Top 10 best papers of the applicant highlighting the important discoveries/contributions described in them briefly

Benu Brata Das laboratory Key contributions in Basic Medical Research:

Prof. Das has identified a novel role of two independent genes PRMT5 and PARP1, which are critical therapeutic target for the repair of DNA breaks linked with Top1-DNA trapped complexes. Das group pioneers the involvement of PRMT5 in the repair of trapped Top1-associated DNA and act as resistance determinants in cancer chemotherapy.

Protein arginine methyltransferase (PRMT5) has emerged as a major symmetric dimethylating enzyme involved in regulating several biological pathways. Impending evidence has indicated a critical role of PRMT5 in tumorigenesis including leukemia, lymphoma, and many solid tumors, making PRMT5 an attractive anticancer target. Prof Das's studies provide the first evidence that PRMT5 is a molecular determinant for Top1cc repair. These studies revealed PRMT5 as a promising druggable candidate whose knockdown creates synergistic vulnerability in cultured cancer cells treated with camptothecin (CPT).

Arginine methylation is an established key epigenetic mark regulating gene expression and cell proliferation, its emerging role in coordinating the DNA damage response pathways (DDR). Prof. Das's group discovers the novel association of PRMT5-an epigenetic factor, in the repair pathway of Top1-trapped DNA lesions. They are the first to show that PRMT5 methylates the DNA repair protein TDP1, which markedly modulates the repair activity of TDP1. PRMT5 knockdown cells exhibit defective TDP1 activity and are hypersensitive to Top1 poison (camptothecin; CPT). The nominee is the pioneer to elucidate the novel role of PRMT5 in Top1cc repair (**Rehman *et al*., *Nucleic Acids Research, 2018***), **which opens new avenues to co-target PRMT5 with Top1 in cancer.** This work was highlighted schematically as a figure in the Nat Rev Mol Cell Biol. 2019 by the pioneers in the field. Prof. Das was invited talk at Gordon Research Conference on DNA Topoisomerase and Biology 2018, USA and ATW2019 meeting, USA.

In a recent study, Prof. Das and his collogues reveal mechanistic crosstalk between two post-translational modifications (Arginine methylation and ubiquitylation of TDP1) that is critical for the repair of trapped Top1cc and maintaining genome stability. Loss of TDP1 arginine methylation results in compromised TDP1 proteostasis, which leads to the accumulation of enzymatically less active TDP1 protein that failed to rescue cells from CPT-induced cytotoxicity (**Bhattacharyya *et al*., *Cell reports, 2022; Bhattacharyya et al., STAR protocols, 2023)***). PRMT5 inhibitor GSK3326595 has been approved as a monotherapy in phase II clinical trials of cancer. Therefore, our current work published in Cell Reports provides a new rationale for the combination of Top1-PRMT5 inhibitors in tumorigenesis. This work has received several international and national attention in the field and has a proposition for clinical trials.

Leishmania donovani, a unicellular protozoan parasite, causes a wide range of human diseases including fatal visceral leishmaniasis. Tyrosyl DNA-phosphodiesterase 1 (TDP1)

hydrolyzes the phosphodiester bond between DNA 3'-end and a tyrosyl moiety of trapped topoisomerase I-DNA covalent complexes (Top1cc). Das group have shown *Leishmania* harbors a TDP1 gene (LdTDP1), however, the biological role of TDP1 remains largely unknown. In the present study, we have generated TDP1 knockout *L. donovani* ($LdTDP1^{-/-}$) promastigotes and have shown that $LdTDP1^{-/-}$ parasites are deficient in 3'-phosphodiesterase activities and were hypersensitive to Top1-poison like camptothecin (CPT), DNA alkylation agent like methyl methanesulfonate, and oxidative DNA lesions generated by hydrogen peroxide but were not sensitive to etoposide. They have also detected elevated levels of CPT-induced reactive oxygen species triggering cell cycle arrest and cell death in $LdTDP1^{-/-}$ promastigotes. $LdTDP1^{-/-}$ promastigotes accumulate a significant change in the membrane morphology with the accumulation of membrane pores, which is associated with oxidative stress and lipid peroxidation. To our surprise, we detected that $LdTDP1^{-/-}$ parasites were hypersensitive to antileishmanial drugs like amphotericin B and miltefosine, which could be rescued by complementation of wild-type TDP1 gene in the $LdTDP1^{-/-}$ parasites. Notably, multidrug-resistant *L. donovani* clinical isolates showed a marked reduction in TDP1 expression and were sensitive to Top1 poisons. Taken together, our study provides a new role of LdTDP1 in protecting *L. donovani* parasites from oxidative stress-induced DNA damage and resistance to amphotericin B and miltefosine (Roychowdhury *et al*., ***The FASEB Journal***, 2022).

Molecular mechanism of Drug action

Poly(ADP-ribose) polymerases (PARP1) is a DNA repair protein and FDA-approved clinical target in BRCA-defective breast and ovarian cancer. PARP1 catalyzes poly(ADP-ribose) (PAR) chains to various proteins including themselves and Top1. The nominee discovered a novel role of PARP1 in the regulation of Top1-nuclear mobility through Top1-PARylation. They establish that inhibition of Top1-PARylation efflux Top1 from the nucleolus to the nucleoplasm results marked increase in Top1 trapping. The CPT-resistant Top1 (N722S) patient mutant shows defective nuclear dynamics due to faulty PARylation (***Das SK., Nucleic Acids Research, 2016***).

Progress has been remarkable in recent years regarding the elucidation of the repair pathways involved in the removal of Top1 cleavage complexes. Our laboratory has made seminal contributions in the field by discovering that TDP1 is epistatic to PARP1 for the repair of Top1cc. PARP1 appears to act as a molecular determinant between TDP1 and the endonuclease pathway for the repair of Top1cc. They also show that TDP1 is PARylated by PARP1. PARylation stabilizes TDP1 and enhances its recruitment to DNA damage sites without interfering with TDP1 catalytic activity. TDP1-PARP1 complexes, in turn, recruit XRCC1 in camptothecin-treated cells. These studies uncover mechanisms of PARP activation and molecular networks of PARP1 for the repair of Top1-induced DNA breaks. TDP1 is recruited to DNA damage sites by PARP1 through TDP1-PARylation. PARP inhibitors are FDA-approved anticancer drugs. Cancer cells exposed to the combination of PARP inhibitors with Top1 inhibitors substantially increased Top1 trapping

which is due to increased unrepaired Top1cc-associated DNA double-strand breaks (DSBs) and lethality. This work provides a molecular mechanism explaining the synergism between PARP and Top1 inhibitors by showing that PARP1 and TDP1 are epistatic which is highly relevant for the ongoing clinical trials combining PARP inhibitors and top1 inhibitors in cancer ((**Chowdhuri and Das.*, NAR Cancer*, 2021**; Das *et al*., ***Nucleic Acids Research*, 2014**). Both these works are highly cited for their clinical relevance and *were highlighted by Galluzzi et al., Cell, 2019; Thomas et al, Clinical Cancer Res 2019.*

Molecular insight into disease pathogenesis: Mitochondrial dysfunction

The mitochondrial DNA damage contributes to several human diseases. However, the mtDNA repair pathway is poorly understood and unexplored. Das laboratory at IACS will continue to focus in understanding the molecular mechanism of mitochondrial DNA damage response pathways, identify novel repair factors in the mitochondria.

Human TDP1 is a neuroprotective enzyme and a homozygous mutation of TDP1(TDP1H493R) is responsible for the neurodegenerative syndrome, spinocerebellar ataxia with axonal neuropathy (SCAN1). SCAN1 leads to cerebellar atrophy, and defects in motor coordination and brain function disorder. We have previously discovered that nuclear encoded TDP1 is imported to the mitochondria. TDP1 is critical for mitochondrial DNA (mtDNA) repair (***Das BB et al., PNAS 2010***), however, the role of mitochondria remains largely unknown for the etiology of SCAN1. However, the molecular mechanism of the SCAN1 disease was unknown, because independent mouse model's generated for TDP1 knockout failed to show the signs of neurodegeneration.

Das group provides the first evidence that mitochondria in cells harboring SCAN1-mutant TDP1 are selectively trapped on the mitochondrial DNA (mtDNA), generate mtDNA damage, and show increased fission rates. $TDP1^{H493R}$-trapping prevents mitochondrial transcription, energy production, and mitobiogenesis. Further, to match the metabolic demand, the neuronal cell expressing SCAN1- TDP1-triggers mitophagy that allows identification and removal of dysfunctional mitochondria in neurons through PINK1-dependent mitophagy. Therefore, the mitophagy operation in SCAN1 neurons is a mechanism of survival. (Ghosh *et al*., ***Science Advances, 2019***). This fundamental work opens a new avenue for understanding the role of mtDNA damage in neurological disorders and is highly cited in the field. The work was highlighted in *Nature Reviews Neurology, 2022, and Mitochondrion 2022.*

Design and development of new drugs

DNA topoisomerase I (Top1) is often exploited as an imperative anticancer chemotherapeutic target due to its critical role in DNA supercoil relaxation. Top1-liked DNA

single-strand breaks (Top1cc-SSB) if unrepaired are the sources for Top1-induced DSBs (Double strand breaks) such as when replication forks collide with Top1-associated DNA lesions.

Despite the clinical success of CPT, the major limitations include its unstable chemical structure, poor aqueous solubility, and rapid cellular efflux via membrane pumps, and the acquisition of cellular resistance of these drugs impelled the designing and investigation of new non-camptothecin Top1 inhibitors.

In this effort, the "**Drug discovery program"** at Prof Benu Brata Das's laboratory has developed promising non-camptothecin chemotype comprising hydantoin and thiohydantoin derivatives as potential human Top1 poison by stabilizing Top1-DNA covalent complexes (***Majumdar, P et al., European Journal of Medicinal Chemistry, 2015***). Unlike CPT, thiohydantoin derivative compound **15**-interacts with the free human Top1 as indicated in the preincubation DNA relaxation inhibition experiments and MOE-assisted molecular docking exercise for the compound **15**/Human Top1-DNA ternary complex provided insight to the SARs and suggested following structural requirements to gain Top1 inhibitory activity: (i) a properly substituted 2-thiophenyl ring as the central moiety and, (ii) an alkene substituted thiohydantoin.

Recently, Prof. Das's group discovered a new class of neutral porphyrin derivative 5,10-bis(4-carboxyphenyl)-15,20-bis(4-dimethylaminophenyl) porphyrin (**compound 8**) as a human Top1 inhibitor at **nanomolar concentrations with compelling anticancer activity**. The study further established that compound 8 binds with the free enzyme and targets cellular Top1 for proteasome-mediated degradation and bolsters ROS-induced apoptotic cell death without stabilizing Top1-DNA cleavage complexes. Persistent with inhibition of human Top1 activity in vitro, compound 8 was effective in killing cancer cells by targeting cellular Top1. In contrast to CPT, the selected porphyrin binds reversibly to the free enzyme and effectively inhibits the formation of Top1-DNA cleavage complexes. The compound 8 abrogates CPT-mediated preformed Top1cc, suggesting a plausibility to overcome the limitations of CPT-resistance ***(Das SK et al., Journal of Medicinal Chemistry (ACS), 2018)***. Selected neutral porphyrin derivative exhibited the highest potency against human Top1 activity both as purified enzyme and as an endogenous protein in the total cellular extracts of human breast adenocarcinoma (MCF7) cells from our synthetic library.

To overcome the chemical limitations of camptothecin (CPT), Prof. Das's group in collaboration with synthetic chemists reported the design, synthesis, and validation of a quinoline-based novel class of topoisomerase 1 (Top1) inhibitors and establish that compound 28 (N-(3-(1 H-imidazol-1-yl)propyl)-6-(4-methoxyphenyl)-3-(1,3,4-oxadiazol-2-yl)quinolin-4-amine) exhibits the highest potency in inhibiting human Top1 activity with an IC_{50} value of 29 ± 0.04 nM. These compounds were originally conceptualized through *in silico* human Top1cc-drug docking analysis to search for potent Top1 poison.

Compound 28 traps Top1-DNA cleavage complexes (Top1ccs) both in the *in vitro* cleavage assays and live cells as evidenced by live cell confocal microscopies coupled with

fluorescence recovery after photobleaching (FRAP) assays (***Das SK., Nucleic Acids Research, 2016***). Dr. Das group further showed that point mutation of Top1-N722S in the catalytic domain of human Top1 fails to trap compound 28-induced Top1cc because of its inability to form a hydrogen bond with compound 28 as evidenced in live cells

Unlike CPT, compound 28 shows excellent plasma serum stability and is not a substrate of P-glycoprotein 1 (permeability glycoprotein) advancing its potential anticancer activity. Finally, we provide evidence that compound 28 overcomes the chemical instability of CPT in human breast adenocarcinoma cells through the generation of persistent and less reversible Top1cc-induced DNA double-strand breaks as detected by γH2AX foci immunostaining after 5 h of drug removal ***(Kundu and Das et al., Journal of Medicinal Chemistry (ACS), 2019)***.

The metabolic instability of lactone has markedly decreased the efficacy of biologically active natural products. Replacement of lactone moiety with α-fluoroether, a novel bioisostere of lactone provides a significant increase in metabolic stability. The utility of the α-fluoroether/lactone substitution was authenticated by the discovery of (20S, 21S)-21-fluorocamptothecin derivatives as stable Top1 inhibitors. Newly identified Top1 poison-based quinoline structure exhibited t1/2 of 69.1 min in human liver microsomes in comparison to compound 1 with t1/2 of 9.9 min. (Kundu *et al.,* ***European Journal of Medicinal Chemistry, 2020)*.**

Top 10 best papers of the applicant as corresponding author:

1. Bhattacharjee S, Rehman I, Basu, S., Nandy S, Richardson J., Das, B.B*. The interplay between symmetric arginine dimethylation and ubiquitylation regulates TDP1 proteostasis for the repair of topoisomerase I-DNA adducts. ***Cell Reports***, 2022,39, 110940 (IF: 9:98)

2. Rehman, I.; Basu, S.; Das, S.K.; Bhattacharjee, S.; Ghosh, A.; Pommier, Y.; and ***Das, B.B****. **2018.** PRMT5-mediated arginine methylation of TDP1 for the repair of topoisomerase I covalent complexes. ***Nucleic. Acids Research***., **46**: 5601-5617. (IP: 16.97)

3. Ghosh, A., Bhattacharjee, S., Paul Chowdhuri, S., Mallick, A, Rehman, I., Basu, S., and ***Das, B.B*.*** 2019. SCAN1-TDP1 trapping on mitochondrial DNA promotes mitochondrial dysfunction and mitophagy. ***SCIENCE ADVANCES***, ***2019***, ***5, eaax9778*** .

4. Roy Chowdhury S., Das SK., Banerjee B., Paul Chowdhuri S., Majumder H.K., and ***Das, B.B****. TDP1 knockout *Leishmania donovani* accumulate Topoisomerase1-linked DNA damage and are hypersensitive to clinically used antileishmanial drugs. ***The FASEB Journal***, 2022, 36(4): e22265.

5. *Majumdar, P*, Bathula C, *Basu S.M., Das, S.K.*, Agarwal R, Hati S, Singh A, Sen, S*, ***Das, B.B.****. 2015. Design, synthesis and evaluation of thiohydantoin derivatives as potent

topoisomerase I (Top1) inhibitors with anticancer activity. ***Eur J Med Chem.*** ;102:540-5.

6.Das SK, Ghosh A, Paul Chowdhuri S, Halder N, Rehman I, Sengupta S, Sahoo KC, Rath H*, ***Das BB*** [#*]. **2018** Neutral Porphyrin Derivative Exerts Anticancer Activity by Targeting Cellular Topoisomerase I (Top1) and Promotes Apoptotic Cell Death without Stabilizing Top1-DNA Cleavage Complexes. ***J. Med. Chem***., 61 (3), 804–817.

7. Kundu, B., Sarkar, D., *Chowdhuri, S. P*., Pal, S., *Ghosh, A.*, *Das, S. K*., Mukherjee, A., Bhattacharya, D., ***Das, B.B***.* Talukdar, A.* **2020**. Development of a metabolically stable topoisomerase I poison as anticancer agent. ***Eur J Med Chem.;202:112551.***.

8. Kundu, B., *Das, S. K*., *Chowdhuri, S. P*., Pal, S., Sarkar, D., *Ghosh, A.*, Mukherjee, A., Bhattacharya, D., ***Das, B.B***.* Talukdar, A. **2019**. Discovery and Mechanistic Study of Tailor-Made Quinoline Derivatives as Topoisomerase 1 Poison with Potent Anticancer Activity. ***Journal of Medicinal Chemistry (ACS)***., **62**: 3428-3446.

9. Das, S.K., Rehman, I., Ghosh, A., Sengupta, S., Majumder, P., Jana, B and ***Das BB*** [*#]**.** Poly(ADP-ribose) polymers regulate DNA topoisomerase I (Top1) nuclear dynamics and camptothecin sensitivity in living cells. ***Nucleic. Acids Res.*** 44, 8363-75. 2016.

10. Chowdhuri SP, Dhiman S, Das SK, Meena N, Das S, Kumar A, Das, B.B*. Novel Pyrido[2',1':2,3]imidazo[4,5- c]quinoline Derivative Selectively Poisons Leishmania donovani Bisubunit Topoisomerase 1 to Inhibit the Antimony-Resistant Leishmania Infection in Vivo. **J Med Chem**, 2023.