http://www.e-journals.in
DOI:10.7598/cst2014.896

Chemical Science Transactions
2014, **3(4),** 1562-1568

RESEARCH ARTICLE

3-[(*E*)-(4-Hydroxy-3-methoxybenzylidene)amino]-2-thioxoimidazolidin-4-one as Snail1 Inhibitor with Anticancer and Anti-migratory Properties Against Colorectal Cancer

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Received 31 May 2014 / Accepted 29 June 2014

Abstract: Colorectal cancer is the third leading cause of cancer related deaths in humans. Research is going on to increase the treatment options of colon cancer with decreased side effects. Four imidazolidinones were synthesized by the cyclization reaction between Schiff bases and ethylchloroacetate and were characterized by advanced spectral techniques and CHN analysis. The cytotoxic and anti-migratory properties of imidazolidinones against HCT116 (human colorectal adenocarcinoma) cells were studied using Sulphorhodamine-B (SRB) assay and wound healing assay respectively. 3-[(*E*)-(4-Hydroxy-3-methoxybenzylidene)amino]-2-thioxoimidazolidin-4-one (**2d**) displayed excellent cytotoxicity with low IC_{50} in SRB assay. The wound gap observed on treatment with **2d** indicated the reduced motility of HCT116 cells. Snail1, an E-Cadherin repressor plays an important role in epithelial to mesenchymal transition which influences metastatic cancer and hence, a molecular target in cancer treatment for anti-invasive drugs. The effective binding of **2d** into the active site of snail1 in docking studies, suggests it to be a promising anti-neoplastic agent in the treatment of colorectal cancer.

Keywords: Oxazepines, Cytotoxicity, Metastases, Snail1

Introduction

Colorectal adenocarcinoma continues to be a major killer in humans in developing as well as developed countries. Metastases is the major cause of cancer-associated deaths that occurs when cancer cells detach from the primary tumor and disseminate via the circulation and invade surrounding tissues to form the secondary tumors. Snail1 induced epithelial-to-mesenchymal transition (EMT), involving repression of E-cadherin, converts epithelial cells into mesenchymal cells with migratory properties that contribute to the acquisition of invasive properties in epithelial tumors[1-3]. In cancer, snail1 protein induced EMT facilitates the delamination of cells from the primary tumor, intravasation into lymph or blood vessels, favoring invasion and confers selective advantage to migratory malignant cells to metastasize[4-7]. Advances in the last few years have led to our understanding that snail1 is a

new potential target of anti-invasive drugs, owing to its association with dedifferentiated metastatic tumors of different origins. Invasion of cancer cells is also favored by the angiogenic properties of snail[8].

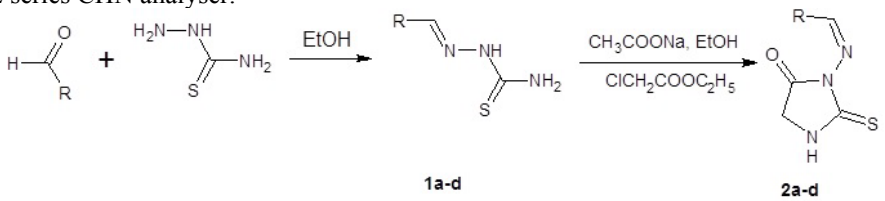
Snail1 inactivation could prevent invasiveness and make invasive cells more susceptible to destruction. Snail physically interacts with the lysine specific histone demethylase (LSD1) via the SNAG domain, and recruits LSD1 to epithelial gene promoters. LSD1 is essential for snail-mediated transcriptional repression and in its absence; Snail fails to repress its targets. These results underline the critical role of LSD1 in snail-dependent transcriptional repression of epithelial markers and suggest that a suitable inhibitor could prevent LSD1from complexing with snail1 and thereby reduce cancer progression.

Schiff bases, compounds that contain an azomethine group, formed by condensation of primary amine with the carbonyl products represent valuable intermediates in organic synthesis and have attracted much interest in the development of pharmacologically active compounds with potent antibacterial[9,10], antifungal[11,12] and anticancer[13] properties. Schiff bases appear to be important intermediates in a number of enzymatic reactions involving interaction of enzyme with an amino or a carbonyl group of the substrate. 2-thioxo-4-imidazolidinone are a class of heterocycles having a ring that contains two groups-thion and carbonyl at positions 2 and 4 respectively that possess wide spectrum of therapeutic activities such as anti-bacterial[14], anti-inflammatory[15]and anti-tumor[16]. Thus, the present study focuses on the synthesis of imidazolidinones with anti-tumor and anti-migratory properties as effective inhibitors of snail1 and could presumably prevent LSD1-Snail1 complex formation, responsible for cancer progression during pathogenic conditions.

Experimental

(2*E*)-2-(Substituted)hydrazinecarbothioamide (Schiff's bases) **1a-d** were synthesized by the amino condensation between hydrazinecarbothioamides and four different aromatic aldehydes[17]. Four new imidazolidinones were prepared by the reaction between Schiff bases and ethylchloroacetate in presence of anhydrous sodium acetate[18]. The synthetic pathway is depicted in Scheme 1.

Thin layer chromatography of imidazolidinones on 0.25 mm silica gel plates was performed using a 1:9 mixture of ethyl acetate and hexane mixture as eluent. Melting points of imidazolidinones were determined by open capillary method and were uncorrected. The elemental analysis of the newly synthesized compounds was carried out in Flash thermo 1112 series CHN analyser.



Comp. No	R
1a, 2a	4-hhdroxy phenyl
1b, 2b	3-indolyl
1c. 2c	2,3-dimedroxy phenyl
1d, 2d	2-methoxy-3-hydoxy phenyl

Scheme 1. Synthetic route for (2*E*)-2-(substituted)hydrazinecarbothioamide (**1a-d**) and 3-{[(*Z*)-(4-substitutedphenyl)methylidene]amino}-2-thioxoimidazolidin-4-one (**2a-d**)

Anticancer activity

The synthesized imidazolidinones were studied for their anticancer properties in HCT-116 cells, procured from NCCS, Pune and cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10 % Fetal Bovine Serum (FBS) at 37 °C in 5 % CO_2 atmosphere.

Sulphorhodamine-B (SRB) assay

Test solutions of imidazolidinones were dissolved in 0.2 % DMSO and diluted with media prior to the experiment. 10^4 cells/well in 100 µL of medium were seeded in 96-well plates and kept overnight for 24 hours at 37°C in CO_2 incubator. The cells were then exposed to different concentrations (12.5-200 µg/mL; 100 µL/well) of imidazolidinones. Control wells were treated with medium containing 0.2% DMSO. After 48 h, 50 µL of ice cold 30 % trichloroacetic acid was added to each well, and incubated at 4 ºC 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 and incubated for 30 min in dark. The wells were rinsed with 1% acetic acid, dried, 10 mM Tris base was added to each well and the absorbance was read at 540 nm in a multi-well plate reader (ELx800, BioTek Instruments Inc., Winooski, VT, USA). The percentage of growth inhibition was calculated using the formula:

$$\% \text{ Cytotoxicity} = [(AC - AB) - (AT - AB)] / (AC - AB)$$

Where, AC, AB and AT are absorbance of control, blank and test respectively[19].

Scratch wound assay

Cells ($1x10^5$ cells/well) were seeded in 6-well plates containing DMEM media supplemented with 10% FBS. After attainment of 80% confluence, media was aspirated and a single scrape was made in a linear pattern through the centre of the plate to create a constant zone width across all wells. Then, cellular debris was removed by washing with PBS and the cells treated with doxorubicin (1 µg/mL) and 2d (3 µg/mL).Wound closure (in µM) in each well after 24 and 48 h was measured using microscope stage micrometer and eye piece micrometer at 45x objective in each well and images were taken using a CCD camera (Nikon Eclipse TS100). The migration distance (average of triplicates) was computed by subtracting the width of injury line at 24 h or 48 h from the initial width of the injury line (at 0 h) and expressed in µm[20,21].

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

Where A = width of injury line at 0 h; B = width of injury line in the control; C = width of injury line at 24 h; D = Width of the injury line at 48 h.

Molecular modeling and docking studies

Human Snail1 crystal structure complexed with FAD (Flavine adenine dinucleotide) with corresponding entry code 2Y48 was recovered from the PDB database with a resolution of 3 Å. Protomol was generated for carrying out docking studies based on already complexed ligand residue, FAD using Surflex dock module of sybylver 1.7 licensed to Manipal Institute of Technology, Manipal University, India (Tripos Inc. St. Louis, USA). The ligands were built using ligand preparation module of surflex descriptors. The best favorable conformation in terms of highest docking score was chosen. The agreement of **2d** to Lipinski's Ro5 was evaluated[22,23].

Results and Discussion

FT-IR spectra of imidazolidinones recorded in KBr pellet using Shimadzu-8400S spectrometer showed a strong absorption band around 1700 cm^{-1} characteristic of C=O stretching vibration. The absence of asymmetric and symmetric stretching vibration of NH_2 groups at 3445 and 3200 cm^{-1} observed in Schiff bases clearly confirmed the formation of imidazolidinones. The ^{1}H-NMR of imidazolidinones recorded in deuterated DMSO solvent and TMS as internal standard using Bruker spectrometer displayed a singlet downfield at 11.9 ppm attributed to the proton attached to the nitrogen in the ring. The mass spectra of the compounds recorded in a Schimadzu GCMS-QP5050 mass spectrometer showed molecular ion peaks which were in accordance their respective molecular masses.

The IC_{50} for standard drug doxorubicin in HCT-116 cell line was 1.5 ± 0.02 µg/mL. **2d** with hydroxyl and methoxy substituents exhibited minimum IC_{50} value of 21.6 ± 0.7 among all the imidazolidinones screened against HCT-116 cell line. **2a** with a hydroxyl substituent and **2c** with a methoxy group displayed comparatively higher IC_{50} values of 79.3 ± 3.8 and 39.5 ± 2.7µg/mL respectively. **2b** with an indole substituent displayed an IC_{50} of 31.3 ± 4.1µg/mL. All IC_{50}values are expressed as mean ± SEM (n = 3).

2d which displayed minimum IC_{50} value was further subjected to *in vitro* scratch wound healing assay to check the motility of HCT116 cells. Figure 1 displays the wound width after 24 and 48 h of wound infliction for control, doxorubicin (1 µg/mL) and **2d** (3 µg/mL). At the end of 24 h, the wound gap in cells treated with **2d** was almost same as that in the case of standard, showing its efficacy in restricting cell migration. Figure 2 illustrates cell migration across the wound for the control, standard (1 µg/mL) and **2d** (3 µg/mL) and clearly indicates the reduced motility of cells after 48 h following application of the wound, without any wound closure ($p < 0.05$). Wound healing was observed with the control cells.

The best docking pose where **2d** lies deep into the Snail1 binding cavity representing the ligand-protein interaction and the binding mode is depicted in Figure 3. **2d** showed hydrogen bonded interaction with the surrounding cage of amino acids within the binding pocket of Snail1. Docking of **2d** resulted in high scoring orientations and favourable docking score of 5.54 presumably due to two H-bonds and also hydrophobic interactions with the receptor molecule. The oxygen and hydrogen of the hydroxyl group at the para position of the phenyl ring were H-bonded to hydrogen attached to the amino group of lysine (LYS) and oxygen attached to carboxyl group of leucine (LEU) respectively.

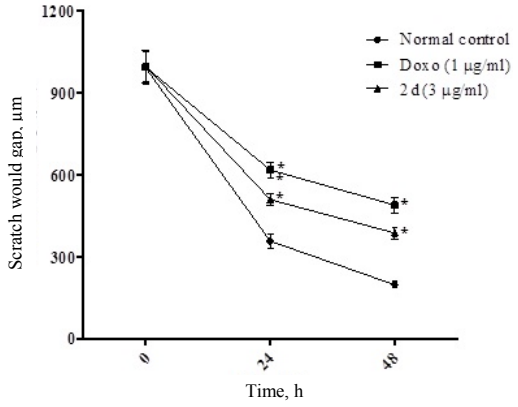


Figure 1. Graph depicting wound gap at the end of 24 and 48 h after wound infliction

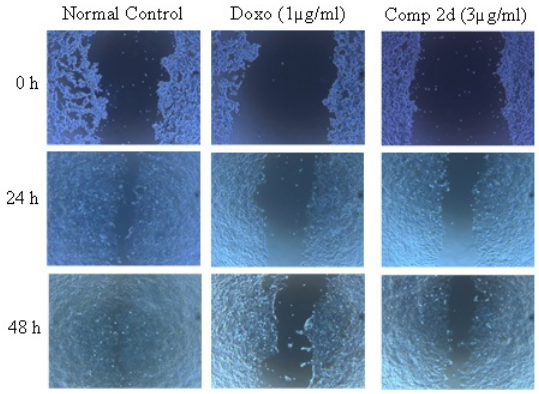


Figure 2. Picture displaying HCT 116 cell motility in *in vitro* wound healing assay. Control cells shows wound healing at 48 h

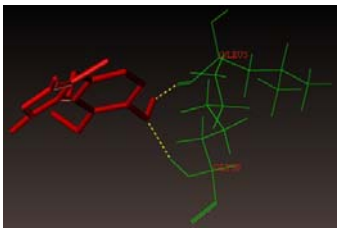


Figure 3. Binding of **2d** into the active site of Snail1 showing the hydrogen bonding interactions with the amino acid cage

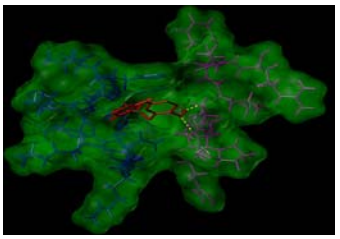


Figure 4. Picture depicting **2d** within the active site of Snail1, preventing the LSD1-snail1 complex formation. LSD1(left), 2d (middle) and Snail1 (right)

The critical role of LSD1 in snail-dependent transcriptional repression of epithelial markers suggests that the LSD1-snail1 complex could be a potential therapeutic target for prevention of EMT associated tumor invasion. **2d** binds with the active site of snail-1 destabilizing the LSD1-Snail1 complex (Figure 4) and this could probably impair the repression activity of snail1[24,25].

Molecular weight greater than 500 Dalton and high numbers of Hydrogen bond acceptors (>10) and donors (>5) may weaken permeability across membrane bilayer. The Lipinski parameters for **2d** (mol. wt- 265, LogP -1.64, HBA -4 and HBD -2) suggests that it might have better oral bioavailability. Polar surface area (PSA) for **2d** was 106.25 $Å^2$, which is an indicator of its better intestinal permeability, absorption and passive transport.

*General procedure for synthesis of 3-{[(Z)-(4-substitutedphenyl)methylidene] amino}-2-thioxoimidazolidin-4-one (**2a-d**)*

0.01 mol of (2*E*)-2-(4-substituted)hydrazinecarbothioamide (Schiff bases) and 0.01 mol of ethylchloroacetate dissolved in ethanol was refluxed in presence of anhydrous sodium acetate on a water bath for 6 hours. The reaction mixture was poured into crushed ice. The solid separated was washed with cold water, filtered, dried and recrystallized from ethanol.

*3-[(E)-(4-Hydroxybenzylidene)amino]-2-thioxoimidazolidin-4-one (**2a**)*

Yellow solid (70%) m.p.216-220 °C; IR (KBr) [cm^{-1}]: 3471 (OH str.), 3201 (NH str.), 3050 (Ar. C-H str.), 1720 (C=O str.), 1612(C=N str.), 1504 (Ar. C=C str.), 1234 (C=S str.);

^{1}H NMR [ppm] DMSO-d_6, 400MHz: 11.93 (1H, NH), 11.41 (1H, CH=N), 7.7-8.2 (4H, Ar. H), 3.75 (2H, CH_2), 7.54(1H, OH); MS (m/z): 235 (M^+); Anal. calcd. for $C_{10}H_9N_3O_2S$; C, 51.06; H, 3.83; N, 17.87. Found: C, 51.20; H, 3.84; N, 17.89.

*3-[(E)-(2,3-Dihydro-1H-indol-3-ylmethylidene)amino]-2-thioxoimidazolidin-4-one (**2b**)*

Yellow solid (73 %) m.p.172-178 °C; IR (KBr) [cm^{-1}]: 3240 (N-H str.), 3109 (Ar. C-H str.), 1704 (C=O str.), 1620 (C=N str.), 1573 (Ar. C=C str.), 1242 (C=S str.); ^{1}H NMR [ppm] DMSO-d_6, 400 MHz: 12.29 (1H, indole NH), 11.89 (1H, NH), 11.50 (1H, CH=N), 7.6-8.1 (5H, Ar. H), 3.76 (2H, CH_2); MS (m/z): 258 (M^+); Anal. calcd. for $C_{12}H_{10}N_4OS$; C, 55.81; H, 3.88; N, 21.71. Found: C, 55.93; H, 3.89; N, 21.73.

*3-[(E)-(2,3-Dimethoxybenzylidene)amino]-2-thioxoimidazolidin-4-one (**2c**)*

White solid (77%) m.p.226-228 °C; IR (KBr) [cm^{-1}]: 3155 (NH str.), 3047 (Ar. CH str.), 2909 (CH_3asym. str.), 2831 (CH_3 sym. str.), 1712 (C=O str.), 1596 (C=N str.), 1542 (Ar. C=C str.), 1265 (C=S str.); ^{1}H NMR [ppm] DMSO-d_6, 400 MHz: 11.92 (1H, NH), 11.43 (1H, CH=N), 7.7-8.2 (3H, Ar. H), 3.79 (2H, CH_2), 3.48 (3H, OCH_3),3.42 (3H, OCH_3); MS (m/z): 279 (M^+) $C_{12}H_{13}N_3O_3S$; C, 51.61; H, 4.66; N, 15.05. Found: C, 51.69; H, 4.68; N, 15.08.

*3-[(E)-(4-Hydroxy-3-methoxybenzylidene)amino]-2-thioxoimidazolidin-4-one (**2d**)*

Yellowish green (76%) m.p.260-262 °C; IR (KBr) [cm^{-1}]: 3556 (OH str.), 3200 (NH str.), 3110 (Ar. CH str.), 2923 (CH_3 asym. str.), 2880 (CH_3 sym. str.), 1697 (C=O str.), 1596 (C=N str.),1550 (Ar. C=C str.), 1288 (C=S str.);^{1}H NMR [ppm] DMSO-d_6, 400 MHz: 11.91 (1H, NH), 11.42 (1H, CH=N), 7.7-8.2 (3H, Ar. H), 3.8 (3H, OCH_3), 3.75 (2H, CH_2), 7.95 (1H, OH);265 (M^+); Anal. calcd. for $C_{11}H_{11}N_3O_3S$; C, 49.81; H, 4.15; N, 15.85. Found: C, 49.92; H, 4.14; N, 15.88.

Conclusion

Four new imidazolidinones were synthesized by cyclization reaction of Schiff bases with ethylchloroacetate. The different spectral techniques and the elemental analysis confirmed the structure of the compounds. This study reveals 3-[(*E*)-(4-hydroxy-3-methoxybenzylidene)amino]-2-thioxoimidazolidin-4-one to be a potent cytotoxic and antimigratory agent as well as a novel antimetastatic snail1 inhibitor against colorectal adenocarcinoma cells.

Acknowledgement

The authors are grateful to Director and Head-Department of Chemistry and Biotechnology, Manipal Institute of Technology, Manipal University for providing us the necessary laboratory facilities.

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