in Jurkat T-lymphocyte proliferation assay. Both oximes exhibited significantly higher activity as compared to CaeA (**1**). However, we were surprized to note that the results obtained in two assays for oxime **123** were very different. In Jurkat T-cell proliferation assay **123** was found to be more active than CaeA (**1**). However, in mitogen induced T-cell proliferation assay, **123** exhibited much inferior activity compared to CaeA. This particular finding raised doubt about the activity of all other oximes as well. Therefore, it was decided to perform mitogen induced mouse T-lymphocytes proliferation assay for all the oximes

IC_{50} values, concentration of oxime at which 50% inhibition was observed for each oximes was calculated by nonlinear regression method through inhibitor Vs normalised response using GraphPad Prism®. The results obtained in the assay were largely similar to those obtained in Jurkat T-cell proliferation assay except following: (i) Oxime **145** exhibited significantly higher activity (ii) oxime **123** was less active as already stated earlier and (iii) oxime **166** bearing 4-OCF_3phenyl group on oxime moiety was found to be inactive.

Final selection of oxime for animal studied: Oxime 146 selected earlier based on Jurkat T-cell proliferation assay method performed equally well in mitogen induced T-cell proliferation method exhibiting IC_{50} value of 0.090 compared to 0.27 for CaeA. In addition oxime **97** and **145** also exhibited activity similar to oxime **146**. The IC_{50} value for **97** and **145** were 0.091 and 0.078, respectively.

Oxime **97** possesses an ethyl group at oxime moiety, whereas **146** and **145** has methyl group at this position. The reasons for selecting methyl derivative in place of ethyl derivative have already been listed above. Oxime **146** and **145** are similar in structure except that two methyl groups at 4,4′-position of bipyridine in **146** have been replaced with methoxyl groups in **145**. The aryl methoxy compounds are usually metabolized by either demethylation to phenols or hydroxylation of ring followed by ring cleavage. The intermediated phenol can cause toxicity through generation of radicals. In comparison, aromatic methyl group are metabolized to hydroxymethyl group, followed by glucuronide conjugation. The conjugation step is expected to help in distribution as well as clearance of drug. Moreover it is well known that a single methyl group substition can significantly improve selectivity and potency of a molecule. In addition, methyl group is also known to provide metabolic stability and improved pharmacokinetics.

In the view of (i) the foregoing discussion (ii) the fact that the activity of oxime **146** and **145** is not much different from each other and (iii) **146** was best molecule in T-Jurkat cell based assay, we decided to retain **146** as molecule choice for animal studies. As already stated earlier, CaeA has an aldoxime structure compared to **145**, which is a ketoxime. Therefore, for comparison purpose **169**, (aldoxime equivalent of **145**) was also retained for animal studies. Although oxime **123** did not perform well in mitogen induced T-cell proliferation assay, we decided to keep this molecule. The reason being that it would provide a molecule of higher lipophilicity than **146** and **169** and may provide better understanding of results of *in vivo* studies. All the selected molecules followed Lipinski's rule of five.

So far, we have demonstrated that several of synthetic analogs of CaeA possessed property of suppression of T-lymphocytes (T cells) *in vitro* and based on (i) efficacy of suppression and (ii) physico-chemical properties, three best candidates, namely *N*-{([2,2'-bipyridin]-6-yl)[4-(trifluoromethoxy)phenyl]methylidene}hydroxylamine (**123**), *N*-[1-(4,4'-dimethyl[2,2'-bipyridin]-6-yl)ethylidene]hydroxylamine (**146**) and *N*-[(4,4'-

dimethyl[2,2'-bipyridin]-6-yl)methylidene]hydroxylamine (**169**) were selected for studies directed towards demonstration of activity *in vivo*. The ability of a molecule to prolong the survival of transplanted organ is an excellent gauge for immunosuppressive activity *in vivo*. Because it is well know that immune system of body recognizes the implant as foreign and mount a response to reject it. Accordingly, we have tested the selected molecules **123**, **145** and **169** for their ability to prolong the survival of skin allograft in mouse.

The selected oximes **123**, **146** and **169** or cyclosporine A was given by oral gavage or intra peritoneal (i.p.) injection daily prior to 5 days of grafting procedure to until the allograft rejection. Trunk skin (1 cm x 1 cm) of donor mice BALB/c ($H2^d$) was implanted onto lateral thorax of recipient mice C57/BL6 ($H2^b$), which is genetically distinct strain. The grafts were secured by bandages. The recipient mice were monitored daily. After 7 days, bandage was removed and allograft region monitored daily until the allograft rejection.

All three selected analogs of CaeA were able to significantly prolong the allograft in mouse model. Whereas placebo group rejected graft in 8-10 days, CaeA analogs treated animals retained the graft for 18-21 days, which compared to control is a very significant prolongation in life of the graft. All the tested analogs performed better than the standard drug cyclosporine. Oxime **169** proved best; it prolonged the life of skin graft by about 21 days, compared to about 18 days by cyclosporine.

T cells play an important role in the immune system. T cells play an important role in the immune system. T cells are of two kinds, T-helper cells (Th) and T cytotoxic (T_C) cells. Th cells differentiate to Th1 cells or Th2 cells. Increase in population of Th1 cells relates with the graft rejection, and on the contrary increase in population of Th2 cells relates with graft tolerance and IL-10 upregulation. Th1 cells secrete IL-2, TNF-β and IFN-γ and their levels in serum increase several fold leading to rejection of allograft.

We assayed IL-2, TNF, INF-γ and IL-10 circulating in the peripheral blood of the animals undergoing skin allograft experiment with oral dose of CaeA analogs or CsA. Compared to placebo group, animals that received CaeA analogs showed 3 to 4-fold decrease in serum INF-γ. Corresponding decrease in INF-γ level in CsA treated group was about 1.4-fold. Compared to placebo group, animals that received CaeA analogs or CsA showed 2.5 to 3-fold increase in serum IL-10. Compared to placebo group, animals that received

CaeA analogs or CsA showed significant decrease in serum TNF. IL-2 levels also decreased with CsA and analog **146**.

Decrease in serum concentration of INF-γ and TNF and to some extent IL-2 as well, and several fold increase in IL-10 concentration clearly indicates that CaeA analogs have been able to significantly decrease Th1/Th2 ratio, thereby tilting the immune response in favour of graft tolerance.

Conclusions

A library of molecules based on CaeA scaffold were synthesised and assayed for T cell anti-proliferation activity using (i) Jurkat T-lyphocytes and (ii) mitogen, concanavaline A (Con A) stimulated T-lymphocyte. Several synthetic analogs of CaeA possessed property of suppression of T-lymphocytes (T cells) *in vitro*. Based on (i) efficacy of suppression and (ii) physico-chemical properties, three best candidates **123**, **146** and **169** were selected for studies directed towards demonstration of activity *in vivo*. All three selected analogs of CaeA that were tested were able to significantly prolong the allograft in mouse model. Compared to placebo group, animals that received CaeA analogs showed 3 to 4-fold decrease in serum INF-γ and 2.5 to 3-fold incease in serum IL-10. A significam decrease in serum TNF level was also observed in treated groups. Decrease in serum concentration of INF-γ and TNF and to some extent IL-2 as well, and several fold increase in IL-10 concentration clearly indicates that CaeA analogs have been able to significantly decrease Th1/Th2 ratio, thereby tilting the immune response in favour of graft tolerance.

Overall, the results clearly demonstrate that CaeA analogs **123**, **146** and **169** are potent immunosuppressive agents *in vivo*. These significantly prolonged the life of allogenic skin graft in mouse model. Therefore, these molecules are ready for induction in to next phase of drug development program, i.e. pre-clinical and clinical studies.