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IN-SILICO AND IN-VITRO STUDIES OF SYNTHESISED MALON SUBSTITUTED THIOSEMICARBAZIDES*

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ABSTRACT

Tuberculosis (TB) is a communicable disease which is spreading worldwide on an alarming rate. The infection primarily target the lungs (a pneumonia) is caused by bacteria known as Mycobacterium tuberculosis (M.TB). There is a progressive increase in multidrug resistant (MDR) tuberculosis along with the recent increase in cases of tuberculosis. Therefore, there is an urgent need for microbiological research and development of new anti-mycobacterial agents. For this purpose, a series of malonic thiosemicarbazide derivatives (3a-3i) was synthesized and evaluated for in-silico and in vitro anti-tubercular screening. These derivatives were prepared by the reaction of substituted phenyl isothiocynates and substituted malonic acid hydrazides. The structure of these synthesized N-(Malon-substituted-anilic)-4-phenyl thiosemicarbazides were confirmed by various spectroscopic techniques like FT-IR, ^{1}H-NMR and Mass spectroscopy. In order to test the inhibitory effect of the synthesized compounds on protein kinase B (PKnB) from Mycobacterium tuberculosis, computational studies were undertaken. Their in-silico screenings demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol. These compounds

have shown moderate to good scores for drug likeness. REMA (Resazurin microtitre assay) method was used for in-vitro anti-tubercular screenings against M.TB $H_{37}Rv$ in which three of the synthesized compounds were found to be active.

KEYWORDS - Drug likeness, In-silico, In-vitro anti-mycobacterial screening, Synthesis, Thiosemicarbazide.

1. INTRODUCTION

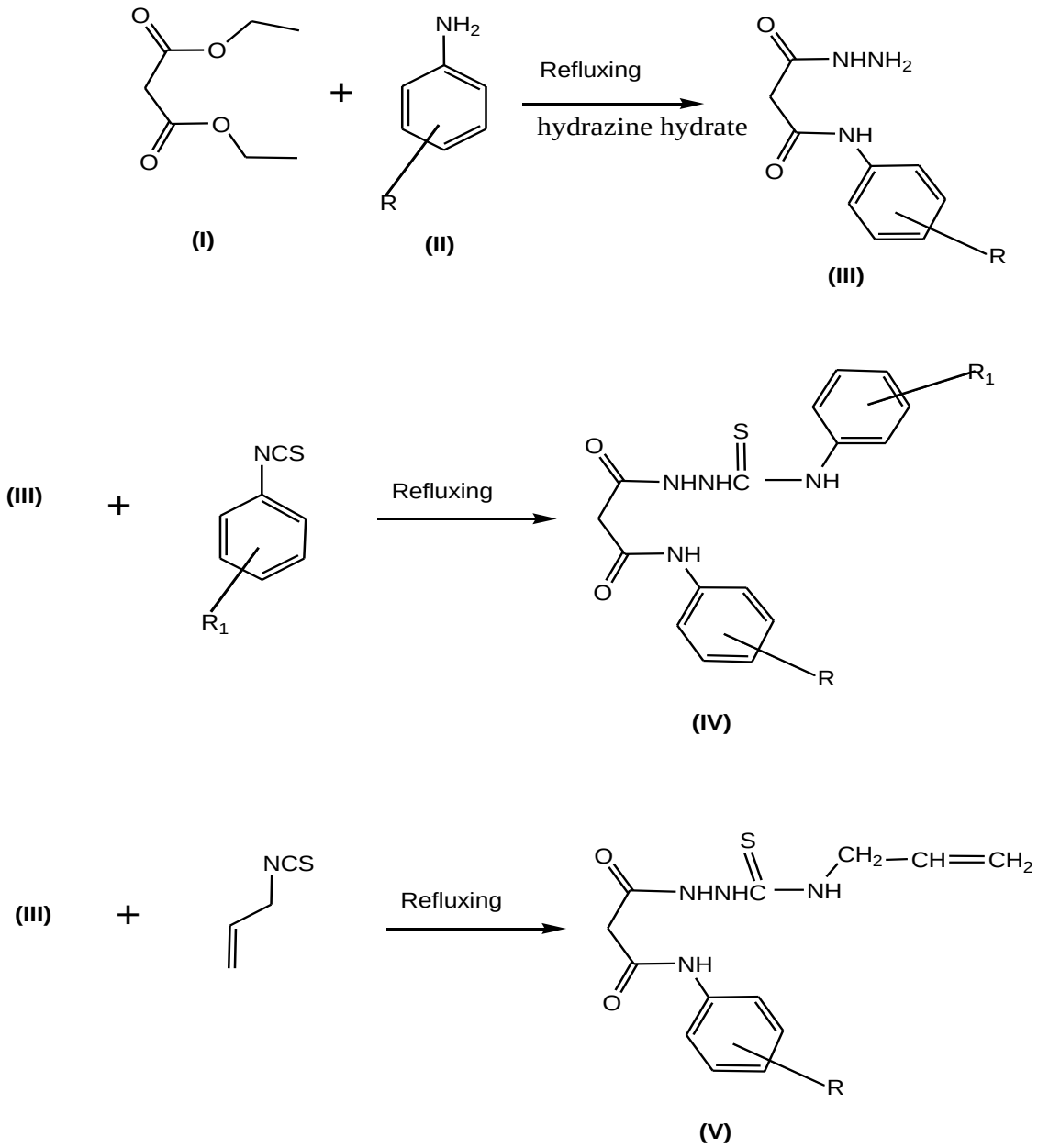
Tuberculosis(TB) caused by a single infectious pathogen is a leading cause of death worldwide. In the present scenario, it is regarded as the most dangerous infective diseases throughout the world. Round about one-third of the world's population has been infected with the causative agent *Mycobacterium tuberculosis* (M.TB). Every year, approximately eight billion people are infected with TB and globally it accounts for almost a million deaths annually [1]. Over the last 15 years [2], there are increase in cases of TB out of which one-fifth of adult deaths are reported from developing countries. In industrialized countries where this disease was almost eradicated, the rise is favored by the pathogenic synergy with Human Immunodeficiency Virus (HIV) infection [3]. It was reported that, if control measure are not effective, in that case around 19-43% of the world population might get infected with M.TB between 2000 and 2020 [2,4]. Moreover the appearance of Single-Drug-Resistant (SDR) and Multi-Drug-Resistant (MDR) accompanied with emergence of TB is particularly alarming [5]. Several fatal out brakes have been caused by MDR-TB [6]. In some parts of the world, where the incidence of MDR-TB is expected to be as high as 14%, it remains to be a public health crisis and poses a significant health security threat to the treatment and control of the disease. As per estimations of WHO (World Health Organization), 600,000 new cases are rifampicin resistant, which is the most effective first line drug [7]. However, in the last 40 years, the Food and Drug Administration (FDA) have approved only few drugs for the treatment of TB due to lack of pharmaceutical industry research in this particular area [6,8]. These serious concerns require particular attention. Thus, stimulating the continued search for new anti-mycobacterial drug candidates. Thiosemicarbazide bearing moities have been reported as anti-tubercular agents [9-11] along with other biological activity like antifungal [12] hypoglycemic [13] and anti-viral [14] activities. Keeping these points in mind, we herein this paper report synthesis of some thiosemicarbazide analogues along with their in-silico virtual screening and in-vitro anti-mycobacterial screenings against *Mycobacterium tuberculosis*.

2. EXPERIMENTAL

The chemicals were purchased from Sigma Aldrich/Merck and used without further purification. Melting points are uncorrected and were determined in open capillary tubes. Silica-gel-coated Al plates purchased from Merck were used to check the purity of the compound. The structures of the compounds was elucidated on the basis of their Infra red spectra (IR) which was done using KBr discs, on a Perkin Elmer Spectrum RX1 infra red spectrophotometer. ^{1}H NMR spectra was recorded in DMSO on Bruker DRX-300 (300 MHz) and Jeol AL300 FT-NMR (300 MHz) systems; chemical shift (δ) are reported in ppm using TMS as an internal reference. Elemental analysis was performed on Elementor Vario EL III. All the compounds gave satisfactory microanalysis. All the synthesized compounds were exploited for their physicochemical properties by web base software, MolSoft 2007. Docking was done onto the nucleotide-binding pocket of the M. tuberculosis PKnB structure (PDB ID: 2FUM) [15] using the program AutoDock4 [16]. In-vitro anti-tubercular screenings were carried out using REMA method [17].

2.1. Synthesis

Synthesis of N-(substituted)phenyl malonamic acid hydrazides was achieved as per our reported method [18]. N-(substituted)phenyl malonamic acid hydrazide (0.001mol) and (4-substituted)phenyl isothiocynate (0.001mol) was dissolved in 7 ml and 3 ml of ethanol respectively. The mixture was refluxed for two hours. The white product obtained on cooling was recrystallized with hot absolute ethanol and was found to be N-(Malon-substituted-anilic)-4-phenylthiosemicarbazide as shown in scheme 1



Scheme 1

2.2. Characterization

Physicochemical characterization of the synthesized compounds was done by the estimation of melting point. Structural characterization was done by using elemental analysis, IR and ^{1}H NMR.

N-(Malon-4-bromo-2-fluoroanilic)-4-(4'-bromophenyl)thiosemicarbazide ***(3c):*** White crystal; Yield: 0.13g, 28.88%; m.p: 178 ^{o}C; IR data for said compound are N-H stretching at 3435 cm^{-1}, C-H stretching at 2994 cm^{-1} (CH_2), N-C=O, stretching of amide-II at 1533 cm^{-1}, (>C=C< / Ar-C-C, stretching at 1646 cm^{-1}, C=S at 1335 cm^{-1}, C-N, stretching of primary aromatic amine at 1262 cm^{-1}, -N-N-, stretching at 1220 cm^{-1}, ArC-Br at 1012 cm^{-1} and mono

substitution of ring at 771 cm^{-1}; Anal. Calc. for $C_{16}H_{13}O_2N_4Br_2FS$: C 38.09, H 2.57, N 11.11, S 6.34; Found: C 38.18, H 2.47, N 11.17, S 6.40.

*N-(Malon-4-bromo-2-fluoroanilic)-4-(4'-fluorophenyl)thiosemicarbazide (**3g**):* White crystal; Yield: 0.17g, 38.46%; m.p: 176 ^oC ; IR (KBr) (cm^{-1}): 3433 (N-H, stret.), 2997 CH_2(C-H stret.), 1529 (N-C=O, stret., amide-II), 1646 (>C=C< / Ar-C-C, stret.), 1339 (C=S), 1262 primary aromatic amine (C-N, stret.), 1219 (-N-N-, stret.), 1110 (ArC-F, stret.), 1012 (ArC-Br, stret.), 771 (mono substituted ring); ^{1}H-NMR (300 MHz, DMSO-d6) (δ ppm): 2.25 (s, H, NH), 2.25-2.30 (s, 1H, HN-C=S), 3.42 (s, 2H, CH_2), 4.02-4.05 (s, 1H, NH), 6.32-6.85(m, 4H, Ar-H), 7.16(s,1H, Ar-H), 7.61(s,1H, Ar-H), 7.22(s,1H, Ar-H), 9.43 (s, 1H, CONH), 10.06(s, 1H, CONH); Anal. Calc. for $C_{16}H_{13}O_2N_4BrF_2S$: C 43.34, H 2.93, N 12.64, S 7.22; Found: C 43.42, H 2.89, N 12.70, S 7.15.

*N-(Malon-4-bromo-2-fluoroanilic)-4-allyl thiosemicarbazide(**3i**):* White; Yield: 0.13g, 33.51%; m.p: 182 ^oC; IR (KBr) (cm^{-1}): 3433 (N-H, stret.), 2997 CH2(C-H stret.), 1529 (N-C=O, stret., amide-II), 1646 (>C=C< / Ar-C-C, stret.), 1339 (C=S), 1262 primary aromatic amine (C-N, stret.), 1219 (-N-N-, stret.), 1110 (ArC-F, stret.), 1012 (ArC-Br, stret.), 771(mono substituted ring);^{1}H-NMR (300 MHz,DMSO-d6) (δ-ppm): 2.25-2.30 (s, 1H, HN-C=S), 3.42 (s, 2H, CH_2), 4.02-4.05 (s, 1H, NH), 4.11 (s, 2H, N-CH_2), 5.06 (s, 1H, >C=CH_a), 5.10 (s, 1H, >C=CH_b), 5.16 (s, 1H, CH=CH_2), 9.43 (s, 1H, CONH), 10.06(s, 1H, CONH); Anal. Calc. for $C_{13}H_{14}O_2N_4BrFS$: C 40.10, H 3.85, N 14.39, S 8.25; Found: C 40.23, H 3.80, N 14.45, S 8.24.

2.3. Physicochemical properties

Molsoft online software (www.molsoft.com) was used to calculated physico-chemical properties like molecular weight, partition coefficient (log P), polar surface area, number of stereocentres, number of hydrogen donor, number of hydrogen acceptor and drug likeness score of the synthesized compounds.

2.4. In-Silico Molecular Docking

ChemDraw Ultra 8.0 (ChemOffice package) was used to draw the ligands. AutoDock Tools were employed for converting these ligands to energy minimized 3D structures for in silico protein–ligand docking. All the heteroatoms were removed from the 2FUM in order to make the receptor ligand free before doing the docking. Lamarckian Genetic Algorithm [19] was used for docking the thiosemicarbazides. 9 molecules were screened and the grid maps representing the protein were calculated using auto grid. The grid size was set to 60*60*60 points with grid spacing of 0.375 Å. Docking was carried out with standard docking protocol

on the basis of a population size of 150 randomly placed individuals; a maximum number of 2.5 *107 energy evaluations, a mutation rate of 0.02, a crossover rate of 0.80 and an elitism value of 1. Ten independent docking runs were carried out for each ligand and results were clustered according to the 1.0 Ǻ rmsd criteria. The molecules were then tested for structure analysis by the visualization tool. UCSF chimera within 6.5 Ǻ region was used to visualize the coordinates of the docked protein along with the ligand.

2.5. In-Vitro Antimycobacterial Activity

In-vitro screenings against M.TB (H37Rv) was done by REMA method. The selected media was middle brook 7H9 supplemented with OADC obtained from Hi-Media. A 100µL of the media was dispensed in each well of a 96 well cell culture plate. The compounds were tested at different concentrations (i.e. 3.25, 6.26, 12.5, 25, 50, 100, 200, 400, 500, 1600µg/ml) for the determination of minimum inhibitory concentrations (MIC's). Ethambutol and rifampicin were used as standard drug.

3. RESULT AND DISCUSSION

The proposed work was carried out with the intention of synthesis of some thiosemicarbazides (scheme 1) and to exploit their potentials via in-silico and in-vitro screenings. The IR spectra depicted the signature peak of NH_2 at 3435cm^{-1}, sec. amide(N-C=O) 1531cm^{-1}, and carbonyl peak(C=O) at 1798-1742 cm^{-1}. The characteristic peak (C=S) of the title compounds i.e. Thiosemicarbazide occurs at 1396-1339 cm^{-1}, sec. amide (N-C=O) at 1541-1529 cm^{-1} and the methylene group(CH_2) peak from malonamic acid hydrazide and thiosemicarbazide occur at 2930-2897 cm^{-1}. The proton spectra revealed the characteristic CH_2 signal at δ 3.46-3.51(ppm) and proton signal of sec. amide (CONH) at δ 7.91-8.30 (ppm) and NH_2 proton of compound occurs at δ 2.52-2.90 (ppm). The in-silico screenings demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol. Out of 9 molecules, 7 protein-ligand complex showed H - bond with the active site residue VAL 95 (Table 1, Figure 1). The synthesized compounds had shown favorable drug-like properties were drug Likeness scores ranged from -0.47 to 0.36 as given in table 1. Lipinski's rule of 5 with additional parameters predicted by Molsoft for these synthesized compounds is given in Table 2. MICs along with melting points and yields of the compounds is reported in Table 3. The obtained result revealed that the change in color from blue to pink shows bacterial growth in culture plate indicating compounds 3a, 3b, 3d, 3e, 3f, 3i are not active at

these concentrations. On contrast, compounds 3c, 3g, 3h were found to be active at 1600μg/ml concentration with no color change in culture plate.

4. CONCLUSION

The present study reports the synthesis, characterization, virtual and biological evaluation of a series of synthesized thiosemicarbazide derivatives (3a-3i). They were characterized by elemental analysis and spectroscopic (FT-IR and ^{1}H NMR) techniques. In-silico molecular docking results revealed the binding mode of the thiosemicarbazides at the active site of 2FUM. The docking results demonstrated that the binding energies were in the range of -7.32 kcal/mol to -8.95 kcal/mol, with the minimum binding energy of –8.95 kcal/mol. 7 compounds has shown H-bond interactions with VAL 95, the active site residue. Drug Likeness scores ranged from -0.47 to 0.36. Compounds 3c, 3g and 3h were found to be active against M.TB (H37Rv) at 1600 μg/ml concentration with no change in color of resazurin solution in culture plates.

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Table 1: Binding Energies (Kcal/Mol) And Drug Likeness Scores Of The Synthesized Thiosemicarbazides.

Compound	Mol. formula	Min Binding Energy (kcal/mol)	H-Bond Info	No. of stereo Centre	Drug likeness score
3a	$C_{17}H_{17}O_2N_4BrS$	-8.16	ASP 96(2.868 Å)	0	0.14
3b	$C_{17}H_{17}O_2N_4BrS$	-8.40	VAL 95(2.740 Å), TYR 94 (3.381 Å), GLY 97 (3.166 Å)	0	-0.05
3c	$C_{16}H_{13}O_2N_4Br_2FS$	-8.95	VAL 95(3.275 Å), TYR 94(3.301 Å)	0	-0.42
3d	$C_{16}H_{13}O_2N_4BrFClS$	-8.27	GLU 93(3.295 Å)	0	-0.38
3e	$C_{16}H_{14}O_2N_4BrClS$	-8.35	VAL 95(2.646 Å), TYR 94(3.126 Å)	0	0.16
3f	$C_{16}H_{14}O_2N_4ClFS$	-7.77	VAL 95(2.753 Å)	0	0.36
3g	$C_{16}H_{13}O_2N_4BrF_2S$	-8.21	VAL 95(2.855 Å)	0	-0.40
3h	$C_{16}H_{13}O_2N_4ClF_2S$	-8.22	VAL 95(2.475 Å), TYR 94(3.254 Å)	0	-0.14
3i	$C_{13}H_{14}O_2N_4BrFS$	-7.32	VAL 95(3.501 Å)	0	-0.47

Table 2: Lipinski's Rule Of 5 With Additional Parameters.

Compound	Mol. Wt.	Log P[a]	Log S[b] [Log(moles/L)]	PSA[c]	HBA[d]	HBD[e]
3a	420.03	2.83	-4.96	69.40	3	4
3b	420.03	2.95	-4.96	70.10	3	4
3c	501.91	3.55	-5.90	69.40	3	4
3d	457.96	3.42	-5.88	70.10	3	4
3e	439.97	3.15	-5.07	69.40	3	4
3f	380.05	2.56	-4.44	69.40	3	4
3g	441.99	2.97	-5.27	69.40	3	4
3h	398.04	2.83	-5.25	70.10	3	4
3i	388	1.80	-3.39	70.84	3	4

[[a]solubility parameter, [b]calculated lipophilicity, [c]polar surface area (A^2), [d]number of hydrogen bond acceptors, [e]number of hydrogen bond donors]

Table 3: Melting Point, % Yield And Anti-Tubercular Activity Screenings

Compound	R	R_1	M.P (^{0}C)	Yield (%)	MIC(µg/ml) (1600µg/ml)
3a	2-CH_3	4-Br	208	30.71	Inactive
3b	4-CH_3	4-Br	205	50.43	Inactive
3c	4-Br, 2-F	4-Br	178	28.88	Active
3d	3-Cl, 4-F	4-Br	174	31.12	Inactive
3e	2-Cl	4-Br	178	25.03	Inactive
3f	2-Cl	4-F	168	50.06	Inactive
3g	4-Br, 2-F	4-F	176	38.46	Active
3h	3-Cl, 4-F	4-F	98	25.20	Active
3i	4-Br, 2-F	Allyl	182	33.51	Inactive

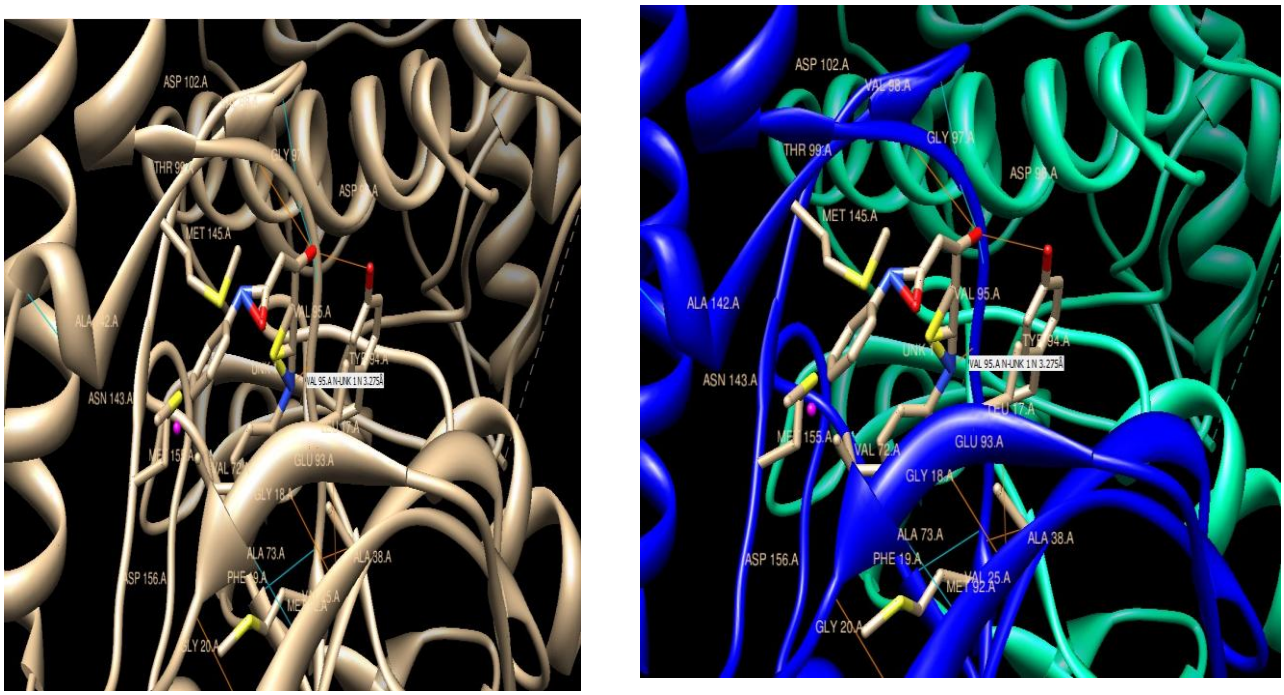

Figure 1: H- Bond Interaction With Active Site Residue