Design, synthesis and pharmacological screening of novel 1,4-benzohetrozine derivatives

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SUMMARY

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Submitted By
Amit Rai

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Supervisor
Dr. Sudipta Saha

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
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DEPARTMENT OF PHARMACEUTICAL SCIENCES
SCHOOL FOR BIOSCIENCES & BIOTECHNOLOGY
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
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Colorectal cancer (CRC), one of the common widespread human malignant diseases, is a major health problem in most developed countries and the most important cause of cancer-related mortality the entire worldwide. An approximate, almost 1.4 million new cases of CRC were recognized in 2012, which produce 10% of all circumstances of cancer around the world. Human cancer has various multi-stage progressions which include stimulation, enhancement and abnormal growth during metastasis. This action is accentuated through several gene mutations via various cellular pathways. Furthermore, adventitious mutations promote tumor magnification where malignant phenotype and metastasis occur at the tumor sites. Factors responsible for the growth of CRC include diet, inordinate corpulence, smoking, diabetes mellitus, and consumption of alcohol. This is prominent with inflammatory bowel syndrome patient, with higher risk of colitis-associated CRC based with intestinal inflammation.

In this regard, 1,4-benzothiazine (1,4-B) is the most effective agent as it has diverse biological responses particularly antifungal, anticancer, immunomulation, antioxidant, antimicrobial, properties. On the other hand, it is noted that 1,4-B are important pharmacophore, which exhibits antitumor activity against the various tumor cell lines. The 1,4-B moiety resemble to phenothiazines, can be predictable to possess biological activities like phenothiazine. The 1,4-B forms significant class of hetrocyclic system. The 1,4-B molecules have enormous significance and consider as important targets because of their broad spectrum of biological activities.

On the basis of the previous literature survey, we designed, synthesized and screened of 1,4-B derivatives for ameliorative activity. We performed *in silico* docking studies of a series of 1,4-B derivatives with various CRC targets like IL-2, IL-6, COX-2, caspase-3 and caspase -8. The docking studies were conducted with 300 compounds, among which 34 compounds showed good binding affinity with ≥-5 kcal/mol. As per results obtained from docking studies, we synthesized 34 compounds and tested them against HT-29 human CRC cell line. Later, we developed pharmacophore model of the novel series of 1,4-B derivatives and build atom-based 3D-QSAR model. Furthermore, we performed the acute toxicity study of more potent compounds i.e. AR13 and AR15 and performed pharmacokinetic profile in rat plasma with the help of high performance liquid chromatography (HPLC). Finally, we conducted *in vivo* study of both compounds at different doses. In this study, we screened molecular pathways of

ameliorative potential of AR13 and AR15 and metabolomic perturbations using ^{1}H-NMR

The purpose of the study is to develop and characterization of 1,4-B derivatives as anti-CRC agents with following objectives.

1. Bioinformatics study to finalize active 1,4-B derivatives.
2. Synthesis of 1,4-B derivatives.
3. *In vitro* study of 1,4-B derivatives using HT-29 human CRC cells line.
4. Pharmacophore, 3D-QSAR models and molecular dynamic simulation of 1,4-B for CRC treatment.
5. Acute toxicity studies of potent compounds.
6. Pharmacokinetics profile.
7. *In vivo* study of 1,4-B derivatives.

The finding of this project establishes a correlation between synthesis and pharmacological screening of novel 1,4-B derivatives. While structural changes leading to different pharmacological effect have been well studied in the perspective of anti-CRC compounds, much less is known about their impact on their toxicity. This study regard as how chemical modification may contribute to both synthesis and pharmacological effects simultaneously. The knowledge generated can help support the future design of efficacious but less toxic anti-CRC agents. In our project, the structural modification involves changing the 1,4-B moiety with the different functional groups. We believe that our data will provide useful information for the wider scientific community as they consider the use of such functional groups for other drug design.

On broader aspects, this study accentuates the value of incorporating both QSAR studies in the early stage of drug discovery, so as to elucidate the key chemical features that correlate with maximal salutary advantage.

Chapter 2 has been focused on *in silico*, synthesis and *in vitro* studies. On the basis of previous literature survey, we designed 1,4-B derivatives. *In silico* molecular docking was performed using five established CRC targets namely IL-2, IL-6, caspase-3, caspase-8 and COX-2. The binding affinity, hydrogen, pi bonds and contracting amino acid were evaluated. Compounds AR13 and AR15 showed good binding affinity

(IL-2, -6.2; IL-6, -6.9; COX-2, -8.9; Caspase-3, -6.0; Caspase-8, -6.0) and (IL-2, -6.2; IL-6, -6.7; COX-2, -8.2; Caspase-3, -6.4; Caspase-8, -6.4).

Prediction of ADME properties was performed via QickProp tool to predict the physiochemical properties of the compounds (AR1-34). Here, we predicted percentage of absorption (% ABS), Predicted apparent Caco-2 cell permeability in nm/sec. All these compounds exhibited the great %ABS ranging from 69.23 to 100%. Further predicted parameter like as QPlog Khsa is a parameter of binding affinity of compounds with human serum albumin, exhibited between accepted range from -1.5 to 1.5.

Molecular dynamic (MD) simulation of both compounds was performed with IL-6. In this study, there were no fluctuations was observed into the RMSD after the 300 ps and 1000 ps time, respectively for AR13 and AR15. After 3000 and 1100 ps time, stability was found for AR13 and AR15 with IL-6 in MD simulation. Potential energy was not shown any fluctuation during MD run, while average binding energy of the complex was approximately -24.84 kJ/mol and 5.44 kJ/mol for AR13 and AR15 respectively.

Synthesis portion was done after the computational study and in this study, we fallowed the standard procedure for the synthesis. After the completion of the reaction performed the spectral analysis for identification of the compounds with the help of FT-IR, MS, ^{1}H and ^{13}C-NMR.

HT-29 human CRC cell line was treated with AR1-34 at varying concentrations (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M) for 48 h. The most active compounds AR13 and AR15 showed that <0.1 and 6.3 respectively. AR5, 22, 31 and 34 showed the moderate activity against HT-29 human CRC cell line

The Structure-activity relationship (SAR) study of most active compounds AR13 and AR15 of this series having 2-chloro and 3,4-dichloro substitution at the position of R, produced significant effect against HT-29 human CRC cell line with GI_{50} < 10µM. Moreover, the replacement of *p*-methoxy group with more electrons withdrawing 2-chloro group at the position R produced more stability and enhanced efficacy. Consequently, *p*-methoxy showed the mild protection against HT-29 Human CRC cell line with GI_{50} 71.5 µM. In addition, substitutions at the position -R with 3-

methoxy, 3,4-dichlorophenyl, and 3,5-dinitrophenyl led to AR22, AR5, and AR34**,** respectively with decreased anticancer activity.

Later, we developed pharmacophore model of the novel series of 1,4-B derivatives and build atom-based 3D-QSAR model. The common pharmacophore model (ADHR26) was developed and the survival score was found to be 3.828. The generated pharmacophore-based alignment was used to develop a predictive atom-based 3D-QSAR model by using partial least square (PLS) method. Phase predictable activity and $LogGI_{50}$ also exhibited the most significant atomic position in the backbone structure of ligands for anti-CRC activity.

The 3D structure feature of atoms is essential for the study of the structure-activity relationship of the ligand against the CRC related targets. The 3D effect of atomic cubes was exhibited the color according to the coefficient values. The cubes were based on the observation effect of acceptor and aromatic ring with the positive coefficient = 1.200e-002 and negative coefficient = -1.200e-002, which is shown by dark blue color for positive coefficient and red color for negative coefficient. Moreover, the positive coefficient determines an increasing in activity, whereas a negative coefficient shows the decreasing activity.

Furthermore, we performed the acute toxicity study of more potent compounds i.e. AR13 and AR15 and performed pharmacokinetics profile in rat plasma with the help of high performance liquid chromatography (HPLC). In the acute toxicity study, there were no variations occurs in physiochemical parameters in all doses (5, 10 and 25 mg/kg), indicated that both AR13 and AR15 were safe for *in vivo* studies.

In vivo study, CRC conditions was developed through dimethylhydrazine (DMH) treatment which altered various physiological parameters including increase in body weight, tumor volume, incidence number and decreased in pH. Altered physiological parameters were restored after AR13 and AR15 and marketed drug, demonstrating it is ability to use as anti-proliferative agent in future, On the other hand, DMH had capacity to damage liver during CRC condition, again enzymes alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) are increased to a greater extent in serum, treatment with those two compounds reduced those hepatic enzyme levels in serum, again protective

action was seen in over experiments. Again, anti-CRC action was further evaluated through measurement of various oxidative stress parameters in colonic tissue.

Reduced glutathione (GSH) showed damaged condition of colon and this condition again normalized after 5FU and treatment groups; found their more effectiveness during CRC condition. In addition, Superoxide dismutase (SOD) and Catalase (CAT) are the antioxidant enzyme which neutralize superoxide's radicals and the catalysis conversion of hydrogen peroxide to water and oxygen, respectively.

Treatment with 5FU and test compounds increased CAT and SOD levels in tissue. We observed the antioxidant properties of newly synthesized compounds and also their anti-proliferative properties. Oxidation of protein and lipid in the another important to be measured during the oxidative system condition, lipid and protein are oxidized to corresponding malonaldehyde (MDA) and protein carbonyl (PC) during the decrease state and both of them are increased during the oxidative cell damage. Consistent with the previous observation, it was obvious that both MDA and PC formation were found to be increased in DMH-treated rats. AR13 and AR15 again reduced their concentration in CRC tissue, signifying their anti-oxidant properties. Altogether, we may conclude that AR13 and AR15 have ability to reduced oxidative stress induced damage in CRC condition.

In addition to various oxidative stress parameters, morphological change is another important parameter to be measured during CRC condition and after treatment histopathology and scanning electron microscope (SEM) analysis revealed that CRC condition was more prominent via formation of tumoral vacuoles, and tumoral stroma, explained the CRC condition in rats. Abolition of tumoral vacuoles and normalization if tissue architecture were found after treatment, indicating the antiproliferative properties of those compounds. SEM analysis recalled the previous observation where damaged colonic tissue became after treatments.

Except antioxidant properties, it is necessary to evaluate the molecular mechanism of anti-CRC potential of new synthesized compounds i.e. AR13 and AR15. Various proinflammatory cytokines such as IL-6 or COX-2 are expressed at inflammatory sites of carcinoma and have important biomarkers for CRC progression. As per literature obtained previously the enhanced expression and secretion of

cytokines can mediate cascade amplification of inflammatory markers during CRC cell damage that can actively contribute to cancer initiation and progression. Consequent with previous observation, we tried to find out the concentration of those proinflammatory markers during CRC condition and drug treatments through enzyme-linked immunosorbent assay (ELISA) assay. Data obtained from ELISA recalled the previous observation where IL-2/6 and COX-2 concentrations were elevated in DMH rats and these elevations again reset back to normal after treatment. Surprisingly, IL-6 and COX-2 showed prominent effect than IL-2 which was similar with *in vitro*. MD simulation experiments previously. qRT-PCR analysis further substantiated the previous findings. Where COX-2 gene was over-expressed at tumerogenic sites, similar trends like ELISA experiment interestingly, treated with AR13 and AR15 predominantly reduced the concentration of these over-expressed genes, rendered their ability to show the suppressive action in molecular level.

Furthermore, this observation, our study confirmed that over expressed COX-2 protein was more prominent in western blot assay, indicating CRC formation in rats. Further, over expressed COX-2 protein again down regulated and explained the protective effect of AR13 and AR15, particularly at 25 mg/kg dose. This action explained the anti-CRC properties of those two compounds via inhibition of COX-2 protein in molecular level. Later, we observed ameliorative potential of AR13 and AR15 where they inhibited over expressed JAK-2 and STAT-3 protein in western blot analysis. This observation clearly explained that these compounds had capacity to block COX-2 mediated JAK-2/STAT-3 molecular pathways.

NMR-based metabolomics analysis provides the more dependable structural and quantitative information of metabolism during CRC condition after treatment. The identification and characterization of perturbed metabolites during CRC may play a significant role in the early diagnosis and therapy. It helps to make the possible map for the drug action into metabolomic pathways during CRC condition. To our best knowledge, we reported for the first time on the impact of 1,4-B derivatives during CRC situation using ^{1}H-NMR-based serum metabolomics. This study coupled with multivariate statistical data analysis to investigate the DMH-induced metabolic alterations and to assess the ameliorate effect of AR13 and AR15 treatment on these alterations. The metabolic pathway involved amino acids, ketone body, choline

metabolism, glycolysis, TCA cycle, phosphatidylinositol, and neoglucogenesis etc which can be associated with the progression of CRC. We observed a significantly increased in glucose level in CC rats as compared to NC. Which indicated that, higher glycolytic pathways, more glucose was utilized by CRC tissue for cell proliferation and therefore, lower level at glucose was observed in serum during experiment, due to Warbug effect.

In addition, myoinositol is also a constituent of cell membranes which releases phosphate ion in serum, thus increases the ATP formation and cell cycle. Myinositol, concentration normalized after AR13 and AR15 administration, expressing their antiproliferative potential again. Glycerol is the major byproduct of lipid metabolism it is more available in body during CRC condition. We detected higher amount of glycerol in serum of CRC rats, demonstrating, lipid metabolism is increased during cancerous condition. Treated with 5FU. AR13 and AR15 decreased glycerol concentration in serum, indicating those agents had ability to enhance glycerol metabolism.

In the present study, betaine was unregulated in serum and over production of betaine was useful for tumor progression. NAG has anti-inflammatory properties and it was down-regulated in CRC condition. However, higher amount was found in treatment group, again protective action was seen at both AR13 and AR15. In addition, glutamate is the byproduct of glutamine which is the precursor of GSH synthesis. GSH serves as natural antioxidant and scavenger free radical during oxidation degradation of cells. Concentration of glutamine decreased in CRC rat's due to oxidative damage and again recovered after treatment due to antioxidant properties of AR13 and AR15. Taurine (Tau) is believed to have an effect on p53-upregulated modulator of apoptosis (PUMA), which is a regulator of apoptosis. When colon can cancer cells were mixed with taurine, there is a blockage of cellular proliferation and an increase in apoptosis of the colon cancer cells. Concentration of the Tau decreased in CRC rat. Lastly cholin and O-acetly cholin are the important constituent of cell membrane formation and was found to be increased in CRC rats, indicating higher cell membrane formation and enhance integrity. Both AR13 and AR15 normalized their concentration, protective action was seen again.

Subsequently, the aforementioned result suggested that AR13 and AR15 had anti-CRC properties. When DMH-treated rats were dosed with AR13 and AR15, the

aforementioned metabolic changes were found which signifying the AR13 and AR15 have potential anti-inflammatory as well as anti-CRC properties.