Original Article

Anti-cancer effects of 1,3-bis (2-ethoxyphenyl) on 4T1 breast cancer cell line, an *in-vivo* study

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Abstract

Background & Aims: Triazene compounds are alkylators with certain chemical, physical, and anti-tumor properties that have been used extensively for producing anti-cancer drugs. In this research, we studied the anti- tumor effects of a new triazene derivate (1,3-bis (2-ethoxyphenyl) on 4T1 breast cancer cell line and induced breast tumors in BALB/c mice.

***Materials & Methods*:** 4T1 cell line was cultured and treated with different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 50µmol) of the 1, 3-bis (2-ethoxyphenyl) triazene in a 24-hour period. Cell survival was evaluated by MTT. Seven-week-old mice were subcutaneously injected with 4T1 cells. Once tumors were observed, mice were randomly grouped and treated with different concentrations of triazene. Tumor size of each group was measured and compared before and after the treatment. Expression of caspases-3 and 9 was investigated by RT-PCR. Statistical analysis was performed by SPSS 22.0 and p-values less than 0.05 were considered significant.

***Results*:** The results of MTT test showed that the triazene induced cell death in a concentration-dependent pattern and decreased cell viability in the 4T1 cell line. Tumor size was significantly decreased in triazene-treated groups compared to control groups. RT-PCR results showed an increase in the expression of caspase genes in the triazene-treated group compared to the control groups.

Conclusion: We found that 1,3-bis (2-ethoxyphenyl) triazene can reduce the survival rate of 4T1 cells *in vitro*, decrease the size of the induced breast tumors, and also increase the expression of caspase genes in the tumor tissues.

Keywords: Breast cancer, Triazene compounds, 1,3-Bis (2-ethoxyphenyl) triazene, Caspase genes, Anti-cancer drugs, Tumor

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Introduction

Triazene compounds were first synthesized about 130 years ago by Grace et al. They are cytotoxic agents

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and their anti-cancer properties have attracted considerable interest (1). Among triazene family, temozolomide and dacarbazine have been used extensively as chemotherapeutic agents. Dacarbazine affects cells through three mechanisms as follows: Alkylation by carbon ions, inhibitory metabolic effects against DNA and RNA synthesis, and alkylating sulfhydryl groups of proteins (2). Temozolomide is another chemotherapeutic agent of triazene family which has alkylating activity. The most influential position in DNA which could be alkylated by dacarbazine and temozolomide is the O^6 position of Guanine (3). Dacarbazine and temozolomide, both methylated DNA by creating methyl diazonium ion. Biological activity and cellular resistance of triazene compounds depend on at least three mechanisms of DNA repair including methyl-guanine methyl-transferase (MGMT), DNA mismatch repair (MMR), and base excision repair (BER) (3).

Caspases, cytosolic proteases with cysteine in their active site, are considered as the main factors involved in apoptosis (4). They are inactive zymogens in cells and become active in response to apoptosis. Apoptosis-involved caspases are divided into two groups: initiator caspases (caspase 2, 8, 9, and 10) and executioner caspases (caspase-3, 6, and 7) (5). In the intrinsic or mitochondrial-mediated pathway of apoptosis, release of cytochrome C leads to alpha-1 oligomerization, which in turn activates caspase-9 and activates downstream caspases, including caspase-3. It should be noted that caspase-3 is activated by both intrinsic and extrinsic pathways of apoptosis (6). Caspase-3 activates DNA fragmentation during apoptosis (7). Moreover, Cell damage, including damage to DNA produced by chemotherapy agents, triggers programmed cell death through a series of cascading events, including the activation of the caspase pathway (8). Several chemotherapy agents, such as Staurosporine and Etoposide, eliminate tumor cells by activating the mitochondrial pathway (9). While a number of other chemotherapy drugs, including Cisplatin and Doxorubicin, initiate cell death by activating cell death and caspase-8 receptors (10). While alkylating agents affect RNA and proteins besides DNA, it is reported that alkylating agents-induced apoptosis is caused by alkylation of the DNA and not alkylation of proteins or RNA (11). Since inhibition of apoptosis is one of the hallmarks of invasive cancer, induction of apoptosis in tumor cells is one of the most important cytotoxic mechanisms of most anticancer drugs (12).

Despite the progress of health care system, late diagnosis of breast cancer has kept death-rates high (13). Breast cancer accounts for 18% of the total cancers in women (14). In this genetic disorder, mutations can be found at 4 to 6 main regulatory genes on different chromosomes in cancer cells. These genes are involved in maintaining physiological balance between proliferation, apoptosis, and differentiation. In fact, about half of all breast cancer cases are hereditary (15).

Materials and methods

1.1. Cell culture

4T1 cell line was purchased from the Pasteur Institute of Iran and then it was incubated in RPMI 1640 medium containing 10% FBS (fetal bovine serum), 100 Unit/ml penicillin and 100 µg/ml streptomycin at 37 ° c and 5% CO2. Subsequently, 4×10^3 cells were cultured in 25 cm^2 flasks and the medium was replaced every 2 days.

1.2. Treatment with 1,3-bis (ethoxyphenyl) triazene

1,3-bis (ethoxyphenyl) triazene was synthetized by researchers in the Faculty of Chemistry, Kharazmi University. 5×10^4 cells were cultured in 24-well plates and incubated for 12 h. Triazene solution was mixed in 96% ethanol and filtered via a 0.2µm filter. After that, cells were treated with different concentrations (5, 10, 15, 20, 25, 30, 35, 40, 50µmol) of the triazene for 24 h.

1.3. MTT assay

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To investigate the viability percentage of cells and also to measure the effect of triazene on cancer cells, 3-(4, 5-dimethylthiazol- 2-yl)-2.5-diphenyltetrazolium bromide (MTT) colorimetric assay was carried out after the treatment. First, cells were cultivated in 24-well culture plates. Then, 100µl of MTT solution (5mg/ml in Phosphate-buffered saline, PBS) was added to each well and incubated at 37°C for 4 h in darkness. Subsequently, the medium was replaced with 1ml Dimethyl sulfoxide (DMSO) and the plate was kept at room temperature for 20 min. The absorbance value was measured at 570nm and the percentage of viable cells was calculated via the following formula: Viability= mean absorbance of sample /mean absorbance of control *100.

1.4. Generation of tumors

Seven- week- old mice Balb-C were selected for initial tumor induction. Mice were kept under 12h light/dark cycle and free access to water and food. This experimental study was conducted under approval of Ethics Committee of Kharazmi University. In order to induce tumor, 4T1 cells were injected subcutaneously at a concentration of 10^6 cells / 100 µl PBS to each mouse. Ten days after injection, primary tumors were visible at the injection site. On the twentieth day after the injection of cells, the tumors were extracted and cut into 3 mm cubes by scalpel. For secondary tumor induction, these parts were transplanted into the right-flank region of the BALB/c mice. Finally, these mice were treated using 1,3-bis (2-ethoxyphenyl) for ten days after the transplant.

1.5. Treatment of cancer-induced mice with triazene

At first, the lethal concentration (LD 50) of the triazene combination was obtained experimentally. Then, 1/3 and 1/5 of the lethal concentration were used to treat cancerous mice. The cancer-induced mice were randomly divided into 5 groups as follows: control, solvent control (alcohol), treatment group 1/5 (treated with 1/5 of the lethal concentration), treatment group 1/3 (treated with 1/3 of the lethal concentration), and positive control (treated with cyclophosphamide). On the 1st, 3rd and 5th days of the test, intraperitoneal injection of 100 µl of 1,3-bis (2-ethoxyphenyl) triazene was conducted in mice.

1.6. Morphometric evaluation of tumor

For morphometric evaluation of tumors, the largest and smallest diameter of each tumor was measured in all tested groups. For this purpose, diameter of the tumors was measured by the caliper on the first (before the first injection) and the fifth days (after the last injection). Significant decrease or increase in tumor size before and after treatment indicates the effect of this agent on tumor growth.

1.7. RT-PCR

To assay the expression of caspases, total RNA was extracted from each tissue sample using a standard RNA extraction kit (Total RNA Purification Kit, PP- 210S; Jena Bioscience, Germany). Primers were designed for amplification of genes and Thermocycler apparatus was used for RT-PCR. Then, the PCR products were separated on agarose (1%) gel and stained with ethidium bromide. The specimens were examined by Gel Doc.

1.8. Statistical analysis

Statistical analysis of data was done using InStat-3 software and one-way ANOVA test. Charts were drawn through Excel and significance was accepted for p-values of <0.05.

Results

Figure 1 shows 4T1 cell line after culturing on RPMI 1640 medium. MTT results showed that 1,3-bis (2-ethoxyphenyl) triazene decreased cell survival in a dose-dependent manner. It is also observed that the concentration of 25 µmol of 1,3-bis (2-ethoxyphenyl) triazene after 24 h treatment could result in 50% cell death in 4T1 cells and it was determined as IC50 (Fig 2).

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Measurement of the largest and smallest diameter of each tumor before and after the treatment period showed a significant decrease in the size of the tumors in treatment groups compared to control groups ($p<0.001$) (Fig 3 and 4).

After Total RNA extraction and designing specific primers for Caspase-3 and 9 genes (Table 1), the expression of these genes in different groups was evaluated by RT-PCR. The results of this study showed that there is a dramatic increase in Caspase-3 expression in groups treated with 1,3-bis (2-ethoxyphenyl) triazene and anticancer drug Cyclophosphamide compared to control groups. The expression of Caspase9 also increased in the groups treated with 1,3-bis (2-ethoxyphenyl) triazene and Cyclophosphamide compared to control groups (Fig 5).

Table 1. List of primers sequence (5'-3')

Target Gene	Forward	Reverse
Caspase-3	CCTCAGAGAGACATTCATGG	GCAGTAGTCGCCTCTGAAGA
Caspase-9	AGTTCCCGGGTGCTGTCTAT	GCCATGGTCTTTCTGCTCAC
β-actin	AGCCATGTACGTAGCCATCC	GCTGTGGTGGTGAAGCTGTA

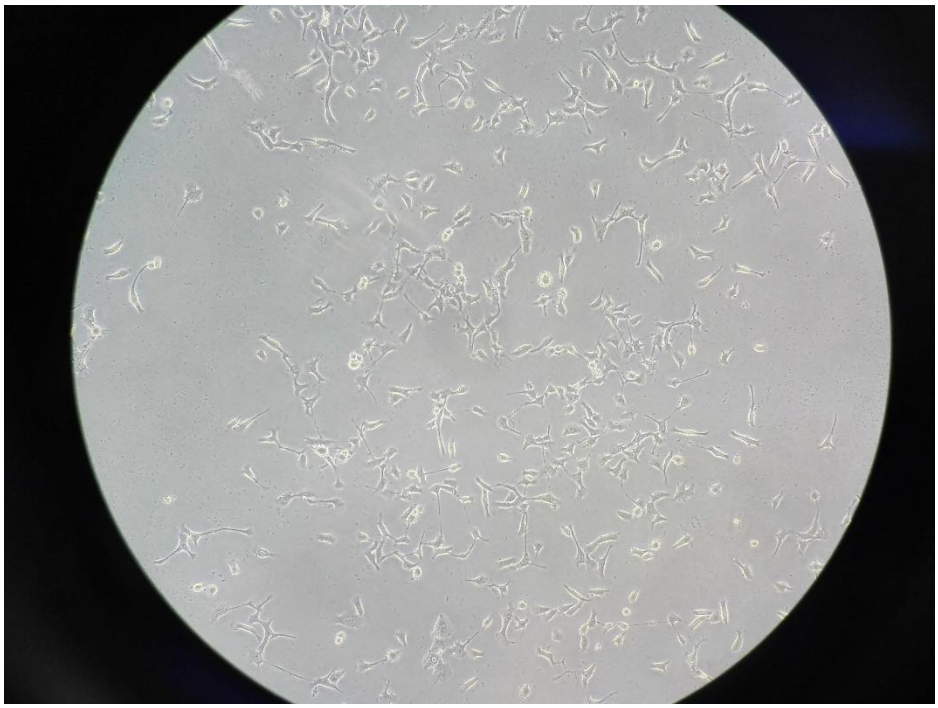

Figure 1. 4T1 cell line. The cells have an epithelial morphology. 4T1 cells will form tumors and spontaneous metastases post implantation into BALB/c mice which very closely mimic stage IV human breast cancer. (Magnification at x400).

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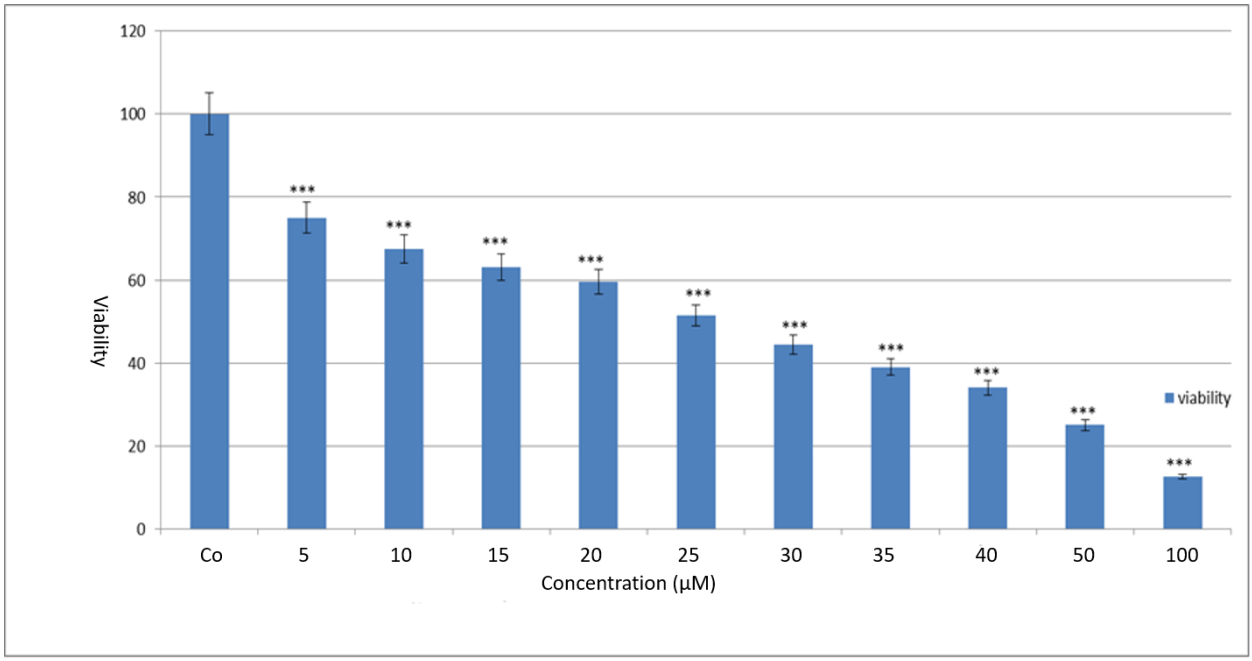

Figure 2. Survival rate of 4T1 cells after the treatment. Viability decreased in a dose dependent manner 24 h after the treatment (Mean ± SE, p <0.001 ***, p <0.01 **, p <0.05 *).

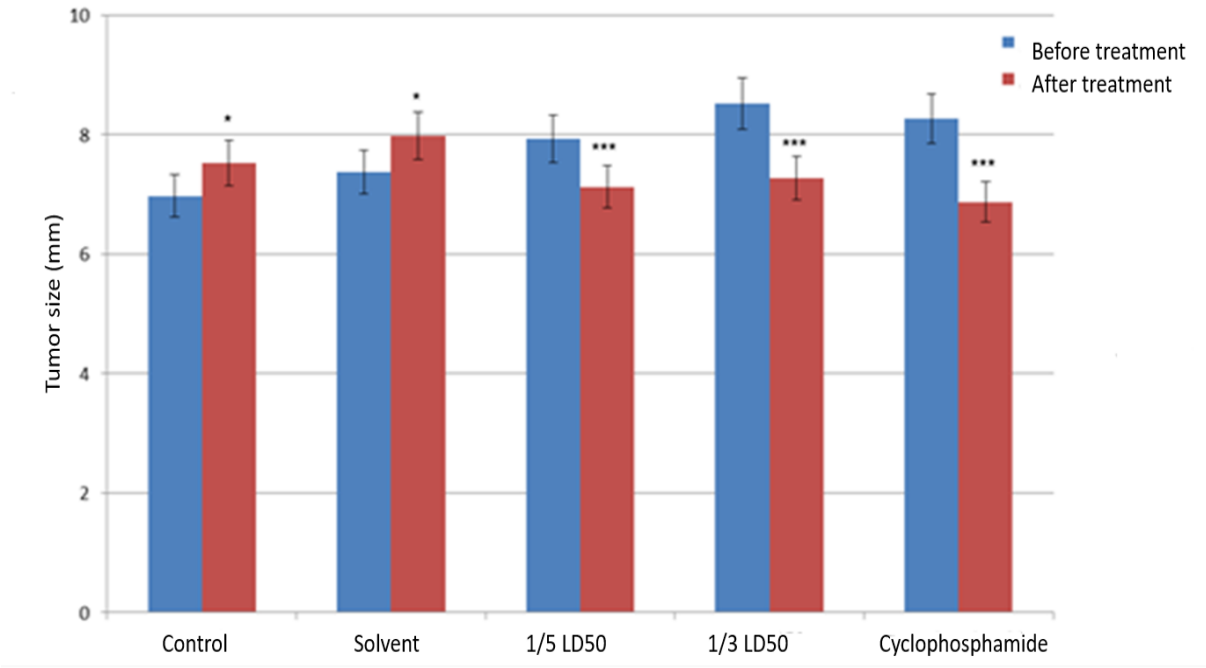

Figure 3. Size of the largest diameter of tumors. Largest diameter of tumors in different groups were compared before and after the treatment.

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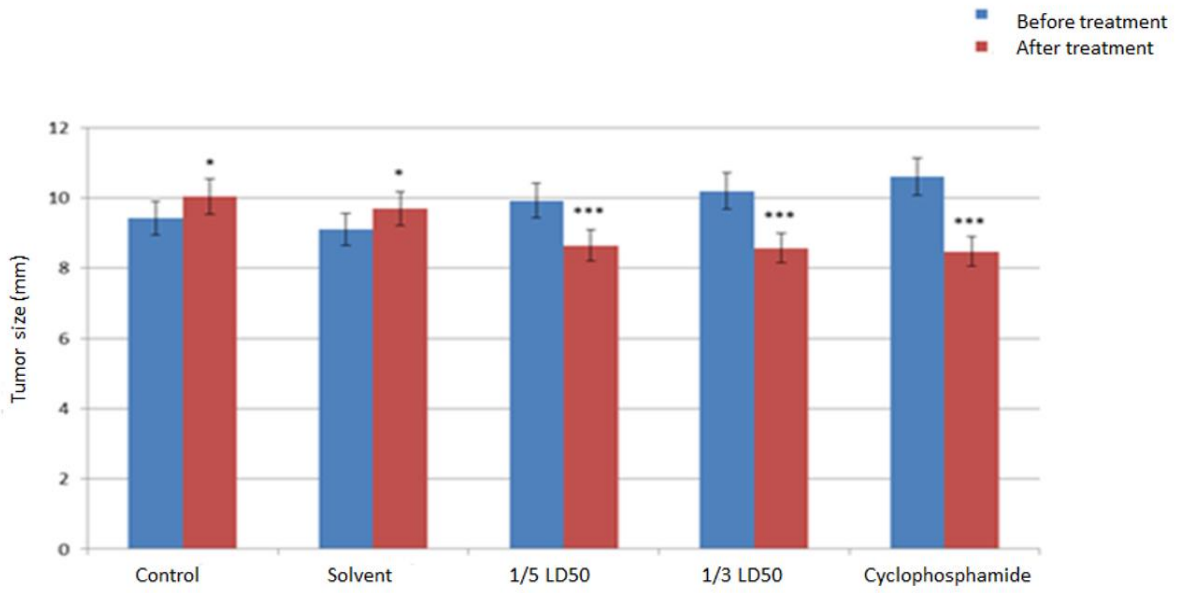

Figure 4. Size of the smallest diameter of tumors. Smallest diameter of tumors in different groups were compared before and after the treatment with triazene.

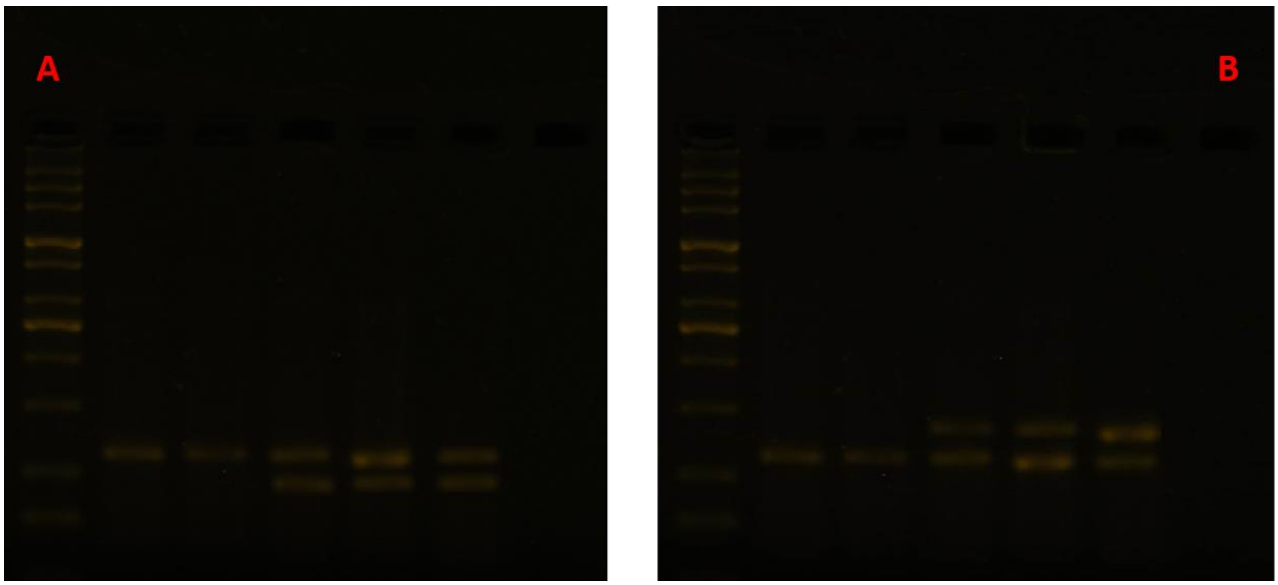

Figure 5. Expression of caspase genes. Caspase-3(A) and 9(B) both were expressed after treatment with triazene

Discussion

It has been previously shown that triazene compounds such as dacarbazine induce apoptosis on various cell lines. Dacarbazine is an alkylating and anti-tumor agent that is activated by cytochrome P450 (2). Our research showed that the newly-synthesized 1,3-bis (2-ethoxyphenyl) triazene reduced viability and induced apoptosis in 4T1 cell line in a concentration-dependent pattern.

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Complementary effect of 1,3 di-aryl triazene and N-acyl 1,3 bis-triazene were studied on several cancer cell lines. IC50 for HeLa, HEp-2, CA3ST, SW480, SW620, MIA PaCa2, and RT-112 were respectively reported 0.63, 0.62, 0.63, 0.53, 0.49, 0.52 and 2/7M. They also observed an increased expression in procaspase-3, 8, and 9 (16). As mentioned, Caspase-9 and 8 are initiatory caspases that trigger mitochondrial-mediated and extrinsic pathways. Our gene expression analysis by RT-PCR showed an increase in the expression of caspase-3 and 9 genes in tumor extracted from triazene-treated mice compared to extracted tissues from control groups, indicating the occurrence of apoptosis in these tissues. Another well-known anti-cancer triazene is Diminazene aceturate, which is a derivative of diaryl triazene and its ability to bind to DNA has been proven. It connects to the DNA through the formation of a complex within the small AT-rich groove in the double-stranded DNA helix structure (17). Diminazene aceturate exhibits cytotoxic effects on the leukemia cell line L1210, with an IC50 of 32 µm (18).

In another study, Matheson et al. examined the effects of new triazene compounds on the A431 cell line. They reported an IC50 of 36 and 59 µm for the two triazene combinations of SMA41 and SMA52 on the cell line, respectively. While IC50 for temozolomide was reported 366 µm on the same cell line (19).

Anti-tumor effects of dacarbazine with a protein called PTD4-apoptin were investigated on several cell lines of melanoma and on melanoma-induced C57BL / 6 mice. The the effects of dacarbazine on induced-melanoma tissues showed a significant decrease in tumor size compared to control group. They also confirmed the occurrence of apoptosis after treatment with dacarbazine (20). According to our findings, treatment of tumorized rats with 1/5 and 1/3 ratios of LD50 of 1,3-bis (2-ethoxyphenyl) triazene resulted in a significant decrease in tumor size before and after treatment. While in control group, the pre-treatment and post-treatment measurements indicated an increase in tumor size. Moreover, 1,3-bis (2-ethoxyphenyl) triazene significantly reduced the growth of *in vivo* induced-tumors by inducing cell death. In fact, 25 µm concentration of 1,3-bis (2-ethoxyphenyl) triazene induced 50% cell death after 24 h. In addition to experimental studies, clinical application of triazene compounds such as temozolomide as an anti-cancer drug showed significant results in patients with leukemia (21). Given that this compound is first synthesized, it is suggested to be examined in other invasive breast cancer cell lines. Also, in animal studies in addition to local tumor parameters, systemic features of this compound such as effect on the liver should be examined.

In conclusion, 1,3-bis (2-ethoxyphenyl) triazene has significant anti-tumor properties. Considering the toxicity and drug resistance related to this therapeutic compound, further studies should be conducted *in vivo* and *in vitro* to detect the thorough mechanism of triazene compounds on cells. As a whole, they could be considered as promising new drugs which can be used to treat a variety of cancers, especially those resistant to current chemotherapy.

Conflict of interest

The authors declare no competing financial interests.

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