A comparative study of combination treatments in metastatic 4t1 cells: everolimus and 5- fluorouracil versus lithium chloride and 5-fluorouracil

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Abstract

Background: Combination therapy has been one of the most pioneering and strategic approaches implemented for malignancy treatment, which can intentionally influence multiple signaling pathways involved in cancer growth and progression. In the present study, the effects of 5-fluorouracil (5FU) in combination with everolimus (EVE) or lithium chloride (LiCl) were evaluated in 4T1 metastatic breast cancer cells and compared to control and each other.

Methods and results: The resazurin assay, CompuSyn, flow cytometry, and real-time PCR were used to investigate cell proliferation, drug synergism, apoptosis, and gene expression. In comparison to the ternary combination of the drugs, the findings showed that cytotoxicity (p-value < 0.0001) and apoptosis (p-value < 0.0001) of two-by-two combinations increased dramatically as a consequence of the extreme synergy between 5FU and EVE or LiCl. Moreover, the hypoxia inducible transcription factor 1-alpha (HIF-1α) and the vascular endothelial growth factor (VEGF) downregulated considerably compared to control (p-value < 0.0001) by combination therapies of EVE-5FU and 5FU-LiCl; however, only VEGF displayed significant downregulation in comparison to single therapies.

Conclusion: The findings showed that the combination of 5FU-LiCl increased cell cytotoxicity and apoptosis significantly more than EVE-5FU but suggests a clinical potential for both to treat metastatic breast cancer encouraging validation of these results in pre-clinical models.

Keywords

Combination therapy, Breast cancer, Everolimus, 5-fluorouracil, Lithium chloride
Introduction

Breast cancer is the most common malignant disease and the primary cause of cancer mortality among women across the world (Ghorbani-Abdi-Saebad et al. 2020) and accounts for 25% of all cases and 15% of cancer deaths worldwide (Bray et al. 2018). Further research in different areas, including improvement and enhancement of drug performance and efficacy, improving drug delivery and decreasing drug resistance and side effects are required to treat this prevalent cancer.

The diseases that we now know as multifactorial disorders such as cancer are caused by multiple genes or environmental factors, not by a single one. Combination therapy is used in multifactorial conditions to achieve more efficient treatment. It targets cancer's key pathways explicitly with a synergistic effect and reduces the dose of chemotherapy (Bozic et al. 2013; Korkut et al. 2015; Amini Chemahini et al. 2020). This method potentially decreases drug resistance while retaining and strengthening the therapeutic potential of anti-cancer reagents such as trapping cells in the cell cycle, inducing apoptosis and limiting the cancer stem cells population, tumor growth, angiogenesis, and metastatic ability (Foucquier and Guedj 2015).

Angiogenesis is a sign of malignancy that plays a significant role in tumor progression, causes an unusual microenvironment, and influences chemotherapeutic conveyance (Nerini et al. 2016). Tumor angiogenesis is an essential and important target that almost all malignancies have in common. In most human tumors, angiogenesis inhibitors have been shown to arrest or halt the tumor's development, but as a single treatment, they will not eradicate cancer. Therefore, for effective tumor treatment, the combination of an anti-angiogenesis agent and chemotherapy may be imperative.

VEGF is a vital factor in angiogenesis, and the most approved anti-angiogenic approaches are blocking this factor and its receptors (VEGFRs) (Jászai and Schmidt 2019). One of the significant inducers of angiogenesis is hypoxia, bringing about the actuation of HIFs. Inhibition of HIF-1α translation by preventing platelet-derived growth factor or its receptors (PDGF/PDGFR) and VEGF/VEGFR can result from the mechanistic target of rapamycin (mTOR) inhibition (Li 2005).

EVE is an mTOR inhibitor; currently undergoing clinical trials for clinical use as an anti-cancer agent alone and in combination with other antineoplastic agents. It targets mTORC1, a multifunctional signal transduction protein that receives many signals and proceeds through multipath regulation (O’Reilly et al. 2011). EVE also functions as an inducer of autophagy (Cerni et al. 2019). LiCl is another autophagy inducer with a different action mechanism to EVE (Ohmuro-Matsuyama et al. 2019).

LiCl uses the mTOR-independent pathway in which lithium inhibits glycogen synthase kinase-3β (GSK3β) (Ha et al. 2014; Motoi et al. 2014; Nakanuma et al. 2020). Lithium can alter the biochemical properties of various transcription factors, interfering and inhibiting many different pathways involved in cancer (Arena et al. 1997; Adler et al. 2010). Evidences has pointed to its potential use as an anticancer agent (Li et al. 2014). Besides, LiCl has been used in combination with other anti-cancer reagents to treat various cancers due to its anti-proliferative and autophagic properties (Adler et al. 2010; Suganthi et al. 2012a).

One of the most commonly used chemotherapy medicines is 5FU, which is useful in curing cancer by inhibiting DNA and RNA synthesis. Since 1957, the drug has played a vital role in the treatment of cancer. 5FU is a heterocyclic aromatic organic compound with a structure resembling a pyrimidine molecule, which is a uracil analog; thus, interfering with nucleotide metabolism and causing cell death by inserting into DNA and RNA structures (Zhang et al. 2008).

Generally, Combination therapy is one of the most successful cancer-fighting techniques available today. This research used murine breast cancer 4T1 cells to investigate, compare the anti-cancer efficacy of various combinations of drugs on metastatic and triple-negative breast cancer cells to advance such potential therapies in the future.

Methods and materials

Materials

4T1 cell line was purchased from the Pasteur Institute of Iran. Cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific, USA), and incubated at 37 °C, in a humidified atmosphere with 5% carbon dioxide. Trypan blue and Resazurin (Sigma-Aldrich, Munich, Germany) were used for cell count and viability assessment, respectively. For apoptosis detection, Annexin V conjugated with fluorescein isothiocyanate (FITC), and PI (Propidium Iodide) kit was used. The high concentration of aqueous LiCl (Sigma-Aldrich) stock and dimethyl sulfoxide (DMSO, Sigma-Aldrich) stock solutions of 5FU (Sigma-Aldrich) and EVE (Novartis, Basel, Switzerland) were stored at 4 °C and −20 °C respectively and finally diluted with fresh culture medium immediately before use.

Single therapy and cell viability

In each well of the 96-well plate, 5×10^3 cells were seeded and incubated for 24 h at 37 °C and 5% CO2. Cells were treated with different concentrations of EVE (0–2 μM), 5FU (0–12 μM) or LiCl (0–60 mM), incubated for 24, 48, and 72 h, and each concentration was set in every 5-well. The Resazurin test measured the half-maximal inhibitory concentration (IC_{50}) of each drug at each time. Then the supernatant was discarded and replaced with 180 μl of the serum-free medium as well as 10 μl of Resazurin reagent solution followed by another 4 h incubation. The viability was calculated by an ELISA
reader at 520–570 nm to measure the optical density (OD) using the following formula.

\[
\text{Viability} = \frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{control}}} \times 100
\]

Combination therapy and drug synergism

In a 96-well plate, 5x10^3 of 4T1 cells were planted. After 24 h incubation, cells were treated with different combination groups of EVE, 5FU, and LiCl for 48 h. The Resazurin test measured cell viability and considered 50%, 75%, and 90% growth inhibition to explore synergism at the maximum drug killing capacity. Synergism evaluated using CompuSyn software (Biosoft, Ferguson, MO, USA), which tested the Chou-Talalay Constant Ratio Program and combination Index (CI). CI = 1 indicates additive effects, CI > 1 indicates antagonist effects, and CI < 1 shows synergism.

Apoptosis

Cells were seeded into 6-well plates at a density of 5 x 10^5 cells per well. 24 h later, treatment groups, alone and combined, were added to every 5-well at around IC50 value for each drug. The cells were checked for apoptosis after 48 h based on the manufacturer’s protocol of Annexin V-FITC/PI kit. Phosphatidylserine migrates to the outer surface of the cell membrane during the early stages of apoptosis and is identified explicitly by phosphatidyl serine-binding proteins (Annexin V-FITC). PI-negative and FITC-positive cells are in the early stages of apoptosis, whereas in the late stages of apoptosis are PI-positive and FITC-positive cells.

Real-time polymerase chain reaction (PCR)

4T1 cells were placed for 48 hours in 10-cm dishes at a density of 1x10^5 and exposed to alone and in combination therapies, which showed better results in previous assays. The cells were harvested, and total mRNA was extracted with RNXplus (Sinacolon, Tehran, Iran) following the manufacturer’s instructions. Extracted RNA concentration was measured by a Nanodrop spectrophotometer (Thermo, USA). The first-stranded cDNA was synthesized with oligo(dT) primers using 1 µg of DNA-free total mRNA. Equal amounts of cDNA were amplified by RT-qPCR using SYBR Green PCR Master Mix (Takara Bio Inc., Japan) and a Rotor-Gene 3000 (Corbett Life Science, USA), which checked the melting curves to check the identity of qPCR primers. The succeeding PCR primers were designed for VEGF (forward: 5’-TGTTGGGGAGGAGGATGTC-3’; reverse: 5’-GTTTGTCGTGTCTTGAACTGTA-3’), HIF-1α (forward: 5’-CCCAACTGACCACACTGA-3’; reverse: 5’-GCCACTGTATGCTGATGCCTTA-3’), β-actin (forward: 5’-GACGGCCAGGTATCACTAT-3’; reverse: 5’-AAGGAAGGCTGGAAAAGAGC-3’). Both primer pairs were checked for the formation of primer-dimers using the three-step process referred to above without the inclusion of the RNA template. Relative mRNA fold changes were identified with the 2^-ΔΔCt method. To normalize the data, actin was separately amplified.

Statistical analysis

All numerical data is recorded as a mean ± standard deviation. All data interpret the results of three independent tests. Data groups were analyzed using the one-way and two-way ANOVA tests by GraphPad Prism version 8.

Results

Cell proliferation and drug synergism

Fig. 1 shows the concentration-dependent inhibition of cell viability by EVE and LiCl, also the dose and time-dependent inhibition of 5FU. The IC50 values of each drug in 4T1 cells at 24, 48, and 72 h were indicated in Table 1. Even though the IC50 value is various in different cell lines, our outcomes showed that the metastatic 4T1 cell line is insensitive to EVE (IC50 > 100nM) (Lane et al. 2009; Huvitz et al. 2015); besides, among treatment time spans consist of 24, 48, and 72 h, the lowest IC50 value was obtained at 48 h that is consistent with the results gained from earlier research (Mendes et al. 2016).

Table 1. IC50 values of EVE, 5FU, and LiCl at 24, 48, and 72 h in 4T1 cells; Data presented as mean ± SD from 3 independent repetitions.

|          | 24 h     | 48 h     | 72 h     |
|----------|----------|----------|----------|
| EVE IC50 | 1.132 ± 0.245 | 0.839 ± 0.162 | 1.141 ± 0.129 |
| 5FU IC50 | 9.518 ± 0.191 | 3.599 ± 1.304 | 3.148 ± 0.364 |
| LiCl IC50| 37.392 ± 4.541 | 21.184 ± 5.662 | 30.223 ± 2.384 |

LiCl (in a single treatment) showed a dual effect at 24 h treatment on 4T1 cells, similar to MCF-7 cells (Suganthi et al. 2012a; Suganthi et al. 2012b). The results showed that treatment with concentrations lower than 5 mM increased cell growth and, at higher concentrations, decreased cell growth in a dose-dependent manner; however, this effect was not observed in 48 and 72 h treatments, and generally, cell death was increased in a dose-dependent manner.

Overall, the lowest IC50 for EVE and LiCl was at 24 h with 0.839 ± 0.162 µM and 21.184 ± 3.662 mM respectively, while it was 3.599 ± 1.304 µM for 5FU at 72 h. It should be noted that the 48-h treatment time was chosen for further cell assessments based on the lower IC50 of EVE and LiCl at this time.
Combination indexes of fixed-dose combinational treatment of EVE, 5FU, and LiCl are indicated in Table 2. Although the 5FU-LiCl combination group showed an additive effect at a 50% cell death rate, there was a considerably more potent synergism at high cell death rates (75% and 90%) rather than other groups. The most successful combination treatments in increasing cytotoxicity were 5FU-EVE and 5FU-LiCl, which showed a substantial rise compared to the single treatments (p-value < 0.0001). Furthermore, binary combination groups showed significantly higher cytotoxic effects than the ternary combination of drugs after 48 hours (Fig. 2a, p-value < 0.0001). The difference in cytotoxicity of cells treated with 5FU-LiCl compared with EVE-5FU was noticeable (p-value < 0.01) so that on average, the former resulted in approximately twice as much cell death as the latter (22% and 40% cell survival, respectively).

As illustrated in Fig. 2b, the treated 4T1 cells have undergone a reduction in number, detachment, elongation, and morphological disarray. Combination therapies caused more intense and radical changes in cell size and shape than single treatments.

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**Figure 1.** 4T1 cells viability after 24, 48, and 72 h of exposure to different concentrations of a) EVE, b) 5FU, and c) LiCl by resazurin assay. Data are expressed as mean ± SD from 3 independent repetitions. Generally, EVE and LiCl showed a dose-dependent and 5FU showed a time- and dose-dependent inhibitory pattern with the lowest IC50 at 48 h and 72 h respectively.

**Table 2.** Combination index (CI) of different combinational groups in 50%, 75%, and 90% cell growth inhibition after 48 h. (CI>1 antagonist, CI = 1 additive, CI<1 synergism; Data presented as mean ± SD from 3 independent repetitions.

| Combination Growth inhibition | 50%       | 75%       | 90%       |
|------------------------------|-----------|-----------|-----------|
| EVE+5FU                      | 0.756 ± 0.421 | 0.139 ± 0.094 | 0.017 ± 0.010 |
| EVE+LiCl                     | 1.614 ± 0.787 | 0.143 ± 0.815 | 0.027 ± 0.013 |
| 5FU+LiCl                     | 1.077 ± 0.342 | 0.090 ± 0.015 | 0.010 ± 0.013 |
| EVE+5FU+LiCl                 | 1.856 ± 1.064 | 0.213 ± 0.116 | 0.039 ± 0.012 |

**Apoptosis**

Flow cytometry evaluated the mortality mechanisms of 4T1 cells treated with all singular and combinational treatment groups for 48 h. Flow cytometry histograms sample (Fig. 3a) as like as results of three independent apoptosis assays in Table 3 indicated that the single treatment of EVE, 5FU, and LiCl had a slight influence on 4T1 cells and induced apoptosis in fewer than 20% of them; however, apoptosis was
**Figure 2.**

a) 4T1 cell viability and b) Microscopic images of untreated 4T1 cells and treated cells after 48 h treatment with IC50 of EVE, 5FU, and LiCl as a single or combinational treatment. Dual-combination therapies generally demonstrated higher cytotoxicity than single and triple-combination therapies. EVE-5FU considerably enhanced the cytotoxicity of cells rather than EVE (p-value < 0.01) and 5FU (p-value < 0.0001). Also, 5FU-LiCl significantly increased it rather than 5FU and LiCl (p-value < 0.0001). Cell size and shape showed more intense changes in combination therapies than single treatments and control as well as detachment, elongation, and morphological disarray; Data are expressed as mean ± SD from 3 independent repetitions. **p-value < 0.01 and ***p-value < 0.0001
Figure 3. a) Flow cytometric dot blot samples of 4T1 cells apoptosis after treatment with drugs as a single and combination for 48 h. A) control, B) EVE, C) 5FU, D) LiCl, E) EVE-5FU, F) EVE-LiCl, G) 5FU-LiCl H) EVE-5FU-LiCl for 48 h (Q1 necrosis, Q2 late apoptosis, Q3 early apoptosis, Q4 viable cells). b) Comparison of apoptotic, necrotic and alive cell percentage in different treatment groups individually and in combination. A significantly higher apoptosis rate was observed in 4T1 cells treated with 5FU-LiCl rather than cells treated with EVE-5FU. Data are expressed as mean ± SD from 3 independent repetitions. **p-value < 0.01.
induced in more than 40% of cells when treated by combination groups. While EVE-5FU could induce more late apoptosis than single treatments, the total apoptosis induced by the 5FU-LiCl was remarkably higher than that of the EVE-5FU (Fig. 3b, p-value < 0.01). The apoptosis and cytotoxicity findings also showed that the triplex combination did not show substantial change compared with the dual treatments.

Table 3. Alive, necrotic, early apoptotic, and late apoptotic percentage of 4T1 cells treated with different treatment groups; Data presented as mean ± SD from 3 independent repetitions. *p-value < 0.05, **p-value < 0.01 and ***p-value < 0.0001 compared to the control.

| Cell type      | Alive cells (%) | Necrotic cells (%) | Early apoptosis (%) | Late apoptosis (%) |
|----------------|-----------------|--------------------|---------------------|-------------------|
| Control        | 97.66 ± 0.36    | 1.38 ± 0.46        | 0.85 ± 0.83         | 0.07 ± 0.01       |
| EVE            | 82.33 ± 1.02*** | 2.77 ± 1.69        | 9.32 ± 1.81**       | 5.58 ± 1.07       |
| 5FU            | 81.60 ± 1.93*** | 2.59 ± 2.79        | 11.84 ± 2.32***     | 3.96 ± 2.77       |
| LiCl           | 89.23 ± 1.97*** | 4.42 ± 1.85        | 5.40 ± 3.73         | 0.92 ± 0.58       |
| EVE-5FU        | 53.06 ± 2.31*** | 9.87 ± 2.22**      | 13.89 ± 1.91***     | 23.16 ± 4.03**    |
| EVE-LiCl       | 57.83 ± 1.06*** | 5.74 ± 3.72        | 29.81 ± 4.90**      | 6.60 ± 1.53*      |
| 5FU-LiCl       | 33.56 ± 3.22*** | 11.78 ± 6.31**     | 41.25 ± 6.07***     | 13.33 ± 3.34**    |
| EVE-5FU-LiCl   | 54.93 ± 2.50*** | 8.17 ± 5.62*       | 30.06 ± 3.58***     | 6.83 ± 0.75*      |

**VEGF and HIF-1α gene expression**

Our results demonstrated a noticeable downregulation of VEGF compared to the control in Fig. 4a when cells were treated with an ~IC₅₀ value of 5FU (3 μM) with p-value < 0.01, while it was upregulated significantly in cells treated with LiCl (20 mM) (p-value < 0.0001). There was no significant change in Mus musculus VEGF expression with EVE (0.8 μM) therapy. At the same time, the combination of 5FU with EVE or LiCl not only reduced the expression of VEGF in comparison with the control (p-value < 0.0001) but also, it declined substantially in 5FU-LiCl therapy relative to 5FU (p-value < 0.05) and dramatically relative to LiCl (p-value < 0.0001). Besides, VEGF downregulated by EVE-5FU combination therapy considerably compared to single therapies (p-value < 0.01).

The relative expression of HIF-1α in 4T1 cells after 48 hours of treatment with EVE, 5FU, and LiCl, as well as two combination groups of EVE-5FU and 5FU-LiCl, was shown in Fig. 4b. The outcomes showed that HIF-1α was downregulated dramatically in both singular and combinational treatments compared to control (p-value < 0.0001), although this was not the case with LiCl.

**Discussion**

Cancer cells try to create mechanisms of survival against the regular chemotherapeutics used for their therapy. Among known factors of improved survival are anti-apoptotic pathways or drug efflux pumps that generate antitumor drug resistance (Fletcher et al. 2010). One of the most effective approaches to overcome these mechanisms and enhance the cellular cytotoxicity is to combine medications or anti-cancer reagents. (Sharma et al. 2004). Numerous studies have indicated that the combination of various anti-cancer agents such as 5FU, Cisplatin, EVE, and LiCl have a dramatic impact on raising drug efficacy, cytotoxicity, triggering of apoptosis and autophagy along with decreasing the tumor size and death rate of mouse cancer models (Matsuzaki et al. 2009; Wang et al. 2014). Although previous researches investigated the drug synergism and effects of these combination therapies in vitro on various cell lines and clinical trials in gastrointestinal cancer have also begun to investigate the possible benefits of combining lithium with capectabine, a prodrug of 5FU, (NCT03153280), limited studies have been done in breast cancers. Our study aimed to evaluate and compare the effects of two mostly used combination therapies on a metastatic triple negative...
breast cancer cell and investigate whether ternary combination excess the effects or not (McKenna et al. 2013).

In our research, the triple combination therapy showed weaker synergism and consequently fewer cellular effects than the other combinations, which are possibly due to drug interactions of LiCl with EVE that lead to excessive induction of autophagy, inhibited chemotherapy-induced apoptosis (Hippert et al. 2006; Jain et al. 2013; O’Donovan et al. 2015). It has been showed that induction of autophagy may contribute to cytotoxicity in apoptosis-deficient cancer cells (O’Donovan et al. 2015), however, induction of autophagy enhances the efficacy of induced apoptosis in malignancies treated with conventional chemotherapies (Li et al. 2009). Autophagy, a multistep lysosomal degradation process that enables nutrition recycling and metabolic adaptability (Levine and Kroemer 2019; Yang and Klionsky 2020) is an intricate image of advanced tumor cells that decreases the ability (Levine and Kroemer 2019; Yang and Klionsky 2020) of cell death (BAD) (Li et al. 2009; Zhou et al. 2014).

Previous studies have indicated the potent combination effects between cytotoxic agents such as 5FU and EVE on increasing cell-induced apoptosis at G0/G1-phase of the cell cycle (Hosono et al. 2010; O’Reilly et al. 2011). Combining these two drugs will boost the efficacy of apoptosis induction, which involves the downstream and upstream proteins of drug target pathways including AKT/mTOR and mitogen-activated protein kinase (MAPK) (Marquard and Jücker 2020). Therefore, using EVE in combination with 5FU can increase the efficiency of EVE and reduce its dose in this insensitive metastatic cell line.

Results of past studies suggest that inhibition of GSK3β by LiCl may bypass drug resistance and increase the anti-cancer therapeutic effects of 5FU (Grassilli et al. 2013) although, LiCl has been shown to has the opposite effect than 5FU and EVE on angiogenesis by inhibiting GSK3β following by stabilizing β-catenin which led to increased expression of VEGFa (McBride et al. 2014; Shi et al. 2016). Our findings revealed a significant reduction in HIF-1α and VEGF expression in cells treated with 5FU and EVE individually and in combination, which did not occur in lithium chloride-containing treatments, possibly due to the same reason. Still, unlike HIF-1α, VEGF expression in combination treatments (5FU-EVE and 5FU-LiCl) was significantly reduced compared to individual treatments, which can be concluded that 5FU in combination with EVE or LiCl can inhibit VEGF expression in both HIF-dependent and HIF-independent pathways (Luo et al. 2009; Maxwell 2005; Poon et al. 2009).

In conclusion, in this study, the combination of 5FU with EVE or LiCl enhanced the efficacy of each drug in 4T1 metastatic cancer cells. It was characterized by increased cytotoxicity, induction of apoptosis, and downregulation of the HIF-1α and VEGF expression. Also, a comparative study of the combinatorial treatments showed that 5FU in combination with LiCl induced more cell death and apoptosis than in combination with EVE, but there was no significant difference in reducing the expression of genes involved in mobility, invasion, migration, and angiogenesis such as VEGF and HIF-1α. Both of these combinations could be used in future studies on a variety of human cell lines and animal cancer models, including human breast cancer models. Furthermore, emerging drug delivery systems such as targeted nanoparticles would be beneficial in reducing adverse effects and increasing efficiency, paving the way for better patient treatment.

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**Supplementary material 1**

**Figures S1–S3**

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Data type: Images (docx. file).

Explanation note: **Fig S1.** Dose effect results of 4T1 cells treated with A) single drugs B) combination groups. **Fig S2.** Fa–CI and Fa-logCI diagrams of different combinations. **Fig S3.** Dose reduction index (DRI)-Fa and logDRI-Fa diagrams of different combinations.

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