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Microwave-assisted synthesis of spirocarbocycle derivatives and their anticancer activity, molecular docking studies

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ABSTRACT

An efficient microwave assisted one pot protocol has been developed to synthesize four spirocarbocyle derivatives from few vinyl malononitriles, substituted aldehydes and indandione. The synthesized compounds were characterized using IR, ^{1}H NMR, ^{13}C NMR, mass spectral studies and elemental analysis. Anti-cancer studies were carried out for compounds 4a-d using (MCF-7) and the docking study of these compounds was also performed in order to verify the efficiency and binding interaction with BRCA1 protein with the PDB ID: 1JNX. For the anti-cancer activity compound 4d showed a maximum activity with an IC50 value of Compounds 4d (4.02±0.98 µg/ml), 4c (3.89±0.82 µg/ml) and 4a (4.42±0.98 µg/ml) were showed almost close activity when compare with the satandard doxorubicin (3.36±0.38). The least binding energy in docking study was found for the compound -16.03 kcal/mol was best among all tested compounds.

Keywords: Spirocarbocycle, Vinyl malononitriles, Multi component reaction, MCF-7, Anticancer activity, Molecular docking.

INTRODUCTION

Spirooxindole signifies vital frames for the synthesis of biologically essential synthetic and natural compounds in drug discovery [1, 2]. Specifically spiro compounds are found in number of biologically active natural and synthetic assets and find application in antimicrobial [3, 4, 8, 16] anticancer [3, 5-7, 9, 10, 15, 19-21] and anti-inflammatory [5, 25] activities. The literature survey reveals that there has been a wide range of active research being carried out in the synthesis of spirooxindoles for the past two decades. The multi component reactions (MCR) are very flexible, atom economic in nature and proceed through a sequence of reaction equilibrium yielding highly substituted derivatives without formation of other byproduct [1, 5, 10, 11, 24, 25]. The development of one-pot MCRs attracted considerable attention due to the various advantages observed in the synthesis5such as simple procedures, minimum time and energy efficiency, high product yields and low cost [24]. The earlier records reported that wide range of compounds like 1,3,4-thiodiazole-2-amide derivatives,1,4-benzodioxan [15], peptide modified thymopentin [18], domino reaction of spirocarbocycle5have been synthesized by following one-pot methodology. As microwave-promoted organic synthesis is an efficient and environment friendly synthetic route in modern synthetic organic chemistry as well as in green chemistry [22, 23, 24] an attempt is taken in this present work to synthesize a set of novel compounds from vinyl malononitriles, substituted aldehydes and indandione and to investigate anticancer

activity. In the present study, the synthesis of bioactive compounds was carried out using green chemistry and microwave assisted which is acting as a powerful alternative method to the conventional synthesis [24]. Compounds 4a-d were synthesized for the evaluation of cytotoxicity using the MTT assay [26]. For the *in silico* studies, the Lamarckian Genetic Algorithm is the primary and promising method for conformations searching [27] and AutoDock have been proved to be a valuable tool capable of accurate prediction of the binding conformations and binding energies of ligands with macromolecular targets [28-30].

MATERIALS AND METHODS

CHEMISTRY

Melting points were determined on Gallen kamp melting point apparatus and are uncorrected. Analytical TLC was performed on pre-coated aluminium sheets of silica gel 60F254 of 0.2 mm thickness (Merck, Germany). Infrared (IR) spectra were recorded on a PerkinElmer FT-IR spectrometer using KBr pellets. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded in DMSO-d_6 solution using TMS as an internal standard on a Bruker Advance DPX-400 MHz instrument. Proton chemical shifts (d) are relative to tetramethylsilane (TMS, δ=0.00) as internal standard and expressed in parts per million. The number of protons (n) for a given resonance was indicated as nH. Coupling constants (J) are given in Hertz. Spin multiplicities are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Mass spectra were recorded under HR-MS (ESI) using Thermo Scientific Exactive Orbitrap mass spectrometer and Thermo Finnigan LCQ Advantage MAX 6000 ESI mass spectrometer and Perkin-Elmer GC-MS. Elemental analysis data were recorded using Thermo Finnegan FLASH EA1112 CHN analyzer.

Synthesis of spirocarbocycle derivatives

The mixture of indandione (1 mmol), substituted aldehydes (1mmol) and alkylidene malononitrile (1 mmol) were stirred in MeOH in the presence of L-proline (20 mol%) and allowed microwave irradiation at 600 Watt for 6-10 minutes. The reaction mixture was stand at room temperature and it was treated with cold water. The solid product was filtered, washed with water and recrystallized using methanol.

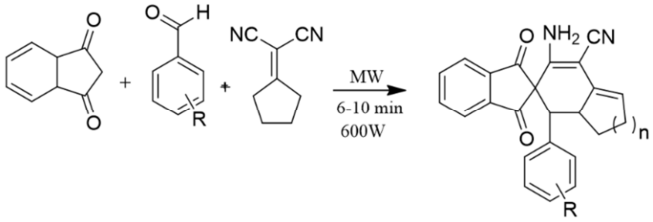

Figure 1. Proposed scheme and achieved compound structures

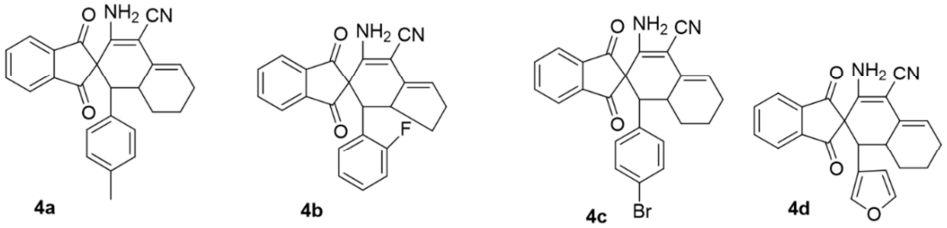

Figure 2. Achieved compound structures 4a-d

3'-Amino-1,3-dioxo-1'-p-tolyl1,3,6',7',8',8a'-hexahydro-1'Hspiro[indene-2,2'naphthalene]-4'carbonitrile (4a). Yellow solid; mp: 224-226 ˚C (Decomposes); IR (cm^{-1}): 755, 1246, 1589, 1661, 1704, 1742, 2205, 2921, 3246, 3345, 3408. ^{1}H-NMR(400 MHz,DMSO-d_6): δ.0.71 (q, 1H, J =12.4 Hz),1.32-1.42 (m, 2H), 1.64-1.66(m, 1H), 1.99 (s, 3H), 2.08-2.20 (m, 2H), 2.98 (d, 1H,J = 12.8 Hz),3.04-3.07 (m, 1H), 5.59-5.61 (m, 1H), 6.12 (brs, 2H, -NH_2,D_2O exchangeable), 6.61 (d, 1H, J = 7.6 Hz), 6.72-6.79 (m, 3H), 7.67 (d,1H, J = 7.6 Hz), 7.75-7.77 (m, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6):δ 20.0, 22.1, 25.4, 27.9, 33.5, 52.2, 63.6, 82.9, 117.4,118.1, 123.1, 123.7,126.6, 129.0, 129.2, 129.2, 131.4, 131.8, 133.1, 136.4, 136.6, 142.7,143.3, 151.9, 199.7, 200.2. HRMS (ESI): Mass calculated for $C_{26}H_{22}N_2O_2Na$ [M+Na] 417.1573, found, $[M+Na]^+$ 417.1573. Anal. Calcd. For: ($C_{26}H_{22}N_2O_2$) C, 79.16; H, 5.62; N, 7.10 Found: C, 79.05; H,5.73; N, 7.01.

6-Amino-4-(2-fluorophenyl)-1',3'-dioxo-1',2,3,3a,3',4hexahydro2,5'spirobi[indene]-7-carbonitrile (4b). Yellow solid; mp222-225 ˚C (Decomposes); IR (cm^{-1}): 761, 1245, 1573, 1646, 1708,1742, 2199, 2829, 3258, 3359, 3445; ^{1}H-NMR (400 MHz, DMSO-d_6):δ 0.25 (q, 1H, J = 11.6 Hz), 1.03-1.04 (m, 1H), 1.57-1.66 (m, 2H), 2.42(d, 1H, J = 12.4 Hz), 2.91-2.97 (m, 1H), 4.71-4.72 (m, 1H), 5.78 (brs,2H, -NH_2, D_2O exchangeable), 6.09 (t, 1H, J = 9.2 Hz), 6.16-6.20 (m, 1H), 6.27-6.34 (m, 2H), 7.00-7.01 (m, 1H), 7.08 (d, 3H, J = 2.9 Hz);^{13}C-NMR (100 MHz, DMSO-d_6): δ 35.0, 36.0, 47.2, 48.9, 69.5, 83.4,120.1, 121.8, 122.6, 128.1, 128.3, 129.6, 133.9, 135.1, 141.6, 141.7, 142.2,147.0, 147.6, 159.2, 163.2, 203.5, 204.0; MS m/z = 385 $[M+H]^+$; Anal. Calcd. for $C_{24}H_{17}FN_2O_2$: C, 74.99; H, 4.46; N, 7.29. Found: C, 75.17; H, 4.35; N, 7.38.

3'-Amino-1'-(4-bromophenyl)-1,3-dioxo-1,3,6',7',8',8a'hexahydro-1'Hspiro[indene 2,2' naphthalene] 4'carbonitrile (4c). Yellow solid; mp 254-256 ˚C (Decomposes); IR (cm^{-1}): 805, 1243,1589, 1636, 1696, 1738, 2200, 2948, 3250, 3361, 3452; ^{1}H-NMR(400 MHz, DMSO-d_6): δ 0.72 (q, 1H, J = 12 Hz), 1.30 (d,1H,J ¼=11.2 Hz),1.39-1.42 (m, 1H),1.65 (d, 1H, J = 9.2 Hz), 2.06-2.07 (m, 1H), 2.15-2.20 (m,1H), 3.03 (d, 1H, J = 11.2 Hz) 3.34-3.41 (m, 1H),5.60-5.62 (m, 1H), 6.18 (brs,2H,-NH_2,D_2Oexchangeable), 6.70 (d,1H, J =7.6 Hz), 7.17 (d, 1H, J = 8.4 Hz), 7.69 (d, 2H, J = 8.4 Hz), 7.69 (d,1H, J = 7.6 Hz),7.74-7.81 (m, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6):δ 22.0, 25.3, 27.7, 33.3, 51.8, 63.4, 82.8, 117.7, 118.0, 120.9, 123.3,123.8, 129.0, 131.4, 131.5, 133.5, 135.6, 136.7, 136.8, 142.5, 143.2,151.6, 199.5, 200.0; MS m/z =459$[M+H]^+$; Anal. Calcd. for $C_{25}H_{19}BrN_2O_2$: C, 65.37; H, 4.17; N, 6.10. Found: C, 65.26; H, 4.27; N, 6.20.

3'-Amino-1'-(furan-3-yl)-1,3-dioxo-1,3,6',7',8',8a'hexahydro1'Hspiro[indene-2,2'-naphthalene]-4'carbonitrile(4d). Yellow solid; mp 247-248 ˚C (Decomposes); IR (cm^{-1}): 1243, 1589,1660, 1704, 2206, 2923, 3253, 3347,3422;^{1}H-NMR(400MHz,DMSO-d_6): δ 0.79 (q, 1H, J = 12 Hz),1.41-1.43 (m, 2H), 1.69-1.72 (m, 1H), 1.94-2.19 (m, 2H), 2.87-2.92 (m, 1H), 2.98 (d, 1H, J = 12.4 Hz),5.57-5.59 (m, 1H),5.87 (s, 1H) 6.14 (brs, 2H, -NH_2,D_2O exchangeable),7.13 (s, 1H), 7.20 (s, 1H), 7.79-7.80 (m, 1H), 7.84-7.85 (m, 1H);^{13}C-NMR (100 MHz, DMSO-d_6): δ 26.7, 30.1, 32.6, 38.2, 47.6, 67.5,87.6, 122.2, 122.8, 125.1, 128.1, 128.6, 136.2, 141.5, 146.5, 147.4, 148.1,148.9, 156.3, 204.7, 204.9; MS m/z =371 $[M+H]^+$; Anal. Calcd. for $C_{23}H_{18}N_2O_3$: C, 74.58; H, 4.90; N, 7.56. Found: C, 74.48; H, 4.79; N, 7.65.

PHARMACOLOGY

***In vitro* Cytotoxicity studies:**

Cell line Culture

MCF-7 (Michigan Cancer Foundation-7), a human breast cancer cell line was used for the cytotoxicity studies. Cells were cultured and preserved as we reported earlier [31]. Normal breast (MCF-7) cells were cultured in 1:1 mixture of DMEM and Ham's F12 medium with 20 mg/ml of epidermal growth factor (EGF), 100 µg/ml cholera toxins, 0.01 mg/ml insulin and 500 µg/ml hydrocortisone, and 5% chelex treated horse serum. 1 ml of homogenized cell suspension was allowed in microtiter plate wells and kept in desiccator under 5% CO_2 atmosphere for bio-assay. Cells were observed in an inverted microscope after 48 hours of incubation. 0.05 ml of the drug was dissolved in 4.95 ml of DMSO to get a working concentration of 1 mg/ml. Drug concentrations were filtered using a 0.45 micron filter before bioassay.

MTT Assay

The anticancer activity of compounds 4a-d on MCF-7 cells was determined by the MTT (3-(4, 5-dimethyl thiazol-2yl) -2, 5-diphenyl tetrazolium bromide) assay the cytotoxicity. Approximately 5000 cells were seeded in 96-well, flat-bottom titer plates and incubated for 24, 48, and 72 hours at 37 ˚ C in 5% CO_2 atmosphere. Different concentrations of compounds 4a-d (50 – 500 µg/mL) were added and incubated further for various time periods. After completion of incubation, the medium was removed. The wells were washed with Phosphate Buffer Solution (PBS). To this 100 µL of the working MTT dye in DMEM media was added and incubated for 2 hours. MTT lysis buffer (100 µL) was added and incubation continued for 4 hrs more. The absorbance was measured at 570 nm and the cell viability was calculated using the following farmula,

Cell viability (%) = Mean OD/Control OD x 100%

Molecular docking studies

In order to get more insight into the binding mode of the compounds, docking studies were performed using Autodock version 4.2.6, Autodock Tools (ADT) version 1.5.6. and the Arguslab version 4.0.1. The structures of compounds 4a-d were generated as ligands as we reported earlier for isoquinoline derivatives in our previous studies

[32] using Chemdraw ultra 10.0 version of Cambridge University. Their 3D atomic coordinates were created utilizing the ACD/Labs – Chemsketch 12.0 software. Compound geometries were cleaned and generated as the corresponding *pdb*. files using the Argus lab software. The three dimensional structure of human BRCA1 enzyme (PDB ID: 1JNX) was retrieved from the protein data bank (PDB) (Source: www.rcsb.org/pdb/). The proteins and ligands in the docking tests were treated using the united-atom approximation and only polar hydrogens were added to the protein, and Kollman united-atom partial charges were assigned. Unless stated otherwise, all waters were removed [17]. The *pdbqt* files for protein and ligands preparation and grid box creation were completed using Graphical User Interface program AutoDock Tools (ADT). AutoGrid was used for the preparation of the grid map using a grid box. The grid size was set to 60 × 60 × 60 xyz points with grid spacing of 0.375 Å and grid center was designated at dimensions (x, y, and z): 1.234, 1.064 and 1.964. The ligands 4a-d was docked into the active sites of BRCA1. The results less than 2.0 Å in positional root-mean-square deviation (RMSD) was clustered together and represented by the result with the most favorable free energy of binding. The docked poses with Lowest Binding Energy, Hydrogen bond, π-π interaction and π-cation interaction results were recorded (Table 1&2) and validated.

Statistical analysis

All biological *in vitro* and *in silico* experiments results were expressed as percentage decrease with respect to control values and compared by one-way ANOVA with Dunnett's post test was performed. GraphPad Prism version 6.07 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used for statistical analysis. A difference was considered statistically significant if $p \leq 0.05$. The 50% inhibitory concentration (IC_{50}) was calculated from the dose response curve obtained by plotting percentage inhibition verses concentrations.

RESULTS AND DISCUSSION

Synthesis of spirocarbocycle derivatives

One-pot multi component reaction of substituted aldehydes with vinyl malononitriles was carried out. The method of microwave assisted synthesis affords the advantage in the convention of getting maximum yield of spirocarbocycle compounds, whereas in the conventional method only using moderate level of yield would be produced. The structure of all the synthesized compounds was elucidated with the aid of IR, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy and elemental analysis. The IR spectrum of all the compounds show peaks at 2200 and 1702 cm^{-1} corresponds to -NH stretching of $-NH_2$ group and –HC=O group of nitrile and amide groups, respectively which clearly indicating the incorporation of both moieties in the product, which was further supported by ^{1}H NMR spectroscopy. Comparing our previous report [5], in this microwave assisted synthesis the compound yields were much improved and also the methods is totally green chemistry.

In vitro **anti–cancer activities**

The human breast carcinoma cells (MCF-7) were incubated with different doses (50 to 500 µg/ml) of compounds 4a-d to evaluate the cytotoxic activity using the our earlier methodology repoted using doxorubicin as the standard [26]. According to the results depicted in the table 1, it was recognized that all compounds 4a-d was able to inhibit the proliferation of the cancer cells (MCF-7). IC_{50} values indicate that some of the tested compounds were as active as doxorubicin. Compounds 4d (4.02±0.98 µg/ml), 4c (3.89±0.82 µg/ml) and 4a (4.42±0.98 µg/ml) were showed almost close activity when compare with doxorubicin (3.36±0.38). While considering percentage inhibition, it was 70.42±7.18 for doxorubicin. Among all the tested compounds, 4d showed (66.81±9.25) comparable % inhibition to doxorubicin. Rest of the compounds were showed a moderate activity. The least activity was found in 4b. None of the compounds showed activity as like as or more to the standard doxorubicin. *p*-value was remakably best for the compound 4d (<0.0001) when comparing to the standard doxorubicin (0.0126).

Table 1. Inhibitory percentage results of MCF7 cell lines*

Entry	% Inhibition Mean±SD	R^2 value	p-value	**IC_{50} (µg/ml)
4a	55.56±7.28	0.9124	0.0176	8.82±1.24
4b	33.14±6.61	0.9371	0.0204	10.32±2.54
4c	60.41±6.27	0.9151	0.0089	5.84±2.78
4d	66.81±9.25	0.9103	0.0098	4.02±0.98
Std[#]	70.42±7.18	0.9062	0.0126	3.36±0.38

*[#]Standard-Doxorubicin, *Data were expressed as mean ±SD (n = 4), statistically significant differences are at P < 0.05, ** Data were expressed as mean ±SD (n = 4).*

Molecular docking studies

Molecular docking studies of 4a-d were carried out to predict the anticancer activity. Crystal structure of doxorubicin was used as the standard for docking analysis. The binding free energy for 1JNX was found between -16.03 and -5.62 kcal/mol. These free energy values indicating that the newly synthesized compounds had shown a fortunate selectivity towards BRCA1. The 2D view of protein–ligand interactions of the best poses generated by 1JNX studied routines are shown in Figure 1-6. All the top docked poses generated by each docking routine exhibited well-established bonds with one or more amino acids in the binding pocket of 1JNX.

Table 2. Docking results of 4a-d with BRCA1 (PDB ID: 1JNX) protein

Entity	BE (kcal/mol)	LE	AA# residue	H-bond Å	π-π interaction	π-cation interaction
4a	-8.23	-0.32	Ser1755	2.961Å	-	Arg1762 to Ar-2
4b	-5.62	-0.38	Ser1755 Gln1846, Arg1751	1.869Å Invisible	-	Arg1762 to Ar-2
4c	-10.56	-0.42	Glu1754 Gln1546	2.991Å 2.867Å	Phe1761 to Ar-3	-
4d	-16.03	-0.64	Asp1840 Leu1831	0.159Å 1.641Å	-	Arg1699 to Ar-3

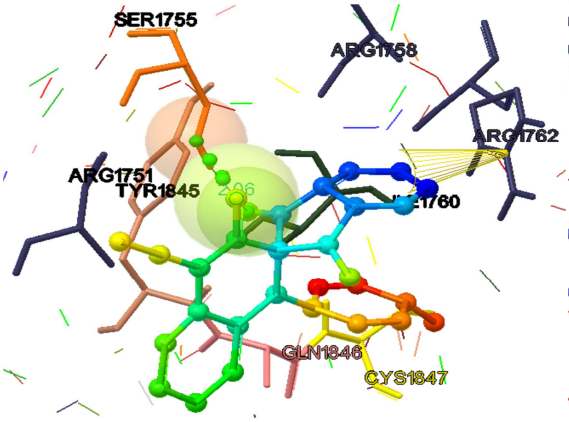

Figure 1. 4a interaction with BRCA1

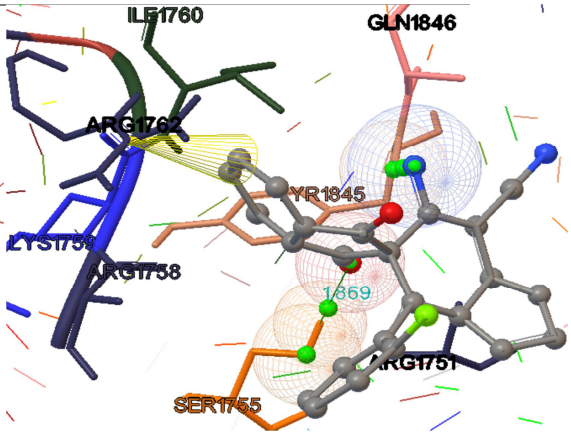

Figure 2. 4b interaction with BRCA1

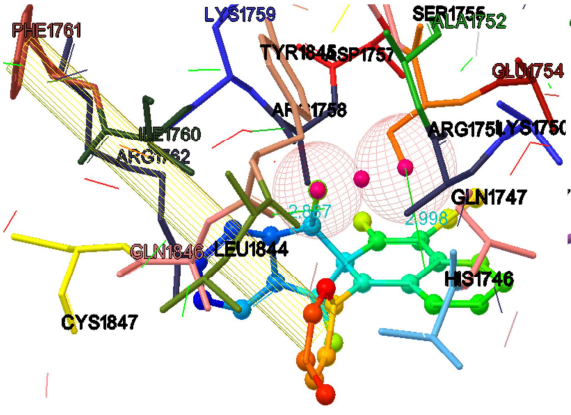

Figure 3. 4c interaction with BRCA1

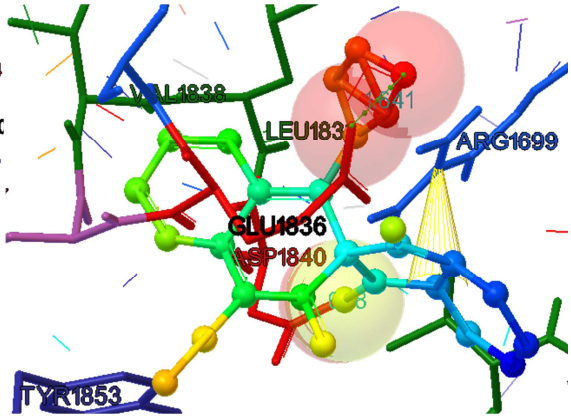

Figure 4. 4d interaction with BRCA1

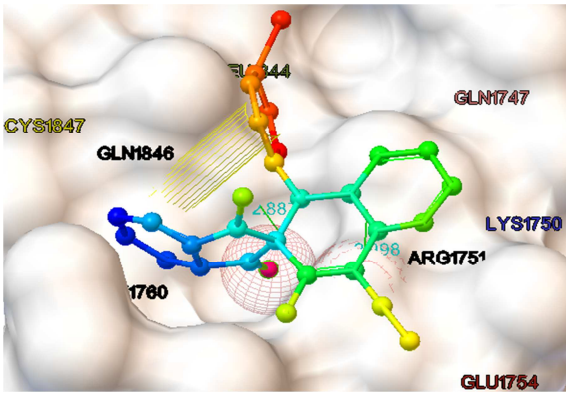

Figure 5. 4c in the binding pocket of 1JNX

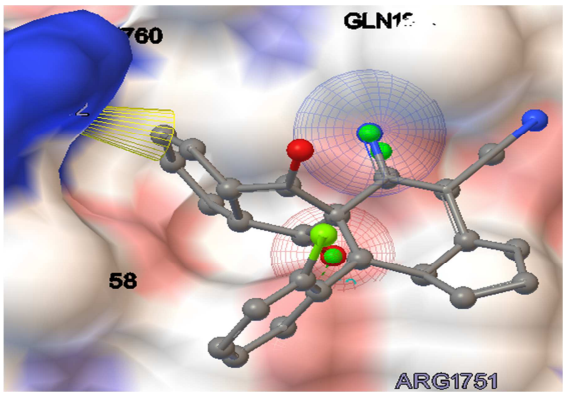

Figure 6. 4d in the binding pocket of 1JNX

CONCLUSION

Bioactive spirocarbocycle compounds namely (4a, 4b, 4c and 4d) were synthesized using substituted aldehydes, alkylidene malononitrile through Michael addition reaction by microwave assisted synthesis method. The new synthetic route developed is found to be efficient and environmentally benign as it involves minimum amount of solvent and there was no formation of byproduct. The bioassay of the compounds was investigated and the results revealed that all the compounds are effective against MCF-7 cell lines for breast cancer. In particular, compound 4d is found to be more efficiency among all the compounds. To authenticate the biological activity and favorable interaction sites docking study of all the compounds was performed against the protein 1JNX (breast cancer) using Auto dock tools. The result obtained from the analysis showed that the compound 4d is the best docked ligand against the target site with the binding energy of -16.03 kcal/mol.

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