Design, Synthesis and Pharmacological Screening of Novel 1,3,4-Thiadiazole derivatives

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SUMMARY

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The goal of the current research study was to describe the design, synthesis of novel 1,3,4-thiadiazole derivatives for the treatment of colorectal cancer (CRC). Over few decades, CRC remains a foremost public health problem. Only a few medications are available in the market for the treatment of CRC, possession of contraindication and side effect are the biggest unacceptability for the patient. As per the current demand in structural modification in 1,3,4-thiadiazole derivatives, we recently synthesized novel 1,3,4-thiadiazole derivatives and discovered herein the cellular functioning of these compounds towards the treatment of CRC at molecular level.

The chemistry of heterocyclic containing 1,3,4-thiadiazole has been an interesting field of study from ancient years and subsequently, this nucleus constitutes a significant class of compounds for new drug development. Thiadiazole belongs to the tri-heterocyclic template which comprises of two electron-deficient carbon, two nitrogen, and one sulfur atoms. This ring is practically an electron-deficient in nature and has pretty high thermal stability. Due to this property, C3 and C5 are the most reactive position of this molecule. The nature of substitutions is that the nitrogen atoms subject to nucleophilic attack and the carbon atoms attack by both nucleophilic and electrophilic substitutions. The existing literature related to structure activity relationship (SAR) suggested that this ring is most essential for the anticancer activity. Substitution of a 3,4,5-trimethoxy group-containing phenyl on the 1,3,4-thiadiazole derivatives possessed superior cytotoxic activity against selected cancer cell lines. Similarly, the introduction of -OCH_3 and -F substituted at phenyl ring produced higher anti-cancer properties. These results revealed that 1,3,4-thiadiazole and substituted electrophilic containing phenyl ring with 1,3,4-thiadiazole are the essential pharmacophores for the anticancer activity. Recently, various 1,3,4-thiadiazole derivatives had been synthesized and evaluated their biological activities including antimicrobial, antituberculosis, antioxidant, anti-inflammatory, anticonvulsants, antidepressant and anxiolytic, antihypertensive, anticancer and antifungal activity. The search for anti-CRC compounds with more selective activities and lower side effect continues to be an active area of intensification examination in medicinal chemistry.

In the first chapter of this thesis, we addressed the overview related to CRC, review literature of 1,3,4-thiadiazoles, and also discussed the research envisaged, hypothesis and plan of work throughout the thesis work. The main objective of present research work was to designed and synthesized novel series of 1,3,4-thiadiazoles and evaluated their anti-CRC properties. Furthermore, it was necessary to evaluate the molecular pathways and determine the impact of titled compounds on metabolomic perturbations during CRC condition.

To address the problem, we first designed a series of novel 1,3,4-thiadiazoles using computational approaches as described in chapter 2. Later, virtual docking studies of 200 designed compounds were performed with various CRC targets like interleukins 2, 6 (IL2, IL6), cyclooxygenase 2 (COX2), caspase-3, and caspase-8. The results from docking study revealed that 35 compounds had better interaction energy >-5 kcal/mol with various assigned molecular targets and the ligand-protein complexes were found to be stable. Later, an *in silico* absorption, distribution, metabolism and excretion (ADME) study was performed for prediction of ADME properties of tilted compounds. It displayed good oral absorption, human albumin protein binding and followed the Lipinski rules. Hence, these compounds might be stable in the form of pharmaceutical doses. Similarly, the molecular dynamic simulation demonstrated the stability of backbone structure of best poses of the ligand-protein complex along with molecular dynamic (MD) simulation run.

After the molecular docking, we synthesized (VR1 to VR35) a novel series of 1,3,4-thiadiazole derivatives from 2-amino-5-(substituted)-1,3,4-thiadiazole condensed with substituted aldehyde or ketone in the presence of glacial acetic acid *via* Wolff-Kishner reduction (in chapter-2). Further, synthesized compounds were characterized by FTIR, NMR (^{1}H and ^{13}C), MS and elemental analyses. After that, these compounds were investigated for anticancer activity against HT-29 human colon cancer cell line using SRB assay. *In vitro* anticancer study revealed that VR24 and VR27 were found to be active against HT-29 cells (GI_{50}<10 µM). Compounds VR5, VR13, VR20, VR28, and VR32 showed the moderate activity with GI_{50}<60 µM. Later, SAR studies further explained that compounds VR24 and VR27 had *p*-methoxy and p-bromo substitution at the position R, 3,4,5-trimethoxy (VR24) at the position R_1 and 4-bromo phenyl (VR27) at the position Ar of backbone structure which produced significant effect against HT-29

human cancer cell line with GI_{50}<10µM. Moreover, the exchanged substituent of *p*-bromo with 3,5-dinitro at the position R and electron withdrawing group of *p*-bromo phenyl with p-chloro, 3-methylphenyl showed the mild protection against HT-29 cancer cells. Substitutions at the position R and R_1 of the parent molecule with 4-CH_3, 3,5-NO_2, and 3,4-dichloro, 3,4,5-trimethoxy, 3-methoxy, 4-hydroxy, respectively for the compounds VR20, VR13 and VR5 exhibited moderate effects with decreasing anticancer properties. The promising anticancer effect was observed during substitution of the less hydrophilic group (-NO_2) at R position and Ar position bearing a para-substitution with the electron withdrawing group along with a hydrophobic (-CH_3) substituent. Other compounds of synthesized series showed the less effectiveness against the human colon cancer cell line as they contained electron withdrawing group at the R position and less hydrophilic groups at the R_1 position of backbone structure. However, the substitution of the lipophilic group (-CH_3) at the R position and electron withdrawing group at R_1 or Ar position of the structure showed the increasing anti-cancer potential.

In vitro enzyme-linked immunosorbent assay (ELISA) (IL2, IL6, and COX2) were performed to explore the molecular targets of VR24 and VR27 and find out whether *in silico* data had a similar pattern at the molecular level. VR24 and VR27 inhibited the assigned molecular targets, imparting their ameliorative effects against colon cancer as showed in chapter 2. Due to these encouraging results, we concluded that both VR24 and VR27 may be effective against CRC.

In the chapter 3, the pharmacophore and three dimensional-quantitative structure-activity relationship (3D-QSAR) studies were performed via data obtained from above HT-29 cells experiment. In this study, common pharmacophore model and atom-based 3D-QSAR were generated of newly synthesized 1,3,4-thiadiazole scaffolds. The dependable common pharmacophore hypothesis (AARR53) and statistically significant 3D-QSAR models were generated, where we found the highest correlation coefficient (r^2=0.94) with the best-fit line: y=0.96x+0.07 using partial least squares (PLS) analysis. This model provided the significant information about the 3D structural feature required for the ligand in order to anti-CRC treatment. In this model, evaluation of statistical analysis indicated the robustness and productivity of the model. The pharmacophore

model showed the optimal chemical structural feature for CRC treatment, whereas the 3D-QSAR model explored the effect of substituted chemical feature of titled compounds. The 3D-QSAR model explored the effect of substituted chemical feature such as H-bond acceptor (ether linkage) which is responsible for increasing the potency against CRC. The basic chemical structures of VR24 and VR27 have the similar substitutional features, reported previously and obtained data *in silico* and *in vivo* study, correlated with each other. It signified that VR24 and VR27 containing 1,3,4-thiadiazole scaffolds are important for CRC treatment. On the other side, saturation of =N- group of 1,3,4-thiadiazole ring decreased the activity against CRC. Furthermore, the molecular docking and dynamic studies supported this 3D-QSAR approach and provided the significant information in order to evaluate the ligand-protein affinity and backbone stability during MD simulation. It also provided clear evidence for the design of best analogs. Finally, the model was developed by 3D-QSAR and pharmacophore in order to ascertain activity against CRC and their targets, which may provide the significant information to the researcher for developing the potential novel lead and searching new scaffold for CRC treatment in future.

After the assessment of *in vitro* anti-CRC properties of VR24 and VR27, acute toxicity studies were carried out to find out the safety profile *in vivo* which is described in chapter 4. During acute toxicity study, we did not observe any significant reduction in body-weight gain persisted over the 15 days following VR24 and VR27 orally administration at three different doses, respectively. The levels of various oxidative and enzyme parameters were normal after the treatment with VR24 and VR27. The results obtained from the histopathological studies revealed that there was no evidence of liver cell necrosis or damage in the treatment groups with respect to the normal control (NC). Therefore, we concluded that the both compounds VR24 and VR27 are safe towards rats.

After that, pharmacokinetic study was further performed to determine the plasma drug profile in rats (in chapter 4). We developed a sensitive and accurate HPLC-UV method for the determination of VR24 and VR27 in rat plasma. The results collectively suggested that LLOQ and %RSD were within the limit which revealed that our optimized method is precious in every aspect. The calculated non-compartment model parameters of

pharmacokinetic study are shown the maximum plasma concentration (C_{max}), time of maximum exposure (T_{max}), and (area under the curve at infinite time) $AUC_{0-\infty}$ were found to be 0.52±0.01 µg/mL, 4.0 h, 20.45±0.45 µg.h/mL for VR24 and 5.55±0.35 µg/mL, 2.0 h, 165.12±9.17 µg.h/mL for VR27, respectively. VR24 and VR27 compounds had slow absorption rate which is useful for CRC treatment as they remain in the gastrointestinal tract (GIT) for the higher amount with the promising effect of VR24. The MRT (mean residential time) was found as 27.48±0.36h and 25.82±1.29h of the VR24 and VR27, respectively. Both had a higher volume of distribution in plasma which could be easily observed from their MRT results. VR24 had higher clearance rate (CL) than VR24 again enlightened the better effectiveness of VR24. Those above results from our experiment clearly showed both compounds had a lower rate of absorption, higher volume of distribution and lower clearance rate which are particularly indicated a good pharmacological response. The newly optimized methods for VR24 and VR27 may be more robust to researcher with respect to accuracy and precision.

These aforementioned results from *in silico*, *in vitro*, acute toxicity, and pharmacokinetic studies suggested to perform *in vivo* activity of VR24 and V27 against DMH induced albino Wistar rat models and determine their molecular mechanism for the CRC treatment. In this experiment, new synthesized VR24 and VR27 were administered in rats to estimate their antineoplastic potential in albino rats as showed in chapter 5. After that, the physiological parameters including body weight, tumor incidence number and volume, pH and total acidity were measured after oral administration of VR24 and VR27 at 10 and 25 mg/kg and 5-FU at 10 mg/kg. The body weight variation was more prominent for DMH treated rats; however, both the drug treatments successfully normalized it to a greater extent. A similar observation was found for tumor incidence number and volume.

In the CC rats group, we observed a significant decreased in colonic pH and increased in total acidity in respect NC. Administration of VR24, VR27, and 5-FU restored to back all these parameters to normal. The enzymatic levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) in the serum were also measured in the similar

experiment. It was noted that the level of these enzymes more significantly ($p<0.001$) attenuated after the oral treatment of VR24 and VR27 at both doses, respectively, compared to the CC rats.

Various oxidative stress parameters including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), thiobarbituric acid reactive substances (TBARS), and protein carbonyl (PC) were evaluated to substantiate anti-CRC effect of both compounds. There was a dramatic reduction for SOD, GSH, CAT and increased malondialdehyde (MDA), PC in the CC, compared to the NC group. Treatment with VR24 and VR27 at different doses was normalized those oxidative stress in CRC condition.

Later, histopathology and scanning electron microscopy (SEM) analysis of colon tissue were performed to estimate the cellular architecture of colon tissue. A number of lesions, epithelial stratification, more vacuolated and damaged cells, goblet depletion, nuclear disparity and structural abnormality were observed in the colon tissue of CC rats after DMH treatment. Treatment with VR24 and VR27 repaired these structural abnormalities. A similar trend was observed during SEM analysis.

In the ELISA assay, we further analyzed the effect of VR24 and VR27 in the proinflammatory cytokines such as IL2, IL6, and COX2 in CRC treatment. We found that IL2, IL6, and COX2 in rat colonic tissue were increased significantly by 2.03, 1.52 and 0.41 folds, respectively in CC rats compared to NC. Both treatments (particularly 25 mg/kg) attenuated the levels of IL2, IL6, and COX2 in CC condition. Both compounds showed the more pronounced effect on IL6/COX2 with respect to IL2. The results obtained from *in vivo* study clearly suggested that both VR24 and VR27 at 25 mg/kg dose reduced oxidative parameters to normal, normalized tissue architecture, and reduced pro-inflammatory cytokines particularly IL6 and COX2.

Quantitative real-time reverse transcription-polymerase chain (qRT-PCR) and western blot analysis were carried out to discover the molecular level. In this experiment, we found the over expressed IL6 and COX2 genes in CC rats which became normal after VR24 and VR27 administration as is discussed in chapter 5. Furthermore, we determined the protein level of those cytokines in CRC using western blot analysis. IL6/COX2, as

well as Janus kinases 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) proteins, was over expressed at tumorigenic sit of CRC. VR24 and VR27 treatment inhibited the expression of IL6/COX2 and JAK2-STAT3. Therefore, both compounds exerted anti-CRC potential via preventing IL6/COX2 mediated JAK2-STAT3 phosphorylation in CRC condition.

In 1D-Carr–purcell–meiboom–gill (1D-CPMG) ^{1}H-NMR metabolomics analysis were performed using combined and pair-wise orthogonal projection to latent structure with discriminate analysis (OPLS-DA) model, OPLS-DA loadings S-plot, box-cum-whisker plots, dendrogram, and pathways analysis to determine the metabolic perturbation in the same experiment (in chapter 5). We found that CRC rat's sera showed the up-regulation of metabolites including myoinositol, glycine, and betaine and down-regulation levels of glucose, isoleucine, very-low-density lipoprotein (VLDL), melatonin, N-acetyl aspartate (NAA), N-acetyl-glutamate (NAG), citrate, sarcosine, citrulline, malonate, choline, tryptophan, lysine, creatinine, and serine. Lastly, ^{1}H-NMR based metabolomic further coined that perturbed few important metabolites turn down to normal after treatment.

In the last chapter 6, summary and conclusion of whole research work were discussed. We may conclude that VR24 and VR27 exhibited the better antiproliferative effect on DMH-induced CRC via blockade of IL6/COX2 mediated JAK2-STAT3 signal transduction pathway and thus, demonstrated the utility of VR24 and VR27 as a useful anti-CRC drug for future anti-cancer therapy. On the other side, the effect of VR27 was more pronounced at 25 mg/kg dose than VR24 against the CC rats. It is necessary to investigate the other molecular targets of CRC, is under the future scope of this study.