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Design, Synthesis, SAR and Anti-Breast Cancer Potential of New Thymoquinone Analogs as PPARγ Ligands

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Abstract: New thymoquinone derivatives bearing thiazolidine moiety (2-6) were designed and synthesized in one pot facile addition reaction. The structure of the newly synthesized compounds was elucidated by elemental analyses and spectral data. The *in-vitro* antitumor activity of the obtained compounds has been evaluated.

Key words: Thymoquinone, thiazolidine, breast cancer, PPARγ, KSA

INTRODUCTION

Breast cancer is the most common type of cancer affecting >1 million women and causing high mortality worldwide. In the Kingdom of Saudi Arabia (KSA), hospital and population based statistics have shown that breast cancer has the highest crude frequency rate among Saudi women. Breast cancer constitutes 18% of all cancers in Saudi women.

A large number of breast cancers express Estrogen Receptor (ER) as well as Progesterone Receptor (PR) (Deroo and Korach, 2006) and respond to hormonal therapy or aromatase inhibitors. However, there is a group of patients (12-17%) who do not respond to such treatment because of the absence of these two receptors as well as the receptor HER-2/neu (ErbB2); this group represents a highly aggressive breast cancer subtype, known as Triple-Negative Breast Cancers (TNBC) that is difficult to treat. In recent years TNBC has gained attention due to its aggressive clinical behavior, poor prognosis and lack of targeted therapies.

(PPARs) are fatty acid-activated transcription factors that belong to the nuclear hormone receptor family. Peroxisome proliferator Receptor (PPARγ) ligands cause breast cancer cells to undergo apoptosis and inhibit tumor angiogenesis and invasion in breast cancer (Fenner and Elstner, 2005; Vallve and Palau, 1998). The important roles of PPARγ ligands in promoting cell cycle arrest was previously reported. PPARγ inhibits the expression of the PP2A phosphatase and thereby increases phosphorylation of the E2F/DP complex resulting in cell cycle withdrawal (Altiok *et al*., 1997). Growth arrest in response to PPARγ activation can also result from the induction of p18 and p21, two CDK inhibitors which inhibit the CDK activities necessary for cell cycle progression. Consistent with a role in cell cycle arrest, activation of PPARγ in various cell types correlates with reduced levels of RB phosphorylation and consequently reduced cell cycle progression. Activation of PPAR-γ has anti-proliferative effects and slow down the progression of cancerous disease (Collins *et al*., 1998; Cobb *et al*., 1998; Azukizawa *et al*., 2008; Demetri *et al*., 1999).

PPAR-γ ligands have been shown to be potent inhibitors of angiogenesis, a process necessary for solid-tumour growth and metastasis.

Among diverse tissue cancers, the breast cancers have significant lipogenic capacity and the modulation of fat metabolism has been associated with alteration of cancer cell growth and apoptosis. PPAR ã expression was confirmed in human breast cancer cell lines as well as primary and metastatic breast adenocarcinomas and PPARγ activation caused inhibition of proliferation, changes in epithelial gene expression associated with a more differentiated, less malignant state and extensive lipid accumulation in cultured breast cancer cells.

Over thousands of years, a large number of natural products have been used for the treatment of different kinds of disease despite lacking scientific verification of their effectiveness and safety. Agents derived from natural sources have attracted great attention in the recent years. In this study, we are concerned with one of those agents; Thymoquinone (TQ). TQ is one of the most active ingredients of Nigella Sativa or black cumin seeds. The most prominent activity of TQ is its antioxidant and anti-inflammatory effects. TQ was also found to exhibit chemopreventive and anticancer activities.

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The potential of thymoquinone has attracted the attention of scientists to investigate the molecular mechanisms involved and evaluate its significance in the treatment of cancer. The anticancer effects of thymoquinone are mediated through different modes of action, including anti-proliferation, apoptosis induction, cell cycle arrest, ROS generation and anti-metastasis/anti-angiogenesis.

In addition, this quinone was found to exhibit anticancer activity through the modulation of multiple molecular targets, including PPAR-γ, activation of caspases and generation of ROS. Thymoquinone was found to induce the activity of PPARs including PPAR-γ in MCF-7 breast cancer cells. By using molecular docking analysis, TQ was shown to form partial interactions with 7 polar residues and 6 non-polar residues in the PPAR-γ receptor (Woo *et al*., 2000) despite of its high potency as anticancer.

Thiazolidinedione (TZD) drugs were found to be ligands for PPARg, some natural ligands for PPARg have been identified and used to elucidate the role of PPARg in cellular functions both in vitro and in vivo. Other derivatives of TZD have also been developed with higher specificities and activities. The reference drug used in this study, efatutazone (also called CS-7017 or RS5444) is a selective high-affinity TZD-class ligand of PPARγ.

The use of natural products to complement conventional medicine has been proposed long ago for the drug toxicity which becomes a common problem in chemotherapy contributing to response failure in certain cases. Moreover, the combination of TQ with clinically used anti-cancer drugs such as isofosfamide, cisplatin and doxorubicin has led to improvements in their therapeutic index and to the protection of non-tumor tissues against chemotherapy inducing damage. Additionally, novel thymoquinone analogs/nanoparticles have been synthesized and were found to possess greater anticancer and antioxidant activities than thymoquinone itself and they are at the same time non-toxic (Banerjee *et al*., 2010).

The above facts and in continuation to our previously reported work in synthesis of new antitumor agents (Ghorab *et al*., 2006) prompted us to design, synthesize and evaluate new thymoquinone analogues as anti-breast cancer agents on different cell lines, through binding and activation of PPAR-γ receptor. Structure Activity Relationship (SAR) study was set to correlate the changes in the structure with the antitumor activity.

We hypothesize that these TQ analogues may modulate the activity of PPAR-γ pathway in breast cancer cells and inhibit tumor cell growth. The activity of PPAR-γ receptor was investigated *in-vitro* also by using PPAR Reporter kits.

MATERIALS AND METHODS

Chemicals and reagents: Thymoquinone and the different reagents used for the chemical reactions, together with the suitable solvents as ethanol, methanol and dioxanewere purchased from Sigma-Aldrich. The designed compounds that was prepared in this study was synthesized by standard procedures.

Chemistry: Melting points are uncorrected and were determined on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK). Elemental analysis (C, H, N) were performed on Perkin-Elmer 2400 analyser (Perkin-Elmer, Norwalk, CT, USA) at the microanalytical laboratories of the Faculty of Science, Cairo University. All compounds were within ±0.4% of the theoretical values. The IR spectra (KBr) were measured on Shimadzu IR 110 spectrophotometer, ^{1}H-NMR spectra were obtained on a Bruker proton NMR-Avance 300 (300 MHz) in DMSO-d_6 as a solvent, using Tetramethylsilane (TMS) as internal standard. Mass spectra were run on HP Model MS-5988 (Hewlett Packard).

5-(3-thioureido N-substituted benzenethymoquinone) -3-methyl-thiazolidine-2, 4-dicarboxylic acid ethyl ester (2-6): A mixture of 1 (TQ) (2 g, 0.01 mol), the appropriate thymoquinones (0.01 mol) in dioxane (20 mL) was stirred for 6 h at room temperature. The reaction mixture was poured into ice/water. The solid formed was filtered while and recrystallized from ethanol. All mass spectral data come into accordance with postulated structures.

Diethyl 3-methyl-5-(3-(4-thymoquinone)thiazolidine)-2, 4-dicarboxylate(2): (98% yield), m.p. 205-207°C, IR(KBr, cm^{-1}): 3472, 3380, 3308 (NH, NH_2), 3080 (CH arom.), 2926, 2858 (CH aliph.), 1680, 1671 (2 C = O), 1236 (C = S), 1370, 1150 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.2 [m, 6H, 2CH_3 ester], 2.4 [s, 3H, CH_3thiophene], 4.2 [m, 4H, 2 CH_2 ester], 7.3 [s, 2H, 2NH D_2O exchangeable], 7.8, 7.9 [2d, 4H, Ar-H], 10.0 [br., 2H, SO_2-NH_2]. Anal. Found: C, 45.50; H, 4.30; N, 8.70%. $C_{18}H_{21}N_3O_6S$ (439) CalcdC, 45.85; H, 4.45; N, 8.91%.

Diethyl-3-methyl-5-(3-(4-(N-(3-methylisoxazol-5yl, thymoquinone)phenyl)thiazolidine-2, 4-dicarboxylate (3): (95% yield), m.p. 220-222°C, IR (Kbr, cm^{-1}): 3500, 3410,

3350 (NH), 3094 (CH arom.), 2984, 2895 (CH aliph.), 1706, 1654 (2 C = O), 1550 (C = N), 1234 (C = S), 1332, 1166 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.3 [m, 6H, 2CH_3 ester], 2.3 [s, 3H, CH_3 oxazole], 2.35 [s, 3H, CH_3thiophene], 4.2 [m, 4H, 2 CH_2 ester], 6.1 [s, 1H, CH oxazole] 7.9-8.2 [m, 4H, Ar-H], 11.5, 11.8, 12.2 [3s, 3H, 3NH D_2O exchangeable]. Anal. Found: C, 47.40; H, 4.04; N, 10.35%. $C_{22}H_{24}N_4O_7S$ (522) CalcdC, 47.82; H, 4.34; N, 10.14%.

Diethyl-3-methyl-5-(3-(4-(N-thiazol-2-yl,thymoquinone) phenyl) thiazolidine--2,4-dicarboxylate (4): (92% yield), m.p. 235-237°C, IR(KBr, cm^{-1}): 3480, 3400, 3350 (NH), 3100 (CH arom.), 2984, 2860 (CH aliph.), 1705, 1680 (2 C = O), 1550 (C = N), 1250 (C = S), 1380, 1150 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.2 [m, 6H, 2CH_3 ester], 2.4 [s, 3H, CH_3thiophene], 4.2 [m, 4H, 2 CH_2 ester], 6.5-6.8 [m, 2H, 2CH thiazole], 7.8-8.1 [m, 4H, CH arom.], 10.2, 11.6, 12.2 [3s, 3H, 3NH D_2O exchangeable]. MS (m/z): 554 (M^+, 1.52%), 299 (100%), 254 (45.19%), 156 (30.07%). Anal. Found: C, 45.60; H, 4.20; N, 10.45%. $C_{21}H_{22}N_4O_6S_4$ (554) CalcdC, 45.48; H, 3.97; N, 10.10%.

Diethyl 3-methyl-5-(3-(4-(N-pyrimidin-2-yl, thymoquinone) phenyl)thiazolidine)-2, 4-dicarboxylate (5): (91% yield), m.p. 218-219°C, IR(KBr, cm^{-1}): 3406, 3292 (NH), 3080 (CH arom.), 2984, 2856 (CH aliph.), 1666 broad (2 C = O), 1582 (C = N), 1230 (C = S), 1330,1160 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.4 [m, 6H, 2CH_3 ester], 2.3 [s, 3H, CH_3 thiophene], 4.2 [m, 4H, 2 CH_2 ester], 7.6-8.0 [m, 7H, CH pyrimidine+Ar-H], 11.7, 12.2 [2s, 3H, 3NH D_2O exchangeable].MS (m/z): 549 (M^+, 1.15%), 257 (100%), 258 (12.41%), 186 (12.60%). Anal. Found: C, 48.14; H, 4.01; N, 12.95%. $C_{22}H_{23}N_5O_6S_3$ (549) CalcdC, 48.08; H, 4.18; N, 12.75%.

Diethyl 3-methyl-5-(3-(4-(N-(4-methylpyrimidin-2 yl) thymoquinone) phenyl) thiazolidine)-2, 4-dicarboxylate (6): (95% yield), m.p. 200-202°C, IR(KBr, cm^{-1}): 3488, 3382 (NH), 3070 (CH arom.), 2966, 2870 (CH aliph.), 1702, 1670 (2 C = O), 1592 (C = N), 1238 (C = S), 1326, 1154 (SO_2). ^{1}H-NMR (DMSO-d_6) δ: 1.3 [m, 6H, 2CH_3 ester], 2.7 [s, 3H, CH_3 thiophene], 2.9 [s, 3H, CH_3 pyrimidine], 4.2 [m, 4H, 2 CH_2 ester], 6.5 [m, 2H, CH pyrimidine], 7.8-8.2 [m, 4H, Ar-H], 10.3, 11.0 [2s, 2H, 2NH D_2O exchangeable], 11.0 [br, 3H, 3NH D_2O exchangeable]. Anal. Found: C, 49.10; H, 4.60; N, 12.40%. $C_{23}H_{25}N_5O_6S_3$ (563) CalcdC, 49.02; H, 4.44; N, 12.43%.

Biological evaluation

Chemicals and facilities: All chemicals and reagents were supplied by Sigma-Aldrich. Facilities including human tumor cell line (MCF-7) biochemical equipments and gamma-irradiation source have been made available by the National Cancer Institute (NCI), Cairo (Egypt).

The cell culture

Cell lines

Cells and reagents: One normal human breast epithelial cell line, HBL-100 and three human breast cancer cell lines, MCF-7 (luminal A), MDA-MB-231 (triple negative) and MDA-MB-453 (HER2 positive) was used in this study. Cell lines was maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 100 U mL^{-1} penicillin–streptomycin at 37°C with 5% CO_2. Antibodies that was used for IHC include anti-PPARγ, antibodies for Western blotting included anti-PPAR, anti-β-actin, anti-PCNA and anti-active-caspase-3.

MTT assay and apoptosis assay: Ninety six-well plates was seeded with 3×103 cells per well. Following overnight incubation, analogs was added at the appropriate concentrations and the cells was incubated for 2 day. Medium was removed and the cells to be washed with PBS. Cell numbers was calculated colorimetrically using the Cell Counting Kit at a test wavelength of 450 nm. Apoptosis was determined using dual-color flowcytometry analysis after staining of cells with Annexin V-FITC and propidium iodide.

Determination of toxicity profile of the novel compounds: The synthesized TQ derivatives was tested for their tolerability. The dose was estimated according to the LD50 of the compounds, LD50 was estimated in 3 different lab animals (mice), the defined dose was used as a guide for the safety testing of the compounds.

Evaluating the tolerability: TQ derivatives was given for six months at 3 dose rate (1/10-1/20-1/40 LD50) by different routes pf administration (oral and IM), blood samples was collected regularly along the whole period of the experiment. The collected samples was subjected to analysis includes whole blood picture and tests for liver and kidney functions, tumor biomarkers and mutagenic effect (bone marrow samples) and histo-pathological investigation was done on tissues (liver, spleen, kidney, brain, stomach and testis) from tested animals (2 animals from each group.

Statistical analysis: Data for *in-vitro* cytotoxic activity expressed as surviving fraction is presented as mean±SE and analysis of variance has been done by using ANOVA

test and multiple comparisons between different concentrations of the compounds alone and with radiation has been done by using multiple comparison test of significance, Duncan test. Mohamed significance was considered at $p<0.05$.

Molecular docking study: An X-ray crystallography structure of PPAR-γ was obtained from the RCSB Protein Data Bank. Molecular docking of TQ analogues to PPAR-γ was then performed using the Dock feature in MOE. The default "Triangle Matcher" algorithm was used to generate about 100 different poses for TQ analogs. We will use London dG scoring function to rank these poses. The top 10 poses was retained and further refined by energy minimization. The MMFF94x force field with calculation of implicit solvation energy using Generalized Born model was used for the energy minimization. Side chains of residues within 6 A° from TQ analogs was allowed to move during energy minimization. After energy minimization, the pose with the best interaction energy with PPAR-γ was retained (Woo *et al*., 2012).

Luciferase assay: The activity of PPAR-γ was investigated using luciferase assay. Briefly, cells was seeded at density of about 6×104 cells per well in 12-well microtiter plate followed by overnight incubation. The cells was incubated in DMEM medium for 1 h before transfection with pPPRE-tk-Luc and Renilla plasmids as an internal control. For PPARs study, the cells was transfected with:

- GAL4-PPAR-a LBD plasmid
- GAL4-PPAR-g LBD plasmid
- GAL4-PPAR-d LBD plasmid

To cover all isotypes of PPAR receptor together with GAL4-Luc and Renilla plasmids. For dominant negative transfection, the cells was transfected with PPAR-γ mutant containing amino acid substitutions in the DNA binding domain that abolish binding to PPAR-γ response elements or pCMX-mPPARg plasmid (a cDNA clone encoding the mouse PPARγ) together with pPPRE-tk-Luc and Renilla plasmid as an internal control.

Cells was transfected with the indicated plasmids using calcium phosphate transfection kit for 12-14 h before recovery with normal RPMI1640 medium for at least 6 h. After TQ analogs treatment, the cells were harvested using ice-cold reporter lysis buffer. The lysate will then undergo centrifugation at 12,000 rpm for 3 min. The supernatant was mixed with luciferase substrate solution for luciferase reading, followed by stops and glow buffer for Renilla reading. Bioluminescence generated was measured using Sirius luminometer. The luciferase reading obtained was normalized to the corresponding Renilla reading and to the protein amount.

Transcriptional activity of peroxisome proliferators-activated receptors: The Cignal PPAR reporter is designed to measure transcriptional activity of peroxisome proliferator-activated receptors (PPARs) 36. The PPAR reporter was prepared as PPAR-responsive luciferase construct and a constitutively expressing Renilla construct (40:1).

The PPAR-responsive luciferase construct was adjusted under the control of a minimal (m) CMV promoter and tandem repeats of the PPAR transcriptional response element.

The number of response elements and the intervening sequence between these response elements was experimentally optimized to maximize the signal to noise ratio.

The constitutively expressing Renilla construct encodes the Renilla luciferase reporter gene acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. By using the simple dual-luciferase assay, we easily monitored the activity of PPAR and determine the effect of the new TQ analogues.

RESULTS AND DISCUSSION

Molecular docking: Figure 1-5 show the binding interactions of newly synthesized TQ analogues into active site of PPARγ receptor pocket. It is obvious from molecular docking results that all the newly synthesized 5 analogues show good binding interactions with the amino acid residues at the active site of PPAR γ receptor.

Chemistry: A single pot reaction under mild conditions was used to synthesize 5 new substituted amino TQ derivatives (2-6). Structure verification via spectral and microanalytical data was carried. All obtained data came in accordance with postulated structures. All new compounds were obtained in excellent yields.

Thymoquinone (TQ) was found to be a strategic starting material for preparing a series of new heterocyclics bearing reactive sites. Thus, the nucleophilic reaction of thiazolidinessulfanilamide, methoxazole, thiazole, diazine and merazine, on the highly positive carbon of the TQ 1 in stirringdioxane afforded the corresponding 5-(3-thioureido N-substituted thymoquinone) 3-methyl-thiazolidine-2, 4-dicaroxylic acid ethyl ester derivatives; 2-6, respectively. The structure of

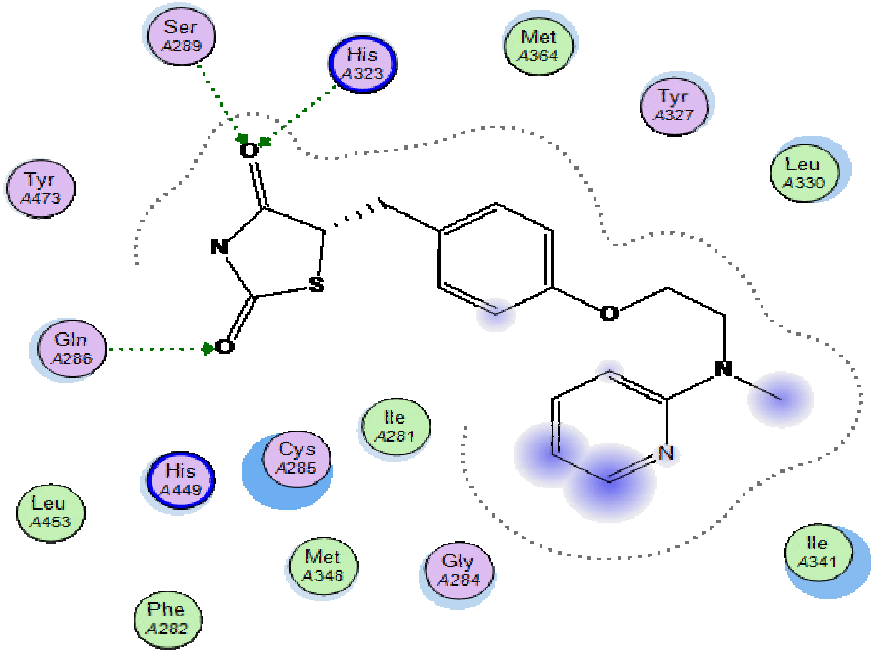

Fig. 1: Binding interaction of compound 2 into PPAR-γ pocket

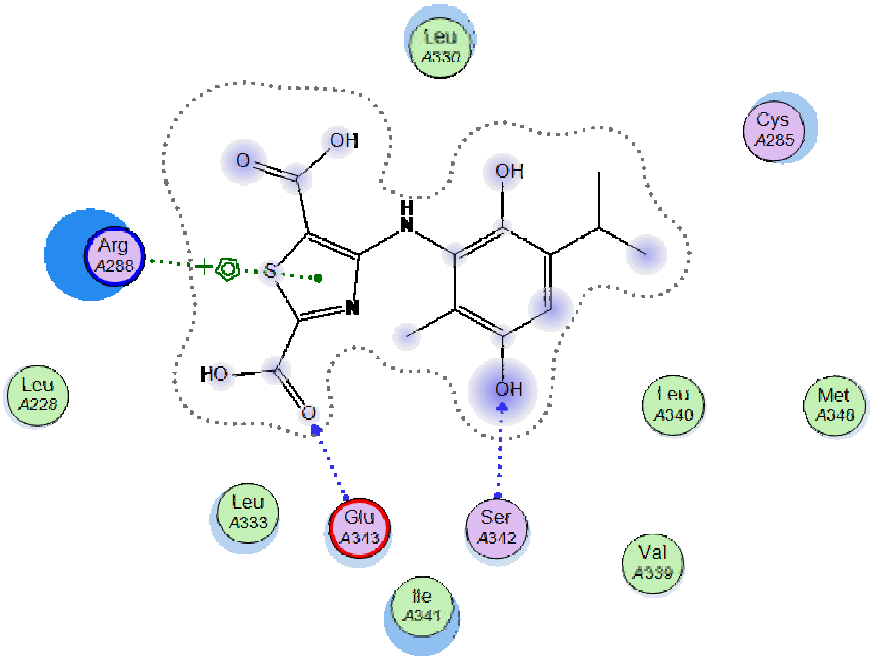

Fig. 2: Binding interaction of compound 3 into PPAR-γ pocket

compounds 2-6 was established on the bases of elemental analyses and spectral data. IR spectra showed the disappearance of the (N = C = S) band and presence of bands for (NH) (CH arom.), (2C = O), (C = S) and (SO_2). IR spectrum of 2, revealed bands at 3472, 3380, 3308 for (NH, NH2), 3080 (CH arom.), 2926, 2858 (CH aliph.), 1680, 1671 (2 C = O), 1236 (C = S), 1370, 1150 (SO2). 1H-NMR spectra of compounds 2-6 revealed multiplet signals at δ 1.2-1.4 ppm and 4.2 ppm corresponding to the six and four protons assigned for (2 CH3 ester) and (2 CH2 ester)

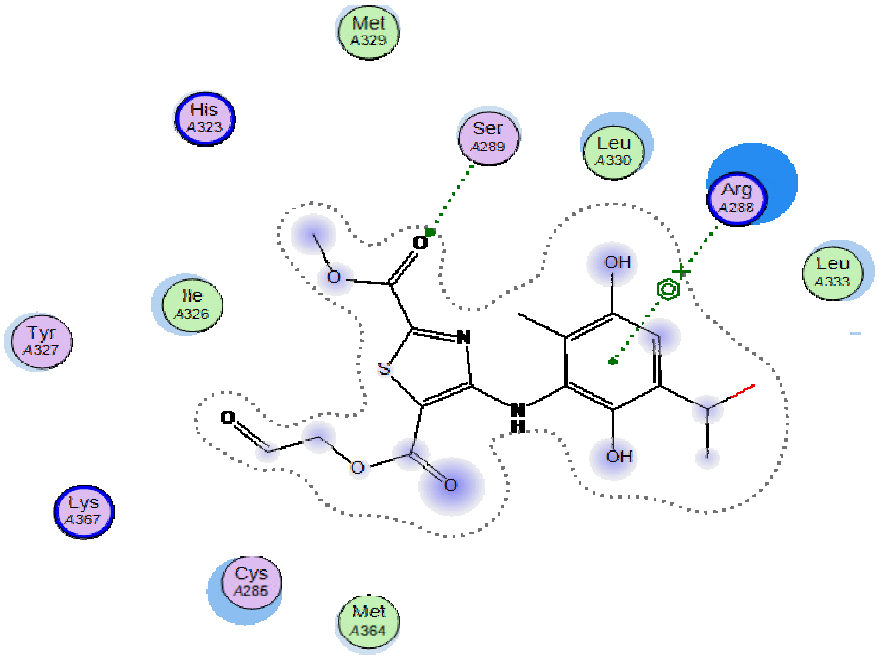

Fig. 3: Binding interaction of compound 4 into PPAR-γ pocket

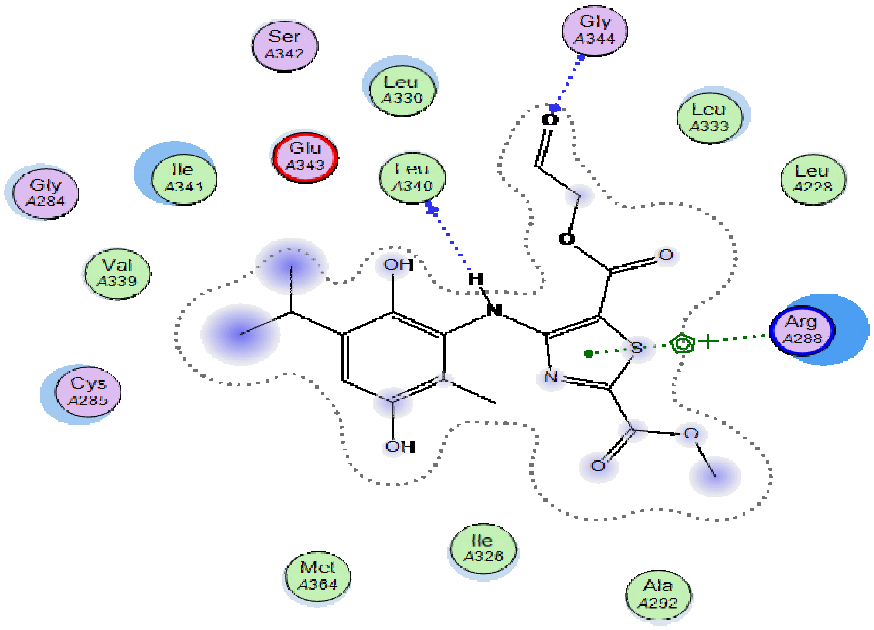

Fig. 4: Binding interaction of compound 5 into PPAR-γ pocket

respectively, multiplet at δ 7-8 ppm for (Ar -H) and singlets at 10-12 ppm for (NH). Mass spectrum of 4 exhibited molecular ion peak m/z at 554 (M+, 1.45%) with a base peak at 299 while that of 5 exhibited a molecular ion peak m/z at 549 (M+, 1.15%) with a base peak at 257. 1H-NMR spectrum of compound 5 showed a multiplet at 7.6 ppm due to the three protons for (3CH pyrimidine) and that of compound 6 exhibited singlet to the two protons of the (2CH pyrimidine) and singlets at 2.7 and 2.9 ppm corresponding to the six protons of the (2 methyl groups of the thiazolidine and pyrimidine rings, respectively).

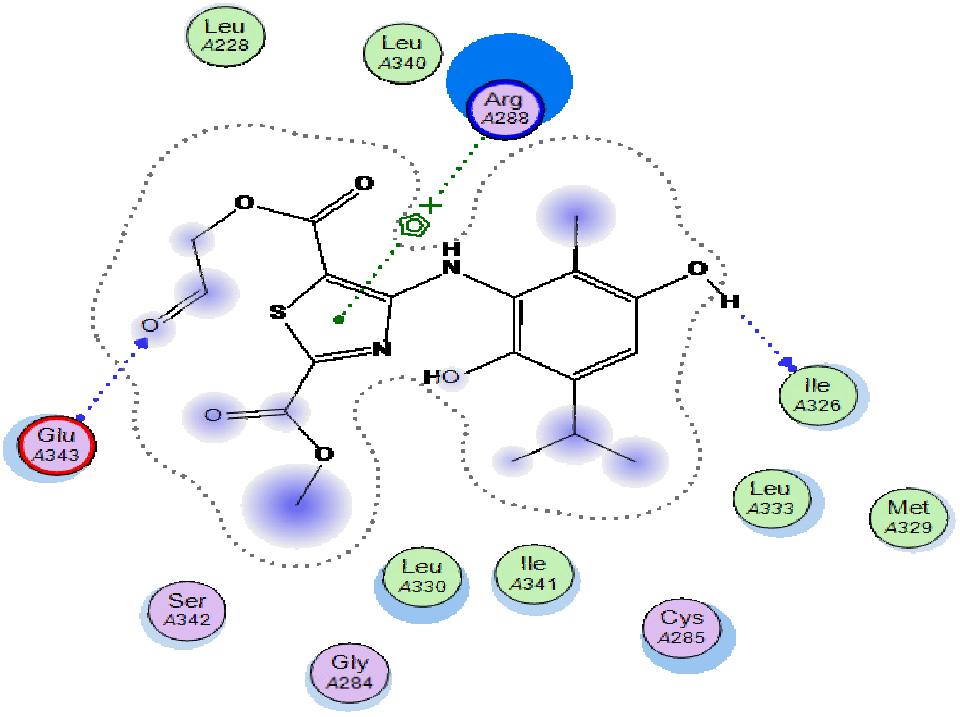

Fig. 5: Binding interaction of compound 6 into PPAR-γ pocket

Table 1: *In vitro* cytotoxic activities of some newly synthesized thymoquinone derivatives against human breast (MCF7) cancer cells

	Cytotoxicity (IC_{50})[a, b] µM	
Compound	(MCF7)	MDA-MB-231
1	3.17	4.05
2	2.8	3.76
3	1.61*	3.20*
4	1.8*	2.75*
5	4.1	4.4
6	3.59	4.07
Efatutazone (CS-7017)	3.2	4.5

[a]IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%; [b] Values are means of three experiments; *Significant at $p<0.05$

Anti-breast cancer and apoptosis evaluation: Table 1 shows the results of anti-breast cancer activity of the newly synthesized TQ analogues on human breastcancer cell line. Efatutazone, the reference drug used in this study is one of the most effective antitumor agents used to produce regressions in different lymphomas (Irvin *et al*., 2008). It is used as positive control in order to compare the results of the synthesized compounds as cytotoxic, relative to it. The relationship between survival ratio and concentration of the tested compounds was plotted to obtain the survival curve of breast cancer (MCF 7) cell lines. The response parameter calculated was IC_{50} value (Table 1) which corresponds to the compound concentration causing 50% mortality in net cancer cells.

Compounds (IC_{50} µM); 2 (2.8) a thiazolidine derivative, 3 (1.6) and 4 (1.8) thienopyrimidine derivatives and 5 (4.1), 6 (3.59), 1 (3.17), TQ derivative, showed significant *in vitro* cytotoxic activity against compared to the reference drug Efatutazonewith IC_{50}3.2 µM (Table 1). The most potent derivatives were 3 and 4 expressing more activity than reference drug. In addition the rest of analogues showed nearly similar activity to the reference drug used. These results agreed with that thiophene, thienopyrimidine, thiazolidine and thymoquinone moieties showed antitumor activity (Ghorab *et al*., 2006; Gali-Muhtasib *et al*., 2006).

Luciferase assay: To investigate whether TQ analogues are good ligands for PPARγ, we carried out a luciferase reporter assay using full-length PPARγ-Luciferase expression vector. PPARγ luciferase activity was enhanced by TQ analogues treatment in a dose dependent manner with the highest efficacy at 1.0 mM and enhanced by 3-folds compared with vehicle (Fig. 1). However, in the presence of compounds 3 and 4, PPARγ activity was increased by 1.0 and 1.6-folds at the concentrations of 1.0 and 10 mM, respectively. The maximal PPARγ luciferase activity induced by analogues 3 and 4 approximately by 45% of that induced by thymoquinone itself, indicating that TQ was a moderate PPARγ ligand (Fig. 1). Compared with other analogues which were specific agonists of PPARγ also.

CONCLUSION

All new analogues tested show cytotoxic activity against breast cancer, the most potent compounds are 3 and 4. The rest of compounds show nearly same cytotoxic activity asEfatutazone reference drug used in this study.

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