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Signed Details of the Research Work

I. Probing Endosomal Toll-like Receptors (TLRs)

Introduction to endosomal TLRs: The human innate immune system is the first line of defense against the invasion of pathogenic microorganisms. Toll-like receptors (TLRs), mostly expressed on antigen-presenting cells such as dendritic cells, are germline-encoded pattern recognition molecules that play key roles in innate immunity by regulating inflammation. Among the family of TLRs, TLR3, TLR7, TLR8, and TLR9 have been identified in humans that are located inside the endosomal compartments (pH = 4.5−6.5) of the immune cells and they recognizes nucleic acids of both pathogenic origin and self-origin. In humans, TLR7 and TLR9 are expressed selectively in plasmacytoid dendritic cells (pDCs) and B lymphocytes. Upon activation, they drive type I interferon (IFN) production from pDCs. TLR activation is one of the initial defensive mechanisms utilized by the host to accumulate innate and subsequent adaptive immunity to fight invading pathogens. However, when self-origin RNA or DNA molecules get access to endosomes and aberrantly activate endosomal TLR7/8/9, it initiates autoreactive inflammation in different autoimmune diseases such as systemic lupus erythematosus (SLE), psoriasis, Sjögren's syndrome and systemic sclerosis. Hence, inhibition of TLR7/9 would play an important role in these clinical contexts. Both TLR7 and TLR9 are structurally similar to each other and initiate common downstream signaling upon activation. This results in the recruitment of the transcription factors NFKB and IRF7, leading to the induction of inflammatory cytokines as well as type I IFNs.

Development of Antagonists. Exploration of potent and selective small-molecule TLR7 and TLR9 antagonists as therapeutic agents in several clinical contexts is of great interest, although many candidates are at different stages of clinical trial but as of now none of them are available for clinical use. Previous reported efforts in this context were mainly random in the *absence of human crystal structure of TLR7 and TLR9*. Among them many candidates are oligonucleotides. Small molecule candidates are always favorable for well-known reason as oligonucleotides suffers from stability issue, poor pharmacokinetics and dual agonism-antagonism profile.

The goal of my lab is to rationally design selective antagonists for the nucleic acid-recognizing TLRs (TLR7 and TLR9) for devising novel therapeutic strategies in relevant clinical contexts. We have an exhaustive pipeline consisting of molecules from Purine, Quinazoline, Benzoxazole and Imidazopyridines. This platform was established through applying various rational design strategies described below. Till now the work resulted in two patents (WO/2019/092739; US 10662177-B2 Granted) and many publications in reputed journals.

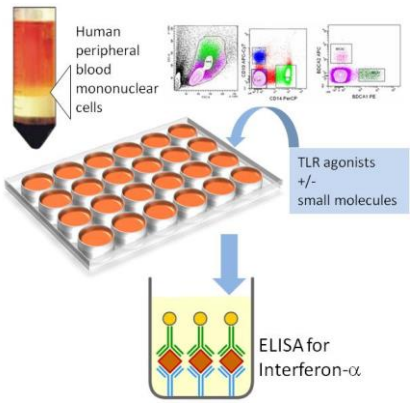
The TLR7 and TLR9 antagonists were designed mainly through

A) Conceptual development agonist-to-antagonists
B) Activity Based Design
C) From known antagonistic scaffold
D) Incorporating essential features into privilege scaffold.

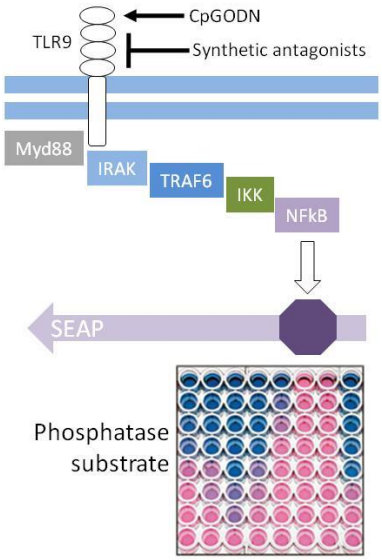
BRIEF DESCRIPTIONS OF VARIOUS ASSAY PLATFORMS FOR VALIDATION

In order to find agonistic or antagonistic activity of synthesized molecules, several assay platforms were developed at IICB, Kolkata for screening and validation purposes in TLR4 (for cross selectivity), TLR7, TLR8 and TLR9.

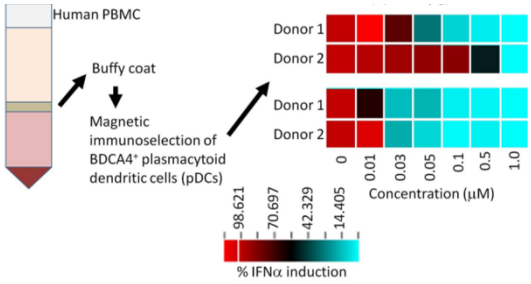
Primary Immune Cell Assay



Reporter Assay



Isolated Human pDCs Assay



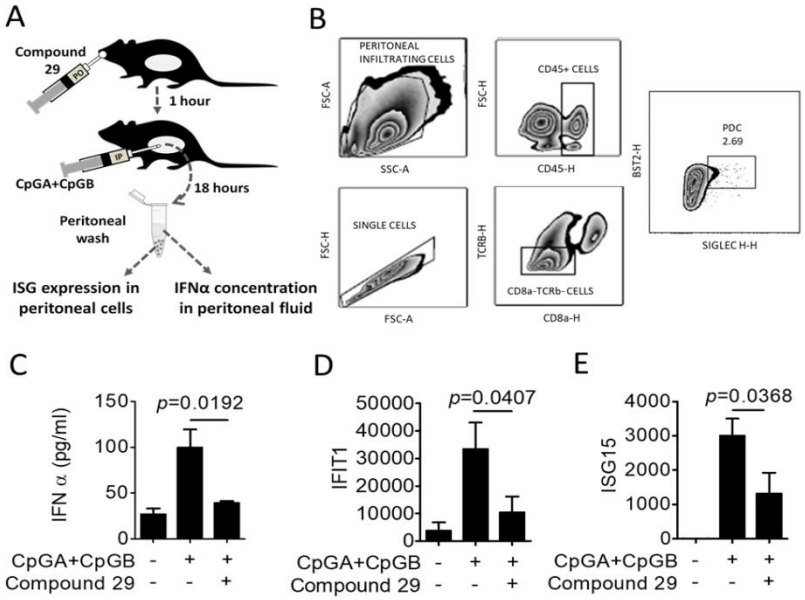
The synthesized compounds were subjected to three-stage **in-vitro screening** assay.

a) **Medium-throughput Antagonism Assay in Primary hPBMCs.** Primary human PBMCs were isolated from blood taken from healthy individuals. This assay was used for initial screening.

b) **Antagonism in Reporter Cell Line**: The molecules were screened for IC_{50} determination using HEK-Blue™-HTLR7/9/8/4 cells, a HEK293 reporter cell line expressing human TLR7/9/8/4 and a secreted alkaline phosphatase (SEAP).

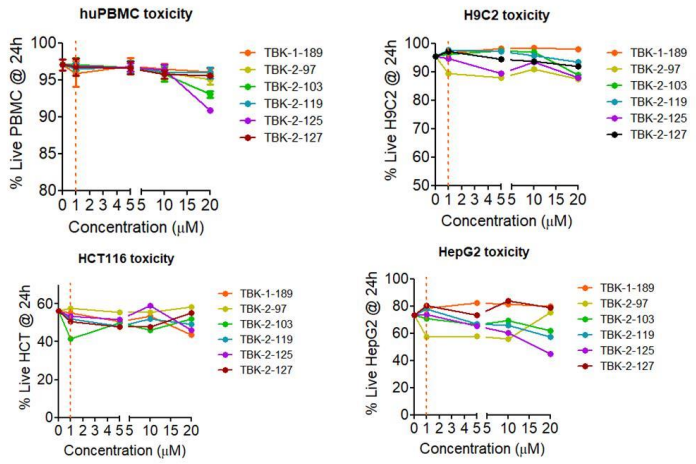
c) **TLR7/9 Antagonism Assay in isolated Human Plasmacytoid Dendritic Cells (pDCs)**: Human plasmacytoid dendritic cells (pDCs) are enriched in TLR7/9. Primary assay was performed using human pDCs to study the TLR7/9 antagonistic activity for validation.

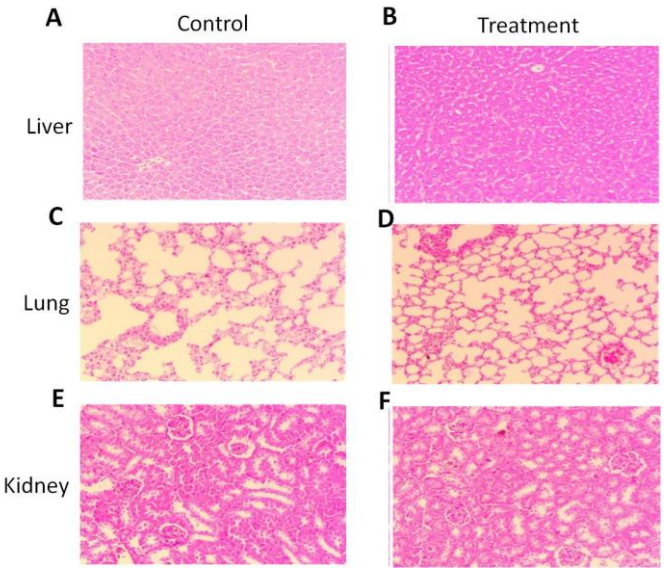
In-vivo Pharmacodynamic Model. The model was developed in-house at IICB, Kolkata to check the in-vivo efficacy. Expressions of interferon signature genes (ISGs) in tissues are reliable biomarkers of IFN signaling and are associated with disease activity in clinical contexts. To estimate the in-vivo efficacy of a representative TLR9 antagonists, a clinically relevant murine model was developed where mice were administered a mixture of CpGA and CpGB, the bonafide TLR9 agonist, to the peritoneal cavity of mice, which caused immune cell (including pDC) infiltration into the peritoneum. This phenomenon causes the induction of interferon stimulated genes (ISGs) and IFNα which were analyzed by IFNα ELISA kit and mRNA expression was evaluated by quantitative PCR and flow cytometry.



(A) Oral administration of lead compound at a dose of 10mg/kg body weight of mice was done in the target group of mice 1 hour before i.p. injection of a mixture of CpGA and CpGB. After 18 hours, peritoneal wash was collected. IFNα ELISA was done using the peritoneal fluid, while expression of target genes was monitored in the cellular component. (B) Flow cytometric analysis was done to determine the infiltration of immune cells, especially pDCs into mouse peritoneum. (C) Concentration of IFNα in mice peritoneal fluid w/o administration of compound 1 was measured by IFNα ELISA (2 independent experiments). (D, E) mRNA expression levels of IFIT1(D) and ISG15 (E) in the peritoneal infiltrating cells of mice w/o exposure to compound 29 was quantified by Real-time PCR .

In-vitro and in-vivo toxicity assessment. The in-vitro toxicity assessment was done several cell lines of different tissue origin. Human peripheral blood mononuclear cells (hematopoietic origin), HCT116 (a colon cancer cell line representing gut epithelium of endodermal origin), HepG2 (a hepatocellular carcinoma cell line representing hepatocytes of mesodermal origin), H9C2 (a rat cardiomyoblast cell line representing mesodermal origin) by propidium iodide staining.



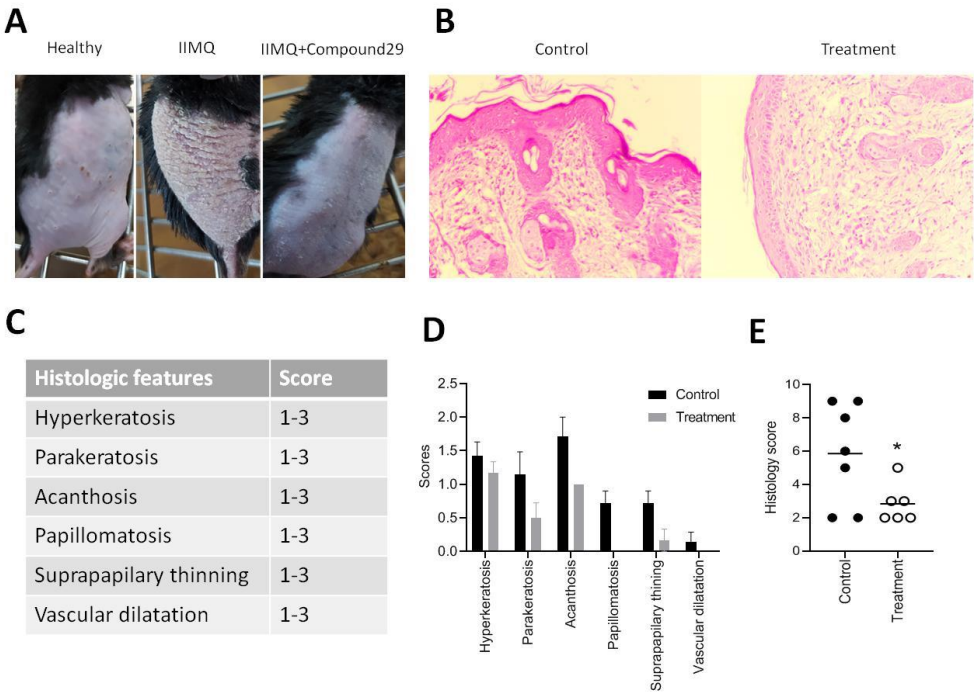


Short-term visceral toxicity assessment. Short-term visceral toxicity assessment was also done for lung, kidney and liver cells from sacrificed mice after administration of the lead candidate for 7 days

In-vitro and in-vivo Pharmacokinetics. The synthesized molecules were subjected to thorough scrutiny of *in-vitro* pharmacokinetics for assessment of Plasma stability in mice and human, Caco-2 permeability and liver microsomal stability for both mice and human etc. Mice were administered with the desired doses (mg/kg) of synthesized compounds orally and intravenously to checked the parameters (C_{max}, t_{max}, $t_{1/2}$, AUC, intrinsic clearance; CL_{int}, Volume of distribution; V_{ss}) and calculation was done for % of oral bio-availability.

Dose (mg/kg)	Route of administration[a]	C_{max}(ng /mL)	t_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-}24h}$ (ng h/mL)	CL (mL/min kg)	Vss(L/kg)	F (%)
15 (n=3)	iv	-	-	0.98	50.86	3.18	4.2	70.8
15 (n=3)	po	2.6	2.0	4.6	36.06	-	-	

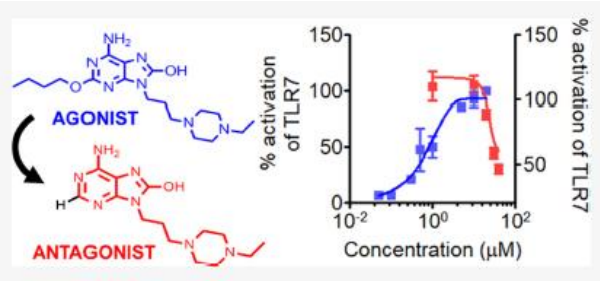
Preclinical Murine Psoriasis Model. The activity of synthesized compounds finally evaluated in pre-clinical murine psoriasis model developed in-house at IICB, Kolkata. This model bears a strong resemblance to human psoriasis and has been used in multiple studies to explore the pathogenesis of the disease. The model displayed all morphological features associated with psoriasiform inflammation. Histopathologic exploration of psoriatic skin sections from control and treated mice were evaluated and a scoring system of each pathogenic feature such as hyperkeratosis, parakeratosis, acanthosis, papillomatosis, suprapapilary thinning, and vascular dilatation was given to visualize the stark differences between psoriatic and treated mice skin.



Efficacy of TLR7 Antagonists in preventing Psoriasis like inflammation in rodent- structure. The ability of our lead compound to significantly reduce disease severity of a TLR7/9 driven autoimmune disease in vivo, could be established.

STRATEGIES:

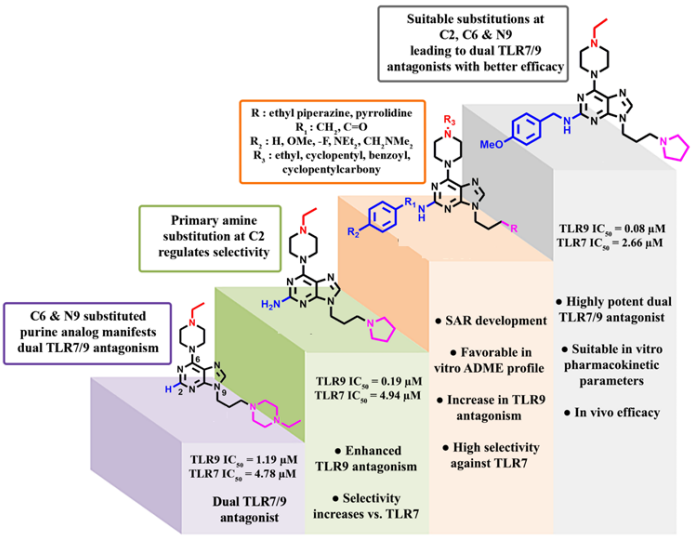
A) **Agonist To Antagonist in Purine Scaffold.** Our approach was to design TLR7/9 antagonist from TLR7 agonist. We hypothizes that both agonist and antagonist of TLR7/9 might bind at the similar site thus, might share similar structural feature for receptor affinity. **We've mapped the path for transforming agonist to antagonist through a single-point change.** We've established that a small structural modification 'Chemical Switch' in TLR7 ligand that can lead to reversal in their functional activity. The removal of the butoxy group at C2 position of the TLR7 purine agonist, transform the resulted compound into TLR7 antagonist. **This is a seminal contribution in the field of medicinal chemistry in general.** Our strategy was included in "Into the Fray! A Beginner's Guide to Medicinal Chemistry' (https://doi.org/10.1002/cmdc.202000929).



A Chemical Switch for Transforming a Purine Agonist for Toll-like Receptor 7 to a Clinically Relevant Antagonist. Mukherjee A, Raychaudhuri D, Sinha BP, Kundu B, Mitra M, Paul B, Bandopadhyay P, Ganguly D,* and **Talukdar A** *. **Journal of medicinal Chemistry**, **2020**, 63, 4776.

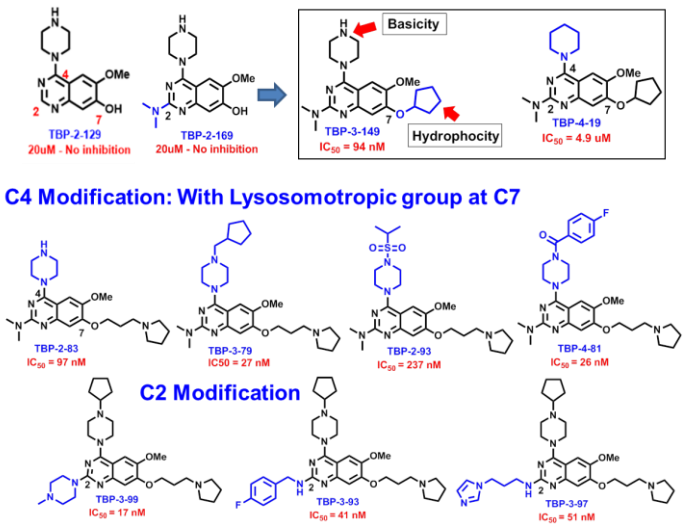
Subsequent study was more of understanding the chemical spaces around our previously designed TLR7/9 purine antagonists through extensive structure-activity relationship (SAR) and lead validation through

systematically optimizing via in-vitro pharmacokinetic, in-vivo pharmacokinetics, in-vitro and in-vivo toxicity assessment, validation in in-house developed pharmacodynamic mouse model and finally establishing the efficacy of the lead candidate in a preclinical psoriasis mouse model of the autoimmune disease.



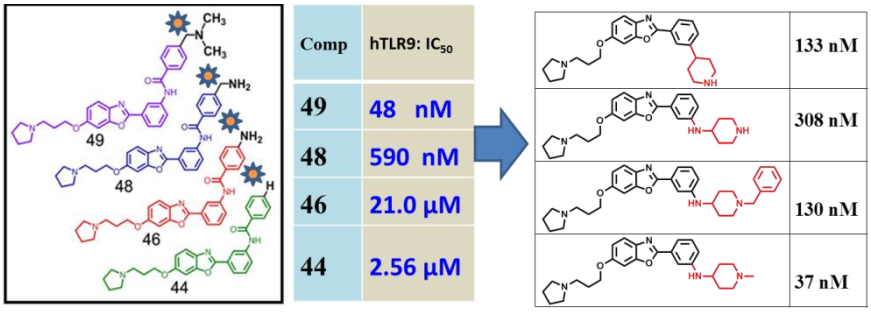
Systematic Optimization of Potent and Orally Bioavailable Purine Scaffold as a Dual Inhibitor of Toll-Like Receptors 7 and 9. Kundu B, Raychaudhuri D, Mukherjee A, Sinha BP, Sarkar D, Bandopadhyay P, Pal S, Das N, Dey D, Ramarao K, Nagireddy K, Ganguly D,* and **Talukdar A***. **Journal of Medicinal Chemistry**, **2021,** *64*, 9279.

B) **Activity Guided Rational Design: Quinazoline Scaffold.** Through an activity-guided approach (based on hTLR9/7-inhibition in primary human immune cells and hTLR9/7 reporter cells), we have identified chemical features in quinazoline core that are not only essential for selective hTLR9 inhibition as well as dual TLR7 and TLR9 inhibition. **Talukdar A*. European Journal of Medicinal Chemistry**, **2018**, *159*, 187.

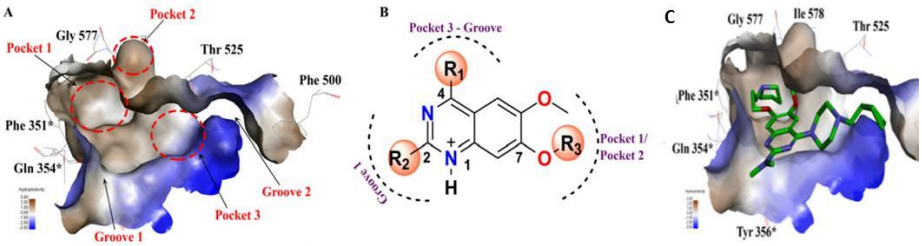


We found that the substitution patterns at C-2, C-4 and C-7 of quinazoline ring act in concerted manner to influence the antagonism. At C-7 position hydrophobicity is important, whereas at C-4 piperazine nitrogen basicity is important. The C-2 position can accommodate different substitutions, which allows late stage modifications to counter any issues during validation. We have also optimized the lead candidate with favorable ADME properties along with favorable oral bioavailability, urinary excretion kinetics and in vivo TLR9 antagonism efficacy for one of the representative lead compound in a clinically relevant rodent model of aberrant TLR9 activation.

C) **Chemical Optimization of existing antagonists: Benzoxazole as model.** The initial study was based on structural exploration of the hTLR9/hTLR7 antagonism from known E6446 hTLR9/hTLR7 antagonist having 2-phenylbenzoxazole core that can strongly inhibit TLR9/TLR7 signaling. From drug designing perspective our antagonists had lesser molecular flexibility. We provided a correlation between our binding mode hypothesis and hTLR9 antagonistic activity for future rational development. **Talukdar A*. European Journal of Medicinal Chemistry**, **2017**, *134*, 334-347.



Universal Binding Model. Due to the unusual topology of the ligand binding surface of TLR9 and TLR7 lacking conventional pockets, the functional mechanism of potential TLR9 antagonism by small molecules is not understood, consequently small molecule TLR9 antagonists have so far been developed by empirical screening. Our lab has proposed hypothetical binding model to design TLR9 and TLR7 antagonists. The proposed ligand-receptor interaction could be which correlate with TLR7 antagonistic activity thus paving the way for rational design using varied chemotypes. **Later our hypothetical model was validated through X-ray co-crystal structure of TLR7 published in Nature Comm. by Tojo et. al. doi: https://doi.org/10.1038/s41467-020-19025-z.**



Talukdar A*. European Journal of Medicinal Chemistry, 2021, 210, 112978.

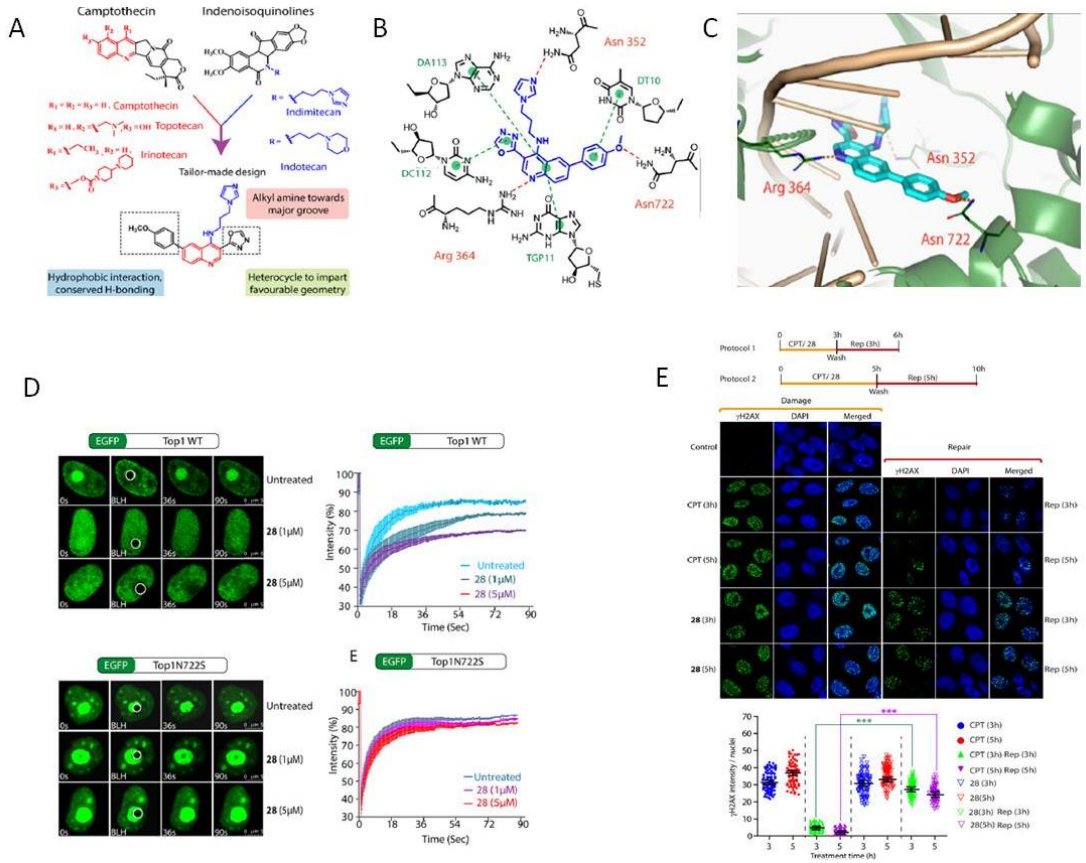
Perspective. Recently, we have published a Perspective in J. Med. Chemistry highlighting rational medicinal chemistry approaches to elucidate the structural attributes of small molecules capable of agonism or antagonism or of elegantly switching between the two. The structural evolution of different chemotypes can provide the framework for the future development of endosomal TLR agonists and antagonists.

Structural Evolution and Translational Potential for Agonists and Antagonists of Endosomal Toll-like Receptors. **Talukdar A**,* Ganguly D, Roy S, Das N, Sarkar D. **Journal of Medicinal Chemistry**, **2021,** 64, 12, 8010.

II. Design and Development of *non-camptothecin'* topoisomerase I (Top1) inhibitors for cancer chemotherapy

In various tumor cells, Top1 is over-expressed than the normal cells; hence, modulating the Top1 activity in tumor cells to block DNA replication and cell division has made Top1 an attractive drug target for anticancer therapy. Anticancer drugs like camptothecin (CPT) and its FDA approved derivatives (Topotecan and Irinotecan) selectively trap Top1-DNA cleavable complexes, which can account for the killing of dividing malignant cells by 'Top1 poisons'. Topotecan and irinotecan are used for advanced colorectal carcinomas and ovarian cancers, which emphasizes the significance of Top1 as a drug target. However, CPTs are not ideal drug molecules due to their toxicity, inherently unstable chemical structure which rapidly inactivated in plasma due to hydrolysis of lactone E-ring and suffer from rapid cellular efflux via membrane pumps (Pgp). As a result there is great interest in the development of '*non-camptothecin*' Top1 inhibitors as anticancer agents.

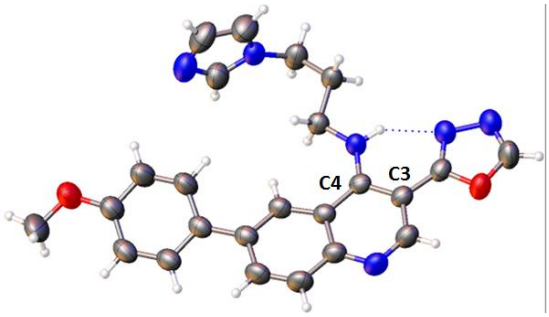
Hypothesis driven Conceptual Design. *Here we have successfully developed and patented (WO2019229765) a new class of potent and selective Top1 poison based on the quinoline core with improved physicochemical properties as well as potency than camptothecin.*



(A) Design of tailor-made analogue (B) 2D binding interactions of lead compound with the important amino acid residues and base pairs Red dotted lines indicate hydrogen bond and green dotted lines indicate π−π stacking interaction (C) hypothetical binding model of the ternary complex of human Top1−DNA−our Top1 inhibitor (generated from PDB ID: 1T8I) (D) Lead Top1 inhibitor-mediated stabilization of the Top1−DNA cleavage complex in live cells (E) Lead

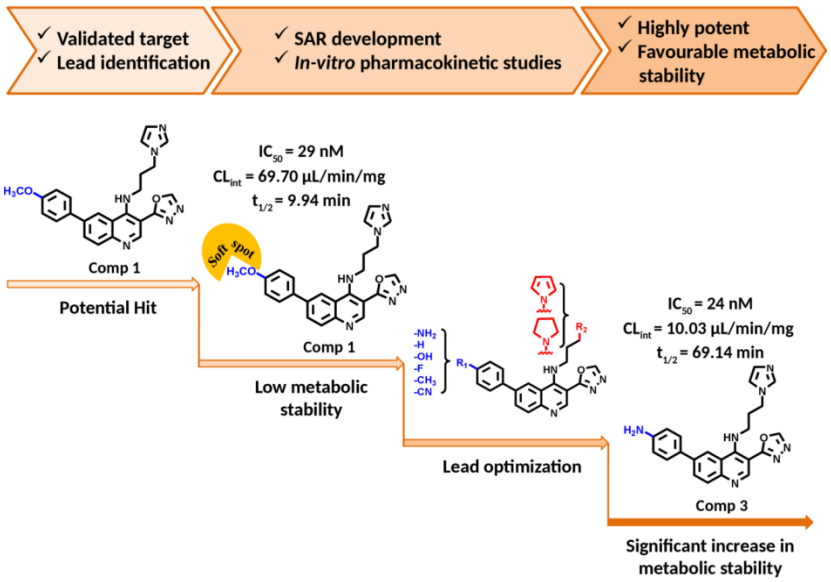
Top1 inhibitor generates persistent and less reversible DNA DSBs as detected by γH2AX staining.

The design was initiated based on the structural features of known ligands/poison that bind through the network of interactions in the active site of human Top1 enzyme. Our selective lead Top1 poison is



bicyclic in nature unlike polycyclic camptothecins or indenoisoquinolines. Strategically placed C4 nitrogen atom along with a heterocycle at the C-3 position would form an intramolecular hydrogen bonding (proved through crystal study), which impart requisite polycyclic geometry and suitable curvature essential for stabilizing the Top1−DNA cleavage complex. *Our Top1 poison A) does not intercalate with DNA nor react with Top1 enzyme but have the ability to stabilize covalent Top1-DNA intermediate to form ternary complex. B) We have also provided mechanistic insight of Top1 inhibition through live cancer cell imaging and through mutation study. C) Unlike CPTs, they can stabilize Top1−DNA cleavage complexes even after 5 h proved by gamma-H2AX assay. D) In-vitro ADME study revealed that unlike CPT and its derivatives, our lead Top1 inhibitors are highly plasma stable with ideal log D value for oral absorption and is not a Pgp substrate*. **Talukdar A*. Journal of Medicinal Chemistry, 2019**, *62*, 3428.

Lead Optimization. The developed Top1 poison with quinoline core was further optimized for greater metabolic stability and efficacy. The present study puts forth strategic modification at the metabolically

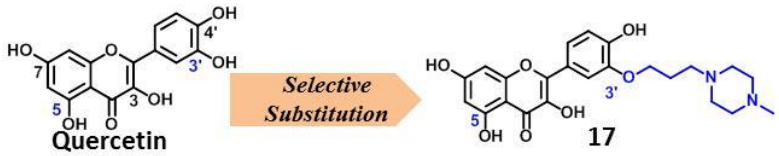


sensitive spot with different groups leading to compound. The metabolite profiling was done and based on the metabolite analysis further development was conducted. Lead compound with -NH_2 substitution, which showed marked increase in the metabolic stability with retention of Top1 inhibitory activity. For lead optimization various in-vitro pharmacokinetic assays were performed such as solubility, Caco2 permiability, metabolic stability, plasma stability, identification of metabolities etc. **Talukdar A*. European Journal of Medicinal Chemistry**, **2020**, *202*, 112551.

In order to envisage structurally diverse novel chemical entity as Top1 poison with better efficacy, Ligand-based-pharmacophore model was developed using 3D QSAR pharmacophore generation (HypoGen algorithm) methodology to identify hit molecules which are now being modified in my lab for designing future class of potential topoisomerase I inhibitor.

Talukdar A*. Computational and Structural Biotechnology Journal, 2019, *17*, 291.

Natural product Based drug design through modulating pharmacokinetics. Quercetin possesses diverse pharmacological properties but its application in the pharmaceutical field is limited due to its poor bioavailability resulting from poor water solubility and poor permeability. We devised a systematic chemical modification through a selective synthetic methodology, which enables the installation of different substitutions at C-3′ and C-5 positions of quercetin. Regioselective substitution of specific hydroxyl group quercetin to modulate its log D value and aqueous solubility through the attachment facilitator moieties led to ~100-fold increase in the cytotoxic activity of our semisynthetic derivatives in colon cancer cells as compared to quercetin. We have also validated the lead derivative in in-vivo CT-26 tumor-bearing mice in a colon cancer model. We believe that the study has an immense potential toward the systemic development of natural products which has poor bioavailability. **Talukdar A*. ACS Omega, 2019,** *4*, 7285.



Compd	Solubility pH=7.4 (µg/mL)	$logD_{7.4}$	IC_{50} HCT116 (µM)	In Vivo Efficacy (CT 26) (Dose: 50 mg/kg)	
				Tumor reduction	Survival (30 days)
Quercetin	0.1	0.76	45.3	15%	0%
17	**41.5**	**2.53**	**0.48**	**60%**	**67%**

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