Design, Synthesis and Pharmacological screening of novel Indole fused benzo and pyrido heteroazepines

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Summary

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SUMMARY

Over the decades, hepatocellular carcinoma (HCC) remains a foremost public health problem with limited treatment option. Sorafenib is only FDA approved drug for the treatment of HCC with limited response rate due to local metastasis and chemotherapeutic resistance. Indole and heteroazepine both are the extensively reputed heterocyclic rings in the area of cancer drug design and the fusion between them has received massive attention in the recent past. Meanwhile, Paullone (Indole-fused benzazepinone) is well documented for its cytotoxicity, however its precise mechanism of cytotoxicity is still unclear. Literatures provide two suggestions: (1) rational modifications in paullone ring structure to enhance cytotxicity, and (2) exploration of a specific target to identify the mechanism underlying paullone-modified ring. We, therefore, designed and synthesized novel paullone-like molecules, namely indole fused benzo and pyrido heteroazepines by rational modification in fusion site, insertion of hetero-atoms in azepine ring, replacement of benzene ring with pyridine ring, and introduction of a flexible phenyl ring on the basis of molecular docking approach and discovered their cellular functioning towards the treatment of HCC at molecular level (Figure 1).

Taking indole fused benzooxazepine (IFBO) as parent molecule, we designed almost 200 derivatives by introducing electron withdrawing and electron donating substituents on the possible positions and subjected them to molecular docking study on HCC-related inflammatory and apoptotic targets. Among all the derivatives, a total of 40 compounds endow possibly better complementarity with the receptor molecules whereas the rest of the compounds showed poor binding profile. Thus, we decided to synthesize and characterize these 40 compounds as per the results obtained from molecular docking studies. An efficient one pot multi-component domino reaction approach was employed for the synthesis of the proposed derivatives.

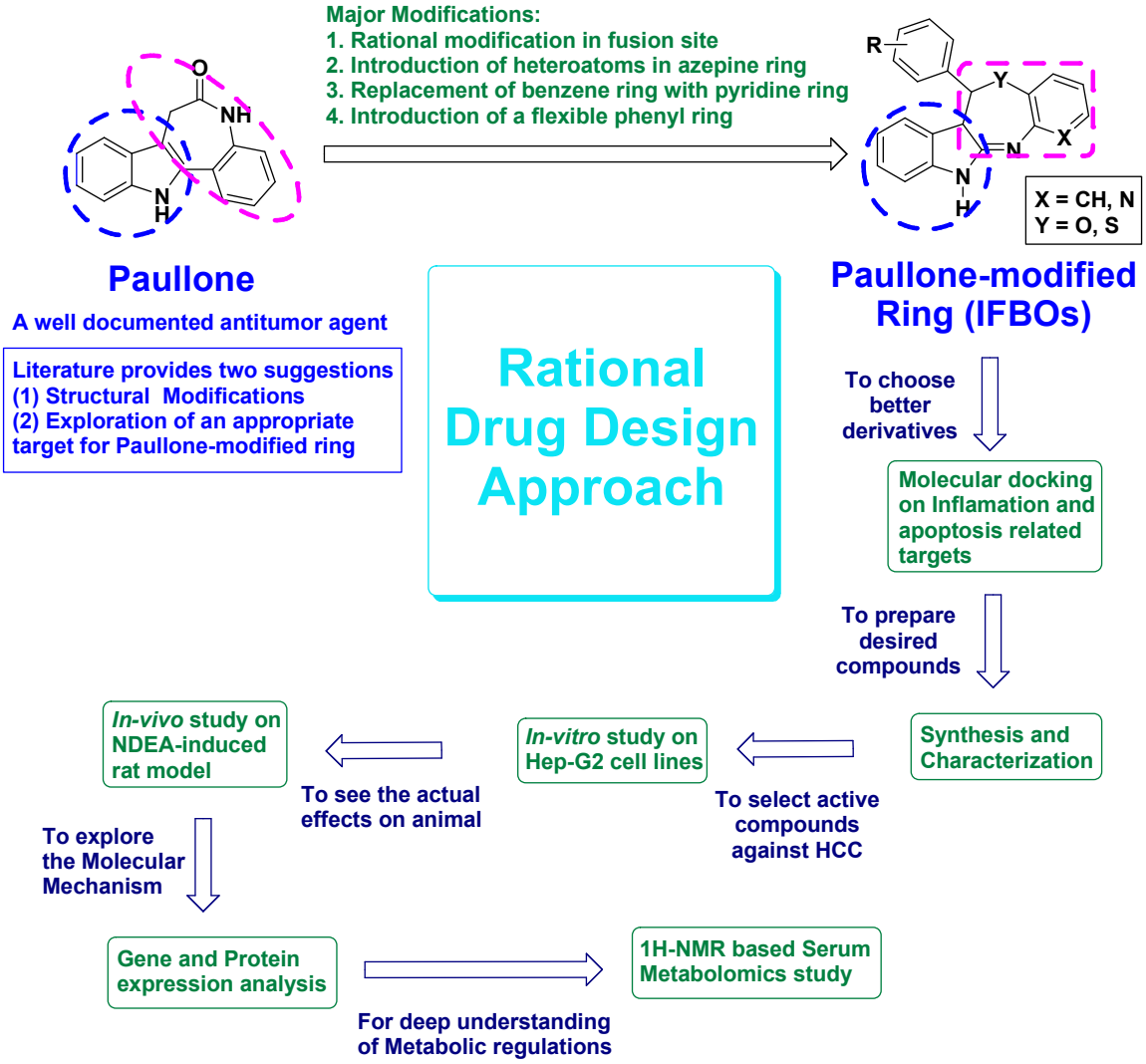

Figure 1: A brief description and rationale of the study

Once the synthesis and characterization have been completed, all the synthesized compounds were subjected to *in vitro* screening on Hep-G2 cell line to investigate their anti-proliferative potential against HCC cells. As per results obtained from *in silico* studies, most of the compounds showed higher interaction energy, crucial number of H- and π- bonds, formation of stable ligand-protein complex with inflammation-associated targets IL2 and IL6 compared to those of the apoptotic targets caspase-3 and caspase-8. Later, all synthesized compounds were subjected to *in vitro* anticancer screening on Hep-G2 human cancer cell line. Considering GI_{50}<10µg/mL to be active, it was predicted that among 40 screened compounds, a total of nine compounds (**6a**, **10a**, **13a**, **14a**, **15a**, **7c**, **10c**, **13c**, and **14c**) were found to be active on this cell line. However, the analysis of the

results were surprising for three compounds **6a**, **10a** and **15a** where they showed an excellent and valuable cytotoxic profile against Hep-G2 cells and thus selected for further *in vivo* study. Although the parent compound **1a** showed the moderate cytotoxic potential (GI_{50} = 48.3µg/mL) against the Hep-G2 cell line, however, 9 of its substituted derivatives exhibited high order of cytotoxicity (GI_{50} < 10µg/mL) towards the Hep-G2 cell line. The structure activity relationship (SAR) on the *in vitro* activity revealed that electronegativity of the substituents attached with flexible phenyl ring greatly influence the cytotoxicity and followed an order of cytotoxicity: F > Cl > Br > OH >OCH_3 > OC_2H_5 > H > CH_3. Activity is also little influenced by the different combination of these substitutions with the inserted heteroatoms in azepine ring.

On the basis of *in vitro* result, three potentially active IFBOs (**6a**, **10a** and **15a**) out of total nine active compounds were selected for detailed *in vivo* pharmacological study. Prior to initiate this study, we performed an acute toxicity study for dose determination and decided an oral dose of 10 mg/kg body weight for *in vivo* pharmacological screening in rats. In addition, a pharmacokinetic study was also performed for the determination of plasma drug concentration profile of IFBOs where a lower rate of absorption, higher volume of distribution and lower rate of clearance were observed, indicating good therapeutic response of IFBOs after oral administration in rats.

We, therefore, conducted *in vivo* anti-tumor activity of these three IFBOs in the NDEA-induced HCC model using male albino rats and provides few notable findings related to the mechanism underlying the IFBO action. In our preliminary investigations, we assured for the induction of carcinogenic condition in NDEA-exposed rats by the reduced body weight of animals and higher incidence number of carcinogenic nodules in the liver tissue. The observed reduction in these changes after IFBO administration was primary indication of its protective effect against carcinogenic condition, which suggests the need of further biochemical and pathophysiological investigations. It has already been reported that there is a decrease in the activities of antioxidants during HCC conditions. The decrease in the enzymatic defense of GSH, SOD and CAT was profoundly observed after the NDEA treatment that unveils their increased utilization during excessive cellular proliferation. Further, the NDEA treatment increased the production of MDA and PC that

validated the damage of the cellular lipids and proteins, respectively. It is notable that IFBO administration reduced the levels of MDA and PC with restoration of enzymatic antioxidant defense of GSH, SOD and CAT, substantiating its tumor protecting ability with remarkable antioxidant effects.

Further, bilirubin and biliverdin are the catabolic by-products of RBCs, and the elevated levels of these biomarkers indicate hepatic damage. The elevation in these markers during NDEA exposure and their restoration after IFBOs administration also supported the hepatic disease control. Moreover, NDEA exposure elevated the levels of liver function enzymes (AST, ALT and ALP) which reflect the advancement of carcinogenesis condition. The observed efficacy of IFBOs to restore these enzyme levels indicated the ability of IFBOs to prevent hepatic damage. Similarly, the high levels of serum LDH obtained in the NDEA-exposed group could be responsible for NDEA mediated liver damage due to occurrence of pre-neoplastic lesions. Treatment with IFBOs led to the improvement in LDH level which probably caused the attenuation in hepatic damage and alleviation of pre-neoplastic lesions and thereby potentiating its anti-tumor property.

Further protective effect of IFBOs was evidenced through histopathology and SEM analysis. The microscopic image of histopathology showed irregular shaped cytoplasm and nuclei in the NDEA-treated rats that were probably due to excessive free radical generation during NDEA exposure. The potential reduction in ruptured and denatured cells (RC and dN) in IFBO-treated groups signified the ameliorative potential of IFBOs against HCC. A similar trend was found in SEM analysis also.

Recent studies suggested that some of the pro-inflammatory cytokines, including IL-2, IL-6, IL-10 and IL-1β are over-expressed at cancer specific sites and particularly linked to induction of HCC conditions. We investigated the altered levels of these markers in the NDEA-exposed group and the effects of IFBO treatment over them through ELISA. IFBOs treatment provided the most efficient restoration of IL-6 concentration than other cytokines that provided an evidence of their cellular functioning through the reduction in the over-expression of IL-6 at cancer sites. This evidence is supported well with our MD simulation study, which led to the formation of a stable

binding complex between IFBOs and the IL-6 receptor. Consequently, we focused our attention towards inflammation dependent IL-6 mediated mechanism of cytotoxicity for our synthesized compounds.

Further, gene expression analysis revealed that the IFBOs treatment provided a rapid reduction in IL-6 mRNA expression which followed a trend similar to those measured in ELISA. Furthermore, it is well reported that IL-6 is released strongly in response to various inflammatory stimuli and serves as a potent JAK/STAT activator. In particular, the HCC-causing oncogenic effect is mediated through persistent stimulation of IL-6, leading to the activation of JAK2 followed by phosphorylation/activation of STAT3. This activated STAT3 undergoes homo or hetero-dimerization leading to nuclear translocation, DNA binding and subsequently gene transcription of several oncogenes involved in initiation and progression of HCC. From the results of protein expression analysis, we confirmed that IFBOs demonstrated its effects through the inhibition of the IL-6/JAK2/STAT3 signaling cascade in NDEA-induced carcinogenic rats. Firstly, IFBO treatment favored the reduction of the IL-6 protein expression which substantiated the gene expression study where a decreased IL-6 mRNA expression was observed. Secondly, IFBO treatment favored the decreased expression of p-JAK2 and p-STAT3 with concomitant reduction in STAT3 expression. Thus, it can be stated that IFBO treatment produces not only the inhibition of JAK2/STAT3 phosphorylation but also the direct inhibition of STAT3. Thus, we herein present the new *in vivo* findings of IFBOs concluding that the anti-tumor effects of IFBOs are due to the favorable regulation of the IL-6-JAK2-STAT3 signaling cascade. This study provides an insight into molecular mechanism of a novel paullone-like ring, IFBO that completely differs from the expected mechanism of antiproliferative action of paullones.

We further implemented ^{1}H NMR based metabolomics to evaluate whether IFBOs have the ability to restore the metabolic perturbations associated to NDEA-exposed HCC condition. OPLS-DA score plots and box-cum-whisker plots using MetaboAnalyst were obtained from 1D ^{1}H CPMG NMR spectral data of rat serum and clearly demonstrated significant metabolic alterations in the NDEA-exposed carcinogenic condition. Interestingly, a decreased glucose level and increased lactate level were observed in the NDEA-exposed group, which was well supported by earlier findings that

demonstrate the carcinogenic condition. These findings completely supported the Warburg effect and may be linked with a higher amount of glucose consumption by cancerous tissues followed by formation of lactate as a by-product. The significant event recorded in this study manifested the excellent ability of IFBOs to restore two major metabolic perturbations, i.e., reduced glucose and elevated lactate levels, a hallmark for tumor progression. Similarly, we noticed that there were significantly elevated levels of pyruvate, glutamine, citrulline, creatinine, tyrosine, and arginine in NDEA-treated rats. Moreover, decreased levels of betaine, valine, and isoleucine were recorded in NDEA treated rats. All of these metabolites were successfully retrieved close to normal after the administration of IFBOs. With all these metabolic modulations and their regulations by IFBOs, it may be concluded that our study is well supported by the reported metabolic alternations showing elevated glycolysis or gluconeogenesis (glucose and lactate), and β-oxidation (lipoproteins and lipids) with reduced tricarboxylic acid (TCA) cycle (pyruvate, glutamine) utilization during the HCC condition.

In conclusion, we herein employed a short rational method to discover the molecular mechanism of our newly synthesized paullone-like skeleton, IFBO. The current study substantiates the biochemical, pathophysiological and molecular link of IFBO treatment and demonstrates the mechanism of their anti-tumor activity. Our *in vivo* result confirms those of the previous *in vitro* study. The IL-6 mediated JAK2-STAT3 inhibitory properties of all the three IFBOs was found parallel to their respective *in vitro* antitumor activity, demonstrating almost similar order of activity in all cases, i.e., **6a** > **15a** > **10a**. Although the molecular insights discovered for IFBO action provides the blockade of a well-known IL-6-JAK2-STAT3 signaling pathway, it completely differs from the controversial cytotoxic mechanisms of paullones. Moreover, using a metabolomics approach in an *in vivo* model, we discovered an advanced mechanistic understanding of IFBO action at cellular level. Altogether, the results indicate that IFBOs have exceptional potential to obliterate HCC and could serve as potential candidate molecules for the development of anti-HCC drugs.

Publications Related to Ph.D. Thesis

Research Papers

1. **Ashok K Singh**, Vinit Raj, Sudipta Saha: Indole-fused azepines and analogues as anticancer lead molecules: Privileged findings and future directions. *European Journal of Medicinal Chemistry* (**Elsevier, IF: 4.519**). 142, 2017, 244-265.

2. **Ashok K Singh**, Umesh Kumar, Vinit Raj, Amit Rai, Pranesh Kumar, Amit K Keshari, Dinesh Kumar, Biswanath Maity, Sneha Nath, Anand Prakash, Sudipta Saha. *Scientific Reports* (**Nature: IF: 4.259**) (Accepted) 2018.

3. **Ashok K Singh**, Umesh Kumar, Vinit Raj, Vimal Maurya, Dinesh Kumar, Biswanath Maity, Anand Prakash, Arnab De, Amalesh Samanta, Sudipta Saha. Novel fused oxazepino-indoles (FOIs) attenuate liver carcinogenesis through IL-6/JAK2/STAT3 signaling blockade with strong metabolic regulations. *Life Sciences* (**Elsevier: IF: 2.702**) (Accepted) 2018.

4. **Ashok K Singh**, Vinit Raj, Amit Rai, Amit K. Keshari, Sudipta Saha: Indole fused benzooxazepines: A new structural class of anti-cancer agents. ***Future Science OA***. 01/2017; 3(1), DOI:10.4155/fsoa-2016-0079.

5. **Ashok K Singh**, Vinit Raj, Amit Rai, Amit K Keshari, Pranesh Kumar, Sudipta Saha. Determination of 5*H*-benzo[2,3][1,4]oxazepino[5,6-*b*]indole in rat plasma by RP-HPLC-UV Method: Application to Pharmacokinetic Studies. ***Asian J Pharm Clin Res.*** (**IF: 0.40**) 10(12), 2017, 425-430.

Patents

6. **Ashok K Singh**, Sudipta Saha, Amit K. Keshari, Shubhini A. Saraf, Dinesh Kumar, Anand Prakash: Indole Fused Pyrido-oxazepine and its derivatives for the Treatment of Hepatocellular Carcinoma. ***Application Number: 201611016493 (Available Online)***, Year: 05/2016.

7. **Ashok K Singh**, Sudipta Saha, Vinit Raj, Amit Rai, Amit K Keshari, Pranesh Kumar: 5*H*-benzo[2,3][1,4]thiazepino[5,6-*b*]indoles for the treatment of liver cancer. ***Application Number 201711026168***, Year: 07/2017.