Synthesis, XRD, HAS, in silico molecular docking studies and biological assessment of novel Schiff base compounds as anti-cancer and antimicrobial agents

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ABSTRACT
In this study, versatile multifunctional Schiff base (SB) derivatives were synthesized. Compounds 1–8 were prepared by a mild condition and were pharmacologically assessed for their role in vitro anti-cancer and their impact on human fibrosarcoma (HT-1080) and cervical cancer cells (HeLa), in addition to their antimicrobial activity regarding fungal strains, gram-positive and gram-negative bacteria. Preliminary in silico study of 1–8 and the standard compound (5-Fluorouracil) was accomplished, using Drug2Way and PASS software. Besides, docking investigations were carried out using Schrödinger software to determine the interaction of p53-MDM2 and pf-DHFR binding affinity for all the compounds. The antimicrobial results exhibited that these novel compounds have modest to good inhibitory action against the tried bacterial and fungal strains. The crystal structures of 2 and 7 have been determined. Hirshfeld Surface Analysis (HSA) is in agreement with the XRD studies. Both compounds have shown enol–imine tautomeric forms as EE isomer.

1. Introduction
Cancer, in addition to heart diseases, is an additional leading cause of death in many countries [1,2]. There is a high demand for new ideas to cultivate unique drugs as a cure for life-threatening infections e.g. cancer and others [3]. Although survival rates have improved due to efficient anti-cancer drugs, many types of a tumour still have no proven effective treatment [4]. Most new therapeutic candidates involve organic compounds because organic compounds are important in many biological processes. For example, most of the drugs have heterocyclic functional groups [5].

The p53—MDM2 interaction created a valuable goal to develop effective antitumor agents. In the cell process, the growth of cancer cells was prevented by avoiding the excessive expression of MDM2 (Murine Double Minutes) that exhibits the important activation of tumour suppressor protein p53 [6]. The unusual interaction between MDM2 and p53, overturns the important activity of p53 in around 50% of all human cancers [7,8]. Therefore, large number of scientists are researching small molecules to be competitive with MDM2 to interact with the active site of p53 protein, hindering MDM2’s binding with p53 [9].

Antimicrobial drugs cause worrying about infectious illnesses due to their great morbidity and mortality. This is due to their resistance towards the existing antibiotic remedy. Moreover, the microorganisms have developed resistance against many treatments and this is a challenge. Scientists witness these days a rise in infections because of microorganisms’ strong resistance to many antimicrobial agents, which have become an additional challenge as well in research [10]. To solve the problem, a desire for a newer class of antimicrobials has encouraged the researcher to propose numerous inhibitors of different key enzymes. For example, Dihydrofolate reductase (DHFR) is one of many crucial enzymes involves in the course of DNA replication, it catalyses the conversion of dihydrofolate into tetrahydrofolate [11]. Recently, researches have revealed that amino acid changes within pf-DHFR are linked with drug developments [12]. Therefore, DHFR enzyme has demonstrated to be trustworthy as a target towards novel antimicrobial drugs. Bearing in mind these facts, the necessity for the improvement of novel chemical entities that will perform as antitumor and antimicrobial agents becomes urgent.

The aim of the current study is to synthesize, characterize, and test novel prospective based drug-like compounds for their potential antitumor and antimicrobial activity using X-ray, HAS and molecular modelling. The presence of OH group(s) at different positions on the phenyl moiety makes this study interesting [13]. In fact, the OH functional group(s) were intentionally...
planned to afford hydrogen bond to the biological sites to guarantee better interactions with the biological receptors. The main feature in most of the compounds in this study is that they have OH group(s) at different positions on the phenyl group to study the effect of that on the docking study, Scheme 1 [14]. A previous SAR (Structure–activity relationship) investigation suggested that the presence of 2-OH is important for high activity in the interaction. This is probably due to the presence of a strong polar substituent OH at the ortho position of the phenyl group in some cases [15,16].

Further related details, SBs are a class of molecules which has been the theme of substantial interest, Figure 1, due to their interesting metal chelating properties, flexibility to adjust the structure to fine-tune it for a specific biological use and essential biological activities [17]. In particular, heterocyclic SBs dominated medicinal-chemistry due to their vast range of biological properties [18,19]. They occupy a range of biological significance as anti-tubercular, anti-cancer, anti-inflammatory, antioxidant, antimalarial, antiviral and antimicrobial so forth [6,10,20–29]. The dynamic canters of cell elements are thought to get interacted with –CH=N via forming a H-bond that affects the regular cell processes [30] and consequence in the demolition of enzymatic activity of tumorous cells, thus SB compounds could be presents as a possible target.

2. Experimental

2.1. General characterization techniques

All chemicals were obtained from Sigma-Aldrich. Chemicals were used as received. The melting points were taken on a Mel-Temp. Capillary melting point apparatus.

**Scheme 1.** Formation of SB compounds 1-8. Reagents and conditions are: bicyclohexyl amine with various aldehydes (1:2 mols), ethanol, stirring 1–3 h and reflux/70–80°C for 1–2 h.

**Figure 1.** Examples of related reported Schiff base (SB) derivatives. (A) [31], (B) [31], (C) [32,33].
and are uncorrected. Carb CHN Model CE-440 Analyzer and on an Elementar Vario EL III Carlo Erba 1108 was used for carbon, hydrogen, nitrogen contents. IRRAffinity-15 Shimadzu instrument was used for infrared spectra (n/cm⁻¹) using KBr disks. Microflex Bruker instrument was used for MALDI mass chromatograms. Bruker AMX-400 spectrometer operating at 400 and 100 MHz using TMS as an internal standard was used for ¹H and ¹³C NMR. (CDCl₃, DMSO-d₆). Thermo Scientific Genesy 10s UV–Vis spectrophotometer.

2.2. Synthesis of SBS

Schiff bases (1–8) were prepared by condensation reaction of 4,4'-methylenebis(cyclohexylamine) with 2 moles of substituted benzaldehydes in ethanol. The products were purified by re-crystallization from different solvents such as EtOH, DCM, CHCl₃ and DMSO to produce crystalline materials in most of the cases, (Scheme 1).

CH₂–(C₂H₁₀–N = CH–C₆H₄–OH–OH₂)₂(1), 2,2'-(1E,1’E)-(4,4'-methylenebis(cyclohexane-4,1-diyl))bis(azan-1-yl-1-yldiene))bis(methan-1-yl-1-yldiene)diphenol, EEZE ratio, 2.10:0.83: yield 80%. M.p. 159–161°C. Analysis of (C₂₇H₃₂Br₂N₂O₂): Calculated: C; 77.48%, H; 8.19%, N; 6.69%. Expt.: C; 77.42%, H; 8.25%, N; 6.65%. IR, cm⁻¹ (KBr): (N = C) 1628, (C = C) 1494, (C = O) 1282. ¹H NMR, δ in ppm (400 MHz, CDCl₃): 14.02 (br, 1H, O–H, ZE), 13.68 (br, 1H, O–H, EE), 8.30 (1H, CH = N–, ZE), 8.28 (1H, CH = N–, EE), 7.44–6.76 (m, 8H, Ar–H), 3.44 (1H, CH = N–, s, ZE), 3.07 (t, 1H, CH = N–, J = 11 Hz, EE), 1.77–0.88 (m, 20H, dicyclohexyl methane). ¹³C NMR (101 MHz, CDCl₃) in ppm: 161.37 (C–OH), 160.31 (C = N), 130.49–115.40 (Ar-5C), 67.17 (C–N), 43.42 (CH₂), 31.19–27.14 (cyclohexyl). UV–vis (λmax nm, DMF): 327, 417 nm. MS (m/z): 418.26. Found: 418.98 [M⁺].

CH₂–(C₂H₁₀–N = CH–C₆H₄–OH–OH₂–Br₂)₂(2), 2,2'-(1E,1’E)-(4,4'-methylenebis(cyclohexane-4,1-diyl))bis(azan-1-yl-1-yldiene))bis(methan-1-yl-1-yldiene)(4-bromo phenol), EEZE ratio, 2.05:2 yield 76%. M.p. 195–197°C. Analysis of (C₂₇H₂₈Br₂N₂O₂): Calculated: C; 67.20%, H; 7.10%, N; 5.81%. Expt.: C; 67.18%, H; 7.22%, N; 5.76%. IR, cm⁻¹ (KBr): (N = C) 1637, (C = C) 1508, (C = O) 1232. ¹H NMR, δ in ppm (400 MHz, DMSO-d₆): δ 13.72 (s, 1H, br O–H, ZE), 13.50 (s, 1H, br O–H, EE), 8.33 (s, 1H, CH = N–, ZE), 8.31 (s, 1H, CH = N–, EE), 7.09–6.17 (m, 4H, Ar–H), 3.68 (s, 1H, CH = N–, ZE), 3.44 (t, 1H, CH = N–, J = 11.6 Hz, EE), 2.51 (DMSO), 1.96–6.17 (m, 20H, dicyclohexyl methane). ¹³C NMR, δ in ppm (101 MHz, DMSO-d₆): 158.9 (C–O), 160.04 (C = N), 133.63–125.22 (aromatic 5C), 63.11 (C–N), 39.93 (CH₂), 34.01–28.28 (cyclohexyl). UV–vis (λmax nm, DMF): 295, 325 nm. MS (m/z): 482.24. Found: 482.97 [M⁺].

CH₂–(C₂H₁₀–N = CH–C₆H₄–Br₂–OH–OH₂)₂(3), 4,4'-((1E,1’E)-(4,4'-methylenebis(cyclohexane-4,1-diyl))bis(azan-1-yl-1-yldiene))bis(methan-1-yl-1-yldiene)dibenzened-1,3-diol, EEZE ratio, 1.0:0.0 yield 66%. M.p. 120–122°C. Analysis of (C₂₇H₃₀N₂O₄): Calculated: C; 83.89%, H; 8.87%, N; 7.25%. Expt.: C; 83.90%, H; 8.86%, N; 7.23%. IR, cm⁻¹ (KBr): (N = C) 1643, (C = C) 1448. ¹H NMR, δ in ppm (400 MHz, CDCl₃): 8.25 (s, 1H, CH = N–, EE), 7.65–7.18 (m, 10H, Ar–H), 3.08 (t, 2H, CH = N–, J = 11.2 Hz, EE), 1.78–0.90 (m, 20H, dicyclohexyl methane). ¹³C NMR, δ in ppm (101 MHz, CDCl₃): 159.35 (C = N), 136.92–128.55 (Ar-5C), 70.74 (C–N), 45.00 (CH₂), 34.21–31.97 (cyclohexyl). UV–vis (λmax nm, DMF): 272 nm. MS (m/z): 386.27. Found: 382.41 [M⁺].

CH₂–(C₂H₁₀–N = CH–C₆H₂–Br₂)₂(4), (NE,N'E)–4,4'-methylenebis(N-benzylidendicyclohexamethine mine), EEZE ratio, 1.0:0.0: yield 66%. M.p. 120–122°C. Analysis of (C₂₇H₃₂N₂O₂): Calculated: C; 83.89%, H; 8.87%, N; 7.25%. Expt.: C; 83.90%, H; 8.86%, N; 7.23%. IR, cm⁻¹ (KBr): (N = C) 1643, (C = C) 1448. ¹H NMR, δ in ppm (400 MHz, CDCl₃): 8.25 (s, 1H, CH = N–, EE), 7.65–7.18 (m, 10H, Ar–H), 3.08 (t, 2H, CH = N–, J = 11.2 Hz, EE), 1.78–0.90 (m, 20H, dicyclohexyl methane). ¹³C NMR, δ in ppm (101 MHz, CDCl₃): 159.35 (C = N), 136.92–128.55 (Ar-5C), 70.74 (C–N), 45.00 (CH₂), 34.21–31.97 (cyclohexyl). UV–vis (λmax nm, DMF): 272 nm. MS (m/z): 386.27. Found: 382.41 [M⁺].
namine), EE:ZE ratio, 0.96:0:06: Yield 63% M.p. 120–123°C. Analysis of (C27H32Br2N2): Calculated; C 59.57%, H 5.93%, N 5.15%. Expt.: C; 59.52%, H; 5.85%, N; 5.20%. IR, cm⁻¹ (KBr): (N = C) 1643, (C = C) 1485, (C – Br) 819. 1H NMR δ in ppm (400 MHz, CDCl3): 8.26 (s, 2H, CH=N=, EE and ZE), 7.51 (d, 4H, Ar-H), 7.61 (d, 4H, Ar-H), 3.38 (s, 1H, CH–N=, EE), 3.15 (t, 1H, CH–N=, J = 10.8 Hz, EE), 1.79–0.93 (m, 20H, dicyclohexyl methane). 13CN M R, δ in ppm (400MHz, CDCl3): 157.41 (C–N), 128.35–110.28 (Ar-5C), 69.37 (C–N), 123.61 (C–Br), 43.81 (CH2), 32.86–27.33 (cyclohexyl). UV–vis (λ max, nm, CH2–(C6H10–N–, EE)), 7.53-6.59 (m, Ar-H), 2.99 (t, 1H, CH–N=, J = 12.0 Hz, EE), 2.91 (s, 6H, Ar-N-(CH3)2), 1.79–0.91 (m, 20H, dicyclohexyl methane). 13CN M R, δ in ppm (101 MHz, CDCl3): 156.50 (C = N), 134.68–128.45 (Ar-5C), 69.39 (C–N), 123.61 (C–Br), 43.81 (CH2), 32.86–27.33 (cyclohexyl). UV–vis (λ max, nm, DMF): 274 nm MS (m/z): 544.09 Found: 544.32 [M]+.

CH2–(C6H10–N=CH–C6H2–N(CH3)2–4)2(8), 4.4’-(1E, 1’E)-(4,4’-methylenebis(cyclohexane-4,1-diyl))bis(azan-1-yl-1-ylidene)bis(methan-1-yl-1-ylidene)bis(N,N-dime thylaniline), EE:ZE ratio, 2.29:0.08: yield 42%. M.p. 180–185°C. Analysis of (C27H32Br2N2O2): Calculated: C; 77.78%, H; 9.38%, N; 11.85%. Expt.: C; 77.88%, H; 9.80%, N; 11.62%. IR, cm⁻¹ (KBr): (N = C) 1605, (C = C) 1527, (C–N) 1178. H NMR δ in ppm (400 MHz, CDCl3): 8.12 (2H, CH=N=, EE), 7.53-6.59 (m, Ar-H), 2.99 (t, 1H, CH–N=, J = 12.0 Hz, EE), 2.91 (s, 6H, Ar-N-(CH3)2), 1.79–0.91 (m, 20H, dicyclohexyl methane). 13CN M R, δ in ppm (400MHz, CDCl3): 157.41 (C = N), 150.85 (Ar-N), 128.35–110.28 (Ar-5C), 69.37 (C–N), 44.00 (CH3), 39.14 (CH2), 39.14–27.56 (cyclohexyl). UV–vis (λ max, nm, DMF): 295, 360 nm. MS (m/z): 544.09 Found: 544.32 [M]+.

2.3. X-ray determination

Single crystals for X-ray analysis were obtained by the slow evaporation of EtOH for compounds 2 and 7. The colourless crystals of the compounds with suitable dimensions were taken on a glass fibre for the X-ray study. Diffraction data were collected on a Bruker AXS Kappa Apex2 CCD diffractometer with crystal size as in Table 1. Data gathered, cell refinements, and data reduction were achieved using the CRYSALISPRO [34] and SAINT [35] software. Molecular graphics used include ORTEP [36] and PLATON [37] programs. The structures of both the SBs (2 and 7) were solved by the direct method by (SHELXL-97) [38]. Positional and anisotropic atomic displacement parameters were refined for all non-hydrogen atoms. Crystallographic data are available as supplementary publication number CCDC 1814237 and CCDC 1814231 for compounds 2 and 7. They can be obtained via http://www.ccdc.cam.ac.uk/conts/retrieving.html.

2.4. Docking studies and biological studies

The active site in the pocket of the protein was found based on the co-crystal structure and their critical amino acids are carefully chosen for our docking study by existing crystal structure. The p53 protein complex (PDB ID: 4OGN) and DHFR receptor (PDB ID:4DPD) were extracted from Protein Data Bank (www.rcsb.com) [39]. The protein complex was prepared by the protein preparation wizard in Maestro of Schrödinger software [40]. Addition of hydrogens and removal of water molecules beyond 5 Å from the active site of the protein using review and modify tool. The reviewed protein has been optimized and minimized using the refine tool. Force field OPLS-2005 used to optimize and minimize the protein until the root mean square deviation (RMSD) reached a value of 0.3 Å [41]. The 2D structures of compounds 1–8 were imported from the project table and the structures were minimized and geometrically refined using the Ligprep module [42]. Configurations were generated using torsional search method with distance-dependent dielectric solvation treatment and OPLS–2005 force field. The extra precision (XP) mode of docking was used to find the best fit molecules in the active site of p53 protein using Glide application of Schrödinger software suite [43]. Details of MTT assay;
cell growth inhibition assay and more are available in the same research paper [44].

2.5. Antimicrobial activity

The bacterial strains viz., Klebsiella pneumoniae, Salmonella Typhi, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli and fungal strains viz., Penicillium chrysogenum, Candida albicans, Aspergillus niger and Aspergillus flavus are obtained from (India), National Chemical Laboratory, Pune.

2.5.1. In vitro antifungal activity and antibacterial

The in vitro biological actions of the SBs were examined following our reported procedure [10].

3. Result and discussion

3.1. Chemistry

The chemical reaction of the obtained SB compounds is shown in Scheme 1. 4,4′-Methylenebis(cyclohexylamine) derivatives are important starting materials used for producing various heterocyclic compounds of biological importance [45]. Thus, these locations in the double branching scaffold could be helpful to produce a big structural diversity in drug motifs using heterocycles. They can also offer favourable biological properties. Therefore, we are interested in synthesizing a variety of new Schiff derivatives from the 4,4′-Methylenebis(cyclohexylamine) moiety.

In Scheme 1, compounds 1–8 were obtained via the condensation chemical reaction of various aldehydes and a commercially available 4,4′-Methylenebis(cyclohexylamine) in ethanol at 70–80°C for 4–5 h, without a catalyst to yield the corresponding SB with good yield. The structures of compounds 1–8 were established as EE and ZE isomeric forms by spectroscopic data (X-ray, IR, Mass, 1H, and 13C-NMR) and CHN elemental analysis. There are only two peaks in this spectrum with a different ratio for the hydroxyl groups, indicating two different isomers only. A change in the deuterated solvent from CDCl3 to DMSO-d6 led to the broadening of all peaks and, furthermore, the enhancement of the EE isomer over the ZE isomer in the 0.84:2 ratio respectively. In fact, there are three possible isomers in these compounds. EE, ZZ and ZE (ZE and EZ are the same). 1H NMR shows two sets of signals which means we have two isomers Figure 2. 1H shows a singlet and triplet indicating a different environment in one case due, probably, to the existence of ZE. The X-ray, single crystal structure confirms the EE isomer, Figures 3 and 4. The three isomers EE, ZZ and ZE were energetically minimized to give total calculated minimize energy (MM2) using Chem. 3D, minimum RMS gradient of 0.01 kcal/Å mol, 21.22, 50.26, 36.11 kcal/mol respectively. This indicates that ZZ isomer is the least favoured isomer whereas EE is the most favoured isomer followed by ZE isomer. This is in agreement with the fact that compounds 2 and 7 were easily crystallized and their structures were measured by single-crystal X-ray crystallography technique. All obtained characterization results are in agreement with the molecular formula of the obtained molecules. The IR spectra of compound 7 exhibited a sharp band at 1641 cm⁻¹ which is due to the azomethine (−CH=N) group. The clear absence of bands at 1720 and 3300 cm⁻¹ for the carbonyl stretching and −NH₂ stretching of amine and aldehyde respectively proves the complete condensation of the aldehydes and amine. Furthermore, the target compound was established by the presence of Br stretching frequency at 826 cm⁻¹. In general, the distinctive feature of the Infrared absorption bands of the free ligands with the wavenumbers 1643–1605 cm⁻¹ due to the C=O stretching vibration. A minor broad-band for the −OH in compounds 3–5 is comparable to those large broad bands for compounds 1 and 2 due, possibly, to the absence of participation of the m-OH and/or p-OH in the intra-molecular hydrogen bonding. The presence of intra-molecular H-bonding in 2 between the amine o-OH and −CH=N is, additionally established by the X-ray crystallography structure, Figure 3. X-ray analysis for compound 2 displays enol−imine tautomeric forms, Scheme 2. This is important because it means that the OH group is available for better binding in the biological system [13]. The 1H NMR spectra for 1–3 and 5 show downfield peaks for −OH (13–14.6 ppm) presenting the ratio of EE to ZE isomers based on proton integration, Table 2 and Figure 2. Compound 6 is only EE isomer, whereas 7 and 8 are mixtures showing a negligible amount of ZE. Compounds 2 and 7 are mixtures as well but EE isomer is dominant. The X-ray structures of 2 and 7 confirm the EE isomer, Figures 2 and 4. This indicates that EE isomer is a favoured conformation because, most likely, of the steric hindrance [46].

The 1H NMR spectra (for 1–8, except 6) show two signals appearing as a singlet around 9 ppm which are attributed to 13C=N (EE and ZE geometric isomers) in addition to aromatic protons at 6.5–7.5. The cyclohexyl group and CH₂ appear like a complex up-field the spectrum, Figure 2.

It is interesting to mention here that an E- and Z-isomers of other compounds, have been isolated and examined for their pharmacological activities; only relatively small differences in the biological activities of the isolated isomers were confirmed [47].

3.2. Single-crystal-XRD study of compounds 2 and 7

The ORTEP diagram of compounds 2 and 7 are shown in Figures 3 and 4. All bond lengths for compound 2 are within the expected ranges [48,49]. Compound 2
lies about a twofold symmetry axis, with C(15) lying on this axis. The bond length of C8-N1 (1.255(9)Å) is within C=N double bond values, while the bond length between C5-N1 (1.468(11)Å) is within C=N single bond values. Moreover, the bond length of C14 and O1 (1.325(10) Å) conform to the value for a carbon–oxygen single bond. The torsion angles of C9–C8–N1–C5, C6–C5–N1–C8, N1–C8–C9–C14 and C6–C5–N1–C8 are −177.0 (6) Å, 99.3 (9) Å, 176.5 (6) Å, and, 138.0 (9) Å, respectively. Besides, the compound 2, all the phenyl and cyclohexyl rings are almost lie in a plane and both cyclohexyl rings exhibit a perfect chair conformation, as well as the substituents, are occupied in the equatorial positions of both cyclohexyl rings. The dihedral angle between the two rings (amine and 2-hydroxy 5-bromo phenyl ring) is 147°. This is in conjunction with the strong intra-molecular H-bonding distance between O(1)-H(1A)...N(1) is 2.585(9) Å. Moreover, compound 7 analyses were done by taking 2 as the representative compound.

**Figure 2.** $^1$H NMR for compound 2 demonstrating the two isomers (EE and ZE).

**Figure 3.** ORTEP View of CH$_2$-(C$_6$H$_{10}$-N = CH-C$_6$H$_3$-OH-2,Br-5)$_2$ 2, showing the atom-numbering scheme. The intra-molecular H-bonds are displayed using dash line. The probability level, Thermal ellipsoids, is drawn at 30%. “a” and “b” are used to distinguish between the carbons of the disordered cyclohexyl group.
Figure 4. ORTEP View of the molecule of \( \text{CH}_2-(\text{C}_6\text{H}_{10}-\text{N}=\text{CH}-\text{C}_6\text{H}_3-\text{Br}-4)_2 \), indicating the atom-numbering scheme; Thermal ellipsoids are drawn at the 30% probability level.

Scheme 2. The likely pseudo-aromatic six membered ring involving hydrogen bonding.

Table 2. The EE and ZE ratio based on \(^1\)H NMR integration.

| Compound | Rate  | Compound | Rate  |
|----------|-------|----------|-------|
| \( (\text{EE and ZE}) \) 1 | 2.10:0.83 | \( (\text{EE and ZE}) \) 5 | 1.00:0.76 |
| \( (\text{EE and ZE}) \) 2 | 2.00:0.52 | \( (\text{EE and ZE}) \) 6 | 1.03:0.00 |
| \( (\text{EE and ZE}) \) 3 | 1.00:0.74 | \( (\text{EE and ZE}) \) 7 | 0.96:0.06 |
| \( (\text{EE and ZE}) \) 4 | 2.12:0.82 | \( (\text{EE and ZE}) \) 8 | 2.29:0.08 |

3.3. Hirshfeld surface analysis (HSA)

The Hirshfeld surface analysis (HSA), mapped over \( d_e \) and \( d_i \) shape index plots, are shown in Figures 6 and 7 for compounds 2 and 7 using Crystal Explorer 17 which can be obtained from Crystal explorer website [50]. This program offers further understanding of the weak inter-molecular interactions influential in the packing of molecules in crystals. This is in agreement with data obtained from the XRD analysis, Figure 5. The blue and red colours specify the positive and negative potentials, respectively, Figure 6(a) and Figure 7(a). The negative electrostatic potential is usually interrelated with the lone pair of electronegative atoms. The HS utilizes the function of normalized distances mapped over \( d_e \) and \( d_i \), where \( d_e \) and \( d_i \) are the distances from a given point on the surface to the nearest atom outside and inside, respectively [51]. HSA mesh diagrams are provided to show additional insight into the actual lay down of the molecules Figure 6(b) and Figure 7(b). The relative contributions of different atomic contacts to the HS are shown in Figure 8. The H...H interaction is found to be dominating in compound 7, accounting for 54.9% of HS area, whereas H...C/C...H, H...Br/Br...H contacts accounted for 20.8% and 18.0% of HSs area respectively Figure 8. The H...H interaction is found to be less dominating in compound 2, accounting for 23.8% of the HS area. Also, H...C/C...H, H...Br/Br...H contacts, unexpectedly accounted much less for 4.2% and 0.2% of HSs area respectively.

Although O...H/O...O contact plays a critical role in compound 2, (38.3%), it is totally absent in compound 2, enol-imine form 2, keto-amine form (not observed)
Figure 6. (a) Hirshfeld surfaces views of the molecule CH$_2$–(C$_6$H$_{10}$–N = CH–C$_6$H$_3$–OH–2,Br–5)$_2$ 2 mapped over $d_i$ (the internal distance) and $d_e$ (the external distance from the HS) with enabled surface transparency. The red and blue regions represent negative and positive electrostatic potentials, respectively. (b) Mesh diagram of 2 showing the lay down of the molecule with the electron density around it.

Figure 7. (a) Hirshfeld surfaces views of CH$_2$–(C$_6$H$_{10}$–N = CH–C$_6$H$_3$-Br–4)$_2$ 7 mapped over $d_i$ (the internal distance) and $d_e$. (the external distance) with enabled surface transparency. The red and blue regions represent negative and positive electrostatic potentials, respectively to the Hirshfeld surface. (b) Mesh diagram of 7 showing the lay down of the molecule with the electron density around it.

Figure 8. Two-dimensional fingerprint plots for compound 7, (a) Total, (b) H...All, (c) H...H, (d) H...C, (e) H...Br, and (f) H...N. The outline of the full fingerprint is shown in grey. $d_i$ is the closest internal distance from a given point on the Hirshfeld surface and $d_e$ is the closest external contacts.
7. The same for N...H/H...N in 2 is high whereas weak in compound 7. In both compounds 2 and 7, C...C interaction is 0.0% indicating negligible π−π stacking interaction. Accordingly, HS results are in agreement with the XRD packing analyses. Table 3 summarizes the relative contributions of internal and external atomic contacts to the HS for 2 and 7 compounds.

3.4. In silico molecular docking studies

To justify the potency of the active compounds (1–8) as anti-cancer and antimicrobial agents, compounds (1–8) were docked against p53 protein (PDB ID: 4OGN) and P. Falciparum dihydrofolate reductase (pf-DHFR) (PDB ID: 4DPD) downloaded from protein data bank (RCSB). The interactions between 4OGN and 4DPD and (1–8) are tabulated in Tables 4 and 5. From docking simulation analysis of p53 protein, the docking scores of compounds 1–8 vary from −8.190 to −5.380, Tables 4 and 5. The evdw and ecoul are the expected van der Waals’ interaction and electrostatic interaction energies between the protein and the ligand. All the synthesized Schiff derivatives have a smooth sliding score, with substantial interaction between the crucial residue of HIE 96 (except compounds 2 and 5) and compounds 1 (TYR 676, LEU 54, THR 15), 2 (SO4 202 (OH & N)), 3 (VAL 93) and 7 & 8 (TYR 63). Moreover, compounds 1, 3, 4, 6, 7, and 8 show good p−p interaction with crucial residues of HIE 96, which is crucial for inhibitory affinity with p53 [7,39]. These π−π stacking interactions were accomplished by the phenyl ring (docking score).

between the p53 and some compounds, and that of a smaller group on the hydroxyl functionalized azomethine shows beneficial for the binding efficacy. Furthermore, the structural examination highlighted that the existence of a hydroxyl-functionalized moiety in the SB confirms the increase in binding ability (−7.741 to −9.148) Figure 9.

Table 4. Docking outcomes for compounds 1–8 with the MDM2 protein (PDB ID: 4OGN).

| Comp | XP GScore | XP HBond | Glide evdw | Glide ecoul | XP Penalties | Interacting Residues |
|------|-----------|----------|------------|-------------|--------------|----------------------|
| 1    | −5.883    | 0.7      | −36.219    | −1.433      | 2.5          | TYR 676, LEU 54, THR 15
| 2    | −7.058    | 0.747    | −31.106    | −11.7       | 0            | SO4 202 (OH & N)
| 3    | −7.037    | 1.33     | −28.633    | −9.056      | 0            | H2O, HIE 96
| 4    | −6.233    | 0.7      | −29.783    | −2.756      | 0            | H2O, HIE 96
| 5    | −8.910    | 3.36     | −24.264    | −16.406     | 0            | VAL 93, H2O
| 6    | −5.727    | 0.7      | −28.042    | −3.151      | 0            | H2O, HIE 96
| 7    | −5.380    | 0        | −36.336    | 3.434       | 0            | HIE 96, TYR 63
| 8    | −5.380    | 0        | −33.033    | −1.866      | 0            | HIE 96, TYR 63

Table 5. Docking results for compounds 1–8 with the dihydrofolatereductase (DHFR) receptor (PDB ID: 4DPD).

| Comp | XP GScore | XP HBond | Glide evdw | Glide ecoul | XP Penalties | Interacting Residues |
|------|-----------|----------|------------|-------------|--------------|----------------------|
| 1    | −4.348    | 1.237    | −44.927    | −1.118      | 3            | THR 107, ILE 164
| 2    | −4.709    | 0.7      | −43.48     | −5.511      | 0            | SER 108
| 3    | −4.163    | 1.821    | −36.397    | −9.494      | 0            | SER 108, TYR 170, ALA 16
| 4    | −7.390    | 1.73     | −28.922    | −11.693     | 0            | THR 130, ARG 129, SER 129, ARG 106, SER 108
| 5    | −9.081    | 4.599    | −37.664    | −15.052     | 0            | SER 111, PHE 196
| 6    | −8.137    | 2.502    | −41.888    | −13.902     | 3            | TYR 170, ALA 16, ARG 106, SER 128, ARG 129, THR 130
| 7    | −0.255    | 0        | −28.525    | 2.066       | 4            | –
| 8    | −3.028    | 0.9      | −31.651    | −3.203      | 0            | ASP 194

3.5. Biological studies

3.5.1. In vitro cytotoxic effect on human cancer cell lines HT-1080 and HeLa

The anticancer activities of the novel compounds were tested for their cytotoxic activity against two different cell lines: a human fibrosarcoma cell line (HT-1080) and a human cervical cancer cell line (HeLa) at several concentrations viz. 5, 10, 20, 30, 40, 50, and 60 μg mL\(^{-1}\), with 5-fluorouracil taken as a standard drug, and the IC\(_{50}\) results are tabulated in Table 6. Figure 10 shows the inhibitory effect of 1–8 on HT-1080 and HeLa cancer cells after 24 and 48 h, respectively. The screening results showed that the tested compounds revealed moderate/good antitumor activity against HT-1080 and HeLa cell lines.

Remarkably, the highest activity in fibrosarcoma cancer cells is displayed by compound 5 in the tested cells with IC\(_{50}\) values 17 ± 0.16 for 24 h and 11 ± 0.83 μg mL\(^{-1}\) for 48 h respectively. This could be due to a large number of hydroxyl groups in compound 5. The compounds 1, 2, 3, 4 and 5 with hydroxyl substitution pattern displayed admirable anti-cancer activity when compared to the standard drug 5-fluorouracil. Whereas the rest of the compounds, 7 and 8 displayed slightly decrease anti-cancer potency when compared to other compounds. This, probably, shows that the hydroxy substitution (−OH) in the SB moiety is significant to improve the anti-cancer activity [53]. Also, in the vitroantitumor evaluation of the synthesized compounds was carried out, with compounds 1–8 being screened for anti-cancer activity using HeLa tumour cells, with 5-fluorouracil as the standard drug. The topmost activity in cervical cancer cells was again shown by compounds 5 and 6 (IC\(_{50}\) = 14 ± 0.40 and 13 ± 0.36 after 24 h and 08 ± 0.36 and 07 ± 0.22 after 48 h μg mL\(^{-1}\)) compared to the standard drug and compounds 1 and 4 (IC\(_{50}\) = 20 ± 0.25 & 819 ± 0.22 for 24 h...
Figure 9. Docking poses for the interactions of (A) compound 5 with the P53 protein (PDB ID: 4OGN) and (B) compound 5 with the DHFR receptor, (PDB ID: 4DPD).

Table 6. IC50 values (expressed in μg/mL) of compounds 1–8 against fibrosarcoma tumour (HT-1080) and cervical tumour (HeLa).

| Comp | IC50 (μg/mL) | HT-1080 cells | HeLa cells |
|------|-------------|---------------|-------------|
|      | 24h | 48h | 24h | 48h | 24h | 48h | 24h | 48h |
| 1    | 23 ± 0.36 | 13 ± 0.16 | 20 ± 0.25 | 11 ± 0.80 |
| 2    | 25 ± 0.48 | 14 ± 0.08 | 21 ± 0.12 | 12 ± 0.30 |
| 3    | 20 ± 0.32 | 12 ± 0.16 | 22 ± 0.80 | 11 ± 0.48 |
| 4    | 22 ± 0.66 | 13 ± 0.36 | 19 ± 0.22 | 10 ± 0.8 |
| 5    | 17 ± 0.16 | 11 ± 0.83 | 14 ± 0.40 | 08 ± 0.36 |
| 6    | 20 ± 0.80 | 12 ± 0.80 | 13 ± 0.36 | 07 ± 0.22 |
| 7    | 34 ± 0.80 | 26 ± 0.20 | 39 ± 0.10 | 30 ± 0.70 |
| 8    | 26 ± 0.16 | 17 ± 1.0 | 25 ± 0.24 | 17 ± 0.45 |
| 5-Fluouracil | 25 ± 0.065 | 14 ± 0.032 | 20 ± 0.01 | 11 ± 0.016 |

and 11 ± 0.80 & 10 ± 0.8 for 48 h μg mL⁻¹) also exhibited good activities.

Moreover, compounds 1 and 4 have revealed similar activity to the reference drug, whereas the rest of the SBs have revealed lesser effectiveness compared to other derivatives, even at the maximum concentration. According to this data, it has been indicated that compounds 5 and 6 were the best among all the examined cancer cell lines and compound 4 also displayed better activity against two cancer lines.

The structural activity profile demonstrations that –OH substitution in the SB is clearly indicated the significance for improving the anti-cancer activity. Also, the tri-hydroxyl substituted SB compound 5 (IC50 = 17 ± 0.16 mg mL⁻¹) was found to be stronger when compared with compound 3 (IC50 = 20 ± 0.32 mg mL⁻¹) against the HT-1080 cell line. However, the replacement of the –OH group at the R3 position in the SB in 4 (IC50 = 22 ± 0.66 mg mL⁻¹) by a halogen (–Br) in compound 7 (IC50 = 34 ± 0.80 mg mL⁻¹) led to decreased activity. The results show that the bromine atom does not affect cytotoxic activity.

3.5.2. In silico cytotoxic effect on human cancer cell lines

Predictions of the cytotoxic effects of the SB derivatives against various cancer cell lines have also been accomplished using the Drug2Way and PASS online software [54,55]. In addition to the above findings from the in vitro, the preliminary in silico studies of 1–8 and the standard compound (5-Fluouracil) may act as a guideline to facilitate the design of more effective analogs and lower side effects for the treatment of drug-resistant cancer. SB 1–6 derivatives demonstrated activities similar to glucan endo-1,6-beta-glucosidase and...
ubiquinol-cytochrome-c reductase (UQCRH) inhibitors at Pa > 0.7, (SI, Table 1). Whereas, SBs 2 and 7 an exhibited expression enhancer called HMGCS2 (3-hydroxy-3-methylglutaryl-CoA synthase 2). Compound 6 showed activity similar to nicotinic receptor, antagonist and phobic disorders treatment (SI, Table 1). No biological activity was predicated for all SB derivatives on non-tumour cells using PASS at Pa > 0.7 scale (SI, Tables 2a and 2b). However, 5-Fluorouracil showed activities similar to antineoplastic, pterin deaminase, testosterone 17beta-dehydrogenase (NADP+) dihydropyridine, dehydrogenase and antieczematic and more inhibitors, (SI, Table 3b).

Predictions of the cytotoxic potential of the SB derivatives and 5-Fluorouracil was also performed using Drug2Way [55] on cancer cell-line. The prediction was carried out at Pa > 0.3 and Pa > Pi scales. Cytotoxicity for BS derivatives 1–6 was shown on cell lines Hs 683 (Oligodendroglioma) and MDA-MB-453 (breast adenocarcinoma). No results of activity at Pa > 0.3 on the non-tumour cell was observed for 1–6. However, activity predicted on non-tumour when Pa > Pi for compounds for all the SBs 1–8 ranged from 0.132 to 0.189 on cell line prostate epithelial cell (PrEC) (SI, Table 3b). Cytotoxicity at low level also on WIL2-NS (Lymphoblastoid cell) for compounds 1 and 5. In addition, low predicted cytotoxicity is shown on embryonic lung fibroblast (WI-38 VA13, IMR-90 and WI-38) for compounds 4, 6 and 8. Cytotoxicity of compounds 2 and 3 was predicted on non-tumour cell lines keratinocyte (Hacat) and embryonic kidney fibroblast (HEK293), respectively, (SI, Table 3b). Cytotoxicity of the standard, 5-Fluorouracil was also performed at Pa > 0.3 and Pa > Pi scales with both tumour and non-tumour cell lines (SI, Tables 4a, 4b, 5a and 5b). Results showed cytotoxicity on different carcinoma cell lines for renal, pancreatic, non-small cell lung, breast, melanoma and childhood T acute lymphoblastic leukaemia. Cytotoxicity was also predicated for 5-Fluorouracil on non-tumour cells such as fibroblast (NHDF) and keratinocyte (HaCaT) cells (SI, Table 4b).

3.5.3. Antimicrobial activity

The prepared SB compounds were screened for their antibacterial efficacy in vitro against a spectrum of Gram-positive and Gram-negative pathogenic bacteria including resistant strains viz. Streptomycin resistant and sensitive Klebsiellapneumoniae, SalmonellaTyphi, Staphylococcus aureus, Bacillus subtilis, Pseudomon as aeruginosa and Escherichia coli following the reported precedent by Dhar et al., and their MIC values are shown in Table 7. A glance at the MIC values in Table 6 indicates that among the Schiff derivatives (compounds 1–8), compound 5 with three hydroxyls functionalized SB showed superior activity against all the bacterial strains except E. coli which were used for this study.

Moreover, compounds 1, 2 and 6 were the doubly active and effective against one bacterial strains, in which compound 1 against S. Typhi, compound 2 against K. pneumoniae, B. subtilis and E. coli, compound 6 against B. subtilis and E. coli as compared with reference drug, whereas other compounds 1, 2, 4, 6, 7, and 8 were equally active against, in which compounds 2, 4, 6, and 7 against S. Typhi, compound 8 against S. Aureus, compounds 4, and 8 against B. subtilis, compound 1 against E. Coli, respectively. The exciting point is that in the bacterial strain, most of the synthesized compounds revealed reasonable inhibitory activities against K. pneumoniaeand less active against P. aeruginosa except compound 5 which were used for this study. Among SBs (1–8), the para-substituted hydroxyl analogy compounds (3, 4 & 5) shown good antibacterial activity (12.5 μg/mL) against K. pneumoniae, respectively, but compound 2 exhibited one-fold
decreased activity against the same strains. This may be due to the hydroxyl with bromo substitutions at ortho and meta-position (R & R3). In general, all the compounds showed higher antibacterial activity towards *K. pneumoniae* and *B. subtilis*, respectively.

Besides, antifungal activities of compounds 1–8 were also screened and their MIC values are listed in Table 8. In this study, Amphotericin B was considered as standard drug. Amongst all the tested compounds 2 against *P. chrysogenum* (6.25μg/mL), *A. niger* (12.5μg/mL) and *A. Flaves* (12.5μg/mL) exhibited significant antifungal activity when compared with the reference drug. The compound 2 having hydroxyl with bromo-substituent on the phenyl ring displayed greater activity than those with methyl and bromo-substituents (compounds 7&8). This may be because of the presence of a more electron-negative bromo atom with a hydroxyl group in the aromatic ring which may enhance the biological effect, metabolic stability lipophilicity and bioavailability. Boosted lipophilicity may lead to easier absorption and transportation of molecules within the biological systems [56,57]. Moreover, compound 8 exhibited less/or no activity in most of the tested fungal strains. This may be because of +M effect of methyl substituent. However, the other compounds displayed modest to good activity. In addition, compound 5 exhibited superior activity against *P. chrysogenum* and *C. albicans* compound 4 showed one fold increased activity against *A. niger* and compounds 3, 4, 6, and 8 comparable activities against *P. chrysogenum* compound 1 against *C. albicans* compounds 3 and 5 against *A. niger* compounds 1, 6, and 7 against *A. Flaves* than the reference streptomycin drug. Overall, all the SB compounds displayed higher antifungal activity towards *P. chrysogenum* except compounds 1 and 7.

The structural activity profile shows that de-protected –OH group and electron-withdrawing (bromo) substitution in the SB ring is important for enhancing the antifungal activity. Besides, the bis-hydroxyl substituted SB compound 5 (MIC’s = 6.25 μg/mL & 100 μg/mL) was found to be more potent when compared with compound 3 (MIC’s = 12.5 μg/mL & 6.25 μg/mL) against the *P. chrysogenum* and *C. albicans* strain. It has been noticed that boosted antitumor (antifungal) activity was established by the additional functionalization of the hydroxyl group in the SB moiety.

4. Conclusion

In this study, several bis-cyclohexyl SBs were synthesized and characterized by various analytical and spectral techniques. The crystal structures of 2 and 7 have been measured and demonstrated the presence of intra-molecular H-bonds with enol–imine tautomeric forms in ZE and EE isomers at different ratios. Hirshfeld Surface Analysis is in support of XRD analysis. *In vitro* anticancer activity against human cancer cell lines: human fibrosarcoma (HT-1080) and cervical cancer cells (HeLa) activity and antimicrobial activity concerning Gram-negative and -positive fungal strains and bacterial of structurally related novel SBs were examined. SB Ligand 5 which possess three hydroxyl group was found to be an outstanding docking agent for p53-MDM2 and pf-DHFR. While the SB 5 and 6 revealed modest activity against cervical cancer (HeLa) cell lines. The antimicrobial results displayed that the newly prepared

Table 7. Antibacterial activities of compounds 1–8 for bacterial strains in MIC (μg/mL).

| Compounds | *K. pneumoniae* | *S. typhi* | *S. aureus* | *B. subtilis* | *P. aeruginosa* | *E. coli* |
|-----------|-----------------|-----------|-------------|---------------|----------------|---------|
| 1         | 50              | 25        | 200         | 200           | 200            | 12.5    |
| 2         | 25              | 50        | 100         | 6.25          | 200            | 6.25    |
| 3         | 12.5            | 200       | 200         | –             | –              | 50      |
| 4         | 12.5            | 50        | 100         | 12.5          | 50             | 25      |
| 5         | 12.5            | 25        | 25          | 6.25          | 12.5           | 50      |
| 6         | 25              | 50        | 200         | 6.25          | 50             | 6.25    |
| 7         | 100             | 50        | 200         | 25            | 100            | 50      |
| 8         | 200             | 200       | 50          | 12.5          | 50             | –       |
| Streptomycin | 50              | 50        | 50          | 12.5          | 50             | 12.5    |

Note: (–) No Inhibition; Control – DMSO.

Table 8. Antifungal activities of compounds 1–8 for bacterial strains in MIC (μg/mL).

| Compounds | *P. chrysogenum* | *C. albicans* | *A. niger* | *A. Flaves* |
|-----------|-----------------|---------------|------------|-------------|
| 1         | 100             | 25            | 100        | 50          |
| 2         | 6.25            | 50            | 12.5       | 12.5        |
| 3         | 12.5            | 100           | 25         | –           |
| 4         | 12.5            | 50            | 12.5       | 200         |
| 5         | 6.25            | 6.25          | 25         | 100         |
| 6         | 12.5            | 50            | 100        | 50          |
| 7         | 25              | 50            | 100        | 50          |
| 8         | 12.5            | –             | –          | 100         |
| Amphotericin-B | 12.5            | 25            | 25         | 50          |

Note: (–) No Inhibition; Control – DMSO.

| Compound | R | R’ | R” | R”’ |
|----------|---|----|----|-----|
| 1        | OH| H  | H  | H   |
| 2        | OH| H  | H  | Br  |
| 3        | OH| H  | OH | H   |
| 4        | H | H  | OH | H   |
| 5        | OH| OH | OH | H   |
| 6        | H | H  | H  | H   |
| 7        | H | H  | Br | H   |
| 8        | H | H  | N(CH3)2 | H |
SB compounds have shown moderate/good inhibitory activity against the tested bacterial and fungal strains. Thus, the present work reports the role of hydrogen bond donor and acceptor groups, steric hindrance and effects of substituents in the core structure of the molecule exhibit a better target, precise potential drug molecule.

Acknowledgment

MAS is thankful to AvH and Dr David L. Hughes, UEA, U.K.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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