International Journal of Pharmaceutical Chemistry

ISSN: 2249-734X (Online)
Journal DOI:10.7439/ijpc
CODEN:IJPCI4 (American Chemical Society)

Research Article

(*E*)-1-(2,3-dimethoxyphenyl)-N-(4-methylpyridin-2-yl)methanimineas a potent anticancer agent against colorectal cancer

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Abstract

Colorectal cancer is the third common malignancy in man, with a significant morbidity and mortality rate.Three Schiff bases were synthesized by aminocondensation of 4-methyl-2-amino pyridine and three aromatic aldehydes and characterized by CHN analysis and advanced spectral techniques. Theywere screened for their cytotoxic properties using Sulphorhodamine-B (SRB) assay,and their antimigratory properties using wound healing assay against HCT116 (human colon cancer)cell lines.(*E*)-1-(2,3-dimethoxyphenyl)-*N*-(4-methylpyridin-2-yl)methanimine (DMPM) exhibited very low IC_{50} in SRB assay with goodantimigratory activity as witnessed by wound healing assay. The protein processing enzyme MetAP2 has been identified as a molecular target for endothelial cell proliferation and angiogenesis and hence plays a key role in cancer therapy. The docking studies of DMPM with the active site of MetAP2 suggest it to bea potent chemotherapeutic agent in the treatment of colorectal cancer.

Keywords: Schiff bases,cytotoxicity,migration anddocking

1. Introduction

Colon cancer is one of the most common cancers diagnosed in both men and women. It also accounts for the leading cause of cancer related deaths. A large proportion of research is focused on understanding the cellular and molecular mechanisms underlying colorectal tumor development[1,2]. The analysis ofthe errors which occur during cell divisionthatlead to carcinogenesis and identification and study of certain natural substances in the body that blocks cancer cell growth are currently hot areas of current research[3,4]. The most recent application of chemistry in anticancer drugs conception has been to generate drug leads for specific cancer-related targets like proteins, enzymes or nucleic acids.

During cancer cell proliferation, tumor growth is accompanied by the formation of new blood vessels called angiogenesis. The blockade of angiogenesis and thereby tumor growth pathway is a promising therapeutic strategy in developing anticancer drugs. Methionine aminopeptidase 2 (MetAP2), a member of the dimetallohydrolase family, is a cytosolic metalloenzyme which plays a key role in angiogenesis. MetAP2 protects eIF-2α (α subunit of eukaryotic initiation factor 2) from inhibitory phosphorylation from the enzyme eIF-2α kinase, inhibits RNA-dependent protein kinase (PKR)-catalyzed eIF-2 R-subunit phosphorylation and also reverses PKR-mediated inhibition of protein synthesis in intact cells. MetAP2 is the target of two groups of anti-angiogenic natural products, ovalicin andfumagillin, and their analogs[5,6].

Schiff bases that constitute an azomethine group display awide variety of biological activities like antibacterial[7], antifungal[8] and anticancer properties[9].The incorporation of bioactive pyridine moiety is found to enhance the anticancer property of molecules[10]. 2-aminopyridine has attained wide applicability in the synthesis of various pharmaceutical products like anticonvulsants, lipid absorption inhibitors, sodium channel modulators, antibacterial and anticancer agents[11-16]. 2-amino pyridine derivatives are used in the production of the drugs Piroxicam andTenoxicam(non-steroidal anti-inflammatory and analgesic drugs), Sulfapyridine (antibacterial drug), and Tripelennamine (antipruritic and first-generation antihistamine).Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone), has entered several phase I and II clinical trials as an antitumor chemotherapeutic agent[17].The present work focuses on the synthesis, of a lead molecule incorporated with bioactive pyridine moiety with *in vitro*antiproliferative and antimigratory properties against colorectal cancer and its*in silico* docking studies with MetAP2.

2. Materials and methods

1.1 Chemistry: The Schiff bases were synthesized by the amino condensation of 2-amino-4-methyl pyridine with aromatic aldehydes in presence of glacial acetic acid. The synthetic pathway is presented in scheme-1.All the chemicals used for the study were obtained from Sigma-Aldrich, USA.

A 2:8 mixture of ethyl acetate and hexane was used as eluent for performing thin layer chromatography to check the purity of the compounds. Melting points were determined by open capillary method and were uncorrected. The elemental analysis was done in Flash thermo 1112 series CHN analyser.FT-IR spectra of Schiff bases were recorded in KBr pellet using Shimadzu-8400S spectrometer. Infrared spectra of the starting compounds- aldehydes and amines displayed the characteristic bands associated with the C=O str. at 1700-1750 cm^{-1} and N–H str. at 3320–3395 cm^{-1} respectively. The absence of these characteristic reactant peaks and the presence of a strong absorption band around 1600 $cm^{-1}$for C=N str. in the product Schiff base spectra confirmed their formation. The ^{1}HNMR spectra recorded using Bruker spectrometer in deuterated DMSO solvent with TMS as internal standard showed the azomethine protons at 9.8 ppm which was a clear proof for the formation of Schiff bases. The mass spectra recorded in Agilent Technologies 1200 series mass spectrometer displayed the molecular ion peaks which were in accordance their respective molecular masses.

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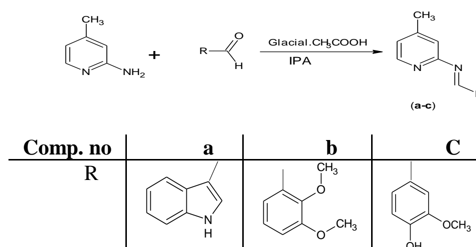
Scheme-1: Synthetic route for *N*-[(Z)-(substituted)methylidene]-4-methylpyridin-2-amine



General procedure for synthesis of *N-[(Z)-(substituted)methylidene]-4-methylpyridin-2-amine*(a-c): To 0.01 mol of 2-amino-4-methyl pyridine dissolved in isopropyl alcohol, 0.01 mol of substituted aromatic aldehyde was added. Catalytic amount (2-3 drops) of glacial acetic acid was added and the reaction mixture was refluxed on a water bath for about 10 hours. The mixture was cooled and evaporated to get the Schiff bases.

(E)-1-(2,3-dihydro-1H-indol-3-yl)-N-(4-methylpyridin-2-yl)methanimine(a) IMPM: Reddish brown solid (87 %) m.p. 194-198 °C; IR (KBr) [cm^{-1}]: 3170 (NH str.), 3145 (Ar. C-H str.), 2931 (CH_3 asym str.), 2880 (CH_3 sym str.), 1635 (C=N str.), 1519 (Ar. C=C str.); ^{1}H NMR ($CDCl_3$), 400 MHz: 9.85 (1H, CH=N), 7.84 (1H, NH), 6.5-7.81 (8H, Ar. H), 2.23 (3H, CH_3); MS (m/z): 235 (M^+); Anal. calcd. for $C_{15}H_{13}N_3$; C, 76.60; H, 5.53; N, 17.87. Found: C, 76.72; H, 5.55; N, 17.91.

(E)-1-(2,3-dimethoxyphenyl)-N-(4-methylpyridin-2-yl)methanimine(b) DMPM: Yellow solid (80 %) m.p.200-202 °C; IR (KBr) [cm^{-1}]: 3160 (Ar. C-H str.), 2947 (CH_3 asym str.), 2831 (CH_3 sym str.), 1666 (C=N str.), 1573 (Ar. C=C str.); ^{1}H NMR ($CDCl_3$), 400 MHz: 9.85 (1H, CH=N), 6.23-7.43 (6H, Ar. H), 3.89 (3H, OCH_3), 3.80 (3H, OCH_3), 2.21 (3H, CH_3); MS (m/z): 256 (M^+); Anal. calcd. for $C_{15}H_{16}N_2O_2$; C, 70.31; H, 6.25; N, 10.94. Found: C, 70.42; H, 6.28; N, 10.97.

(E)-1-(3-methoxy-4-hydroxyphenyl)-N-(4-methylpyridin-2-yl)methanimine (c) HMPM: Yellow solid (80 %) m.p. 226-230 °C; IR (KBr) [cm^{-1l}]; 3402 (OH str.), 3163 (Ar. C-H str.), 2931 (CH_3 asym str.), 2880 (CH_3 sym str.), 1666 (C=N str.), 1535 (Ar. C=C str); ^{1}H NMR ($CDCl_3$), 400 MHz, 10.43 (1H, OH), 9.83 (1H, CH=N), 6.23-7.42 (6H, Ar. H), 3.81 (3H, OCH_3), 2.23 (3H, CH_3); MS (m/z): 242 (M^+); Anal. calcd. for $C_{14}H_{14}N_2O_2$; C, 69.42; H, 5.79; N, 11.57. Found: C, 69.53; H, 5.81; N, 11.60.

2.2 Anticancer activity

2.2.1 Sulphorhodamine-B (SRB) assay: HCT116 (colon-carcinoma) cellswere procured from National Centre for Cancer Studies (NCCS), Pune. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) in 5 % CO_2 atmosphere at 37 °C. SRB assay was performed to assess the cytotoxicity of the synthesized Schiff bases. Test solutions of Schiff bases were prepared in 0.2 % DMSO and diluted with media. 10^4 cells/well in 100 µL of medium were seeded in 96-well plates. The cells were treated with different concentrations (12.5-200 µg/mL; 100 µL/well) of Schiff bases after 24 h of seeding.The control wells were treated with medium containing 0.2 % DMSO. After 48 h, 50 µl of ice cold 30 % TCA was added, incubated at 4 °C for 1 h and washed with distilled water. 50 µl of 0.05 % w/v (in 1% acetic acid) SRB solution was added to each well, incubated further for 30 min in dark, rinsed with 1 % acetic acid and dried. 10 mMTris base was added and the absorbance was read at 540 nm on a scanning multi-well plate reader (ELx800, BioTek Instruments Inc., Winooski, VT, USA). The percentage of growth inhibition for SRB assay was calculated[18,19].

2.2.2 Monolayer wound healing assay: Cell migration is relevant to many processes like tissue repair, as well as cancerinvasion and metastasis. HCT116 migration was assessed using wound healing assay[20]. 1×10^5 cells/well were seeded in 6-well plates containing DMEM media supplemented with 10 % FBS. After attainment of 80 % confluence of monolayer of cells, media was aspirated and a single scrape was made in a linear fashion throughout the center of the plate using a sterile 1 mL micropipette tip to create a denuded zone (width constant across all wells). Then, cellular debris was removed by washing with phosphate buffer saline (PBS) and the cells were exposed to various concentrations of DMPM in DMEM media. Wound closure (in µm) were measured using microscope stage micrometer and eye piece micrometer at 45× objective in each well at 24 h and 48 h. The digital images were captured using a CCD camera attached to inverted microscope (Nikon Eclipse TS100). Analyses were performed in triplicates. The migration distance was computed by subtracting the width of the injury line (at 24 h or 48 h) from the initial width of the injury line (at 0 h) and expressed in µm.

$$\frac{A - (B \text{ or } C \text{ or } D) \times 100}{A}$$

Where A = initial width of the injury line at 0 h; B = width of the injury line in the control; C = width of the injury line at 24 h; D = width of the injury line at 48 h[21,22].

2.3 Statistical analysis: Results were analyzed by one-way ANOVA.

2.4 Molecular modeling and docking studies: Human MetAP2 crystal structure complexed with A797859 with a resolution of 2.35 Å and corresponding entry code 2EA4 was recovered from the PDB database(www.pdb.org). All the water molecules were removed and hydrogen atoms were added to the protein using the Accelrys Discovery Studio ver 1.7 (Accelrys, Inc., San Diego) for carrying out docking studies. The proprietary software is licensed to Manipal Institute of Technology, Manipal University, India. The ligands were built; energy minimization was done using CHARMM force field and was docked into the active site using the complexed A797859 as a reference molecule. One hundred conformations of ligands and their docking modes into the active pocket of the protein (binding cavity) were generated using CDOCKER protocol.

The best favorable conformation was chosen in terms of lowest interaction energy, which was derived from ligand-receptor binding free energies. The agreement of DMPM to Lipinski's rule of five was evaluated[23,24]. In addition, the polar surface area (PSA or steric factor) of DMPMand the number of rotatable bonds was also calculated.

3. Results and discussion

The IC_{50} for IMPM, DMPM and HMPM in SRB assay was 6.3 ± 0.5,4.8 ± 0.2 and 20.1 ± 0.5 µg/mL respectively.All values are expressed as mean ± SEM (n = 3). The IC_{50} for standard drug doxorubicin in HCT-116 cell lines was 1.5 ± 0.02 µg/mL.Schiff basesIMPMwith an indole ring and DMPMwith two methoxy groups attached to the phenyl ring showed excellentinhibition to the proliferation of HCT116 cells.The methyl group, which is often considered as chemically inert can deeply alter the pharmacological properties of a molecule. The presence of a methyl group might have increased the lipophilicity and hence enhanced the hydrophobic bonding possibilities advantageous for ligand-receptor

(DMPM-MetAP2) interactions. This might have made the molecule more compact thereby decreasing the crystal lattice energy, which is energetically favorable. The fixation of large aromatic substituents like indole also aids in the enhancement of its cytotoxicity as seen from the low IC_{50} value of IMPM, due to substantial size of the cavity and the strong interactions between the aromatics and the binding cavity of the receptor macromolecule[25]. The low IC_{50} of DMPM can be attributed to the presence of two methoxy substituents which increases the cytotoxicity and antimitotic activity[26].

DMPM which displayed minimum IC_{50} value was selected for wound healing assay and docking studies with MetAP2. To determine the role of DMPM in the motility of HCT116 cells, the in vitro scratch wound healing assay was carried out.Fig.1. displays the wound gap after 24 h and 48 h of wound generation for control, standard Doxorubicin (1 μg/mL) and DMPM (5 μg/mL). Fig.2 shows cell migration across a wound inflicted in an HCT116 cell monolayer for the control, standard Doxorubicin (1 μg/mL) and DMPM (5 μg/mL). Fig 2 clearly indicates thatafter allowing the cells to grow for 48 h following application of the wound, the HCT116 cells were less motile and could not close the wound ($p < 0.05$), whereas in the control the parental cells could completely heal the wound.

Fig.1: Graphical representation of wound healing assay depicting the wound gap of control, standard and DMPM at the end of 24h and 48 h

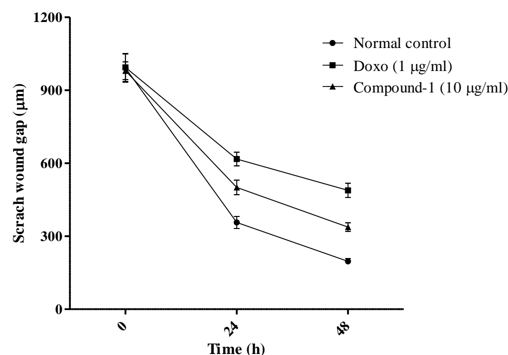
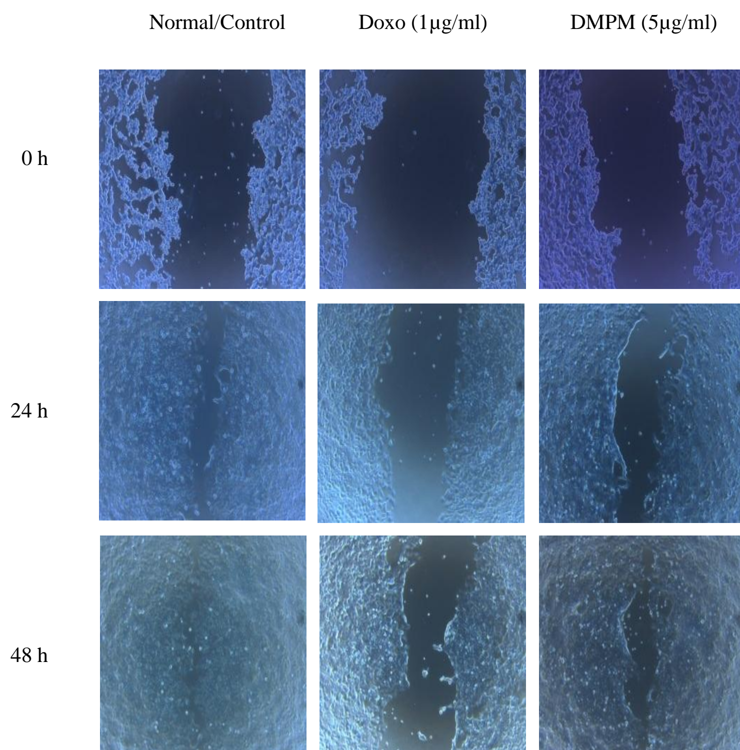
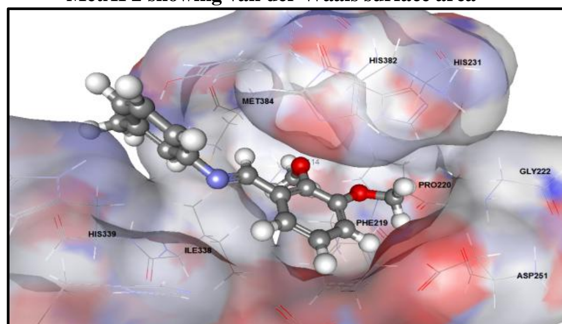


Fig.2: Presentation of cell migration across a wound inflicted in a HCT116 cell monolayer on exposure to vehicle, control and DMPM



The best docking pose where DMPM lies deep into the MetAP2 binding cavity representing the ligand-protein interaction and the binding mode is depicted in Fig.3. DMPM have non bonded interaction with the surrounding cage of amino acids within the binding pocket of MetAP2. Docking of DMPM resulted in high scoring orientations and favourabledocking interaction energy of -34.94 Kcal/mol, presumably due to hydrophobic interactions with the receptor molecule.Higher binding affinity of a compound is often indicative of its selectivity and less interference with other, possibly unwanted off-target sites inducing undesirable or toxic effects. High molecular weight (>500 Dalton) and high numbers of Hydrogen bond acceptors (>10) and donors (>5) may impair permeability across membrane bilayer. The Lipinski parameters for DMPM (mol. wt-255.31, LogP -3.87, H-bond acceptors-3 and H-bond donors-0) suggests that DMPM may have better oral bioavailability. The number of rotatable bonds in DMPM was found to be 4.Polar surface area (PSA) is a property linked to drug bioavailability, where passively absorbed compounds with a PSA > 140 °A are thought to have low bioavailability. The PSA value of DMPM was found to be 29.183$Å^2$.

Fig.3: Docking pose of DMPM having non bonded interaction with the surrounding cage of amino acids within the binding pocket of MetAP2 showing van der Waals surface area



4. Conclusion

In the present study, three Schiff bases with bioactive methyl pyridine moiety were synthesized. DMPM with two methoxy substituents exhibited excellent cytotoxicity and antimigratory properties. DMPM when subjected to docking with MetAP2 displayed favourable interactions with good oral bioavailability, revealing its promising antiangiogenic and anticancer potential against human colorectal cancer.

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