

Indian Science Technology and Engineering facilities Map (I-STEM) Invites application for

1. Partial financial support for using R&D facilities in other institutions through the I-STEM web portal to Users from Academic Institutions and Start-ups (Format-1)

Format for requesting support for accessing and using facilities through the I-STEM web portal.

(i) Details of the Personal Information

- a. Name of the Researcher/Faculty Member:** MUNUGALA CHANDRAKANTH
- b. Name of the Institution/Start-up:** National Institute of Technology Calicut
- c. Name of the Department:** Department of Chemistry
- d. Degree registered for/Product being developed (for start-up):** Ph.D
- e. Mob. No.:** 9133423279
- f. Email ID:** chandrakanth_p210004cy@nitc.ac.in

(ii) Project/Research topic Details:

- a. Project Title/Research Title:** Human Dual Toll-like Receptor (TLR) 2/8 Agonists: Design, Synthesis, Characterization and Structure-Activity Relationship Studies
- b. Sub area:** Heterocyclic Chemistry and Immunology
- c. Area Description:** Organic Chemistry and Medicinal Chemistry
- d. Aims & Objective (in Bullets):**
 - Design a series of novel heterocyclic small molecules from the reported most potent human TLR2 and TLR8 agonists.
 - Synthesis and characterization of all the designed molecules using modern spectroscopic techniques such as FTIR, NMR and HRMS.
 - Development of SAR studies and identification of few most potent analogs from each series using hTLR2 and hTLR8-specific reporter gene assay.
 - Counter-screening of all the active molecules using hTLR-3/-4/-5/-7/-9 and NOD-1/-2- cellular assays to identify the specificity of the compound towards hTLT2/8.
 - To identify candidate compounds that induce high levels of cytokines and chemokines in *ex vivo* assay for future adjuvanticity study.
- e. Duration (MM/YYYY):** 04/2023 (6 months)

f. Project Summary:

Modern vaccines have often required the incorporation of an appropriate immune potentiator (also termed adjuvant) along with the antigen. Adjuvants initiate the early innate immune responses which lead to the generation of more robust and long-lasting adaptive immunity. Toll-like receptors (TLRs) play a key role in the innate immune system. They recognize highly conserved molecules derived from microbes such as bacteria, fungi, viruses, and parasites. Thus, drugs that target TLRs are of significant interest as vaccine adjuvants, inflammatory regulators and immunotherapeutics. Agonists targeting TLR4 and TLR9 have been approved for clinical use as vaccine adjuvants and agonists targeting TLR7 and TLR8 are used for treating infectious diseases and skin cancers. Different cellular localized TLRs trigger different signaling pathways. Therefore, combining two or more TLR-agonists (intracellular TLRs with extracellular TLRs) can act in synergy to promote more robust and long-lasting immunity, which is supported by several recent vaccination studies. Hence, design and development of novel multi-TLR agonists as vaccine adjuvants is an emerging area in the field of TLRs.

In view of the importance of TLR2 and TLR8 and multi-TLR agonists as vaccine adjuvants, we have designed several new chemotypes by combining a most potent small molecule TLR1/2-agonist *i.e.* thiourea-linked fused thiophene (**5**, Figure 1) with a pure-TLR8 agonist *i.e.* substituted benzimidazole (**8**, Figure 1) *via* a well-known drug design molecular hybridization approach. All the designed hybrids will be synthesized and characterized using spectroscopic techniques. All compounds will be examined for their TLR agonistic activity using hTLR2 and hTLR8-specific reporter gene assay (NF- κ B induction) followed by counter-screening using hTLR-3/-4/-5/-7/-9 and NOD-1/-2 cellular assays. The most active dual-TLR agonists will be taken further for cytokine and chemokine induction profiling with an *ex vivo* assay. Compounds that strongly stimulate cytokine and chemokine secretion will be selected as candidates for future adjuvanticity studies. SAR study will also provide structural information toward the formulation of these candidates for future *in vivo* studies. Overall, the compounds to be investigated in this proposal will create new classes of small molecule dual-TLR agonists that have not been studied so far.

g. Technical Details

a. TRL

The proposed project will be executed within six months. The component-wise work plan has been given below.

- 1st month: Required chemicals will be procured and initiated the synthesis of the intermediates.
- 2nd month: Synthesis of the intermediates and their characterization using spectroscopic techniques. Optimizing the reaction conditions for the formation of the target compounds
- 3th month: Preparation of the target compounds and structural elucidation using spectral studies.
- 4th month: Evaluation of TLR-agonistic activity including counter screening, and development of SAR.
- 5th month: Identify the compounds that induce high level of cytokines and chemokines.
- 6th month: Submission of the project report to I-STEM. Submission of the manuscript and filing a patent (if the activity is in the range of sub-micromolar).

b. Design & development

Immune activation initiated by pattern recognition receptors (PRRs) especially TLRs is essential to provide protection to the host from various infectious diseases.¹ Recent vaccination studies on multi-TLR agonists demonstrated their potential applications as vaccine adjuvants when compared with individual TLRs.²⁻⁴ In addition, different cellular localized TLRs trigger different signaling pathways. Therefore, combining an intracellular TLR with an extracellular TLR can act in synergy to promote more robust and long-lasting adoptive-immunity. Moreover, to acquire synergy to promote Th1-based immunity (Th2-based immunity has a theoretical risk of vaccine-associated enhanced respiratory diseases), it is essential to design novel multi-TLR agonists especially combining two or more different cellular TLRs. Hence, the design and development of novel multi-TLR agonists as vaccine adjuvants by combining two or more different TLRs, especially intra and extra-cellular TLRs is an emerging area in the field of vaccine adjuvants and TLRs.

Among the 10 human TLRs, TLR2 is specifically required for recognizing components derived from pathogens such as bacteria, fungi, and parasites.⁵ In spite of its functional importance, there is only limited studies have been done so far. Out of which, only a few small molecule hTLR2-agonists have been reported in the literature. Among them, 1,4-diaryl substituted imidazole (**2**, Figure 1) and thiourea-linked fused thiophene (**5**, Figure 1) are the most potent hTLR1/2-agonists.^{6,7} SAR studies on compound **4** (Figure 1) resulted in the

formation of a potent agonist **5**. During the SAR studies, it has been observed that the substitutions on urea-NH, ring-size variation of thiophene-fused ring as well as replacement of the ethyl ester with methyl/isopropyl ester tolerate the TLR1/2 agonistic activity. Whereas, compound **2** does not have many tolerable functional groups. Thus, agonist **5** has been selected for the design of a dual-TLR agonist. Similarly, TLR8 (intracellular TLR) has a unique role among all the TLRs and it can detect *ss*RNA of pathogens such as bacteria and viruses. Several small molecule TLR8 agonists have been reported in the literature. Among them, benzimidazole (**8**, Figure 1) is a bioisostere of nucleic acids such as guanine and adenine and can be synthesized in large quantities very easily. Thus, among the TLR8 agonists, compound **8** has been selected for the design of a dual-TLR agonist. A few examples of small molecule TLR1/2 and TLR8 agonists has given in **Figure 1**.

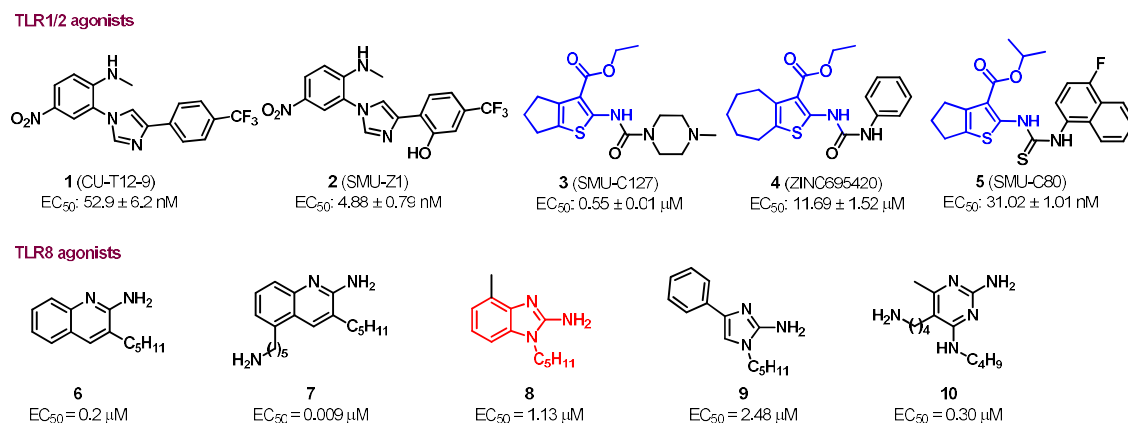


Figure 1. Representative examples of potent small molecule TLR1/2 and TLR 8 agonists

The hybrid molecules **11** and **12** (Figure 2) were designed by replacing 4-fluoronaphthyl group of hTLR1/2-agonist **5** with hTLR8 agonist **8**. One or two potent molecules from each series will be identified after performing a detailed SAR studies by varying the substitutions at all the possible positions, which suggest the tolerable functional groups for dual-TLR agonists. The most active compounds from each series will be counter-screened and evaluated in *ex vivo* and *in vivo* experiments. The best compounds identified from this proposal will serve as novel small molecule dual-TLR agonists which could be useful as potential vaccine adjuvants. The design strategy for the dual-TLR agonists has shown in **Figure 2**.

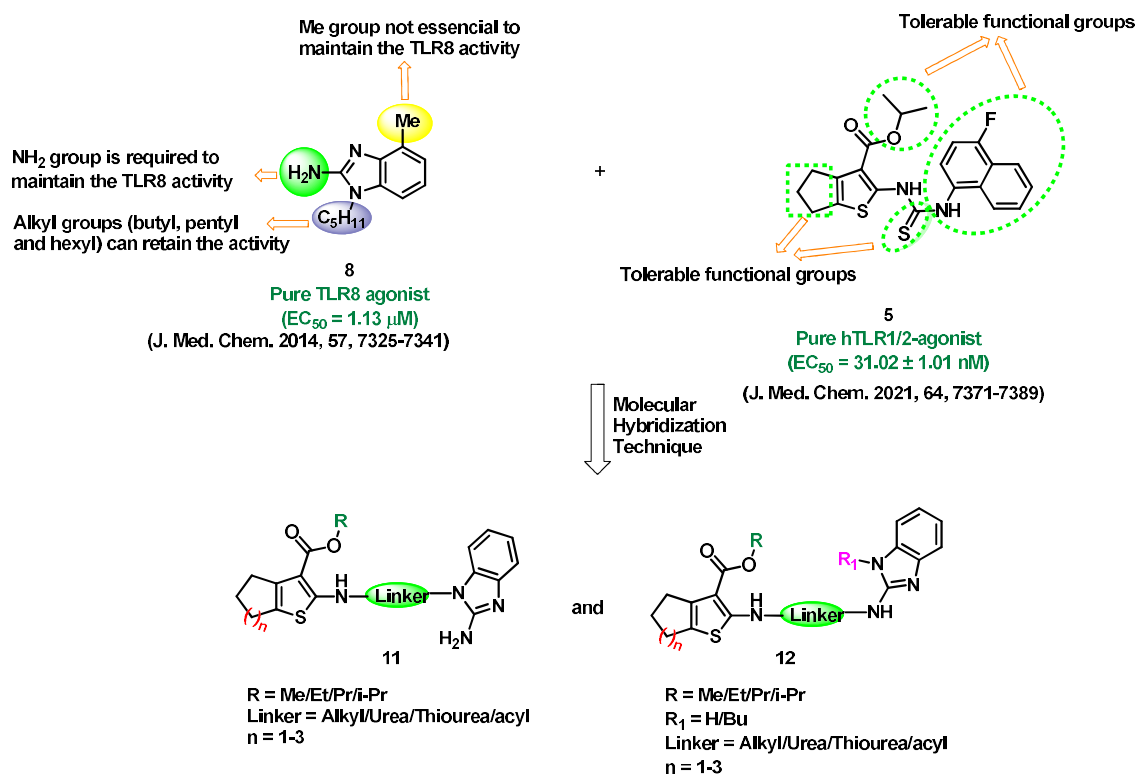
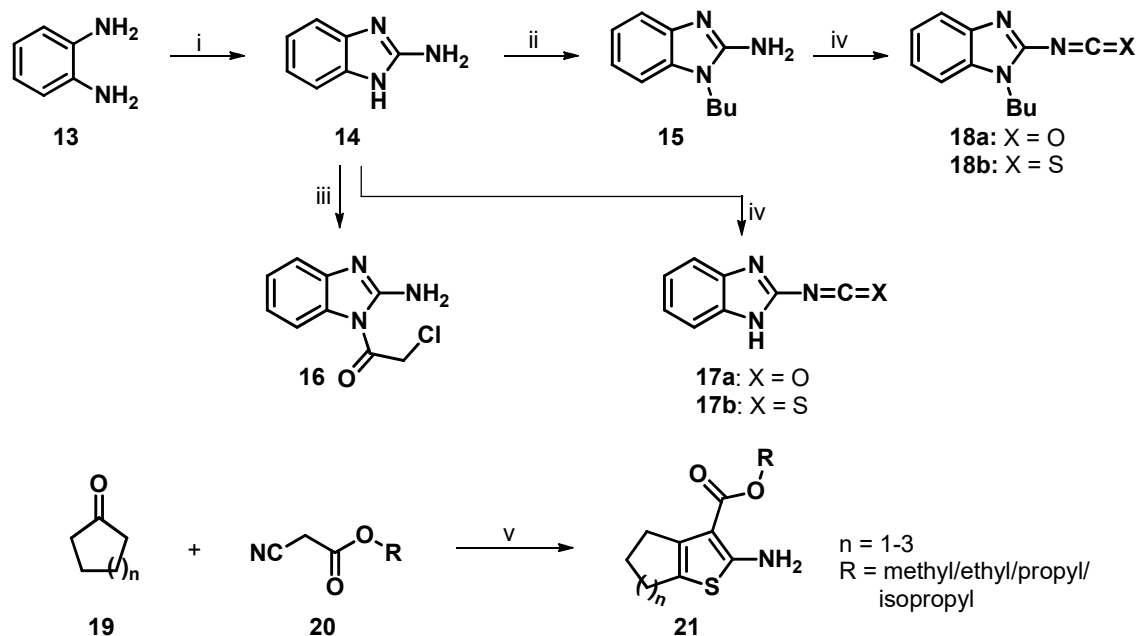


Figure 2. Designing dual-hTLR2/8 agonists from the reported potent hTLR1/2 and hTLR8 agonists.

c. Methodology

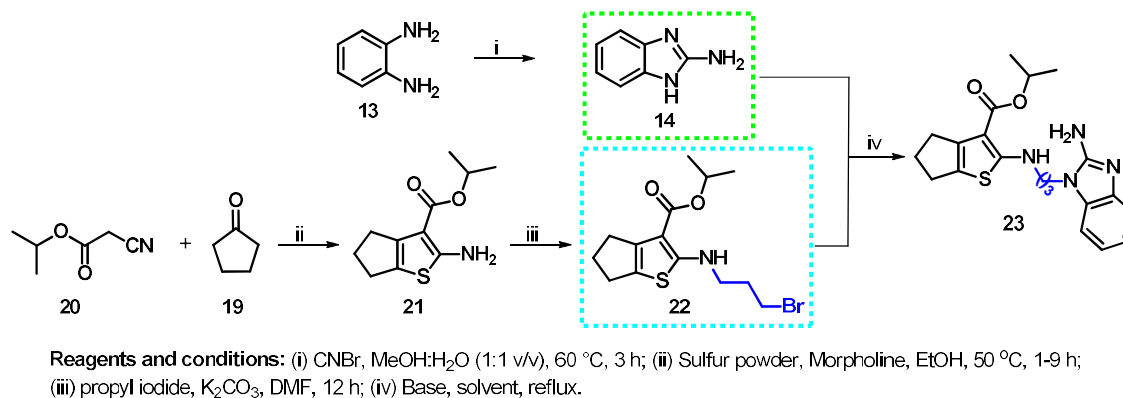
In view to identify the most potent molecules and to acquire the comprehensive knowledge on each of the designed chemotypes, it is essential to understand the effect of substitutions. Thus, the intermediates (**14-18** & **21**) having wide variety of substitutions that required to obtain the target hybrid molecules (**11** & **12**) can be prepared as shown in **Scheme 1** by following the literature procedures.⁸⁻¹¹ *o*-Phenylenediamine (**13**) on treatment with cyanogen bromide in a mixture of methanol and water results in the formation of 2-aminobenzimidazole (**14**). Compound **14** on *N*-alkylation and *N*-acylation leads to the generation of the corresponding compound **15** and **16**, respectively. Compounds **14** and **15** individually on further treatment with triphosgene and thiophosgene gives the corresponding isocyanate (**17a** & **18a**) and thioisocyanate (**17b** & **18b**). On the other hand, fused-thiophene derivatives (**21**) can be obtained *via* a three-component Gewald reaction involving cyclic ketone (**19**), alkyl cyanoacetate (**20**) and Sulphur powder in the presence of a base. Finally, the desired urea/thiourea/alkyl/acyl-linked hybrid molecules **11** and **12** can be prepared by refluxing the intermediate **21** with other intermediates (**14-18**) in a suitable solvent and a

reagent. For example, propyl-linked hybrid molecule (**23**) can be obtained by treating the *N*-alkyl derivative of the intermediate **21** with 2-aminobenzimidazole (**14**) in a suitable solvent and base under refluxing conditions as shown in **Scheme 2**.



Reagents and conditions: (i) CNBr, MeOH:H₂O (1/1, v/v), 60 °C, 3 h; (ii) *n*-BuI, KOH, Acetone, 60 °C, 3 h; (iii) Chloroacetyl chloride, THF, r.t., 5 h; (iv) Triphosgene, DMC, 90 °C (for **17a** & **18a**) / thiophosgene, Et₃N, DCM, 0 °C (for **17b** & **18b**); (v) Sulfur powder, Morpholine, EtOH, 50 °C, 1-9 h.

Scheme 1. Synthetic route for the preparation of the intermediates.



Scheme 2. Synthesis of a target hybrid molecule as an example.

Reference:

- Hussein, W. M.; Liu, T. Y.; Skwarczynski, M. et al. Toll-like receptor agonists: a patent review (2011 - 2013). *Expert. Opin. Ther. Pat.* **2014**, *24*, 453-470.

2. Troy, Q.; Soumaya, B.; Sefik, A. et al. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J. Exp. Med.* **2006**, *203*, 413-424.
3. Travis, B. K.; Donald, L. M.; Delphine, J. L. et al. Combined intralesional Bacille Calmette-Guérin (BCG) and topical imiquimod for in-transit melanoma. *J. Immunother.* **2012**, *35*, 716-720.
4. Sudhir, P. K.; Ioanna, S.; Randy, A. A. et al. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **2011**, *470*, 543-547.
5. Luchner, M.; Reinke, S.; Milicic, A. TLR Agonists as Vaccine Adjuvants Targeting Cancer and Infectious Diseases. *Pharmaceutics* **2021**, *13*, 142.
6. Xiaohong, C.; Gengzhen, Z.; Junjie, Y. et al. TLR1/2 specific small-molecule agonist suppresses leukemia cancer cell growth by stimulating cytotoxic T lymphocytes. *Adv. Sci.* **2019**, *6*, 1802042.
7. Zhipeng, C.; Lina, Z.; Junjie, Y. et al. Design, synthesis, and structure–activity relationship of *N*-aryl-*N'*-(thiophen-2-yl)thiourea derivatives as novel and specific human TLR1/2 agonists for potential cancer immunotherapy. *J. Med. Chem.* **2021**, *64*, 7371-7389.
8. (a) Kokatla, H. P.; Sil, D.; Tanji, H. et al. Structure-based design of novel human toll-like receptor 8 agonists. *ChemMedChem* **2014**, *9*, 719-723. (b) Beesu, M.; Caruso, G.; Salyer, A. C. et al. Structure-based design of human TLR8-specific agonists with augmented potency and adjuvanticity. *J. Med. Chem.* **2015**, *58*, 7833-7849. (c) Beesu, M.; Malladi, S. S.; Fox, L. M. et al. Human toll-like receptor 8-selective agonistic activities in 1-alkyl-1*H*-benzimidazol-2-amines. *J. Med. Chem.* **2014**, *57*, 7325-7341. (d) Beesu, M.; Salyer, A. C.; Trautman, K. L. et al. Human toll-like receptor (TLR) 8-specific agonistic activity in substituted pyrimidine-2,4-diamines. *J. Med. Chem.* **2016**, *59*, 8082-8093. (e) Beesu, M.; Caruso, G.; Salyer, A. C. et al. Identification of a human toll-like receptor (TLR) 8-specific agonist and a functional pan-TLR inhibitor in 2-aminoimidazoles. *J. Med. Chem.* **2016**, *59*, 3311-3330.
9. Yoo, E.; Salunke, D. B.; Sil, D. et al. Determinants of activity at human toll-like receptors 7 and 8: Quantitative structure–activity relationship (QSAR) of diverse heterocyclic scaffolds. *J. Med. Chem.* **2014**, *57*, 7955-7970.
10. Zhipeng, C.; Lina, Z.; Junjie, Y. et al. Design, synthesis, and structure–activity relationship of *N*-aryl-*N'*-(thiophen-2-yl)thiourea derivatives as novel and specific

human TLR1/2 agonists for potential cancer immunotherapy. *J. Med. Chem.* **2021**, *64*, 7371-7389.

11. Nikunj, M. S.; Cole, A. M.; Rehman, U. et al. Syntheses of fluorescent imidazoquinoline conjugates as probes of Toll-like receptor 7. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 6384–6386.

d. Facilities and Instrumentation required

- Liquid Chromatography-High Resolution Mass Spectrometry to check the purity of the compounds.
- Single crystal XRD for the structural elucidation of the compounds.

Sl. No	Characterization	Institute with the facility	Charge per sample analysis (Rs.)	No. of samples	Total amount (Rs.)
1	HRMS	IIT Madras	1200/-	50	60,000/-
2	Single Crystal XRD	STIC, CUSAT	3826/-	3	11,478/-
Total					71,478/-

e. Consumables (Scientific materials): Not required

h. Expected output and outcome

- (i) **Industry/Institute Collaboration:** Yes
- (ii) **Name of the Collaborating Industry/Institute:** CSIR- Central Drug Research Institute, Lucknow
- (iii) **Collaboration Details**
For toxicity studies
 Dr. Mukesh Pasupuleti
 Principal Scientist (Sc EII) & Associate Professor (AcSIR)
 Division of Microbiology
 CSIR- Central Drug Research Institute, Lucknow 226031, India
 Mobile 9721464595
 Office number: 0522-2772450
- (iv) **Additional Details if any**

i. Funding required from I-STEM (with justification:

Details of Requirements & Mode of the Engagement: (Attach the quotation for the consumables)

(i) Short term Engagement:

a. Usage of testing/characterization facilities across the country (Characterization Mode)

- i. **Testing Charges:** 71,478/-
- ii. **Consumable charges:** Nil
- iii. **Travel Cost (Boarding and Lodging, if any):** 20,000/-

b. Usage of fabrication and testing facilities across the country (Fabrication Mode): **Not applicable**

- i. Fabrication and testing Charges
- ii. Consumable charges (if any)
- iii. Travel Cost (Boarding and Lodging, if any)

(ii) Medium term Engagement: **Not Applicable**

a. Execution of project in any one of the Host Institution (Project Mode)

- (i) Fabrication and or testing Charges
- (ii) Consumable charges (if any)
- (iii) Travel Cost (Boarding and Lodging, if any)

b. Execution of project in Multi Host Institutions (Project Mode)

- a. Fabrication and or testing Charges
- b. Consumable charges (if any)
- c. Travel Cost (Boarding and Lodging, if any)

(Round-trip to the Host Institution by 2nd class (Non-AC) railway via the nearest route)

Details of User's Bank Account:

(Please send a copy of the cancelled cheque/copy of the passbook carrying all the information needed)

Beneficiary Name (User's name) : Munugala Chandrakanth

PAN: KRAPK0997M

Bank Account No.: 62409474513

Name of the Branch: IITH Kandi

IFSC Code: SBIN0014182

Note: Proposed proposal must be executed within stipulated duration (max. 6 month) to avoid fund availability issues. Travel form will be provided by the I-STEM office. The users will submit the technical report, original invoice (duly signed by the Head of the Institution)

raised by the Host Institution through I-STEM portal only along with a copy of the travel forms and original tickets through post to the Nodal Officer, I-STEM, Indian Institute of Science, Bangalore. A scan copy of all the documents may be sent in advance.

Generally used abbreviations			
a/c = Account	dep = Deposit	Pr = Principal	
Adj = Adjustm	Draft	Proc = Processing Charge	
Amt = Amount	Dishonour	rd = Recurring Deposit	
Ar = Arrear	Debit	Ret/Rtn = Return	
bal = Balance	Date of Birth	Rnd = Round of	
Capn = Capital	Electronic Fund Transfer	SB = Savings Bank	
chg/ch = Charge	Inoperative	SC = Short Credit	
Chq = Cheque	Insurance	SI/So/SORD = Standing Instruction	
Clos = Closure	Interest	S/D/W/H/o = Son/Daughter/Wife/Husband of	
Coll = Collecti	Loan	tr/trf/xfer = Transfer	
Comm = Commis	Minimum	TT = Telegraphic Transfer	
CDR/CORR = Correction	Outstanding	txn = Transaction	
CR = Credit	P & T = Postage & Telegram	Wdl = Withdrawal	
csh = Cash	Pos = Point of Sale	+MOD Bal = total balance(SB+linked MOD a/c)	

स्टेट बैंक ऑफ हैदराबाद

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State Bank of Hyderabad

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S/D/W/H/o: M SAMPATH KUMAR

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SBIN0014182 Branch Manager

M. Chandrakanth

Signature of the applicant

Tanesh

Signature of the Guide

Dr. Janardhan Banothu

Assistant Professor