Design, Synthesis and Pharmacological Screening of Novel Substituted Thiazolo[3,2-a]pyrimidine and Thiazolo[2,3-b]quinazoline Derivatives

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INTRODUCTION

In our efforts to address the rising incidence of hepatocellular carcinoma (HCC), we have made a commitment to the synthesis of novel molecules to combat HCC. Thiazolo[2,3-b] quinazoline and thiazolo[3,2-a]pyrimidine scaffolds are known to have an anti-tumor effects on certain types of human malignancies; however, their effect on HCC remains unclear. A facile and highly efficient one-pot, multicomponent reaction has been successfully devised utilizing a *p*-toluenesulfonic acid (*p*-TSA)-catalyzed domino Knoevenagel/Michael/ intramolecular cyclization approach for the synthesis of novel 5H-benzo[h]thiazolo[2,3-b]quinazoline and indeno[1,2-d]thiazolo[3,2-a]pyrimidine analogs bearing a bridgehead nitrogen atom. This domino protocol constructed one new ring by the concomitant formation of multiple bonds (C–C, C–N, and C=N) involving multiple steps without the use of any metal catalysts in one-pot, with all reactants efficiently exploited.

All the newly synthesized compounds were authenticated by means of Fourier transform infrared spectroscopy, liquid chromatography–mass spectrometry, proton nuclear magnetic resonance spectroscopy, and carbon-13 nuclear magnetic resonance spectroscopy, together with elemental analysis, and their antitumor activity was evaluated in vitro on a Hep-G2 human cancer cell line by sulforhodamine B assay. Computational molecular modeling studies were carried out on cancer-related targets, including interleukin-2, interleukin-6, Caspase-3, and Caspase-8. Two compounds (4A and 6A) showed growth inhibitory activity comparable to the positive control Adriamycin, with growth inhibition of 50%, 10μg/mL. The results of the comprehensive structure–activity relationship study confirmed the assumption that two or more electronegative groups on the phenyl ring attached to the thiazolo[2,3-b]quinazoline system showed the optimum effect. The in silico simulations suggested crucial hydrogen bond and π–π stacking interactions, with a good ADMET (absorption, distribution, metabolism, excretion and toxicity) profile and molecular dynamics, in order to explore the molecular targets of HCC which were in complete agreement with the in vitro findings.

Further, we investigated the in vivo antitumor activity and the mechanism underlying the effects of 4A and 6A in N-nitrosodiethylamine (NDEA)-induced HCC using male Wistar rats. NDEA was administered weekly intraperitoneal injections of 100 mg/kg for 6 weeks. Various physiological and morphological changes, oxidative parameters, liver marker enzymes and cytokines, were assessed to evaluate the antitumor effect of 4A and 6A.

In addition, Proton nuclear magnetic resonance (^{1}H-NMR)-based serum metabolomics were performed to analyse the effects of 4A and 6A against HCC-induced metabolic alterations. Significant tumour incidences with an imbalance in carcinogen metabolizing enzymes and cellular redox status were observed in carcinogenic rats. Tumour inhibitory effects of 4A and 6A were noted by histopathology and biochemical profiles in NDEA-induced hepatic cancer. Compounds 4A and 6A had potential role to normalize the elevated levels of inflammatory mediators such as interleukin-1β (IL-1β), IL-2, IL-6, and IL-10. In molecular level, the real-time quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) analysis revealed that 4A and 6A attenuated the IL-6 gene over-expression in hepatic cancer. Further, orthogonal partial least squares discriminant analysis (OPLS-DA) scores plot demonstrated a significant separation of 4A and 6A treated groups from carcinogen control group. Both the compounds have potential to restore the imbalanced metabolites due to HCC, signifying promising hepatoprotective activities.

SUMMARY

Hepatocellular carcinoma, especially in the later stages, is a major problem in the clinic and serious complication of cirrhosis or other chronic liver disease. At present, the treatment strategies are limited and there is a clear need for new therapies. Only a few medications are available in the market for the treatment of HCC, possession of contraindication and side effects are the biggest unacceptability for the patient. Therefore, the main objective of my research work is to design, synthesis and evaluation of novel 5H-benzo[h] thiazolo[2,3-b]quinazoline (1A–15A) and indeno[1,2-d]thiazolo[3,2-a]pyrimidine (1B–15B) analogs for the treatment of HCC. Recent studies have delineated the underlying mechanism and discovered novel drug-like small molecules that show promising effects for the treatment of HCC.

These novel synthesized molecules are an untapped resource for novel therapeutic strategies against HCC. Therefore, the current research focused on:

- Design and develop advanced one-pot as well as two-step synthetic routes to novel drug-like molecules
- Applying molecular modelling studies and in vitro evaluation
- Also applying SAR study to uncover and design novel drug-like molecules and
- Finally, in vivo antitumor activity together with proton NMR-based metabolomic studies to optimize the promising drug candidates.

Design and development

These two core structural motifs are formed by the fusion of three biodynamic privileged heterosystems in such a way that one nitrogen atom occupies a bridge head position, therefore being common to both the heterocyclic rings, that is, the thiazole and the pyrimidine rings, and possessing unique structural diversity. We have successfully devised a highly proficient and operationally simple metal-free, one-pot MDR for obtaining a series of novel, hitherto unreported, 5H-benzo[h]thiazolo[2,3-b]quinazoline (1A–15A) and indeno[1,2-d]thiazolo[3,2-a]pyrimidine (1B–15B) analogs displaying potent anticancer activity against Hep-G2 cells as an alternative approach for the treatment of HCC.

Considering this library of novel compounds, we concluded that 5H-benzo[h]thiazolo[2,3-b]quinazoline, along with a substituted phenyl ring (hydrophobic side chain) establishes an important pharmacophoric structure, and the R1, R2, and R3 positions of the phenyl ring, as well as the X1 and X2 positions of the tetralone ring system, are the key reactive sites that could be modified with various groups to elicit greater antitumorigenic potential.

These reactions presumably proceed through a Knoevenagel condensation between substituted α-tetralone or α-indanone and some appropriate aromatic aldehydes in the first step to construct α,β-unsaturated ketones, respectively, which undergo a Michael-type addition approach with the nucleophilic endocyclic nitrogen of the distinctive 2-aminothiazole under the maintained reaction conditions. Then, successive intramolecular cyclization occurred with the loss of a water molecule to give 5H-benzo[h]thiazolo[2,3-b] quinazolines (1A−15A) and indeno[1,2-d]thiazolo[3,2-a]pyrimidines (1B−15B). In this setting, the domino approach and the reaction sequence of Knoevenagel condensation/Michael-type addition/intramolecular cyclization were done in a single step in a one-pot procedure in EtOH.

We have also demonstrated that substitutions with more electronegative groups (–OH, –OCH3) on the hydrophobic side chain directly linked to thiazolo[2,3-b]quinazoline led to active members 4A and 6A, eliciting enhanced antitumorigenic activity, with GI50, 10 μg/mL, which was confirmed by docking analyses. Additionally, 3-methoxy-4-hydroxyphenyl–substituted 5H-benzo[h]thiazolo[2,3-b]quinazoline led to 4A, which displayed excellent antitumorigenic activity among a library of 30 novel synthesized compounds with values of GI50 ,10 μg/mL.

The growth curve of the in vitro findings suggested that the percentage growth inhibition values of the potent compounds 4A and 6A was ≤50% at 10 μg/mL concentration, but they did not move toward a negative value. Therefore, it might be expected in future that both compounds could lead to the death of cancerous cells while minimizing that of normal cells.

This approach, using a one-pot, multicomponent reaction sequence involving a domino Knoevenagel condensation/Michael-type addition followed by an intramolecular cyclization, where the desired molecules are obtained in a one-flask domino manner with atom and step economy in impressive yields (up to 86%) from readily available and low-priced starting

materials, is a resource-effective and desirable route. Additionally, the target compounds were also synthesized by conventional two-step reactions, which were compared with this novel approach on the basis of obtained yields (40%–50%).

Molecular docking studies

Various computational approaches demonstrated effective oral absorption and protein binding. These compounds, therefore, might be stable in some form of pharmaceutical dosage. A study of pharmacokinetic parameters was carried out utilizing QikProp version 4.5 tools to predict the ADME properties of both series (1A–15A) and (1B–15B) and the ranges for the calculated properties of all members, along with their average values are summarized in Table. In addition, we also calculated % ABS, number of H-bond acceptors (n–OH), number of H–bond donors (n-OHNH), octanol/water partition coefficients (QPlogPo/w), and Lipinski's violation.

Interestingly, it was found that the % ABS obtained for all members was 100% and the QPlogPo/w prediction was found to be within the accepted range of -2.0 to 6.5. Moreover, all members followed the violated Lipinski parameters.

Additionally, MD simulation supported our hypothesis regarding the stability of compound 4A with IL-6 protein during the simulation run. The results from the MD simulation run of the active inhibitor showed very less fluctuation with the active site domain of IL-6 and it achieved an almost steady state. We concluded that the compounds would be bound stably to IL-6.

Acute toxicity studies

In this way, next, acute oral toxicity study of both 4A and 6A was carried out to find out the safety profile in albino Wistar rats. Oral administration of 4A and 6A was well tolerated in rats at doses of 5 and 10 mg/kg and also we were not observed any significant reduction in body weight gain persisted over the 15 days. The results obtained from this study implied that both compounds were safe up to 10 mg/kg body weight dose in rats. Therefore, we further decided to perform in vivo anti-HCC activity at a dose of 10 mg/kg body weight in albino Wistar rats.

In-vivo anticancer screening

Synthesised compounds 4A and 6A, which displayed excellent anti-proliferative activity on Hep-G2 cells with GI_{50} < 10 μg/mL and in silico studies revealed that 4A and 6A have impressive binding energy with crucial hydrogen bond and π–bonds to various HCC biomarkers like IL-2, IL-6, caspase-3 and caspase-8. The present investigation was carried out to evaluate the ameliorative effects of NDEA-induced hepatocellular carcinogenesis in albino Wistar rats by 5H-benzo[h]thiazolo[2,3-b] quinazolines and the role of specific cytokines, oxidative and metabolic stress manifestations during cancer progression. Overall, these findings first time clearly suggested the hepatoprotective effect of 4A and 6A against NDEA-induced HCC and its ability to bind with important markers more significantly.

In contrary, NDEA-exposed groups showed decrease in body weight, increase in liver weight and higher number of carcinogenic nodules which indicates critical HCC condition in rats. However, chemo-drugs treatment with 4A, 6A and 5-FU significantly regained the various biochemical parameters near to normal. Exposure of animals to 4A, 6A and 5-FU after NDEA administration significantly normalized the reduced weight, increased % incidence of carcinogenic nodules and enhanced the protective action of 4A and 6A against carcinogen-induced metabolic alterations in hepatic cells.

Further, oxidative stress associated biochemical processes diminished the GSH, SOD and CAT levels and conversely, the formation of MDA and PC were induced in carcinogen-injected rats. Hence, alterations of these parameters confirmed the onset of hepatic oxidative stress and correlation of transformed cells during cancerous conditions. The formation of excess reactive oxygen species (ROS) occurs during the various stages of metabolic biotransformation of NDEA exposure and leads to carcinogenesis by upregulation of several biochemical, intracellular signaling pathways, and gene expression. Endogenous anti-oxidative defence system like, GSH is a free radical scavenger and both the SOD and CAT are antioxidant enzymes which inactivate hydrogen peroxide and dismutase superoxides, respectively. Previously, it was reported that there was a decrease in the activities of enzymatic antioxidants during HCC conditions. Cancerous cells have been reported to sequester vital antioxidants from the systemic circulation, in order to achieve the demands of developing solid tumours.

Treatment with 4A and 6A revealed preventive action to retrieve the levels of GSH, SOD and CAT near to normal and enhanced the anti-oxidative physiological processes. The levels of MDA and PC were attenuated significantly in drug treatment groups as compared to NDEA-exposed HCC rats. Bilirubin and biliverdin are the catabolic by-products of RBCs and the elevated levels of these biomarkers indicate hepatic disease state. During this study, it was observed that the levels of bilirubin and biliverdin were elevated in carcinogen-exposed group, whereas 4A and 6A treatment significantly attenuated the levels of these two specific markers.

Moreover, various enzymes like ALT, AST, LDH and CK are notably increased in human serum with liver metastases of HCC. Similar observations were noted in NDEA-exposed rats and the levels were brought down to normalcy with 5-FU, 4A and 6A drug treatments and predominantly, the impact of 4A is comparable to standard 5-FU.

In addition, intact liver architecture, histopathology and SEM analysis were assessed to analyse the morphological changes associated to NDEA administration and drug treatment. Both histology and SEM analyses revealed that degenerated tumour cells, loss of architecture, and tumoral vacuoles were formed in NDEA-induced rats as previously reported. The histological investigation of the rats treated with 4A, 6A and 5-FU showed cells with architecture more or less similar to control (group I), representing the hepatoprotective activity of the compounds in cancerous condition. In the SEM analysis, we also observed degenerated necrotic tissues in carcinogen exposed rats, which were regularized to a great extent in 4A and 6A treatment groups.

The serum cytokines are key mediators for several pathological and physiological modulations involving inflammation and cancer progression. Further, recent studies indicate that expression of inflammatory mediators like IL-1β, IL-2, IL-6 and IL-10 are involved in initiation and progression of HCC conditions. As per several reports, the regulation of expression and secretion of various cytokines and their receptors have been already described in patients with severe liver conditions. For example, circulating blood interleukins-1β (IL-1β), IL-2, IL-6, and IL-10 concentrations were increased in patients with hepatic cancers.

In consequent with this information related to potential biomarkers, we wanted to know whether 4A and 6A may be active against these HCC specific inflammatory cytokines. The investigation further proved via various ELISA assays where we observed that all these inflammatory cytokines were elevated in NDEA-exposed rats. In addition, the levels of

cytokines regularised after the treatment with 4A, 6A and 5-FU, indicating that both the test compounds exhibited anti-HCC property via inhibition of IL-1β, IL-2, IL-6 and IL-10 over expression at cancer sites. Both the test compounds manifested potential inhibition with IL-6 rather than IL-1β, IL-2 and IL-10.

Similar pattern was also observed in quantitative RT-PCR analysis. The level of IL-6 gene expression was spectacularly decreased with NDEA-exposed rats (group II) and returned to normal control with 4A, 6A and 5-FU treatments which strongly supported potent anti-HCC activity of 4A. Interleukin 6 (IL-6) is a pleiotropic four-helical cytokine that modulates the inflammation-associated cancers by activating the phosphorylation of STAT3 to promote tumor initiation, invasion and metastasis. However, increased levels of IL-6 have been reported to be related to HCC prognosis with elevated cancer risk.

These results of in vivo investigation suggest that 4A and 6A exerted a chemo-preventive effects against experimentally NDEA-induced in vivo HCC in albino Wistar rats and this effect could be attributed to an increased antioxidant profile, restored liver-specific enzymes and decreased expression of oncogenes. Correlations of inflammatory cytokine levels with biochemical markers of HCC were also observed. Effectual treatment with 4A and 6A reflected reduction in the development of carcinogenic hepatic nodules and restored the normal histological architecture of system. 4A and 6A alter the inflammatory signature to reduce significantly the overexpression of IL-6 and attenuate carcinogenic condition.

^{1}H-NMR based metabolomics

Additionally, metabolic profiling established that 4A and 6A have the ability to normalize several metabolites that were significantly disturbed in NDEA-exposed rats, supporting the anticancer activities of 4A and 6A for preventing the endogenous metabolic disorders associated with NDEA-induced liver carcinogenesis.

However, various physiological and morphological changes, oxidative parameters, liver marker enzymes, cytokines and proton-NMR based serum metabolite profiles were assessed to evaluate the antitumor effect of 4A and 6A against NDEA-induced HCC. All these metabolites were successfully retrieved after the administration of 4A and 6A, particularly at 10 mg/kg dose.

While considering all the research findings together, it may be concluded that the newly developed method is both more useful and more profitable in each and every aspect,

compared with the conventional route. Due to the importance of these two core structural motifs, that is, 5H-benzo[h]thiazolo[2,3-b]quinazoline and indeno[1,2-d]thiazolo[3,2-a]pyrimidine, especially in the areas of pharmaceutical and medicinal chemistry, we suggest that the protocols that we have outlined should open up new avenues of investigation, with enormous implications for achieving diversity in chemical synthesis. And our research findings also reveal that the antitumor effect of compound 4A is more prominent than 6A.

Finally, this study provides evidences toward the potency of both 4A and 6A treatment in the amelioration of NDEA-exposed HCC in albino Wistar rats through IL-6 downregulation along with oxidative and metabolic stress reduction and suggests that they can be considered for a range of therapeutic interventions of hepatic cancer in future.