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Original article

Novel 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides: Synthesis, antimycobacterial activity and QSAR investigations

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ABSTRACT

A series of 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides were synthesized using appropriate synthetic route. Antimycobacterial activity of the synthesized compounds (**5a–5j**) was carried out and percentage reduction in relative light units (RLU) was calculated using luciferase reporter phages (LRP) assay. Percentage reduction in relative light units (RLU) for isoniazid was also calculated. The test compounds showed significant antitubercular activity against *Mycobacterium tuberculosis* H37Rv and clinical isolates: S, H, R, and E resistant *M. tuberculosis*, when tested in vitro.

Quantitative structure–activity relationship (QSAR) investigation with 2D-QSAR analysis was applied to find a correlation between different experimental or calculated physicochemical parameters of the compounds studied and 3D-QSAR analysis and to indicate the exact steric and electronic requirements in the ranges at various positions around pharmacophore.

In general Schiff bases exhibit antimycobacterial activity and morpholine ring is important for antimicrobial activity. So we have synthesized 10 different 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides. The structures of new compounds were characterized by TLC, FTIR, ^{1}H NMR, mass spectral data and elemental analysis.

Amongst the compounds tested **5d** and **5c** were found to be the most potent, while **5i**, **5e**, and **5j** were found to have an average activity against *M. tuberculosis* H37Rv and **5a**, **5f**, **5h**, **5g**, and **5b** were found to have a greater activity against clinical isolates: S, H, R, and E resistant *M. tuberculosis* as compared to *M. tuberculosis* H37Rv.

1. Introduction

The deterioration of human population due to the enhanced prevalence of infectious diseases is becoming a worldwide problem. Over the last few years, tuberculosis is retrieving its place among these infectious diseases and today, nearly one-third of the world's population is infected with *Mycobacterium tuberculosis* with approximately three million patients deceasing every year [1]. The contemporary treatment of these infectious diseases involves administration of multi-drug regimen over a long period of time [2,3], which lead to patient noncompliance and rapid emergence of multi-drug resistance strain [4–7]. The resistance problem demands to seek antimycobacterial agents effective against pathogenic microorganisms resistant to current treatment [8]. Furthermore, treatment of infectious diseases is more difficult in immunodeficient patients such as those infected with human immunodeficiency virus (HIV) [9]. Recent studies showed that the application of appropriate dosage regimen with highly potent antimicrobial agents not only eradicates bacterial growth but also minimizes the probability of resistance formation [10–12]. The biochemical basis of both intrinsic and acquired resistance are now known [13–15] and has contributed significantly towards the design of new entities by rational strategies that can be used to counteract the resistance. The development of new potential drugs will be one of the possible solutions to treat various infectious diseases with multi-drug treatment over a long period of time.

Schiff bases exhibit a wide variety of biological activities such as antimycobacterial [16–18], antimicrobial [19], and anticonvulsant [20], etc. Literature survey reveals that morpholine ring is important for antimicrobial activity [21]. In view of the fact that morpholine ring is important for antimicrobial activity and Schiff bases exhibit antimycobacterial activity, we have synthesized some novel

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4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides with the aim of obtaining the new antimycobacterial agents which will be active against some of the resistant strains. Thus in the present investigation, 10 different 4-(morpholin-4-yl)-N′-(arylidene)-benzohydrazides were synthesized and evaluated for their antimycobacterial activity.

2. Results and discussion

2.1. Chemistry

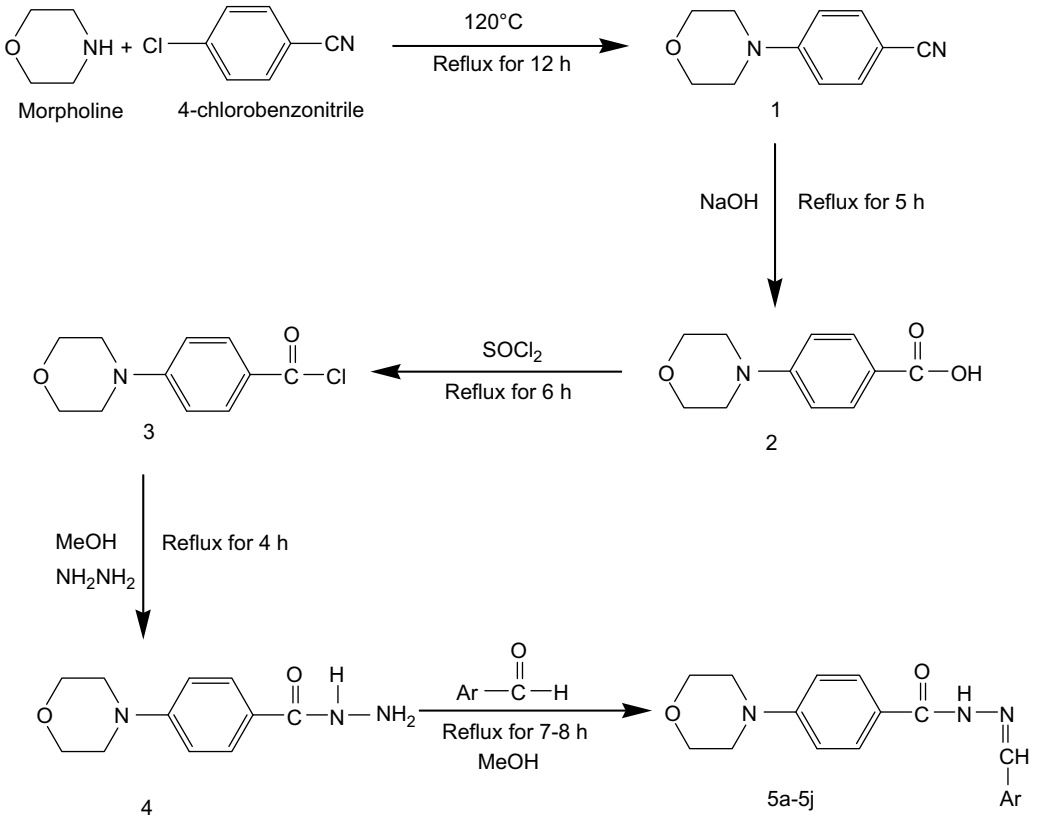
The synthetic route used to synthesize title compounds is outlined in Scheme 1. 4-(Morpholin-4-yl)benzonitrile (**1**) and was prepared according to the method reported in the literature, using 4-chlorobenzonitrile as a starting material. The 4-(morpholin-4-yl)benzoic acid (**2**) was prepared by basic hydrolysis of 4-(morpholin-4-yl)benzonitrile (**1**). 4-(Morpholin-4-yl)benzoic acid hydrazide (**4**) was prepared by hydrazination of 4-(morpholin-4-yl)benzoic acid chloride (**3**) which was prepared by reacting 4-(morpholin-4-yl)benzoic acid (**2**) with thionyl chloride. It was condensed with different substituted aromatic aldehydes in methanol to give corresponding 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides (**5a**–**5j**) in very good yields. The structures of various synthesized compounds were assigned on the basis of different chromatographic, spectral studies and qualitative and quantitative organic analytical studies. The physical data, FTIR, ^{1}H NMR and mass spectral data for all the synthesized compounds are reported in experimental protocols.

The FTIR spectra of the Schiff bases exhibited very similar features and showed the expected bands for the characteristic groups which are present in the compounds such as C–H and the C═N stretching vibrations. The Schiff bases (**5a**–**5j**) showed C═O stretching bands at 1705–1677 cm^{-1} and N–H stretching bands at 3362–3236 cm^{-1}. In the proton NMR spectral data, all protons were seen according to the expected chemical shift and integral values. The aromatic protons appeared as multiplet peaks within the range 6.8–7.8 δ ppm, singlet signals derived from hydrazide (–NH–NH$_2$) structure appeared at 4.23 ppm. The ^{1}H NMR spectra of compounds (**4**) and (**5a**–**5j**) displayed broad singlet due to NH proton of amide (probably due to their ability to get exchanged with D_2O) and each signal showing integration for one proton. For the compounds (**5a**–**5j**), the signals belonging to benzylidene group were observed at aromatic region, while the signals belonging to –NHNH$_2$ disappeared indicating functionalization of hydrazide to hydrazones with substituted aromatic aldehydes.

2.2. Biological investigation

All the newly synthesized Schiff bases were assayed in vitro for antitubercular activity against *M. tuberculosis* H37Rv and clinical isolates: S, H, R, and E resistant *M. tuberculosis*. In case of antimycobacterial activity % reduction in relative light units (RLU) was calculated using luciferase reporter phages (LRP) assay at four different concentrations (0.05, 0.5, 5, and 10 μg/ml) using isoniazid as a reference standard and the observed percentage inhibition is tabulated in Table 1. A compound is considered to be an antimycobacterial agent if fifty percent reduction in the relative light units (RLU) is observed when compared to the control using a luminometer.

The obtained results revealed that the nature of the substituent and substitution pattern on the benzene ring may have a considerable impact on the antitubercular activity of the synthesized compounds. Literature survey reveals that electron-withdrawing or donating groups amend the lipophilicity of the test compounds, which in turn



Scheme 1. Synthetic route for title compounds **5a**–**5j**.

Table 1
% Reduction in relative light units (RLU) against *M. tuberculosis* H37Rv and clinical isolates: S, H, R, and E resistant *M. tuberculosis*.

Sr. No.	Comp	*M. tuberculosis* H37Rv				Clinical isolates: S, H, R, and E resistant *M. tuberculosis*			
		% Reduction in relative light units (RLU)							
		0.05 μg/ml	0.5 μg/ml	5 μg/ml	10 μg/ml	0.05 μg/ml	0.5 μg/ml	5 μg/ml	10 μg/ml
1	**5a**	15.04	96.90	98.41	97.55	58.54	86.92	92.52	93.72
2	**5b**	9.118	98.04	98.40	98.40	29.92	87.17	90.07	88.86
3	**5c**	78.09	98.02	98.38	99.26	67.15	89.91	91.19	94.29
4	**5d**	96.78	98.22	98.98	99.09	83.22	86.33	93.28	95.09
5	**5e**	51.79	77.81	97.53	97.24	43.92	73.58	88.08	88.45
6	**5f**	30.04	96.24	98.10	97.18	56.53	82.97	89.33	90.36
7	**5g**	20.85	98.09	98.49	98.82	48.14	90.72	93.18	95.02
8	**5h**	24.19	97.29	97.75	98.18	48.49	87.24	90.83	91.37
9	**5i**	63.16	98.96	97.82	97.32	55.70	85.67	91.61	92.10
10	**5j**	49.68	96.51	97.59	97.77	35.83	96.88	90.29	90.24
11	INH	96.00	98.42	99.01	98.99	84.37	89.6	95.85	94.70

alters permeability across the bacterial cell membrane. Further, the presence of electron-withdrawing groups (both halogen) in **5d** and **5c** showed maximum antimycobacterial activity.

2.2.1. Antimycobacterial activity

Amongst the compounds tested **5d** and **5c** were found to be the most potent, while **5i**, **5e**, and **5j** were found to have an average activity against *M. tuberculosis* H37Rv.

Amongst the compounds tested **5a**, **5f**, **5h**, and **5g** were found to have a greater activity against clinical isolates: S, H, R, and E resistant *M. tuberculosis* as compared to *M. tuberculosis* H37Rv.

2.3. 2D-QSAR study

The antimycobacterial activities of the compounds tested, presented in pIC_{50} (Table 2), were used in the 2D-QSAR studies. pIC_{50} values were calculated using the following equation.

$$pIC_{50} = -\log C + \log it$$

where C = molar concentration = [concentration (μg/ml) × 0.001/molecular weight]

$$\log it = \log[\%\text{inhibition}/100 - \%\text{inhibition}]$$

A 2D-QSAR study was performed using multiple linear regression (MLR) method. The results obtained are presented in Table 2.

The residuals (actual–predicted activity) were found to be minimal and are presented in Table 3.

Also the graph for actual vs predicted activity for the series is plotted in Graph 1 which shows good correlation.

The regression equation obtained for the different series of compounds is given below.

$$pIC_{50}(\text{M. tuberculosis H37Rv}) = -0.4739\text{T\_2\_N\_5} + 3.1198 \quad (1)$$

$n = 8$, $r^2 = 0.7346$, $q^2 = 0.6523$, F-test = 16.6048.

Table 2
Results of 2D-QSAR equation obtained by multiple linear regression method.

Sr. No.	Statistical parameter and contributing descriptors	Results
1	r^2	0.7346
2	q^2	0.6523
3	Pred_r^2	0.6637
4	Pred_r^2SE	0.4173
5	F-test	16.6048
6	Alpha	0.01000
7	Best-Ran_q^2	0.43524
8	Z-score	1.81367

From the above equation it is clear that the alignment independent descriptor T_2_N_5 contributes negatively for the antimycobacterial activity against *M. tuberculosis* H37Rv which corresponds to number of double bonded atoms (i.e. any double bonded atom, T_2) separated from nitrogen atom by 5 bonds.

The regression equation so obtained will be useful for the prediction of biological activities of the designed series of compounds, in future. The 2D-QSAR study for the synthesized compounds was carried out using multiple linear regression (MLR) method to study the structural activity relationship. In all, 10 compounds along with the standard were used for the 2D-QSAR studies. The compounds were divided into training set (8) and test set (2) respectively using manual selection method by considering the biological and chemical variation.

2.4. 3D-QSAR study

The 3-D QSAR studies were carried out using the Step Wise K Nearest Neighbour Molecular Field Analysis [(SW) kNN MFA] method which can be discussed as follows.

For the kNN MFA method, the alignment of the molecules was carried out using Field Fit alignment. KNN-MFA models were generated using 10 molecules.

The most common structure was used as a template for the alignment of all the analogues.

The careful analysis of comparison of biological activities (pIC_{50}), predicted activities for training and test set molecules indicate very less significant differences (lower values of residuals). Therefore it can be said that the predictive abilities of SW kNN MFA model is good. This comment is supported by the statistical values presented in Table 6.

Table 3
Predicted activity obtained from multiple linear regressions of 2D-QSAR study.

Comp	*M. tuberculosis* H37Rv		
	Biological activity pIC_{50}	Predicted activity	Residual
5a	−0.89956	−0.143408	−0.756152
5b	−1.1445	−1.082856	−0.061644
5c	0.4057	−0.143408	0.549108
5d	1.3312	[a]	
5e	−0.1157	−0.143408	0.027708
5f	−0.5135	−0.143408	−0.370092
5g	−0.7253	−0.613132	−0.112168
5h	−0.643	−0.143408	−0.499592
5i	0.0877	−0.143408	0.231108
5j	−0.1503	−0.143408	−0.006892
INH	1.2249	1.265764	−0.040864

INH – isoniazid.
[a] Outlier.

In all, it can be said that the results obtained by 3D kNN MFA studies by SW method are in agreement with the general requirements of selective and ideal pharmacophore required for potential antimycobacterial activity. The graph for actual vs predicted activity for the series is plotted in Graph 2, which shows good correlation.

Result of 3D data points generated, gives information of steric and electronic interaction energy of a probe atom with each molecule at the grid points. This leads to identification of various molecular features responsible for activity variation and hence aid in design of novel potential molecules. In addition, it provides a mathematical model, which can be used to quantitatively predict the activity of newly designed molecules.

Fig. 2 shows the relative position and the range of the corresponding important electrostatic field in the model generated. Negative range indicates that negative electrostatic potential is favorable for increase in the activity and hence a more electronegative substituent group is preferred in that region. So from the 3D counter plot i.e. Fig. 2 obtained using the SW kNN MFA method it was found that electronegative atoms like –Cl, –F, were essential for the potential antimycobacterial activity.

3. Conclusion

In the present investigation, 10 different 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides were synthesized and evaluated for their antimycobacterial activity. Most compounds exhibited significant antimycobacterial activity. A remarkable activity was found in compound **5d** carrying 4-fluro moiety. Compound **5c** with 4-chloro substitution also exhibited good antimycobacterial activity. Amongst the compounds tested **5d** and **5c** were found to be most potent, while **5i**, **5e**, and **5j** were found to have an average activity against *M. tuberculosis* H37Rv and **5a**, **5f** and **5h** were found to have good activity against clinical isolates: S, H, R, and E resistant *M. tuberculosis* as compared to *M. tuberculosis* H37Rv.

From the detailed analysis of the results of above studies, we conclude that antimycobacterial activity of the synthesized compounds significantly depends on the presence of electronegative group. The result obtained show that out of the synthesized and tested compounds, especially **5d**, **5c**, **5i**, **5a**, **5f** and **5h** may be considered promising for the development of new antimycobacterial agents. Various physicochemical indices are helpful for the understanding of microbiological results, as shown by our 2D- and 3D-QSAR study. As the process of antimycobacterial activity is not clearly defined in terms of the target biomacromolecules, the 2D-QSAR results could not be addressed to a concrete drug–receptor interaction, but they can reveal trends in the relationship between ligand structures and their activities for our set of antimycobacterial agents. Analysis of 3D counter plot generated in 3D-QSAR study provides details on the fine relationship linking structure and activity and offers clues for structural modifications that can improve the activity. These trends should prove to be an essential guide for the future work.

4. Experimental

Melting points were determined by open capillary tubes and are uncorrected. FTIR spectra of the powdered compounds were recorded using KBr on a Jasco FTIR V 430+ spectrometer using Diffuse Reflectance Attachment and are reported in cm^{-1} and ^1H NMR spectra were recorded on a Varian Mercury YH300 (300 MHz FT NMR) spectrophotometer using TMS as an internal reference (chemical shift represented in δ ppm). Mass spectra were recorded on GC–MS QP5050A System (benchtop quadrupole mass spectrophotometer). Microanalyses of compounds were also performed for the determination of percentage of C, H, and N. Purity of the compounds was checked on TLC plates using silica gel G as stationary phase and iodine vapours as visualizing agent. The 4-chlorobenzonitrile was purchased from S.D. Fine-Chem Ltd, Mumbai.

4.1. Synthesis of 4-(morpholin-4-yl)benzonitrile (*1*)

The compound, 4-(morpholin-4-yl)benzonitrile was prepared as per procedure reported in literature [22] and was purified by recrystallization from 50% aqueous ethanol. Yield: 53.28% (solid); mp 82–83 °C. FTIR (KBr) cm^{-1}: 3050 (Ar C–H); 2980 (C–H); 2215 (C≡N); 1495 (C=C); 1115 (C–O–C). ^1H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.28 (t, 4H, CH_2); 3.75 (t, 4H, CH_2); 6.78 (dd, 2H, phenyl); 7.52 (dd, 2H, phenyl). Mass spectra of compound exhibited molecular ion peak at m/z 188 (M^+).

4.2. Synthesis of 4-(morpholin-4-yl)benzoic acid (*2*)

The compound, 4-(morpholin-4-yl)benzoic acid was prepared as per procedure reported in literature [22]. Yield: 98.34% (solid); mp 275-277 °C. FTIR (KBr) cm^{-1}: 3241 (O-H); 3065 (Ar C–H); 2992 (C–H); 1694 (C=O); 1467 (C=C); 1113 (C–O–C). ^1H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.25 (t, 4H, CH_2); 3.62 (t, 4H, CH_2); 6.82 (dd, 2H, phenyl); 7.68 (dd, 2H, phenyl); 12.23 (s, 1H, OH). Mass spectra of compound exhibited molecular ion peak at m/z 207 (M^+).

4.3. Synthesis of 4-(morpholin-4-yl)benzoyl chloride [23,24] (*3*)

To 4-(morpholin-4-yl)benzoic acid (2) (0.01 mol), thionyl chloride (0.015 mol) was added and refluxed on water bath for 6 h. Excess of thionyl chloride was removed under reduced pressure and the residue so collected was used for the next step.

Yield: 79.6% (Solid); mp 130–132 °C. FTIR (KBr) cm^{-1}: 3077 (Ar C–H); 2972 (C–H); 1774 (C=O); 1475 (C=C); 1120 (C–O–C); 870 (C–Cl). ^1H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.31 (t, 4H, CH_2); 3.6 (t, 4H, CH_2); 6.79 (dd, 2H, phenyl); 7.71 (dd, 2H, phenyl). Mass spectra of compound exhibited molecular ion peak at m/z 225.5 (M^+).

4.4. Synthesis of 4-(morpholin-4-yl)benzoic acid hydrazide (*4*)

To the solution of 4-(morpholin-4-yl)benzoic acid chloride (**3**) (0.05 mol) in 75 ml of methanol, 99% hydrazine hydrate (0.1 mol) was added and the mixture was refluxed on water bath for 4 h. After cooling, the precipitate was collected, washed with distilled water and recrystallized from ethanol [25].

Yield: 95.23% (solid); mp 282–284 °C. FTIR (KBr) cm^{-1}: 3325 (N–H); 3102 (Ar C–H); 2988 (C–H); 1638 (C=O); 1455 (C=C); 1115 (C–O–C). ^1H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.28 (t, 4H, CH_2); 3.69 (t, 4H, CH_2); 4.23 (s, 2H, NH_2); 6.98–7.12 (m, 4H, phenyl); 8.27 (s, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 221 (M^+), 222 ($M+1$).

4.5. General procedure for the preparation of 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides (*5a–5J*)

The 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides were prepared as per the reported procedure [15,20], and purified by recrystallization from ethanol.

4.5.1. 4-(Morpholin-4-yl)-N′-(furan-2-ylidene)benzohydrazide

Yield: 94.1% (solid); mp 224–226 °C. FTIR (KBr) cm^{-1}: 3285 (N–H); 3085 (Ar C–H); 2972 (C–H); 2860 (N=CH); 1695 (C=O);

1585 (C=N); 1459 (C=C); 1108 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.3 (t, 4H, CH_2); 3.7 (t, 4H, CH_2);); 6.28–6.33 (dd, 1H, furanyl, $J_{3,4} = 3.5$ Hz, $J_{3,5} = 0.8$ Hz); 6.38–6.44 (dd, 1H, furanyl $J_{3,5} = 3.5$ Hz, $J_{4,5} = 3.5$ Hz); 6.52–6.59 (m, 4H, phenyl); 7.36–7.42 (dd, 1H, furanyl, $J_{3,5} = 0.8$ Hz, $J_{4,5} = 3.7$ Hz); 8.66 (s, 1H, CH); 11.12 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 354 (M^+), 355 (M + 1) Anal. Calcd for $C_{16}H_{17}O_3N_3$: C, 64.21; H, 5.84; N, 16.05. Found: C, 64.83; H, 5.04; N, 16.83.

4.5.2. 4-(Morpholin-4-yl)-N′-(3-nitrobenzylidene)benzohydrazide

Yield: 87.5% (solid); mp 157–159 °C. FTIR (KBr) cm^{-1}: 3288 (N–H); 3065 (Ar C–H); 2976 (C–H); 2862 (N=CH); 1698 (C=O); 1605 (C=N); 1532 (asymm NO_2); 1460 (C=C); 1347 (symm NO_2); 1111 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.22 (t, 4H, CH_2); 3.62 (t, 4H, CH_2); 6.94–7.1 (m, 4H, phenyl); 7.22–7.29 (m, 4H, phenyl); 8.86 (s, 1H, CH); 11.28 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 354 (M^+), 355 (M + 1) Anal. Calcd for $C_{18}H_{18}O_4N_4$: C, 61.01; H, 5.08; N, 15.81. Found: C, 61.55; H, 5.86; N, 15.12.

4.5.3. 4-(Morpholin-4-yl)-N′-(4-chlorobenzylidene)benzohydrazide

Yield: 85.5% (solid); mp 216–218 °C. FTIR (KBr) cm^{-1}: 3380 (N–H); 3092 (Ar C–H); 2982 (C–H); 2864 (N=CH); 1701 (C=O); 1615 (C=N); 1462 (C=C); 1116 (C–O–C); 766 (C–Cl). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.26 (t, 4H, CH_2); 3.79 (t, 4H, CH_2); 7.20-7.23 (m, 4H, phenyl); 7.50-7.68 (m, 4H, phenyl); 8.91 (s, 1H, CH); 11.36 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 343.5 (M^+), 344.5 (M + 1). Anal. Calcd for $C_{18}H_{18}O_2N_3Cl$: C, 62.88; H, 5.24; N, 12.33. Found: C, 62.24; H, 5.06; N, 12.19.

4.5.4. 4-(Morpholin-4-yl)-N′-(4-fluorobenzylidene)benzohydrazide

Yield: 85.6% (solid); mp 216–218 °C. FTIR (KBr) cm^{-1}: 3289 (N–H); 3085 (Ar C–H); 2986 (C–H); 2868 (N=CH); 1705 (C=O); 1621 (C=N); 1465 (C=C); 1231 (C–F); 1120 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.32 (t, 4H, CH_2); 3.81 (t, 4H, CH_2); 7.49–7.58 (m, 4H, phenyl); 7.68–7.79 (m, 4H, phenyl); 8.95 (s, 1H, CH); 12.1 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 327 (M^+), 328 (M + 1). Anal. Calcd for $C_{18}H_{18}O_2N_3F$: C, 66.06; H, 5.51; N, 12.84. Found: C, 66.28; H, 5.66; N, 12.13.

4.5.5. 4-(Morpholin-4-yl)-N′-(4-hydroxybenzylidene)benzohydrazide

Yield: 81.8% (solid); mp 148–150 °C. FTIR (KBr) cm^{-1}: 3289 (O–H); 3255 (N–H); 3056 (Ar C–H); 2974 (C–H); 2830 (N=CH); 1670 (C=O); 1569 (C=N); 1449 (C=C); 1106 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.26 (t, 4H, CH_2); 3.51 (t, 4H, CH_2); 6.94–7.16 (m, 4H, phenyl); 7.39–7.46 (m, 4H, phenyl); 8.42 (s, 1H, CH); 10.12 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 325 (M^+), 326 (M + 1). Anal. Calcd for $C_{18}H_{19}O_3N_3$: C, 66.46; H, 5.85; N, 12.92. Found: C, 66.12; H, 5.05; N, 12.21.

4.5.6. 4-(Morpholin-4-yl)-N′-(4-methoxybenzylidene)benzohydrazide

Yield: 73.9% (solid); mp 233–235 °C. FTIR (KBr) cm^{-1}: 3265 (N–H); 3072 (Ar C–H); 2982 (C–H); 2844 (N=CH); 1678 (C=O); 1672 (C=N); 1451 (C=C); 1174 (Ar C–O); 1109 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.36 (t, 4H, CH_2); 3.66 (t, 4H, CH_2); 6.91–7.12 (m, 4H, phenyl); 7.31–7.36 (m, 4H, phenyl); 8.65 (s, 1H, CH); 11.2 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 339 (M^+), 340 (M + 1). Anal. Calcd for $C_{19}H_{21}O_3N_3$: C, 67.26; H, 6.19; N, 12.38. Found: C, 67.02; H, 6.82; N, 12.76.

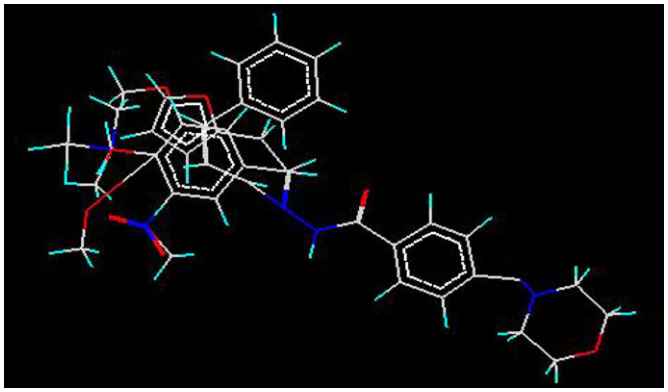


Fig. 1. Overlain of all 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides aligned using alignment tool of V-Life MDS.

4.5.7. 4-(Morpholin-4-yl)-N′-(4-(N,N-dimethylamino)benzylidene) benzohydrazide

Yield: 92.1% (solid); mp 165–167 °C. FTIR (KBr) cm^{-1}: 3245 (N–H); 3061(Ar C–H); 2975 (C–H); 2830 (N=CH); 1680 (C=O); 1572 (C=N); 1454 (N $(CH_3)_2$); 1446 (C=C); 1105 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.27 (t, 4H, CH_2); 3.54 (t, 4H, CH_2); 7.1–7.18 (m, 4H, phenyl); 7.2–7.34 (m, 4H, phenyl); 8.45 (s, 1H, CH); 10.6 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 352 (M^+), 353 (M + 1). Anal. Calcd for $C_{20}H_{24}O_2N_4$: C, 68.18; H, 6,82; N, 15.09. Found: C, 68.70; H, 6.42; N, 15.52.

4.5.8. 4-(Morpholin-4-yl)-N′-(4-methylbenzylidene)benzohydrazide

Yield: 63.9% (solid); mp 138–140 °C. FTIR (KBr) cm^{-1}: 3244 (N–H); 3059 (Ar C–H); 2981 (C–H); 2896 (N=CH); 1678 (C=O); 1569 (C=N); 1450 (C=C); 1108 (C–O–C), 741 (C–H). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.12 (t, 4H, CH_2); 3.48 (t, 4H, CH_2); 6.94–7.16 (m, 4H, phenyl); 7.32–7.45 (m, 4H, phenyl); 8.48 (s, 1H, CH); 10.71 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 323 (M^+), 324 (M + 1). Anal. Calcd for $C_{19}H_{21}O_2N_3$: C, 70.58; H, 6.50; N, 13.00. Found: C, 70.02; H, 6.72; N, 13.66.

4.5.9. 4-(Morpholin-4-yl)-N′-(3-phenylallylidene)benzohydrazide

Yield: 88.1% (solid); mp 186–188 °C. FTIR (KBr) cm^{-1}: 3269 (N–H); 3076 (Ar C–H); 2988 (C–H); 2856 (N = CH); 1688 (C=O); 1582 (C=N); 1462 (C=C); 1113 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.34 (t, 4H, CH_2); 3.58 (t, 4H, CH_2); 6.95-6.98 (d, 1H, $J = 6.5$ Hz, CH=CH-Ph); 7.43-7.48 (m, 4H, phenyl); 7.55–7.61 (d, 1H, $J = 2.2$ Hz, N=CH–CH); 8.71 (s, 1H, CH); 10.09 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 335 (M^+), 336 (M + 1). Anal. Calcd for $C_{20}H_{21}O_2N_3$: C, 71.64; H, 6.27; N, 12.54. Found: C, 71.05; H, 6.42; N, 12.88.

4.5.10. 4-(Morpholin-4-yl)-N′-(3,4,5-trimethoxybenzylidene) benzohydrazide

Yield: 74.1% (solid); mp 209–211 °C. FTIR (KBr) cm^{-1}: 3272 (N–H); 3079 (Ar C–H); 2992 (C–H); 2864 (N=CH); 1690 (C=O); 1594 (C=N); 1464 (C=C); 1176 (Ar C–O); 1116 (C–O–C). ^{1}H NMR chemical shifts at (300 MHz, $CDCl_3$, δ ppm): 3.38 (t, 4H, CH_2); 3.59 (t, 4H, CH_2); 7.4–7.46 (m, 4H, phenyl); 7.65–7.68 (m, 4H, phenyl); 8.76 (s, 1H, CH); 11.22 (bs, 1H, NH). Mass spectra of compound exhibited molecular ion peak at m/z 339 (M^+), 340 (M + 1). Anal. Calcd for $C_{21}H_{25}O_5N_3$: C, 63.16; H, 6.27; N, 10.53. Found: C, 63.78; H, 6.89; N, 10.84.

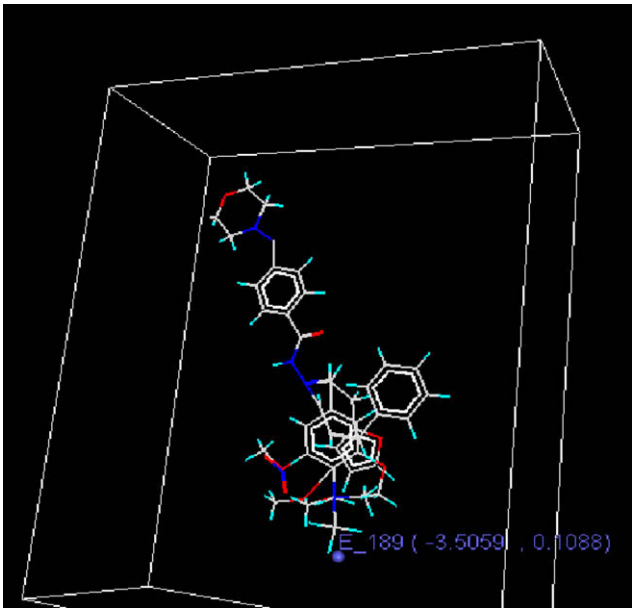


Fig. 2. Stereo view of the molecular rectangular field grid around the superposed 4-(morpholin-4-yl)-N′-(arylidene)benzohydrazides series of Molecular units using SW kNN MFA Method.

4.6. 2D-QSAR studies

All computational studies were performed using V-Life Molecule Design Suite version 3.0 [26]. The compounds were constructed using the fragments in the V-Life molecular database with standard bond lengths and bond angles. Geometry optimization was carried out using standard Merck Molecular Force Filed (MMFF) method with distance dependant dielectric function set to 1.00 and energy gradient of 0.001 kcal/mol. The synthesized compounds (10) along with the standard were used during the 2D-QSAR study.

In case of QSAR study performed for synthesized compounds and reference standard against *M. tuberculosis* H37Rv, expressed as pIC_{50}, two compounds were used in test set, viz., **5g** and **5h** and remaining eight compounds were used in training set and compound **5d** was considered as an outlier.

4.7. 3D-QSAR studies

All computational studies were performed using V-Life Molecular Design Software Version 3.0 [26]. The compounds were constructed using the fragments in the V-life molecular milder database with standard bond lengths and bond angles and geometry optimization was carried out using the standard Merck Molecular Force Field (MMFF) method [27] with distance dependent-dielectric function and energy gradient of 0.001 kcal/mol Å. Alignment of the compounds is a very important feature for any 3D-QSAR method and so was for of Step Wise (SW) kNN MFA method [28]. The overlain of the aligned molecules using MMFF method is shown in Fig. 1. The initial conformations were selected and minimized using the Powell method till root-mean-square deviation of 0.001 kcal/mol Å was achieved.

The compounds were divided into training set (8) and test set (2) respectively using manual selection method by considering the biological and chemical variation. Training set and test set used for developing kNN MFA models using SW kNN MFA model are shown in Tables 4 and 5 respectively. All 8 molecules in the training set were considered as observations to generate QSAR equation using the Step Wise (SW) kNN MFA method.

Table 4
Predicted activity of training set of molecules for 3D-QSAR study.

Sr. No.	Molecule no.	Biological activity pIC_{50}	SW-kNN MFA	
			Predicted activity	Residual
1.	**5a**	−0.89956	−0.705814	−0.1937
2.	**5b**	−1.1445	−0.528838	−0.6157
3.	**5c**	0.4057	0.485445	−0.0797
4.	**5d**	1.3312	0.164762	1.1665
5.	**5e**	−0.1157	0.216743	−0.3324
6.	**5f**	−0.5135	−0.421828	−0.0916
7.	**5h**	−0.643	−0.899957	−1.5429
8.	**5i**	0.0877	0.575019	−0.4873

Table 5
Predicted activity of test set of molecules for 3D-QSAR study.

Sr. No.	Molecule no.	Biological activity pIC_{50}	SW-kNN MFA	
			Predicted activity	Residual
1.	**5g**	−0.7253	−0.714470	−1.4397
2.	**5j**	−0.1503	−0.311808	−0.4621

5. Biological evaluation

All the newly synthesized 4-(morpholin-4-yl)-N′-(arylidene)-benzohydrazides were assayed in vitro for antitubercular activity against *M. tuberculosis* H37Rv and clinical isolates: S, H, R, and E resistant *M. tuberculosis*. In case of antimycobacterial activity, percentage reduction in relative light units (RLU) were calculated using luciferase reporter phages (LRP) assay [29–31] using isoniazid as a reference standard.

5.1. Luciferase reporter phages (LRP) assay [29,30]

Fifty-microliter bacterial suspension equivalent to MacFarlands No.2 standard was added to 400 μl of G7H9 with and without the test compound. For each sample, two drug-free controls and four drug concentrations were prepared and this setup was incubated for 72 h at 37 °C. After incubation 50 μl of the high titer luciferase reporter phage (phAE129) and 400 μl of 0.1 M $CaCl_2$ were added to all the vials and this setup was incubated at 37 °C for 4 h. After incubation, 100 μl of the mixture was taken from each tube into a luminometer cuvette and equal amount of working D-luciferin (0.3 mM in 0.05 M sodium citrate buffer, pH 4.5) solution was added. The RLU was measured after 10 s of integration in the Luminometer (Monolight 2010). Duplicate readings were recorded for each sample and the mean was calculated. The percentage reduction in the RLU was calculated for each test sample and compared with the control. The experiment was repeated when the mean RLU of the control was less than 1000.

Table 6
Statistical parameters giving details of SW kNN MFA method.

Sr. No.	Parameter	SW kNN MFA method
1	q^2	0.5071
2	Pred r^2	0.9102
3	q^2_SE	0.5614
4	Pred_r^2 SE	0.1619
5	k nearest neighbour	5
6	*Contributing descriptor*	
	Electrostatic	E_189 (−3.5059 0.1088)
	Steric	–

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2009.04.023.

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