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Discovery of Novel Inhibitors of Tumor Necrosis Factor-Alpha and Evaluation their Activity in Rheumatoid Arthritis Patients Using Pharmacophore and Virtual Screening

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Abstract: Tumor Necrosis Factor-alpha (TNFα) is one a pro-inflammatory cytokine which plays a major role in the progress of different autoimmunity diseases such as rheumatoid arthritis (RA) is characterized by chronic arthritis. Critical cell functions including cell proliferation, survival, differentiation, and apoptosis are regulated by TNFα signals through two transmembrane receptors, TNFR1 and TNFR2. In this study, employed Computer-Aided Drug Design-based (CADD) approach to identify the drug compounds which use in other diseases, and are able to inhibit the TNFα (It was not previously observed as a TNFα inhibitor). search in databases through an online tool "ZINC Pharmer" based on pharmacophore features, PubChem and drug bank. then evaluation of molecular docking-based screening, and the selection of screening ligand complex with TNFα based on Score and root-mean-square deviation (RMSD) value using the Molecular Operating Environment (MOE) system as well as evaluated by the ADMET. Resultantly, three compounds (Mebeverine, Doxazosin and Nebivolol) were identified which showed the highest binding energy with TNFα and a strong inhibitory effect (compare with reference inhibitors). The results of laboratory Evaluation showed that the three compounds produced $\Delta Tm > 2.0$ °C and therefore it is indicated as the TNF-alpha hit potential, on the other hand, the values of Kd were (0. 805 µM,0.348 µM and 0.704 µM) and IC50 were (4.29 nM, 4.39 nM, and 2.77 nM) respectively. ΔG has also calculated the values (-8.85 Kcal/ mole), (-8.43 Kcal/ mole) and (-8.44 Kcal/ mole (. The results of (LE) showed a ligand capable of significantly inhibiting TNF-alpha. ($LE \leq 0.3$).

Keywords: Virtual Screening، TNF-alpha, Thermos shift assay, RA, CADD.

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic arthritis. It is an unknown cause, prevalent almost all over the world. It usually affects women at a higher rate than men and mainly affects the joints, resulting in damage to cartilage and bone. This disease can destroy various body systems. Treatment options include lifestyle changes, physical therapy, nutritional therapy, medication, and surgery (1) . It affects 0.5 to 1% of the world's population (2). It mainly affects joints' synovial membranes, leading to cartilage and bone damage. This disease can damage different body systems, such as the skin, eyes, lungs, heart, and blood vessels. In addition to chronic

pain, fatigue, and possible disability, RA patients also exhibit increased morbidity and mortality, primarily from cardiovascular disease (3).

Tumor necrosis factor (TNF) has a wide range of biological activities. The cytotoxicity of cancer cell lines was one of the first functions discovered that led to its name (4). TNF-α is mainly produced by monocytes and macrophages, but also by B cells, T cells, and fibroblasts. It is one of the major inflammatory cytokine molecules in RA and is an autocrine (5) . Consists of 157 amino acids. TNF-α receptors are present on almost all nucleated cells. Two distinct membrane receptors that have been identified and cloned are tumor necrosis factor-receptor 1 (TNFR1) and tumor necrosis factor-receptor 2 (TNFR2). Both these receptors are typical transmembrane proteins with extracellular and intracellular domains of about equal size and a single transmembrane domain. Both TNFRI and TNFRII are bound to the surface of cells such as macrophages, lymphocytes, keratinocytes, and endothelial cells (6). Therefore, this study aims to suggest that these candidate 'pharmaceutical compounds' have a greater ability to inhibit TNF, and on the other hand know the effect of these drugs on the immunity of patients who use them to treat other diseases.

Materials and method

1. Active site prediction

The research referred that the complex crystal structure of TNF-α 5with SPD304 ligand (PubChem CID: 5327044) and with UCB-9260 ligand (PubChem CID: 72700327) showed effective inhibition of TNF-alpha (7) therefore used in the study TNF-α dimer complex structure with a small molecule inhibitor as references to determine pocket on the surface of TNF-alpha. From PDB (Protein Data Bank) obtained a complex of TNF-alpha and ligand (PDB ID: 2AZ5) and crystal structures of TNF-α (PDB ID: 6OP0). The active amino acid sites of the complex structure of TNF-α were predicted using structure comparison and site finder algorithms by using MOE software.

2. Pharmacophore-based virtual screening

By create hypotheses of pharmacophore models comprising diverse chemical features including Aromatic, Hydrophobic, Hydrogen bond acceptor and exclusion volume (molecular weight between 200 to 500 g/mole and rotatable bonds $\geq$ 6). All hypothesis models screened the ZINC drug database

3. Molecular docking

Molecular docking studies are used to examine ligands designed or compounds obtained from pharmacophore-based screening. All hits are docked at the active site of the target protein. Used the MOE docking algorithm to bind SPD304 to the active site. The most effective hit is selected based on the S score value of the PSD34 inhibitor and root mean square deviation (RMSD). The S value is a score that measures the affinity between the ligand and the receptor and is calculated by the default score construction function in the MOE.

4. Validation and ADMET analysis

The compounds were further evaluated by the ADMET (metabolism, distribution, excretion, absorption, and toxic properties by using the ADMET SAR server. The ADMET algorithm was used to predict the properties of the designed compounds and drug candidates.

5. Thermos shift assay

TNF-alpha used in this assay was imported from Cusabio company with a purity of >90%. Prepare protein assay stock in HEPES buffer (0.01 M HEPES stock solution at pH 7.4). Thermal Shift Assay (TSA) test run for the determination of the optimal amount of protein. Prepare a dilution series of your protein sample in buffer /DMSO ranging from 0.03 to 0.5 µM final concentration in a total volume of 25 µl. . Export the file into Microsoft Excel analyses (8). Fit fluorescence intensity curve to a Boltzmann sigmoidal curve using TSA_CRAFT service to obtain the melting temperature (Tm) of the protein Calculate: ΔTm = Tm ligand - Tm buffer/DMSO. A positive Tm indicates that the ligand stabilizes the protein from denaturation. Data Analysis to Determine the Dissociation (kd). . Analyze the data using the following equation.

Y= Bottom + ((Top-Bottom) (1-((P- Kd - X+ sqrt (((P+X+Kd) ^2-(4PX))) /(2P))))

Where P: protein concentration. **Kd:** dissociation constant (has the same unit as P). **T:** melting temperature at high inhibitor concentration; **B:** melting temperatures of no inhibitor concentration" (8).

Calculate ΔG and LE

Recommended to assess binding affinity in relation to the number of heavy atoms in a molecule and The calculation of the binding energy(ΔG) of the ligand and ligand efficiency (LE): Calculate Free energy of ligand binding: ΔG = −RT.lnKd. Calculate Binding energy per atom (ligand efficiency(LE)): LE = ΔG/Nnon-hydrogen atoms

6. Inhibitory concentration (IC50)

Serum Allow serum to clot for 10-20 minutes at room temperature. Centrifuge at 2000-3000 RPM for 20 minutes. Collect the supernatant without sediment. Using the manufacturer's ELISA analysis method (Human tumor necrosis factor -alpha Elisa kit). Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature. IC50s values were determined by fitting a dose-response curve (four parameters) to inhibition (%) to the data, using Graph Pad Prism software (9)

Result and discussion

The matching tool in the structure comparison algorithm in the chimaera program showed the overlap in the binding site for both the complex structure of the TNF-α dimer with SPD304 and with UCB-9260. Also used the site finder algorithm to produce a catalytic site of TNF-α. The pocket was predicted size (95), and hydrogen (49). side chain (60), site amino acids (Leu57, Tyr59, Tyr119, Gly121, Val123, Ile155, Leu157) of Chain-A, (Leu57, Ile58, Tyr59, Tyr119, Gly121, Gly122, Leu157) of Chain-B, (Leu57, Tyr59, Ser60, Gln61, Tyr119, Leu120, Gly121, Tyr151, Ile155) of Chain-C.

After identifying the active site and isolate the pocket, the molecules are designed by Pharmacophore-based virtual screening**.**

In CADD (computer-aided drug design), virtual screening is an efficient and quick method to discover novel drug compounds (10). Best hit compounds with a similar feature of reference complex of receptor TNF-alpha with SPD304 inhibitor were screened from the ZINC database with the pharmacophore model which was achieved by ZINC pharmacophore. generated hypothesis models using different combinations of shared

features of active compounds table (1). As a result, 100 out of hit compounds were selected through exclusion similar chemical structure. All hits were shifted into a test database by minimizing the energy through the energy minimization algorithm in the MOE Test database one hundred (100) hits compounds were used for further processed through molecular docking and the ADME profile.

Compounds were docked with the catalytic site of TNF-α. The 35 small molecules with the lowest S value and RMSD were selected for further study. After analysis of the ADMET profiling and exclusion of compounds known to inhibit TNF-alpha, compounds affecting the nervous system and those used in chemotherapy based on the drug bank information, so only 3 compounds were selected as shown in Table (2).

Table (1): Hypotheses of pharmacophore models

Hypothesis	Features					Hits
	type	x	Y	z	Radius	
1	Aromatic	17.5	-3.65	0.00	1.10	148
	Hydrophobic	9.34	-3.85	0.00	0.50	
	Hydrogen -A	12.64	-0.85	0.00	1.00	
2	Aromatic	12.64	-0.85	0.00	1.10	55
	Hydrophobic	-4.00	-3.85	0.00	0.50	
	Hydrogen -A	9.34	-3.85	0.00	1.00	
3	Aromatic	17.5	-3.65	0.00	1.10	83
	Hydrophobic	18.91	-6.82	0.00	1.10	
	Hydrogen -A	9.34	-3.85	0.00	1.00	
4	Hydrophobic	12.64	-0.85	0.00	1.10	92
	Hydrophobic	18.91	-6.82	0.00	1.10	
	Hydrogen -A	5.33	-4.62	0.00	0.50	
5	Aromatic	1.33	-539	0.00	1.10	145
	Hydrophobic	-1.33	-5.39	0.00	1.00	
	Hydrogen -A	9.34	-3.85	0.00	1.00	

A= accepter

Table (2): The selected compounds

ZINC ID	Drug Bank Accession Number	Drug Name
ZINC3813087	DB12554	Mebeverine
ZINC94566092	DB00590	Doxazosin
ZINC4213946	DB04861	Nebivolol

Molecular docking studies are used to examination ligands designed or compounds obtained from pharmacophore-based screening. All hits are docked at the active site of the target protein. Used the MOE docking algorithm to bind SPD304 to the active site. The most effective hit is selected based on the S score value of the SPD304 inhibitor and root mean square deviation (RMSD). The S value is a score that measures the affinity between the ligand and the receptor, calculated by the default score construction function in the MOE (11) . The RMSD is used to compare the docking confirmation with the docking reference configuration. Depend on lower S and RMSD values when selecting compounds (12). The S score $\geq$ -7.5 and RMSD $\geq$2 were selected for screening small molecules. The design compounds showed a good interaction compared to the reference ligand showed in Table (3).

Table (3): The docking score of compounds and interaction types

Compounds	S-Score	RMSD	Number of interaction types		
			Hydrophobic	Hydrogen	Other
Mebeverine	-9.25	-1.34	5	6	0
Doxazosin	-9.05	1.31	8	7	1
Nebivolol	-9.58	-1.51	10	7	1
SPD304 (Reference)	-8.4	-1.79	9	4	0

The compounds (Doxazosin, Nebivolol) shouted affinity (S score) highest than reference ligand. Molecular docking of compounds was displayed in Figures (1); (2) and (3).

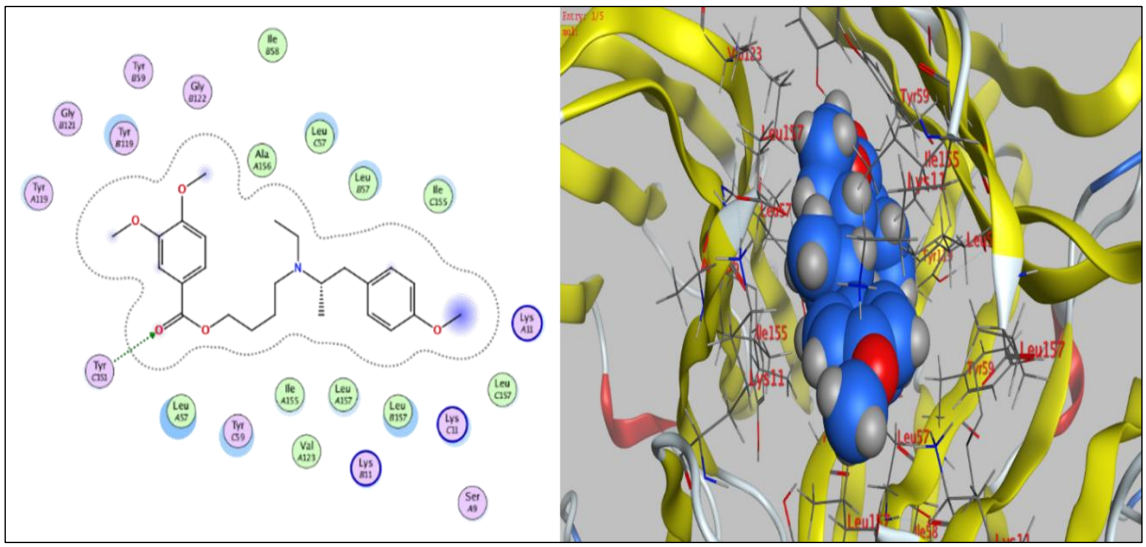

Figure (1): Molecular Docking of Molecule (Mebeverine).

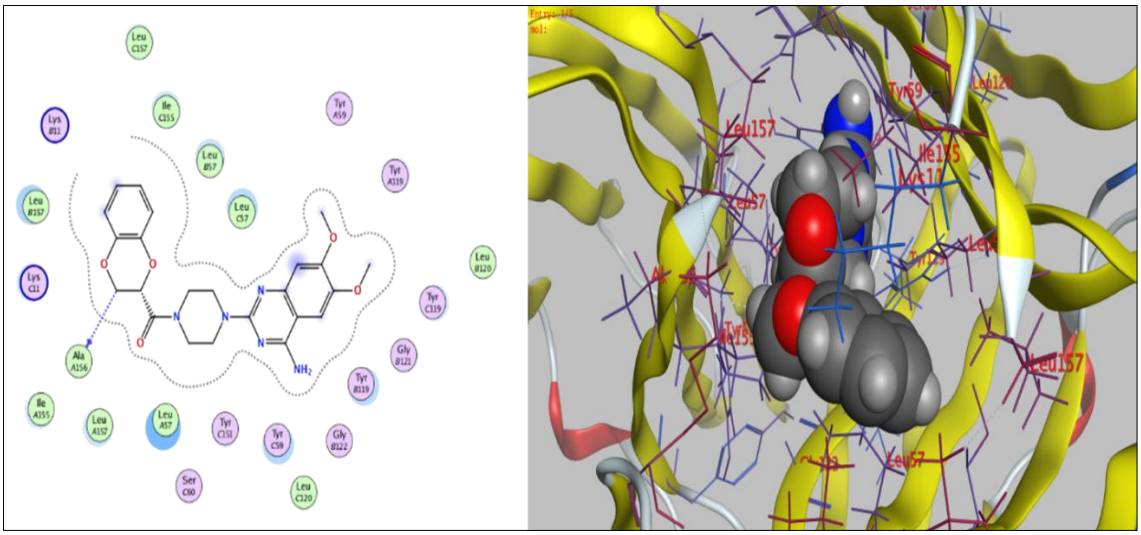

Figure (2): Molecular Docking of Molecule (Doxazosin).

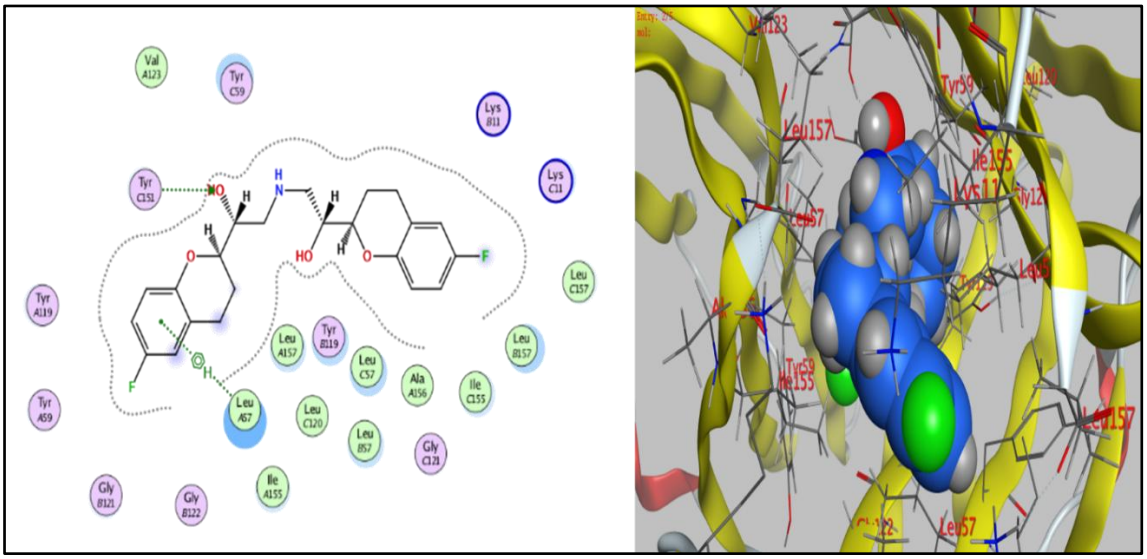

Figure (3): Molecular Docking of Molecule (Nebivolol)

To distinguishing the drug-like and non-drug-like properties compounds were evaluated based on the Lipinski rule. Table (4) showed that all designed molecules Where molecular weight 'MW' of three descriptors were ranged (< 500 g/mole). Meanwhile logarithm of lipophilicity 'log P' (octane-water partition coefficient) was within normal range of known drugs.

The compounds were further evaluated the ADMET (metabolism, distribution, excretion, absorption, and toxic properties by use admet SAR server. The ADME algorithm was used to predict the properties of the designed compounds and drug candidates. Blood-Brain Barrier (BBB) is an important factor in the field of drug discovery. BBB is the resister barrier within the endothelial cells and stops the brain from uptake any pharmaceutical. therefore, the ADMET test for a good and effective drug compound consisted parameters include the compound must pass the barrio of BBB also, oral bioavailability is considered an important factor for the selection of active drugs (13).

Absorb in the human intestine, absorb the Caco-$_2$ permeability, be non-toxic, non-carcinogenic were toxicity studies showed Mebeverine and Nebivolol belonged to toxicity class 4, while Doxazosin belonged to toxicity class 5, out of six classes class 1 represented a toxic molecule while class 6 represented a safety molecule. Non-inhibitor to CYP (Cytochromep450) enzyme. CYP is a group of isoenzymes involved in the catabolism of various chemicals, most drug interactions caused by CYP inhibition can cause serious adverse events, which can lead to poor patient health and failure of drug development. (14). The compounds significantly accepted these parameters of ADMET (Table (5)). Only those ligands were considered to be the potential drug candidates that accomplished all the ADMET models successfully.

Table (4): Drug likeness scores of selected compounds

Five roles	Mebeverine	Doxazosin	Nebivolol	Range
MW	429.65	451.48	404.44	< 500
HBD	0	1	3	< 5
HBA	5	7	5	< 10
LogP	4.60	1.72	2.36	< 5
tPSA	57.25	112.27	70.95	< 140

MW= Molecular Weight ,HBD= Hydrogen Bond Acceptor , HBA = Hydrogen Bond Donor , tPSA = topological Polar Surface Area, LogP = Hydrophobicity parameter

Table (5): ADMET predictor profile and classification

	Molecules		
Absorption	Mebeverine	Doxazosin	Nebivolol
Caco-2 Permeability	+	+	-
Blood-Brain Barrier	+	+	+
Human Intestinal Absorption	+	+	+
P-glycoprotein Inhibitor	NI	NI	NI
Metabolism	Mebeverine	Doxazosin	Nebivolol
CYP450 1A2 Inhibitor	NI	NI	I
CYP450 2C9 Inhibitor	NI	NI	NI
CYP450 2D6 Inhibitor	NI	NI	NI
CYP450 2C19 Inhibitor	NI	NI	NI
CYP450 3A4 Inhibitor	I	NI	NI
Toxicity	Mebeverine	Doxazosin	Nebivolol
AMES toxic	NO	NO	NO
Carcinogenic	NO	NO	NO
Toxicity Class	4	5	4

I= Inhibition , NI= No-inhibition , NO= Non-mutagenic and Non -Carcinogenic

The results showed that the melting temperature Tm increased with the increase in the concentration of the inhibitor, which indicates the binding of the inhibitor to the protein at the site where the stability of the protein increases. Indicating that the inhibitors positively correlate with TNF-alpha. Figures (4), (5), and (6) shows shifts in the melting temperature curves mebeverine, Doxazosin and Nebivolol respectively. The resulting ΔTm values were determined compared to the control. The values of ΔTm are significant. where any small molecule yielding ΔTm > 2.0 °C was indicated as a hit potential (15). The increase in ΔTm was also observed with increasing bonding concentration, this means the stability of the TNF-alpha increases with the increasing concentration of the inhibitor, indicating the affinity of the compounds to the protein the researchers pointed to that ΔTm is large for the higher affinity inhibitor and decreased when the affinity decreases (16).

Kd was calculated using the equation in the methods. Values were as follows 0. 805 μM ,0.348 μM and 0.704 μM for mebeverine, Doxazosin and Nebivolol respectively. In biochemistry or pharmacology, the binding affinity range of Protein interactions is considered to have high affinity if Kd is less than 10 nM (for antibody-antigen complex), medium affinity in the 10 nm-100 μM range, and low affinity if Kd is above 100 μM (17). The dissociation constant represents the partial saturation as a function of the free bonding concentration. Once the Kd of a particular protein-ligand composition has been determined, it is possible to predict partial saturation at any ligand concentration (18).

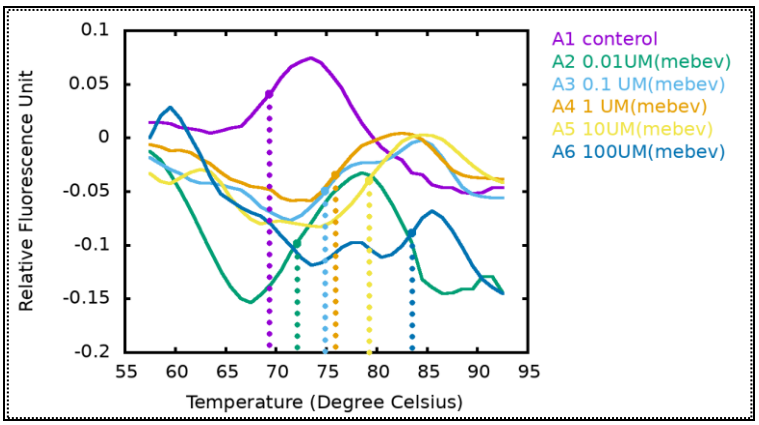

Figure (4): Thermal shift curves of unfolding transition of TNF-alpha in the presence of 100 µM, 10 µM, 1 µM, 0.1 µM 0.01 µM, and 0 µM of mebeverine. Data fit Boltzmann equation gave midpoint Tm of 83.54˚C, 79.26˚C, 75.92˚C, 74.90˚C,72.14˚C and 69.53˚C, respectively, (R2 ≥ 0.9).

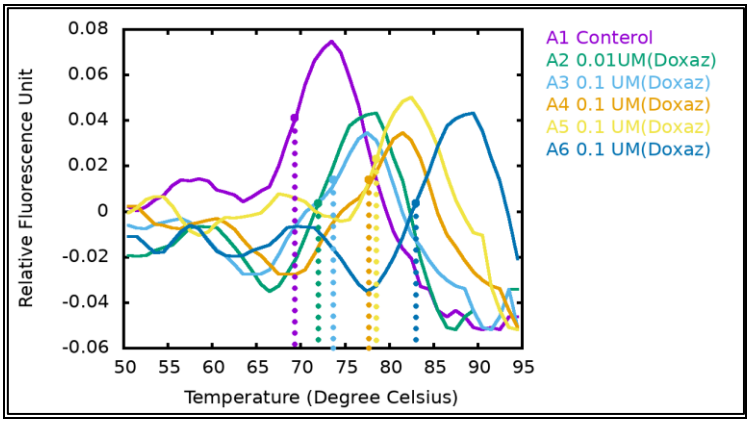

Figure (5): Thermal shift curves of unfolding transition of TNF-alpha in the presence of 100 µM, 10 µM, 1 µM, 0.1 µM 0.01 µM, and 0 µM of Doxazosin. Data fit Boltzmann equation gave midpoint Tm of 82.98˚C, 78.55˚C, 77.68˚C, 73.68˚C, 71.98˚C and 69.35˚C, respectively, (R2 ≥ 0.9).

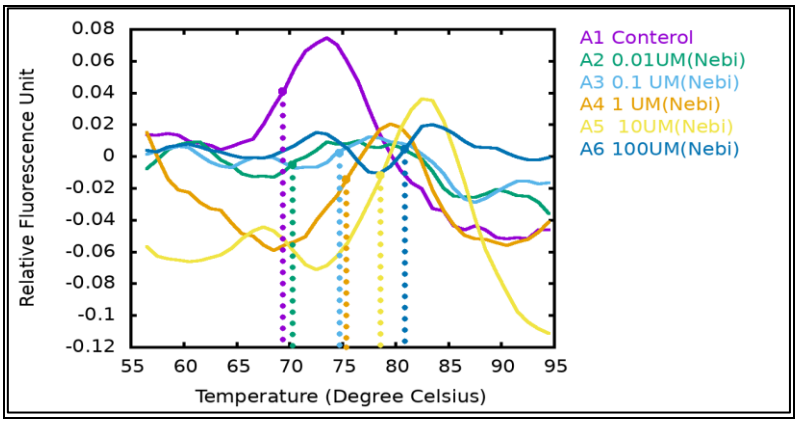

Figure (6): Thermal shift curves of unfolding transition of TNF-alpha in the presence of 100 µM, 10 µM, 1 µM, 0.1 µM 0.01 µM, and 0 µM of Nebivolol. Data fit Boltzmann equation gave midpoint Tm of 80.90˚C, 78.60˚C, 75.35˚C, 74.69˚C,70.29˚C and 69.53˚C, respectively, (R2 ≥ 0.9).

The value of ΔG was calculated based on the kd values, by converting the Kd into the free energy of binding at 300K, LE is useful in ligand assessment and can be calculated by dividing ΔG by the number of heavy atoms (non-hydrogen atoms) (19).as shown in table (6).

The binding free energies obtained by the experiment agree with the docking values of all inhibition within an acceptable range, and this greatly supports the computational studies researches refers to the range between 5 to 15 kcal/mole is considered a strong interaction between the ligand and receptor (20) .The results of LE showed ligands capable of significantly inhibiting TNF-alpha. Evaluated based on ligand efficiency scores (LE $\geq$ 0.3) (21) . the results also showed the effectiveness of all compounds that inhibit TNF-alpha, and the interaction between the ligand and protein spontaneous. were optimization into clinical candidates with good drug-like properties (22).

Table (6): The value of (ΔG) and the value of (LE)

Inhibitor Nam	(ΔG) Kcal/ mole	Heavy atoms	(LE) Kcal/ mole/ HA
Mebeverine	-8. 85	31	0.285
Doxazosin	-8.44	33	0.255
Nebivolol	-8.43	29	0.29

IC50 is the most widely used and most informative measure of drug effectiveness. Refers to the amount of drug required to inhibit half of the biological activity (23). A patient serum sample was used in this analysis to measure the effect of the compounds on TNF-alpha, and dilutions of the ligand added to the serum. The serum - ligand was left for 30 min at 37 °C to allow the interaction to occur. The (IC50) results for mebeverine, Doxazosin and Nebivolol were 4.29 nM, 4.39 nM, and 2.77 nM, respectively. IC50s values were determined by fitting a dose-response curve (four parameters) to inhibition (%) to the data, using GraphPad Prism software. The curves (Figures (7)) showed that all compounds were highly effective in inhibiting TNF-alpha, as the percentage of protein inhibition increased when the concentration of the compounds increased, this occurs as a result of the binding of the inhibitors at a position that prevents the protein from binding with an antibody coating in the Elisa plate.

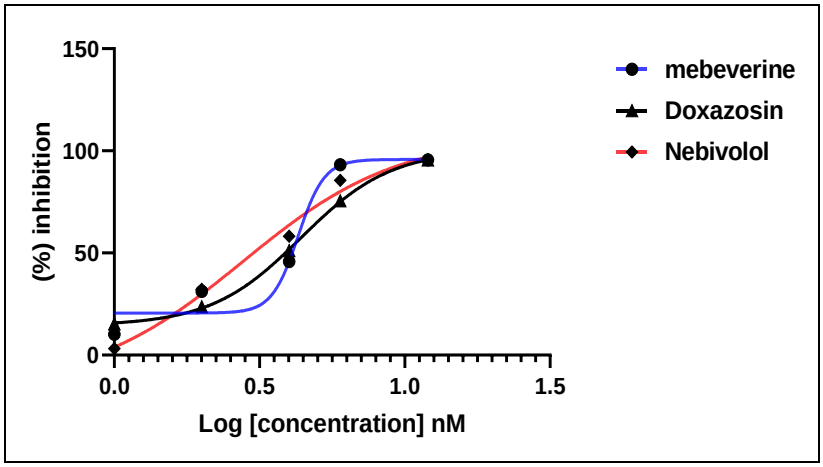

Figure (6): Curve Fitting percentage inhibition versus log of ligand concentration to determine IC50
compounds (R2 ≥ 0.9).

Conclusions

In this study, TNF-α inhibiting compounds were selected by computer methods, these compounds were evaluated in vitro. Experimental verification indicated that the three compounds classified within the medium toxicity also showed a significant ability to bind with TNF-α. It also showed the ability to inhibit TNF-α. The compounds can regulate the levels or activity of TNF-α.

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