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Molecular docking study, synthesis and biological evaluation of Mannich bases as Hsp90 inhibitors

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ABSTRACT

The ubiquitously expressed heat shock protein 90 is an encouraging target for the development of novel anticancer agents. In a program directed towards uncovering novel chemical scaffolds against Hsp90, we performed molecular docking studies using Tripos-Sybyl drug designing software by including the required conserved water molecules. The results of the docking studies predicted Mannich bases derived from 2,4-dihydroxy acetophenone/5-chloro 2,4-dihydroxy acetophenone as potential Hsp90 inhibitors. Subsequently, a few of them were synthesized **(1–6)** and characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral analysis. The synthesized Mannich compounds were evaluated for their potential to suppress Hsp90 ATPase activity by the colorimetric Malachite green assay. Subsequently, the molecules were screened for their antiproilferative effect against PC3 pancreatic carcinoma cells by adopting the 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay method. The activity profile of the identified derivatives correlated well with their docking results.

1. Introduction

The 90 kDa, heat shock protein (Hsp90) is an important member of a class of proteins known as molecular chaperones [1]. These chaperones play a critical role in maintaining the structure and function of other "client" polypeptides within a cell. Among all the chaperone proteins, Hsp90 has emerged as an attractive target for cancer chemotherapy because of its involvement in the repair of multiple oncogenic proteins [2,3]. Furthermore this protein is over expressed in carcinoma cells in association with co-chaperones whereas Hsp90 of normal cells resides in a free uncomplexed form [4,5]. Moreover several proteins responsible for the development of resistance to antineoplastic agents are also customers of Hsp90. Hence inhibitors of this chaperone are considered to be broad spectrum in activity with fewer toxic side effects and reduced liability for acquired drug resistance [6,7]. The healing function of Hsp90 is guided by hydrolysis of ATP to ADP within the N-terminal domain of the protein. Blocking of this ATPase activity leads to attenuation of Hsp90 function [8]. Hence, majority of Hsp90 inhibitors under preclinical and clinical investigation competitively dock to this cleft of the protein [9,10]. The druggability of Hsp90 chaperone was first established in 1994 using the natural product geldanamycin [11]. Since then 17 agents have entered various phases of clinical trials. However till date none of them were approved for human use by any regulatory agencies [12]. Hence rational design of novel class of compounds against Hsp90 is the need of the hour. Furthermore an enormous amount of time and capital is involved in bringing a new molecule to the market. Hence cost effective discovery of small molecular weight Hsp90 inhibitors remains an active field of research and has been reviewed extensively.

The dihydroxy phenyl scaffold was found to be involved in prominent hydrogen bonding and hydrophobic interactions with various amino acid residues at the N-terminal ATP binding cleft of Hsp90 [13–16]. Furthermore, the study of structure activity relationship for Hsp90 inhibitors indicated that removing any one of the hydroxyl group leads to molecules with much lesser binding efficiency [17,18]. Moreover, the resorcinol moiety is suggested to

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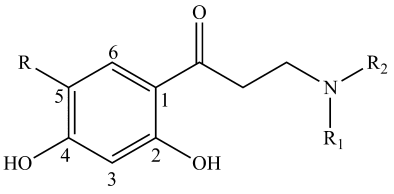


Fig. 1. General structure of Mannich bases synthesized with numbering system used in this work.

be devoid of any toxic adverse effects commonly associated with other fragments like quinone, halopyrimidine, diiodo phenyl, etc. [19]. Literature survey on anticancer agents revealed that Mannich bases possess antiproliferative properties with no concrete evidence of their mechanism of killing the cancer cells [20–28].

Structure based drug design is an efficient approach that predicts the activity of molecules within a short span of time [29]. In view of the fact that dihydroxy phenyl group is an established template for Hsp90 antagonism and considering the importance of Mannich bases in cancer chemotherapy, we performed structure guided drug designing via molecular docking studies for a series of 2,4-dihydroxy acetophenone and 5-chloro-2,4-dihydroxy acetophenone derived Mannich bases (Fig. 1). The ligands (**1–6**) which fared well in the docking program were synthesized and characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic studies. The malachite green colorimetric assay was used to measure the extent of Hsp90 ATPase activity attenuation by our synthesized compounds. This assay accurately determines the amount of free inorganic phosphate liberated due to ATP hydrolysis and is based on the reaction of phosphomolybdate complex with malachite green. The synthesized dihydroxy phenyl propanone analogues were further screened for their antiproilferative effect on PC3 prostate cancer cell lines by utilizing the well-established 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. The results of the present studies open the possibility to expand the molecular diversity space of Hsp90 inhibitors.

2. Materials and methods

2.1. Structure based drug design

The receptor based drug designing process was achieved with Surflex Geom X docking tool of Sybyl X-1.2 version software installed on a Dell Precision T-1500 workstation. A co-crystal structure of 4-{[(2R)-2-(2-methylphenyl)pyrrolidin-1-yl]carbonyl}benzene-1,3-diol and human Hsp90 submitted to protein data bank (PDB ID: 3EKR, resolution = 2 Å) was utilized for the docking procedure [30]. The four conserved waters (902, 903, 981 and 1026) were retained during protein preparation as they provide key interactions in stabilizing the ligand at the active site [31,32]. This was followed by addition of hydrogens for the amino acid residues and waters. The AMBER7 FF99 force field was utilized for the purpose of energy minimization which was followed by protomol generation [33]. A protomol is an idealized representation of a ligand that makes every potential interaction with protein's binding site. The protomol was created by extracting the original ligand (PDB ID: 3EKR) with the help of MOLCADD program of the software. A threshold [Threshold is a factor (from 0 to 1) determining how much the protomol can be buried in the protein. Increasing this number decreases the volume] of 0.5 and a bloat (bloat is used to inflate the protomol and include the nearby crevices) of 0 was maintained during the process of protomol generation [13]. The Mannich bases were first drawn in Chem draw ultra 8.0 and exported as mol files to Maestro, 9.1 versions of Schrodinger software for converting into Sybyl compatible SD file format. Subsequently, a clean, energy minimized, 3D conformation of the ligands were generated using Concord program (random perturbation of the position and conformation of the supplied ligand followed by bump relaxation) and filtered based on drug-likeness. Finally, 50 recacetophenone/5-chloro resacetophenone derived Mannich compounds were docked at the virtual active site via Geom X method by considering 20 poses per ligand [34–36]. The binding affinity of the ligands is predicted by the software in terms of total score which is expressed as—log K_d, where K_d is binding constant. A high value of total score indicates good protein–ligand binding.

2.2. Synthesis of compounds

All the chemicals and organic solvents used in the synthesis of compounds were purchased from Hi-media Laboratories Private Limited, Merck specialties Private Limited and SD fine-chem limited. The reactions were monitored by analytical thin layer chromatographic analysis (stationary phase: florescent F_{254} containing silica gel 60 G coated on aluminium sheets; mobile phase: 10% methanol in DCM). Uncorrected melting point values were determined by using a DRK Digital melting point apparatus. Infra red (IR) spectra were recorded in powder form on Shimadzu IR-Affinity spectrophotometer using Shimadzu diffuse reflectance attachment (DRS-800). ^{1}H NMR (300/400 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Bruker Avance instrument. Chemical shift values are reported in parts per million (δ, ppm) downfield from tetramethyl silane (TMS) as an internal reference standard and coupling constants (J) are given in Hz. Mass spectra were run using a atmospheric pressure-electron spray ionization 6120 Quadrupole LC/MS mass spectrometer (Agilent Technologies, California, USA).

2.2.1. Preparation of 5-chloro resacetophenone [37]

12.7 g (0.0040 mol) of freshly fused anhydrous zinc chloride is dissolved with the aid of heat in 6 ml of glacial acetic acid in a beaker. This was followed by addition of 5 g (0.0340 mol) of 4-chloro resorcinol to the hot solution with constant stirring. The reaction mixture is then heated on a sand bath until it just begins to boil (about 120 °C). The flame is then removed and the reaction is allowed to complete itself at a temperature not exceeding 140 °C. The reaction mixture was allowed to stand on the sand bath for 20 min without heating. This was followed by addition of 25 ml of 0.1N hydrochloric acid to the reaction mass. The resulting dark red solution upon cooling to 5 °C resulted in the formation of a precipitate which was filtered and washed with dilute hydrochloric acid. It was further purified by column chromatography (ethyl acetate/petroleum ether solvent system as the eluent and 60–120 mesh size silica gel as the stationary phase) to obtain light brown color product (Fig. 2).

Resacetophenone was procured from commercial vendors.

2.2.2. General procedure for the synthesis of dihydroxy phenyl propanone Mannich base [38]

A mixture of the resacetophenone/5-chloro resacetophenone (0.0054 mol), paraformaldehyde (0.0059 mol) and appropriate secondary amine salts (0.0054 mol) was transferred to a round bottom flask. To it 30 ml of 95% ethanol was added followed by addition of 1–2 drops of concentrated hydrochloric acid. The reaction mixture was then refluxed for 3 h. The completion of the reaction was ascertained by ascending thin layer chromatographic technique. Thereafter the solvent was removed under vaccum to obtain the product (Fig. 2). The Mannich bases were further purified by using column chromatographic method over 100–200 mesh silica gel using methanol/dichlromethane solvent system as the eluent. The general structure of the ligands along with their numbering

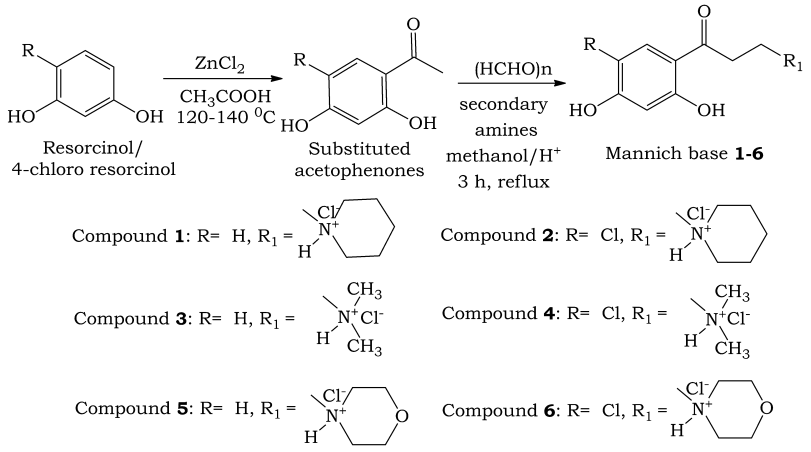


Fig. 2. Preparation of Mannich bases **1**–**6**.

system is depicted in Fig. 1. The individual structures of the compounds are presented in Table 1.

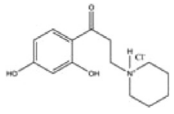
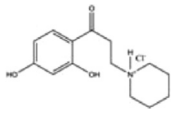
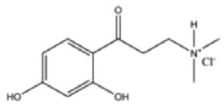
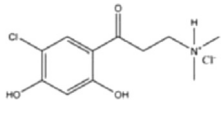
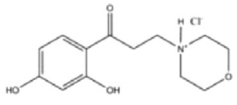
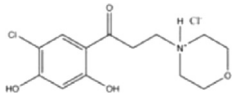
2.3. Binding affinity study

The binding efficiency of the ligands with Hsp90 protein was determined by employing the malachite green ATPase assay. Initially the Histidine-tagged human Hsp90 β (kind gift from Dr. Chrisostomos Prodromou, University of Sussex, United Kingdom) was expressed and purified according to a previously reported procedure [39,40]. The assay procedure was performed by incubating pure hHsp90 β (10 μg) along with 1 μl aliquot of the compounds in DMSO for 10 min at 20 °C in a buffer containing 50 mM Hepes at pH 7.5, 6 mM $MgCl_2$, 20 mM KCl, and 1 mM ATP. The incubation was stopped by the addition of two volumes of malachite green reagent and half a volume of 34% sodium citrate solution to the wells of the plate. Subsequently, the plate was shaken and left to stand at room temperature for about 25 min. This was followed by measuring the absorbance of the solution at 630 nm [41–44]. Geldanamycin was taken as the standard reference molecule.

2.4. Antiproilferative MTT assay [45,46]

The compounds were screened on androgen independent PC3 human carcinoma cell lines for their growth inhibitory activities. Initially, the cells were cultured in RPMI1640 (Invitrogen)/10% fetal bovine serum (Gibco) medium supplemented with 0.007% streptomycin and 0.002% penicillin. The cells were then counted and incubated at 37 °C with 5% CO_2 in a 96 well micro plates until 80% confluence is achieved. The compounds were dissolved in DMSO at a concentration ranging from 0.1 to 5 μM. Then the cells were treated with the test and the standard compound solution. Control wells contained equivalent volume of the vehicle alone. Treated cells were further incubated for 48 h after which 5 μl of MTT reagent

Table 1
Structure and molecular docking results of the synthesized Mannich compounds.

Ligand code	Structure	Total score	Crash	Polar	G score	PMF score	*D* score	Chem score	*C* score
1		7.37	−1.57	3.00	−187.2	−15.14	−108.2	−18.2	2
2		6.24	−1.36	2.43	−202.5	−17.67	−119.5	−15.0	4
3		6.17	−0.43	2.94	−153.4	−20.48	−95.84	−11.2	1
4		5.93	−1.58	2.33	−170.8	−19.03	−108.5	−16.1	2
5		7.06	−2.02	3.99	−173.5	−13.50	−118.6	−19.3	2
6		6.39	−1.00	2.88	−149.8	−20.82	−112.9	−16.7	2

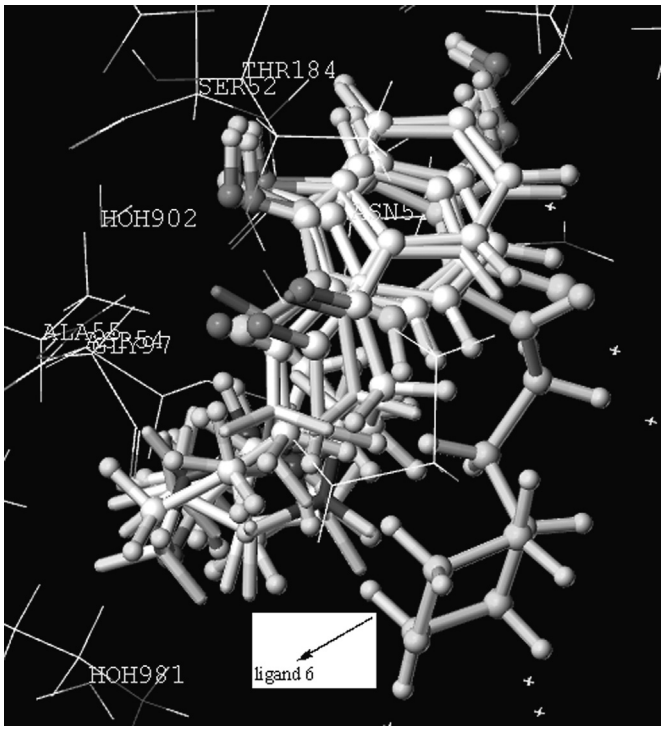


Fig. 3. Alignment of **6** with respect to other Mannich bases.

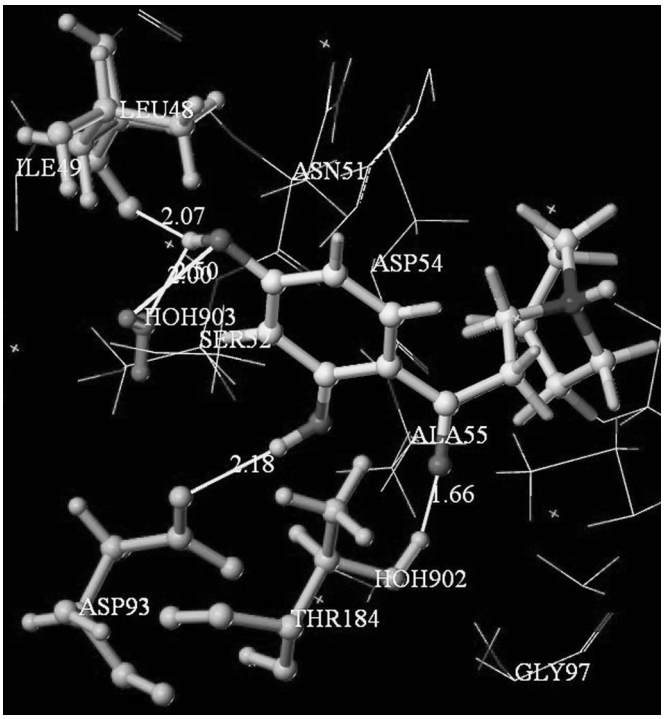


Fig. 4. Hydrogen bond contact (white bold lines) of **6** with amino acids (ball and stick model) and water molecules (ball and stick model) of Hsp90 chaperone.

along with 45 μl of phenol red and FBS free DMEM was added to each well. Thereafter the plates were incubated at 37 °C for 4 h under 5% CO_2 atmosphere. The resultant formazan granules produced by the reduction of MTT was dissolved in DMSO. After 48 h, the absorbance was measured at 550 nm using spectrophotometer in a microplate reader (Bio-Rad, USA). Geldanamycin was used as the standard reference molecule in this antiproliferative study.

3. Results and discussion

3.1. Molecular design

The Geom X method predicted the binding affinities of the synthesized Mannich bases in terms of docking score. Out of 50 molecules docked, 6 were predicted to be potent Hsp90 inhibitors in terms of Total score (total score > 4; total score for geldanamycin = 4.0). The results of ligand–protein interaction studies are summarized in Table 1. The synthesized molecules formed hydrogen bond contact with amino acid Asp 93, Asn 51, Leu 48, Thr 184, water molecule 902 and 903. The following H-bond contact was found to be common or all the Mannich bases (except **6**):

- 2′-hydroxyl group with Asp 93.
- 4′-OH with water molecule 903.

Compound **6** displayed hydrogen bond interaction with Asp 93 and water molecule 903 via its 4′-OH and 2′-OH group, respectively. This discrepancy in binding pattern for **6** can be attributed to its different alignment mode at the active site of the protein (Fig. 3). The hydrogen bonding interaction of the top scored compound (**1**) is illustrated in Fig. 4.

The docking studies further revealed the following hydrophobic interactions between:

- The phenyl propanone part and Leu 48, Leu 107, Ile 49, Ile 91, Leu 107, Met 98, Phe 138, Val 150/Val 186 (Fig. 5).
- The 4- chloro group (for **2**, **4**) and Phe 138, Val 186 and Val 150. This interaction was strong which was reflected in terms of increased D score compared to the non-chlorinated compounds.
- The chlorine of **6** and Ala 55, Ile 96 and Met 98. This variance for **6** is due to its different fashion of occupying the active pocket compared to other chlorinated derivatives (Fig. 3).

3.2. Synthesis

The various Mannich base analogues derived from 2,4-dihydroxy acetophenone and 5-chloro 2,4-dihydroxy

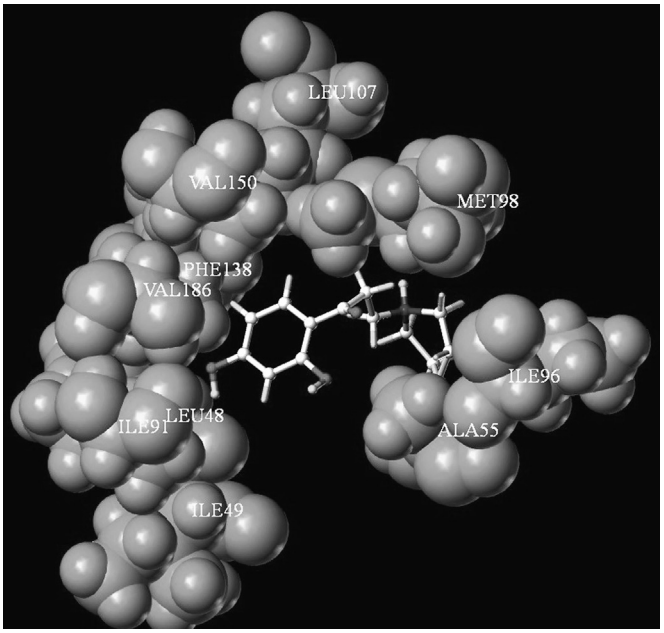


Fig. 5. Hydrophobic interaction between **6** (ball/stick model) and Hsp90 protein. The spheres indicate hydrophobic amino acids.

Table 2
Percentage yield and physical properties of the synthesized compounds.

Compound code	Molecular weight	Melting point (°C)	R_f[a] Meth:Dcm (1:9)	% Yield	Color/state
1	285	172–174	0.75	35	Light brown crystalline
2	320	220–222	0.80	10	Colorless crystalline
3	245	175–177	0.45	52	Light brown crystalline
4	280	135–137	0.60	45	Colorless crystalline
5	287	170–172	0.45	34	Colorless crystalline
6	322	206–208	0.50	25	Light orange crystalline

[a] R_f = retention factor. Dcm = dichloro methane. Meth = methanol.

acetophenone were characterized by melting point and R_f values. All the relevant data along with the percentage yield of the molecules is presented in Table 2. The structure of the compounds was confirmed from IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic studies.

In the FT-IR spectra of the compounds, the absence of C=O stretching vibration (starting acetophenone) at 1645 cm^{-1} and the appearance of bands between 1629 and 1597 cm^{-1} corresponding to the ketonic carbonyl group confirmed the formation of Mannich bases. An absorption band between 2900 and 2853 cm^{-1} due to ethylenic stretching further confirmed the formation of Mannich compounds. The O—H, C=C aromatic and C—OH (phenolic) stretching bands were observed between 3741–3028, 1598–1425 and 1253–1056 cm^{-1}, respectively. The chlorine containing bases (**2**, **4, 6**) displayed corresponding C—Cl stretching bands between 1068 and 1059 cm^{-1}.

In the ^{1}H NMR spectra of the molecules, the absence of three methylic protons signal (for the starting acetophenones) at 2.50 and the presence of four ethylenic protons between 4.23 and 2.63 ppm confirmed the formation of Mannich compounds. All the compounds displayed two hydroxyl proton peaks between 13.56 and 8.80. The signal corresponding to the aromatic protons were observed between 6 and 8 for all the molecules.

The ^{13}C NMR spectra of all the Mannich bases revealed a peak between 203.63 and 199.31 ppm, corresponding to the ketonic carbonyl moiety. The two phenolic C—OH displayed chemical shift ranging from 167.94 to 161.46. A chemical shift value in the range of 42.08–53.01 was attributed to the ethylenic carbons. The remaining aromatic carbons for all the Mannich compounds showed peaks between 134.85 and 103.70 ppm.

In the mass spectra of the Mannich molecules, base peak at M^+ and M^++1 corresponding to the molecular weight of the compounds were observed. The chlorinated compounds (**2, 4, 6**) displayed two peaks at M^+ and $(M+2)^+$ peak (percentage abundance ratio: 3:1) corresponding to the isotopes of chlorine.

3.2.1. 5-Chloro-2,4-dihydroxy acetophenone

IR (KBr cm^{-1}): 3440 (OH stretching); 3100 (aromatic CH stretching); 1645 (C=O stretching of ketone); 1600, 1400 (C=C aromatic stretching); 1230, 1168 (C—OH stretching); 1050 (C—Cl stretching). ^{1}H NMR (DMSO, 400 MHz) δ: 12.34 (s, 1*H*, OH); 11.43 (s, 1*H*, OH); 7.88 (s, 1*H*, Ar—H); 6.46 (s, 1*H*, Ar—H); 2.50 (s, 3*H*, CH_3). Mass (*m*/*z*): 185 $(M-1)^-$.

3.2.2. 1-(3-(2,4-Dihydroxyphenyl)-3-oxopropyl)piperidin-1-ium chloride (**1**)

IR (KBr cm^{-1}): 3741 (OH stretching); 3045 (aromatic C—H stretching); 2760 (ethylene stretching) 2360 (N—H stretching, tertiary amine salts); 1629 (C=O stretching ketone); 1496 (C=C aromatic stretching); 1159, 1116, 1056 (C—OH stretching). ^{1}H NMR (DMSO, 300 MHz) δ: 9.46 (s, 1*H*, OH); 8.79 (s, 1*H*, OH); 7.94 (d, 1*H*, ArH, *J* = 6.9); 6.72 (d, 1*H*, ArH, *J* = 8.8 Hz); 6.20 (s, 1*H*, ArH); 2.97–2.63 (m, 4*H*, ethylene); 1.57–1.69 (m, 10*H*, piperidine). ^{13}C NMR (75 MHz, DMSO) δ: 202.32 (C=O); 167.37, 162.39 (ArC—OH); 132.17, 111.36, 108.64, 106.33 (ArC); 53.01 ($2CH_2$N, piperidine); 51.96, 26.01 (O=C—CH_2—CH_2—N); 24.96 ($2CH_2$ piperidine), 23.02 ($1CH_2$ piperidine). Mass (*m*/*z*): 248 $(M-1-HCl)^-$.

3.2.3. 1-(3-(5-Chloro-2,4-dihydroxyphenyl)-3-oxopropyl)piperidin-1-ium chloride (**2**)

IR (KBr cm^{-1}): 3650 (OH stretching); 2995 (aromatic C—H stretching); 2879 (ethylene stretching); 2654, 2542, 2355 (N—H stretching, tertiary amine salts); 1616 (C=O stretching ketone); 1481 (C=C aromatic stretching); 1253, 1149, 1080 (C—OH stretching); 1049 (C—Cl stretching). ^{1}H NMR (DMSO, 300 MHz): 13.41 (s, 1*H*, OH); 11.08 (s, 1*H*, OH); 7.84 (s, 1*H*, ArH); 7.26 (s, 1*H*, ArH); 4.23-3.55 (m, 4*H*, ethylene); 1.87-2.87 (m, 10*H*, piperidine CH_2). ^{13}C NMR (75 MHz, DMSO) δ: 202.53 (C=O); 167.13, 161.73 (ArC—OH); 133.24, 113.04, 111.66, 105.84 (ArC); 52.17 ($2CH_2$-N, piperidine); 49.02, 26.19 (O=C—CH_2—CH_2—N); 22.34 ($2CH_2$ piperidine), 21.09 ($1CH_2$ piperidine). Mass (*m*/*z*): 284 $(M-HCl)^+$.

3.2.4. 3-(2,4-Dihydroxyphenyl)-N,N-dimethyl-3-oxopropan-1-aminium chloride (**3**)

IR (KBr cm^{-1}): 3028 (OH stretching); 2900 (aromatic C—H stretching); 2877 (ethylene stretching); 2700, 2300, (N—H stretching, tertiary amine salts); 1616 (C=O stretching ketone); 1504, 1477, 1425 (C=C aromatic stretching); 1161, 1136, 1058 (C—OH stretching). ^{1}H NMR (DMSO, 300 MHz): 13.56 (s, 1*H*, OH); 11.99 (s, 1*H*, OH); 7.93 (d, 1*H*, ArH, *J* = 8.9 Hz); 6.69 (d, 2*H*, ArH, *J* = 8.9 Hz); 4.16 (4*H*, Mannich CH_2—CH_2); 2.49 (s, 6*H*, $2CH_3$). ^{13}C NMR (75 MHz, DMSO) δ:203.63 (C=O); 164.40, 163.40 (ArC—OH); 134.85, 112.89, 107.61, 103.70 (ArC); 48.60, 42.47 (O=C—CH_2—CH_2—N); 26.12 (2N—CH_3). Mass (*m*/*z*): 210 $(M+1-HCl)^+$.

3.2.5. 3-(5-Chloro-2,4-dihydroxyphenyl)-N,N-dimethyl-3-oxopropan-1-aminium chloride (**4**)

IR (KBr cm^{-1}): 3650 (OH stretching); 3035 (aromatic C—H stretching); 2877 (ethylene stretching); 2628, 2347, 2287 (N—H stretching, tertiary amine salts); 1602 (C=O stretching ketone); 1549, 1485, 1431 (C=C aromatic stretching); 1222, 1141 (C—OH stretching); 1068 (C—Cl stretching). ^{1}H NMR (DMSO, 300 MHz): 13.45 (s, 1*H*, OH); (11.65, s, 1*H*, OH); 7.76 (s, 1*H*, ArH); 6.45 (s, 1*H*, ArH); 4.47–4.36 (m, 4*H*, CH_2- CH_2, *J* = 2.4 Hz); 2.89 (s, 6*H*, $2CH_3$). ^{13}C NMR (75 MHz, DMSO) δ: 199.31 (C=O); 167.94, 162.72 (ArC—OH); 131.80, 115.00, 107.43, 104.40 (ArC); 51.59, 42.08 (O=C—CH_2—CH_2—N); 25.34 (2N—CH_3). Mass (*m*/*z*): 245 $(M+1-HCl)^+$.

Table 3
Binding and antiproliferative assay results of the synthesized molecules[a].

Compound code	Malachite green assay (IC_{50}, μM)	MTT assay (IC_{50}, μM)
1	0.38	11.23
2	2.51	6.96
3	0.06	12.63
4	1.68	12.04
5	9.07	2.70
6	23.73	5.18

[a] A mean of three independent determinations.

Table 4
Predicted cell permeability and partition coefficient of the prepared Mannich bases*.

Mannich base code	Caco cell permeability	MDCK cell permeability	Log $P_{o/w}$
1	117.405	54.035	1.524
2	134.682	146.459	2.037
3	146.026	68.404	0.857
4	167.314	185.53	1.531
5	115.8	53.237	0.583
6	139.703	152.475	1.134

* Schrodinger software's QuickProp programme was employed for these calculations. Caco cell: cell line derived from human colorectal carcinoma. MDCK cell: Madian–Darby canine kidney epithelial cells. $P_{o/w}$ = partition coefficient calculated for *n*-octanol/water system.

*3.2.6. 4-(3-(2,4-Dihydroxyphenyl)-3-oxopropyl) morpholin-4-ium chloride (**5**)*

IR (KBr cm^{-1}): 3650 (OH stretching); 3047 (aromatic C—H stretching); 2900, 2853 (ethylene stretching); 2304, (N—H stretching, tertiary amine salts); 1597 (C=O stretching ketone); 1489, 1438, 1431 (C=C aromatic stretching); 1116, 1062, (C—OH stretching); ^{1}H NMR ($CDCl_3$, 300 MHz): 13.24 (s, 1*H*, OH); 11.00 (s, 1*H*, OH); 7.62 (d, 1*H*, ArH, *J* = 6.9 Hz); 6.46 (d, 2*H*, ArH, *J* = 6.6 Hz); 3.82–3.95 (m, 4*H*, morpholine; m, 4*H*, Mannich CH_2—CH_2); 2.55–2.69 (m, 4*H*, CH_2, morpholine); ^{13}C NMR (75 MHz, DMSO) *δ*: 202.99 (C=O); 165.04, 162.19 (ArC—OH); 132.29, 112.21, 107.94, 107.53 (ArC); 65.98 ($2CH_2O$, morpholine) 52.49 ($2CH_2N$, morpholine) 51.72, 26.18, (O=C—CH_2—CH_2—N). Mass (*m*/*z*): 252 $(M+1-HCl)^+$.

*3.2.7. 4-(3-(5-Chloro-2,4-dihydroxyphenyl)-3-oxopropyl)morpholin-4-ium chloride (**6**)*

IR (KBr cm^{-1}): 3700 (OH stretching); 3043 (aromatic C—H stretching); 2875 (ethylene stretching) 2306 (N—H stretching, tertiary amine salts) 1598 (C=O stretching ketone); 1598, 1483 (C=C aromatic stretching); 1249, 1134 (C—OH stretching); 1051 (C—Cl stretching).^{1}H NMR ($CDCl_3$, 300 MHz): 13.09 (s, 1*H*, OH); 10.59 (s, 1*H*, OH); 7.70 (s, 1*H*, ArH); 6.30 (d, 1*H*, ArH *J* = 2.7 Hz); 3.87–4.02 (m, 4*H*, morpholine CH_2; m, 4*H*, Mannich CH_2—CH_2); 2.55–2.69 (m, 4*H*, CH_2, morpholine); ^{13}C NMR (75 MHz, DMSO) *δ*: 200.77 (C=O); 165.67, 161.46 (ArC—OH); 131.05, 113.32, 109.18, 105.56 (ArC); 64.76 ($2CH_2O$, morpholine) 52.66 ($2CH_2N$, morpholine) 51.48, 25.70 (O=C—CH_2—CH_2—N). Mass (*m*/*z*): 286 $(M-HCl)^+$.

3.3. Malachite green assay for Hsp90 ATPase suppression

The primary screening assay carried out to substantiate our docking protocol revealed that all the compounds inhibited 50 percent of the Hsp90 protein below 25 μM concentration (Table 3). The chlorine bearing derivatives were less active compared to their non-chloro analogues. The Mannich base derived from resacetophenone and dimethyl amine hydrochloride (**3**) was found to be the most active one with an IC_{50} value of 0.06 μM. However this compound did not top scored in the theoretical binding studies (Total score = 6.17). Furthermore the highest scored Mannich base (**1**, total score = 7.37) of the docking study demonstrated less inhibitory potential in the malachite green assay (IC_{50} = 0.38 μm). This mismatch can be explained from the H bond interaction pattern of the compounds. The 2′-OH, 4′-OH and C=O group of **3** formed hydrogen bond contact with Asp 93, Asn 51/water 903 and Thr 184, respectively (Fig. 6). The binding affinity decreases with change in any one of the above interaction pattern. For example the 4′-OH of **1** formed H-bond contact with Leu 48 instead of Asn 51 that resulted in decreased Hsp90 suppression. Additionally it was observed that hydrogen bond contact of the ligands with Asn 106 (compound **6,** IC_{50} = 23.73 μm) and Lys 58 (Mannich base **5**, IC_{50} = 9.07 μm) leads to significant decrease in their potency. Hence hydrogen bonding with Asp 93, Asn 51/water 903 and Thr 184 leads to highly potent compounds.

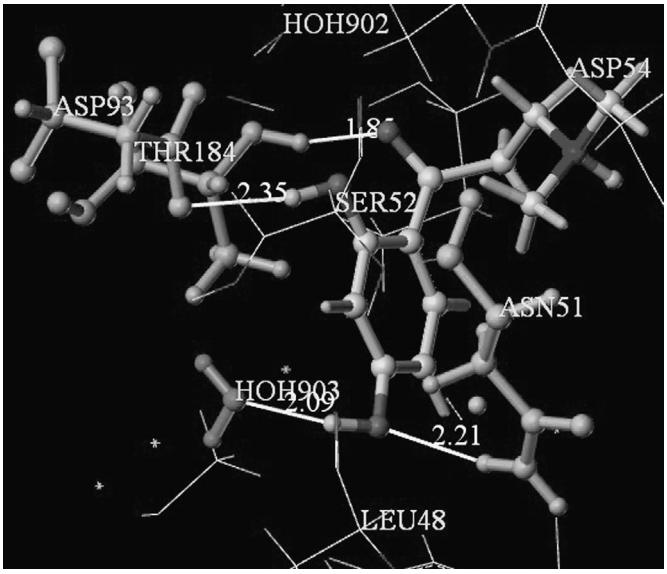


Fig. 6. Hydrogen bond interaction (white bold lines) of **3** at the active site of Hsp90. The amino acids and water molecules involved in H-bond contact are highlighted in ball and stick models.

3.4. In vitro cell growth assay

The MTT assay on PC3 carcinoma cells revealed that Mannich base **1**–**4** displayed less antiproliferative effect compared to their Hsp90 inhibition potential (Table 3). This difference in activity profile can be partly attributed to their permeability properties as all of them were found to be moderately permeable through Caco and MDCK cells (Table 4). This may be due to over expression of multi drug resistance associated MRP and gluthione-*S*-transferase (GST) proteins in PC3 cells which are not clients of Hsp90 [47,48]. However, the aforementioned hypotheses need to be further validated by practical experimentations. The inhibitory effects of compounds **5** and **6** on PC3 cells demonstrated a reverse phenomenon. They suppressed the growth of carcinoma cells more than Hsp90 chaperone (Table 3). An extensive study is required to explain this activity difference, but partly could be due to their different mechanism of attenuating the growth of carcinoma cells.

4. Conclusion

In summary, our research effort led to the discovery of Mannich bases derived from resacetophenone/4-chloro resacetophenone as a novel class of Hsp90 inhibitors with significant antiproliferative effect. This study was guided by structure based drug design technique which identified the amino acids and water molecules (Asp 93, Asn 51, Thr 184 and water 903 for hydrogen bond interaction; Ile 49, Ile 91, Leu 48, Leu 107, Met 98 for hydrophobic contact) critical for Hsp90 suppression by the Mannich bases. The research work further revealed the amino acids and water molecules (Leu

48, Lys 58 Asn 106 and water 902 for H-bond contact; Phe 138, Val 186 and Val 150 for hydrophobic interactions) detrimental for Hsp90 antagonism for this class of compounds. The simple and facile synthetic procedure will stimulate medicinal chemist worldwide to discover more selective Mannich base Hsp90 inhibitors with improved antiproliferative efficacy. A comprehensive structure activity relationship study for this series of molecules will be established in the near future.

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