Anti-angiogenic targeted therapy in triple negative breast cancer HCC-1806 cell line.

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Abstract— Triple negative breast cancer (TNBC) is an aggressive tumor with poor prognosis, however, chemotherapy is the most important treatment for TNBC. It has been demonstrated that TNBC is markedly sensitive to anti-angiogenic therapy. In this context, PI3K/AKT/mTOR pathway may have a regulatory role in angiogenic output. Meanwhile, TORIN 1, PI3K/mTOR inhibitor, has been found to inhibit cell proliferation and it has been suggested that it may enhance the sensitivity of human TNBC cell line to doxorubicin. The present study was taken to explore the possible anti-angiogenic effects of TORIN 1, alone or in combination with therapeutic agent, doxorubicin (DOX) in HCC-1806 TNBC cell line. Cells were treated with two different concentrations of TORIN 1 (10% of IC50 and IC50). Also, cells were treated with concentrations equivalent to IC50 value of DOX. After each treatment, viability of cells was assessed by MTT assay in addition to quantification of VEGF gene expression and ES level, using RT-PCR and ELISA techniques, respectively. Treated cells were examined morphologically and the cell cycle was analyzed by flow cytometry. Moreover, evaluating CD34 protein expression using Western blotting technique was carried out. The results of the present study may lead to the suggestion that treating of TNBC patients with combination of TOR/DOX would have its impact on clinical outcome and low opportunities of developing adverse effects of chemotherapy.

Index Terms— Angiogenesis, cluster of differentiation 34, Doxorubicin, Endostatin, Torin 1, Triple negative breast cancer Vascular endothelial growth factor.

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1 INTRODUCTION

Triple-negative breast cancer (TNBC), as a subtype of breast cancer, is negative for estrogen (ER), progesterone (PR) and human epidermal growth factor (HER2) receptors, with aggressive manner, poor prognosis and present lack of targeted therapies [1], [2].

TNBC generally occurs in younger women, and is associated with a high risk of distant recurrence and death [3]. Generally, the only current systemic treatment for TNBC is chemotherapy, anthracycline/taxane-based therapy which is inadequate alone. Therefore, alternative targeted therapies are urgently required [4].

On the other hand, TNBC is a highly proliferative neoplasm that needs constant angiogenesis throughout all the phases of its development, invasion and metastasis [5].

Angiogenesis is the process of new vessel formation. it is the most essential step in cancer progression [6]. Accordingly, several pro-angiogenic growth factors and endogenous inhibitors of angiogenesis have been identified in breast cancer, among which the most important angiogenic regulators are considered to be vascular endothelial growth factor (VEGF) and endostatin (ES) [7]. Thus, patients with TNBC have high levels of intratumoral VEGF compared with non-TNBC pa-

tients, so It has been suggested that TNBC is highly sensitive to antiangiogenic inhibitors [8].

Doxorubicin (DOX), an anthracycline, has a high efficacy in treating TNBC but it can result in poor outcomes due to chemoresistance induction [9].

On the other hand, the phosphoinositide 3-kinase (PI3K) pathway, is perhaps the most commonly activated signaling pathway in human cancer. TNBC was found to be highly sensitive to PI3K/mTOR inhibitors [10] e.g., Torin1 (TOR), an ATP-competitive inhibitor of mTOR. Torin1, has been reported to inhibit cell proliferation more effectively than rapamycin [11], [12], therefore it has an important role in the regulation of cell growth, proliferation and survival

Accordingly, the present study is an attempt to throw more lights on the proposed antitumorgenic role of TOR through evaluating its influence on angiogenic output in TNBC. Also, the study aimed to explore the effect on angiogenesis in TNBC when combined with other therapeutic agent such as doxorubicin (DOX).

2 Materials and Methods

2.1 Chemicals and Reagents

Doxorubicin: Adriblastina PFS (Doxorubicin Hydrochloride Injection, USP). Torin-1: supplied by TOCRIS Biotechnibr. MTT Assy: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was produced by Serva Electrophoresis; GmbH; Germany. Vascular endothelial growth factor (VEGF): was produced by Biolegio; Lagelandsweg 56, 6545 CG Nijmegen; Netherlands. Endostatin (ES): ELISA Kit, Cat. No. 201-12-1713, was a product of Sunred (Shanghai, China). Antibodies utilized in western blot analysis: Anti-β-actin IgG, Cat. No. mAbcam 8226 and Anti-CD34 antibody, Cat. No. ab81289, were produced by Abcam, (USA), Coomassie brilliant blue R-250, Cat. No. B-0770, was purchased from Sigma- Aldrich (St. Louis, MO, USA). Sodium lauryl sulphate (SDS), Cat No. 05928; was produced by LobaChemie (Colaba, Mumbai, India). Bovine serum albumin (BSA), Cat. No. A9647, bisacrylamide, Cat. No. 146072.2 and mercaptoethanol, Cat. No.100897039, were products of Sigma- Aldrich (St. Louis, MO, USA).

2.2 Cell Line

Human triple negative breast cancer cell line; HCC1806, was purchased from the American Type Culture Collection (ATCC; (ATCC® CRL-2335TM), Manassas, VA, USA). This cell line was characterized as triple-negative/Basal-B mammary carcinoma.

Cells were maintained at 37°C, 5% CO2 in 10% DMEM (Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (Hyclone, Salt Lake City, UT, USA) and 1% penicillin/streptomycin (Invitrogen).

2.3 Experimental Design and Treatments

Untreated human triple negative breast cancer cell line was used as control. Cells were treated with different concentrations of torin-1 (TOR), (10% of IC_{50} and IC_{50};1 and 10 µg/mL), Also, cells were treated with concentrations equivalent to IC_{50} values of DOX, 61.9 µg/mL.

2.4 Combination Treatment

TNBC cell line was further treated with a combination of TOR with DOX. It should be recognized that in these combinations high or low TOR concentration was combined with DOX, i.e., TOR_H-DOX or TOR_L-DOX.

After each treatment, viability of cells was assessed by MTT assay in addition to quantification of VEGF gene expression and ES level, using RT-PCR and ELISA techniques, respectively. Treated cells were examined morphologically and by flow cytometry. Moreover, CD34 protein expression was carried out using Western blotting technique.

2.5 MTT Cell Proliferation Assay

HCC1806 Cells were seeded in medium and incubated at 37°C in a 5% CO2 incubator overnight before being treated with each test compound at different concentrations. After further incubation for 24 h under the same conditions, the cells were treated with MTT solution and incubated for another 24 h. The cell monolayer in each well was added with 20 µL DMSO to dissolve the formazan formed and the optical density was measured at 570 nm with background subtraction at 630 nm. Cell viability of treated samples was calculated in reference to the untreated control that was defined as 100% viability. IC50 values (the concentration of the tested compound causing 50% growth inhibition) were estimated from dose response curves. All experiments were performed in triplicate with internal triplicates

2.6 Morphology

Treated and untreated HCC1806 Cells were morphologically examined under an inverted microscope.

2.7 Cell Cycle Analysis by Flow Cytometry

For cell cycle analysis, HCC1806 cells were plated overnight in 10% DMEM and treated with tested agents for 24 hours. Both floating cells and trypsinized adherent cells were collected and combined for analysis. cells were fixed by dropwise addition into ice cold ethanol and stored at -20° overnight. Cells were then pelleted, washed, and stained for one hour with 50 µg/ml propidium iodide in PBS containing 0.1 mg/ml

ribonuclease A and 0.05% Triton X-100 (Sigma, St. Louis, MO, USA). After gating to exclude debris, the cell cycle distribution was measured using a LSR-II flow cytometer (BD Biosciences). Data were analyzed with ModFit LT software (Verity Software House, Topsham, ME, USA) [13].

2.8 Vascular Endothelial Growth Factor Gene Expression Quantification

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol and reverse-transcribed by using a reverse transcriptase PCR (RT-PCR) kit (Invitrogen). Real-time PCR was performed on an ABI 7500 sequence detection system (Applied Biosystems). The primer sequences were as follows

Forward: 5′-GCA GAA TCA TCA CGA AGT GG-3′

Reverse: 5′- GCA TGG TGA TGT TGG ACT CC-3′

β-actin gene was used as an internal control and the sequences of the primers were

Forward:

5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′

Reverse;

5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′

The applied program was as follow; 1 cycle at 95˚C for 30 seconds during denaturing, 40 cycles at 95˚C for 5 seconds, 1 cycle at 95˚C for 15 seconds. Gene expression was calculated using (CT) method [14].

2.9 Western Blotting

For determination of CD34 protein expression, triple negative human breast cancer cells were collected and lysed in RIPA lysis buffer after 24h of treatment. Membranes were re-probed with β-actin protein antibody to confirm equal loading of protein samples.

2.10 Endostatin (ES) Determination

TNBC cells were treated with different tested agents for 24 hours, ES protein levels were measured by ES ELISA kit, produced by Sunred (Shanghai, China); according to the manufacturer's instructions.

2.11 Statistical Analysis

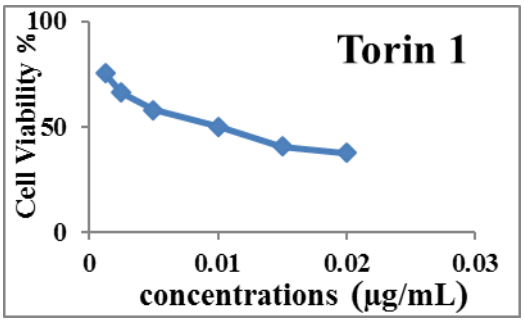
Data were obtained from six independent experiments. Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis was carried out using SPSS package for social science version 16 (SPSS Inc., USA). Multiple comparisons between studied groups were performed by one-way ANOVA. The values of (p) were considered statistically significant at level ≤ 0.05. It should be noticed that the results of CD34 protein were analyzed, 3 samples/group, using ANOVA with Tukey as post-hoc.

3 Results

3.1 Assessment of Cytotoxicity

For analyzing the anti-proliferative effect of DOX and TOR on HCC1806 cell viability, cells were treated with different concentrations of the tested agents. The data showed a dose-dependent decrease in the viability, **Fig. 1**. IC_{50} values of TOR and DOX are 10 and 61.9 µg/mL, respectively.

Moreover, to investigate the effect of targeting angiogenesis in TNBC with combination of TOR with DOX, Chou–Talalay approach for drug combination [15] was adopted to quantitatively define their pharmacological interactions. The data of the present study revealed that the combination of these agents was additively interacted.



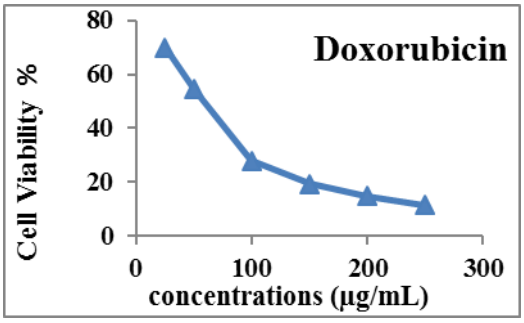


Fig. 1: Effect of treatment with DOX or TOR on HCC1806 cell viability

3.2 Morphological Investigations

Untreated cells appeared elongated, and adherent showed cellular crowding, suggestive of normal proliferation. On the other hand, by treating cells with DOX and TOR, the inhibition in cells growth, and loss of cellular characteristic morphology were observed. **Fig. 2.**

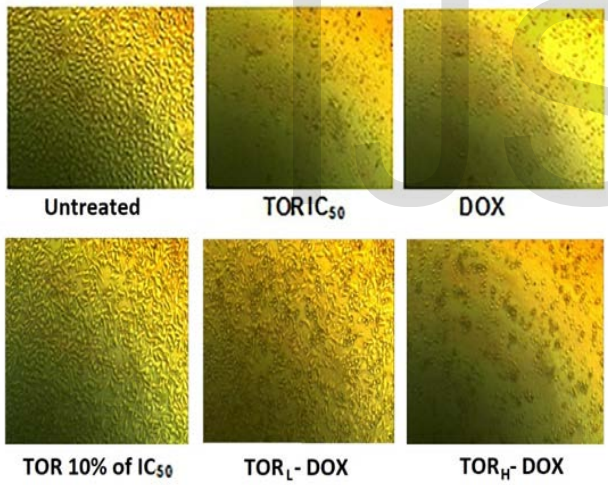


Fig. 2: The effect of treatment with TOR, DOX and their combinations on the Morphology of HCC1806 cells after 24 Hrs of Exposure (by inverted microscope).

3.3 Cell Cycle Analysis:

Flow cytometric (FCM) analysis for TNBC cells before and after treatment with different concentrations of TOR and DOX was carried out. Untreated HCC1806 cells showed presence of cells at sub G1 peak (8.21%) and increase of cells in G0/G1(59.6%) cell cycle phase compared with other phases. treatment of TNBC cells with different concentrations of TOR revealed different effects on cell cycle. Treating TNBC cells with 10% of IC_{50} of TOR showed no obvious changes on cell cycle distribution with respect to untreated cells. Meanwhile treatment of TNBC with either IC_{50} of DOX or TOR resulted in an increase in cell count in sub-G1 phase (60.35% ,50.4%) respectively. **Fig. 3.**

FCM analysis for TNBC cells treated with different combinations of TOR and DOX was carried out. Investigating the combination of TOR and DOX was performed using high and low TOR concentrations. Treating TNBC cells with TOR_L -DOX revealed no obvious changes on cell cycle distribution compared to untreated cells, while Treating TNBC cells with a combination of TOR_H-DOX, induced a further increase of the sub-G1 population up to almost 73%, **Fig. 3.**

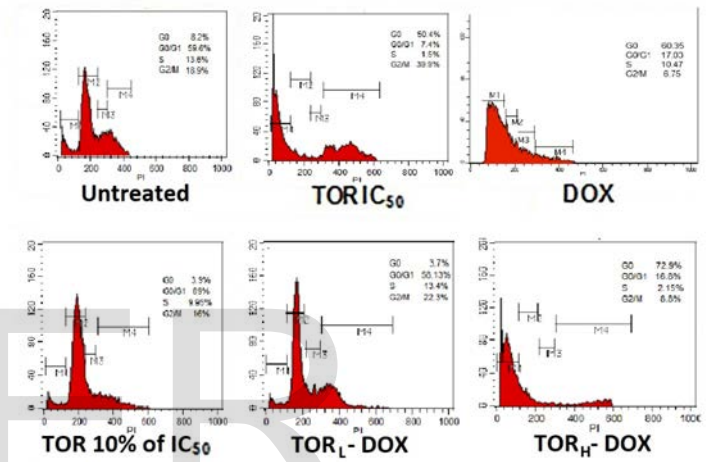


Fig. 3: Flow cytometric analysis of cell cycle distribution of untreated and treated HCC1806 cells. Treatment was carried out with TOR at concentration (10 and 1 µg/mL), with DOX at concentration (61.9 µg/mL) and their corresponding combinations for 24 hrs.
M1 represents sub G population, M2 represents G0/G1phase, M3 represents S phase and M4 represents G2/M phase.

3.4 Assessment of Angiogenic Markers

In regards to treatment of TNBC cell line with either DOX or TOR IC_{50} or 10% of IC_{50}, resulted in a statistically significant down regulation of VEGF gene expression ($p = 0.0001$) for all. The VEGF gene expression down-regulation was more pronounced in TNBC cell line treated with IC_{50} of TOR in respect to that treated with 10% of IC_{50}, without reaching a significant level. **Table 1**

In the present study the effect of treating TNBC cell line with TOR-DOX combinations showed significant down regulation of VEGF gene expression (p = 0.0001) for both when compared with untreated cells. **Table 2**

In respect to treatment of TNBC cell line with either DOX, TOR IC_{50} or 10% of IC_{50}, resulted in a statistically significant elevation in ES protein levels (p = 0.007,0.0001 ,0.0001) respectively. The increase in ES protein level was significantly more pronounced in TNBC cell line treated with 10% of Torin-1 IC_{50} when compared with that treated with DOX or TOR IC_{50}. **Table 1**

Treatment of TNBC cell line with TOR-DOX high or low combinations revealed a significant increase in ES protein level (p = 0.0001) for both. **Table 2**

In regards to treatment of TNBC cells with IC_{50} concentration of DOX, TOR IC_{50} or 10% of IC_{50}, resulted in a statistically significant down regulation in CD34 expression (p = 0.00001) for all**, Fig. 4A, Table 1**

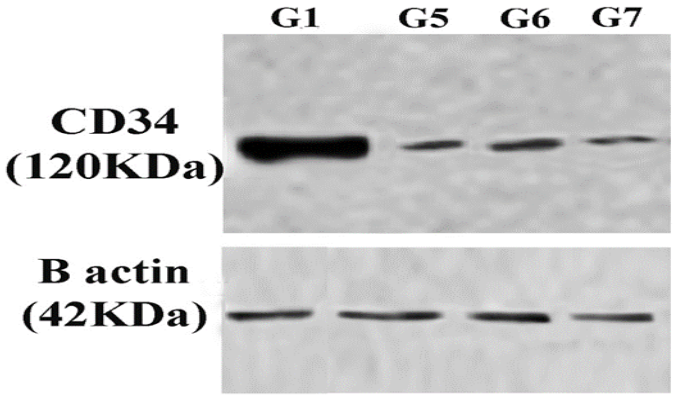


Fig. 4A Western Blot analysis of CD34 protein expression relative to β actin protein (internal control). Effect of DOX and TOR on CD34 protein expression in TNBC cells, G1: Untreated, G5: DOX, G6: TOR IC_{50}, G7: TOR 10% of IC_{50}.

Treatment of TNBC cell line with TOR-DOX combinations showed significant down regulation of CD34 expression (p = 0.00001) for both when compared with untreated cells. **Fig. 4B, Table 2**

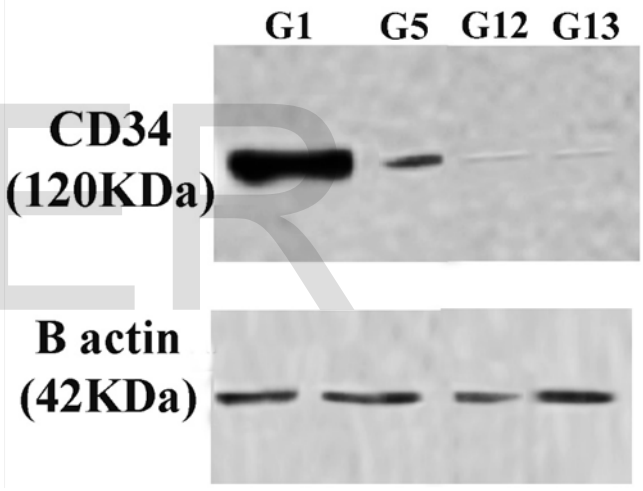


Fig. 4B Western Blot analysis of CD34 protein expression relative to β actin protein (internal control). Effect of TOR and DOX cominations on CD34 protein expression in TNBC cells, G1: Untreated, G5: DOX, G12: TOR_{L}_DOX G13: TOR_{H}_DOX.

Table 1: Assessment of angiogenic markers in TNBC cell line treated with DOX and different concentrations of TOR

	Control	DOX	TOR 10%	TOR IC_{50}
VEGF Gene Expression(relative expression x $^{10-6}$)				
X ± SE	207.7 ± 25.9	33.7 ± 1.3	95.7±5.5	64.3 ± 11.3
Min – Max	147 – 286	30 – 37	82–112	39 - 99
p1		0.0001*	0.0001*	0.0001*
p2			0.021*	0.816
p3				0.794
CD34 Protein Expression(relative protein expression)				
X ± SE	1 ± 0.02	0.509 ± 0.033	0.499 ±0.03	0.519 ± 0.021
Min – Max	1–1	0.457 - 0.571	0.430-0.546	0.492 - 0.560
%of reduction		49.1	50.1	48.1
p1		0.00001*	0.00001*	0.00001*
Endostatin Level(ng/mL)				
X ± SE	23.6 ±0.2	27.0 ± 0.43	34.4 ±0.4	29.0 ± 0.3
Min - Max	23.0 –24.5	25.7 – 28.6	3206-35.2	27.7 – 30.3
p1		0.007*	0.0001*	0.0001*
p2			0.0001*	0.077
p3				0.0001*

p1: Comparison vs. Control
p2: Comparison vs. Cells treated with DOX
p3: Comparison vs. Cells treated with 10% of the TOR
* (p) value was considered significant at level ≤ 0.05

Table 2: Assessment of angiogenic markers in TNBC cell line treated with TOR IC_{50} and its combinations with DOX.

	Control	TOR IC_{50}	TOR_L-DOX	TOR_H-DOX
VEGF Gene Expression(relative expression x 10^{-6})				
X ± SE	207.7±25.9	64.3 ±11.3	73.0±2.6	46.7±4.2
Min - Max	147 – 286	39 - 99	67- 81	39-60
p1		0.0001*	0.0001*	0.0001*
p2			1.000	0.990
p3				0.927
CD34 Protein Expression (relative protein expression)				
X ± SE	1 ± 0.02	0.519 ± 0.021	0.309±0.031	0.280±0.026
Min - Max	1–1	0.492 - 0.560	0.249-0.352	0.231-0.320
%of reduction		48.1	69.1	72
p1		0.00001*	0.00001*	0.00001*
Endostatin Level (ng/mL)				
X ± SE	23.6 ± 0.2	29.0 ± 0.3	32.2±1.1	31.4±0.8
Min - Max	23.0 –24.5	27.7 –30.3	27.7-36.0	29.0-34.0
p1		0.0001*	0.0001*	0.0001*
p2			0.009*	0.044*
p3				0.527

p1: Comparison vs. Control
p2: Comparison vs. Cells treated with TOR IC_{50}
p3: Comparison vs. Cells treated with a combination low concentration of TOR and DOX
* (p) value was considered significant at level ≤ 0.05

4 Discussion

Triple negative breast cancer (TNBC) has exceptionally proliferative and aggressive manners with poor prognosis as results of resistance to currently available treatments [16]. Therefore, it is of a critical issue to develop a novel therapeutic strategy that enhances chemotherapeutic efficiency and overcomes drug resistance [17]. On the other hand, the targeting of angiogenesis in treating TNBC may be of great importance since it has been suggested that this subtype of breast cancer is markedly sensitive to antiangiogenic therapy [8]. In this aspect, significant advances in cancer treatment have been achieved with the development of antiangiogenic agents, the majority of which have focused on inhibition of the vascular endothelial growth factor (VEGF) pathway [18].

A recent gene expression analysis of TNBC identified at least six tumor molecular subtypes, that were highly sensitive to PI3K/mTOR inhibitors in vitro and in vivo [19]. A novel ATP-competitive inhibitor of mTOR, Torin1, has been reported to inhibit cell proliferation more effectively than rapamycin [11]. Torin 1 has been found to impair cell growth and proliferation through a mechanism involving mTORC1 inhibition other than mTORC2 inhibition, in which the rapamycin-resistant functions of mTORC1 is suppressed [12].

Cytotoxic chemotherapy represents the landmark of current treatment strategies for early and metastatic TNBC. In this regard, third generation chemotherapeutic agents, including anthracyclines and taxanes, are considered the most effective available tools. Doxorubicin is a potent chemotherapeutic agent, and its use is part of several standard regimens for different cancers, including TNBCs [16].

In the present study, the anti- tumorigenic effect of TOR, on TNBC cell line was investigated through its influence on angiogenic output in this breast cancer subtype. Furthermore, the study is an attempt to investigate whether targeting two drugs among TOR and DOX could exhibit better clinical outcomes.

In consistence with previous studies [20], [21]. The results of present study revealed that treatment of TNBC with either IC_{50} of DOX or TOR resulted in an increase in cell count in sub-G1 phase reflecting a pro-apoptotic effect of these two therapeutic agents.

Interestingly, the TOR/DOX combination induced a further increase of the sub-G1 population up to 72.9%. Overall, the above data suggest that TOR potentiates the DOX-induced antiproliferative effects by inducing apoptosis of TNBC cells.

It has been reported that the activated form of mTOR, phospho-mTOR, detected at nuclear level, was expressed more frequently in TNBC compared with non- TNBC [22] suggesting that mTOR may play a more important role in the progression of TNBC and could be considered a new target for the treatment of this tumour sub-type [23], [24] Also, it has been demonstrated that PI3K/AKT/mTOR pathway can regulate angiogenesis by modulating expression of nitric oxide and angiopoietins. Thus, inhibition of the PI3K/AKT/mTOR pathway in tumor cells can decrease VEGF secretion by both HIF-1 dependent and independent mechanisms. Vascular endothelial growth factor (VEGF) is a key angiogenic mediator that stimulates endothelial cell proliferation and regulates vascular permeability [25].

Several agents have been found to inhibit PI3K and/or mTOR signaling in tumor cells and efficiently can affect angiogenesis as well as on tumor cell proliferation and survival

[26]. In this aspect Torin1, an ATP-competitive inhibitor of mTOR, has been reported to inhibit cell proliferation more effectively than rapamycin [11].

In the present study and in agreement with previous research [27], treatment of TNBC cell line with different concentrations of TOR was found to significantly down-regulate VEGF gene expression in a dose-dependent manner. The degree of down-regulation of VEGF gene expression was more pronounced in case of IC50 of TOR when compared to that when 10% of IC50 was applied. This was attributed to that mTORC1 as well as Erk1/2 phosphorylates 4EBP1, the inhibitor of eIF4E1. Phosphorylation of 4EBP1 keeps it from inhibiting eIF4E1 and allows eIF4E1 to promote angiogenesis. Eukaryotic translation initiation factor 4E (eIF4E1) along with EGFR have been identified as proteins expressed in brain metastatic cells originating from breast cancer. Once eIF4E1 is activated it also activates hypoxia inducible factor alpha (HIF1α), which then binds with HIF1β, and together they function as transcription factors (TF) for genes involved in angiogenesis, namely matrix metalloproteinases (MMPs) and cyclooxygenase 2 (Cox-2). These proteins function together to remodel the extracellular matrix. HIF1α also acts as a TF for the growth hormone VEGF which when bound to its receptor, VEGFR also aids in angiogenesis [11].

In addition to VEGF, CD34, cluster of differentiation 34, is an important indicator of tumor angiogenesis.CD34 is particularly sensitive to tumor angiogenesis, as it can clearly represent the state of neo-vascularization during the growth of a tumor [28]. In the present work, significant reduced expression in CD34 is observed in TNBC cells treated with different TOR concentrations (48.1% and 50.1% for IC_{50} and 10% of IC_{50} respectively). It should be noted that, the reduction degree of CD34 was almost the same on treating TNBC cell line with DOX (49.1%).

Moreover, in the present study the level of endostatin (ES) as an endogenous inhibitor of angiogenesis [29] was evaluated. the results also showed that both TOR concentrations exhibited anti-angiogenic properties through significant elevation of the level of ES. It seems that the concentration of TOR is a crucial determinant since ES level was significantly higher in low dose (10% of IC_{50}) than in high dose (IC_{50}) and also significantly higher than ES level in cells treated with DOX.

These observations may be in consistence with that observed in respect to VEGF and point out to the anti-angiogenic effect of TOR [26].

Although anti-angiogenic agents targeted to one specific angiogenic stimulator such as Bevacizumab (anti-VEGF) have shown improvements when used in combination with chemotherapeutic agents such as Paclitaxel, 5-Fluorouracil, and Doxorubicin (in certain tumors such as colorectal), their effectiveness seems to decrease in other cancer types, namely breast cancer and non-small cell lung cancer. This may simply be due to the availability of a host of other angiogenic factors to the tumor cell as described above, or by either synthesizing them themselves or by recruiting endothelial cells for angiogenesis. Therefore, a successful antiangiogenic therapy would either target multiple angiogenic factors or an angiogenic factor which is critical to angiogenesis or specifically control a host of other angiogenic factors [30].

In this aspect, we further analyzed the expression of VEGF in TNBC cells to investigate whether the combination therapy could induce protein expression changes. RT-PCR results revealed that the expression of VEGF, further reduction was observed in the combination Group TOR_H-DOX.

the degree of down-regulation of CD34 expression as well as the elevation in ES level in the TOR/ DOX combination groups were more pronounced than that when TNBC cell line was solely treated with DOX. Keeping in mind that the concentration of DOX in the combination with TOR was less than that of IC_{50}, it could be suggested that the effect of chemotherapeutic drugs may be attainable with less needed cytotoxic dose when combined to TOR.

5 Conclusion:

The results of the present investigation revealed that, TOR (mTOR inhibitor) significantly arrests the growth of TNBC cells. TOR-treated cells show an increase of The sub- G1 population compared to control cells. The above data suggest that the anti-proliferative effect caused by TOR in TNBC cells is mainly due to apoptosis induction and that TOR can have discrete effects on the cell cycle depending on concentration.

In addition, it significantly reduced angiogenesis through targeting multiple angiogenic factors as evidenced by the significant reduction of proangiogenic vascular endothelial growth factor, cluster of differentiation 34, and significant increase of anti-angiogenic endostatin. so TOR proved that it is powerful inhibitor with anti-angiogenic properties, suppressing cancer cell growth.

Moreover, on the light of results of the present study it could be suggested that treatment of TNBC patients with TOR/DOX combinations would have its impact on clinical outcome and low opportunities of developing adverse effects of chemotherapy.

6 Funding:

The study was not funded by any supporting organization.

7 Declaration of interest

Authors declare that there is no conflict of interest.

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