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*Research Article*

# Repurposing of Triazole Drugs to Combat Common Cancer Cases

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ABSTRACT

Cancer with its prevalence and high mortality rates is an important problem that health authorities are trying to deal with all over the world. According to the data of the International Agency for Research on Cancer, 19.3 million new cancer cases occurred worldwide in 2020, and unfortunately, about 10 million of them resulted in death. The discovery of new drugs is compulsory due to restrictions such as the side effects of currently used anti-cancer drugs, drug resistance developing over time, and so on. Unfortunately, the development of novel drugs requires especially a long time and a huge amount of cost. At this point, the repurposing approach, which refers to the secondary use of various drugs that was approved by FDA for other purposes, has a vital importance. In this study, the anti-proliferative effects of itraconazaole and fluconazole (ICZ and FCZ), which are actually antifungal drugs, on breast (MCF-7 and MDA-MB-231) and lung (A549) cancer cells were evaluated. Results revealed that both of the drugs had a low inhibitory effect on A549 cell proliferation (inhibition rate was determined as 34.6% and 25.3% for 48 hours exposure to ICZ and FCZ, respectively), whereas they caused promising inhibition on MCF-7 (inhibition rate was determined as 44.1% and 38.3%, respectively for the same conditions) and MDA-MB-231 cell proliferation (Inhibition rate was determined as 35.4% and 43.3%, respectively again for the same conditions).

***Keywords:*** *Breast cancer, Fluconazole, Itraconazaole, Lung cancer, Repurposing.*

# Triazol İlaçlarının Yaygın Kanser Vakalarıyla Mücadelede Yeniden Kullanımı

ÖZET

Yaygınlığı ve yüksek ölüm oranları ile kanser, tüm dünyada sağlık otoritelerinin başa çıkmaya çalıştığı önemli bir sorundur. Uluslararası Kanser Araştırmaları Ajansı'nın verilerine göre 2020 yılında dünya genelinde 19.3 milyon yeni kanser vakası meydana gelmiş ve ne yazık ki, bunların yaklaşık 10 milyonu ölümle sonuçlanmıştır. Halihazırda kullanılan anti-kanser ilaçlarının yan etkileri, zamanla gelişen ilaç direnci vb. kısıtlamalar nedeniyle yeni ilaçların keşfi zorunludur. Ne yazık ki, yeni ilaçların geliştirilmesi özellikle uzun bir zaman ve büyük bir maliyet gerektirmektedir. Bu noktada FDA tarafından farklı amaçlarla onaylanan çeşitli ilaçların başka amaçlarla sekonder kullanımını ifade eden yeniden kullanım yaklaşımı hayati bir önem taşımaktadır. Bu çalışmada aslında antifungal ilaçlar olan itrakonazol ve flukonazolün (ICZ ve FCZ) meme (MCF-7 ve MDA-MB-231) ve akciğer (A549) kanser hücreleri üzerindeki antiproliferatif etkileri değerlendirilmiştir. Sonuçlar, her iki ilacın da A549 hücre proliferasyonu üzerinde düşük bir inhibitör etkiye sahip olduğunu (48 saat süreyle ICZ ve FCZ maruziyeti

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için inhibisyon oranı sırasıyla %34,6 ve %25,3 olarak belirlenmiştir), buna karşın MCF-7 (aynı koşullar için inhibisyon oranı sırasıyla %44,1 ve %38,3 olarak belirlenmiştir) ve MDA-MB-231 hücre proliferasyonu üzerinde umut verici bir inhibisyona neden olduklarını ortaya koymuştur (inhibisyon oranı yine aynı koşullar için sırasıyla %35,4 ve %43,3 olarak belirlenmiştir).

***Anahtar Kelimeler:*** *Meme kanseri, Flukonazol, Itrakonazol, Akciğer kanseri, Yeniden kullanım.*

# I. INTRODUCTION

Cancer is a principal health problem worldwide which cause high incidence of morbidity and mortality. According to annual analysis of American Cancer Society, approximately 3% of annual deaths in the world are associated with cancer disease [1]. It was declared that approximately 14 million newly diagnosed cancer cases occurred in the world in 2012 and with more than half of them resulted with death. In addition, with regret to say that it has been estimated the annual new cases will exceed 20 million by 2025 [2]. Despite such a serious picture and many studies have been conducted on related disease; unfortunately, it is not known exactly why this disease is caused. On the other hand, it is known that unrepaired DNA damage caused by various factors in cells is effective in the development of cancer [1,3]. Once cancer cells are formed, rapidly multiplied malignant cells take the control of the body. Hence, the primary target of chemotherapeutic drugs is to induce the programmed cell death in tumoral cells [3-5]. Not only the pharmacotherapy, but also the surgery and radiation therapy are used alone or in combination in order to deal with cancer cases [6]. The main challenges of using chemotherapeutic drugs in such cases are the resistance of cancer cells against the used drugs by time and the side effects caused from the relative similarities between malignant and healthy cells [4,5,7]. Since the use of selective drugs with minimum side effects is very crucial in terms of pharmacotherapy, there is a great need to develop effective and selective anticancer drugs. Repurposed drugs could be the promising alternative at this point. Actually, repurposing strategy refers to the secondary use of drugs besides their approved treatments [8-13]. Since drug development studies require very high costs and a long process, the repurposing strategy is very important for both to accelerate the process and reduce the cost. In addition to these advantages, this strategy provides the advantage of skipping the toxicity and safety test stages and doing directly clinical trials as a result of the use of currently used approved drugs in drug repositioning [8]. The expected value of €26.6 billion in global market for drug repurposing by 2020 also highlights the importance of related subject in the current pharmacological research area [6,14]. It has been well documented that many drugs with known pharmacology such as aspirin, itraconazole, verapamil and chloroquine that had not been produced for cancer therapy have exhibited at least one anticancer activity [6,12].

Itraconazaole (ICZ), an antifungal antibiotic, has an antitumor activity against various types of cancer such as acute myeloid leukemia [15], non-small cell lung cancer [16], breast cancer [17], recurrent prostate cancer [18,19], glioblastoma [20], gastric cancer [21], melanoma [22,23] and colon cancer [24]. The mechanism of action of itraconazole is mainly based on the inhibition of both angiogenesis and the Hedgehog signalling pathway, decreased Bcl-2 expression and apoptosis induced by increased caspase-3 activity [17,18,23,25]. Although there are not many studies on the anticancer activity of fluconazole (FCZ), a triazole antibiotic which is also in the same class with ICZ, it is generally used for the prevention of invasive fungal infections in immunocompromised individuals due to cancer treatment, HIV and similar reasons. Oude Lashof et al. (2004) compared the efficiency of ICZ and

FCZ in the prevention of oropharyngeal candidiasis infection and found that FCZ was more effective than ICZ [26].

Breast and lung cancers are the most common type of cancer in women and men, respectively [27]. In fact, breast cancer ranks second among all cancer cases in the world [6].

Although much advances have been made in the treatment of breast cancer especially in recent years and the chance of life is gradually increasing, death rates are still very high and it is the fifth among all cancer types. Both the existence of different types of breast cancer and the lack of valid treatment protocol for some types have a big share at high mortality rate. For instance, while estrogen-dependent breast cancer types respond to a high rate of treatment with hormone-specific drugs, these drugs are ineffective in triple-negative species [28-30]. Lung cancer, the most common cancer type in men, is the leading cause of cancer related deaths in the world. The American Cancer Society predicted that approximately 229,000 new lung cancer cases would occurred in the United States in 2020, and approximately 60% of these cases would resulted in death (As of 2021, it is not known whether it is the case). With this high mortality rate, lung cancer ranks first among all cancer types with a share of approximately 25% [31]. As is well known, the initial step in establishing potential novel anticancer drugs is to determine the cytotoxic effect of the relevant components on cancer cells. Therefore, the present study aims to investigate the cytotoxic effect of two triazole antifungal drugs named ICZ and FCZ on breast and lung cancer cell lines.

# II. MATERIALS and METHODS

## A. CELL CULTURE

Human breast adenocarcinoma (MCF-7 and MDA-MB-231), human lung carcinoma (A549) and human lung fibroblast (MRC-5) cell lines were obtained from American Type Culture Collection (ATCC; USA). While MCF-7, MDA-MB-231 and MRC-5 cell lines were grown in DMEM high-glucose medium, A549 cell lines were grown in DMEM-F12 medium. All the media used were supplemented with 20% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere containing 5% $CO_2$ at 37°C.

## B. MTT ASSAY

Cytotoxicity assessment of ICZ and FCZ on studied cell lines was performed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This method is based on the detection of cell proliferation of studied cells which are exposed or not exposed to potential inhibitor by the reduction of MTT into formazan dye by active mitochondria [32]. For the experimental design, firstly $5x10^3$ cells/well were seeded into well plates and incubated in above described culture conditions for 24 hours. Following this step, all the cells were exposed to various concentrations of ICZ and FCZ for 24 and 48 h and 5 mg/mL MTT solutions were added to each well at the end of incubation period. After the new 4 hours incubation with MTT solution, the MTT media were aspirated from all wells. The produced formazan crystals were dissolved in DMSO and the cell proliferation were subsequently detected by measuring absorbance at 570 nm. Cell viability was expressed as percentage survival in relation to control groups treated with 0.1% DMSO alone.

### C. STATISTICAL ANALYSIS

Graphpad prism 5.0 statistics sofware was used for statistical analysis (GraphPad, La Jolla, CA, USA). One-way ANOVA followed by Tukey's test was used to evaluate the experimental data. The error bars in the figures show the standard deviations (±SD).

## III. RESULTS and DISCUSSIONS

Due to the fact that the drugs that can be used in the fight against various types of cancer have vital importance in preventing the proliferation of related cells, the potential cytotoxic effects of two antifungal drugs, ICZ and FCZ, on breast and lung cancer cells were evaluated in the present study. For this purpose, both the breast and lung cancer cell lines were exposed to the drugs in the concentration range of 10-250 μM for 24 h and 48 h. The effects of used drugs on two different breast cancer cell lines, MCF-7 and MDA-MB-231, were presented in Figure 1 and 2, respectively.

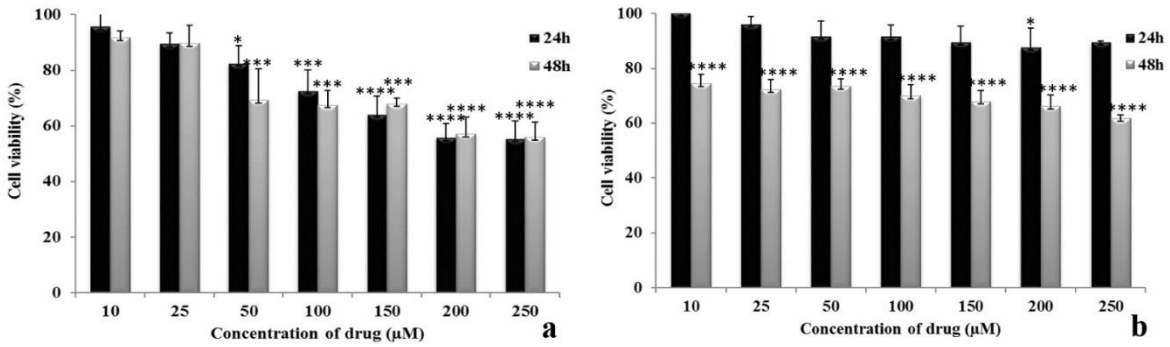


***Figure 1.*** *The effects of itraconazole (a) and fluconazole (b) on cell viability of human breast cancer cell line MCF-7. The percentages of viability were calculated compared with the control group. The results were presented as mean ± SD of three independent experiments. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001 indicate significant differences between control and other studied groups by Tukey's multiple range tests.*

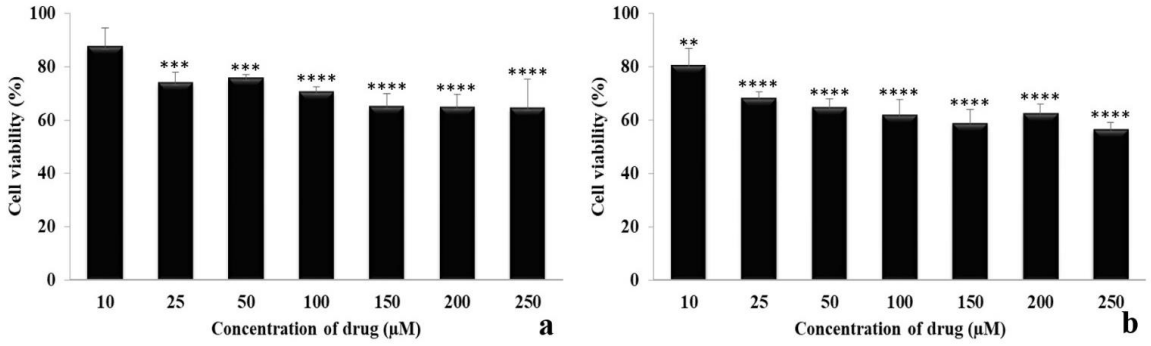


***Figure 2.*** *The effects of itraconazole (a) and fluconazole (b) on cell viability of human breast cancer cell line MDA-MB-231. The percentages of viability were calculated compared with the control group. The results were presented as mean ± SD of three independent experiments. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001 indicate significant differences between control and other studied groups by Tukey's multiple range tests.*

While ICZ did not show cytotoxic effects on MCF-7 cells at low concentrations for 24 h exposure, its effect increased with increasing concentration. However, no statistically significant difference was observed between the data obtained for concentrations of 150 µM and above in the experimental conditions ($p>0.05$). Similar to 24 h drug exposure, the effectiveness of ICZ increased with increasing concentration at 48 h exposure. However, the statistically significant difference could not be detected between the results obtained for 24 and 48 h treatment ($p>0.05$). When evaluated in terms of FCZ, it has been clearly seen that the cytotoxic effect of the drug on MCF-7 cells was negligible under 24 h exposure conditions. Although the efficacy of FCZ at low concentrations was higher than ICZ at 48 h of drug exposure, there was no statistically significant difference in the efficacy of drugs at high concentrations. This was the result of that no statistically significant change was observed in the efficacy of FCZ used in increasing concentrations. For all that, the cytotoxic effect of ICZ against MCF-7 cells was higher than FCZ under the condition of 24 h exposure with 100, 150, 200 and 250 µM of the drug ($p<0.01$, $p<0.001$, $p<0.0001$, $p<0.0001$, respectively).

Both antifungals were found non-toxic on MDA-MB-231 cell line for 24 h drug exposure, therefore the related data were discarded. As stated before, the triple-negative MDA-MB-231 strain is a much more aggressive cell type than MCF-7 cells, which could explain the ineffectiveness of both drugs applied for 24 h. In the case of 48 h exposure, the cytotoxicity of both drugs was statistically significantly different at all concentrations compared to the control group. ($p<0.05$). On the other hand, the results obtained at 25 µM and above for both drugs did not show a statistically significant difference from each other ($p>0.05$). Unfortunately, no difference was observed between the effectiveness of drugs on this aggressive type of breast cancer. Briefly, both drugs had moderate effects on the two different breast cancer cell lines in terms of proliferation inhibition. However, it should be emphasized that ICZ was found to be more effective than FCZ, especially on estrogen-dependent MCF-7 cells. Bae et al. (2018) investigated the effects of four main azole drugs (clotrimazole, ketoconazole, fluconazole and itraconazole) on cell proliferation, apoptosis, cell cycle, migration and invasion of MCF-7 and MDA-MB-231 cancer cells. They reported that all the imidazole drugs inhibited the proliferation of both cell lines. Despite that, only a little inhibition of proliferation was determined for ICZ in MDA-MB-231cells while it was almost negligible for FCZ in both cell lines. The authors also declared that both 50 µM ICZ and FCZ induced apoptosis only in MCF-7 cells [33]. Similar to Bae et al. (2018), Somchit et al. (2002) and Somchit et al. (2004) also

showed that ICZ induced a greater cytotoxicity than FCZ in rat hepatocytes *in vitro* and rat liver *in vivo*, respectively [33-35]. In another study evaluating the cytotoxic effect of ICZ on MCF-7 and SKBR-3 breast cancer cell lines, it was found that ICZ dramatically decreased cell viability depending on time and concentration. It was reported that ICZ inhibited cell growth by arresting cells in the G0/G1 phase. In addition, when MCF-7 and SKBR-3 cell lines were treated with ICZ, it was observed that both mitochondrial membrane potential changed and Bcl-2 protein expression decreased depending on the dose in both cell lines. Furthermore, caspase-3 activity was increased in the SKBR-3 cells. In the light of all these data, it was reported that apoptosis was induced as a result of ICZ treatment [17]. Correia et al. (2018) evaluated the effects of various combinations of verapamil or ICZ with the reference drug, 5-Fluorouracil, on the proliferation of MCF-7 cells in order to test that whether the combining drugs could be more effective in cancer treatment. The effects on non-tumoral cell line of both the individual and combining drugs were also investigated by using MCF-10A cell line. The authors emphasized that although both drug combinations used had promising effect in breast cancer treatment along with very little effect on MCF-10A cell line, the results pointed to ICZ, and particularly its combination with 5-Fluorouracil was the most effective one [6]. Santos Correa et al. (2018) investigated the cytotoxic effects of FCZ on African Green Monkey Kidney (Vero) cell line since the cytotoxicity studies are controversial. They found that 24 h exposure to 2612.1 µM FCZ reduced cell viability to 35% compared with the control. They also reported that FCZ induced necrosis in Vero cell line both for all concentrations used and tested harvest times as compared with the negative control [36]. Despite that, Rodriguez et al (1995) reported that FCZ had no significant effect on the cell viability in a primary culture system of rat hepatocytes for 0.5-6 h exposure to 25-200 µM of FCZ [37]. Similar to latter investigation, De Logu et al. (2005) stated that no significant change was observed in the viability of Vero cells exposed 3265 µM FCZ for 72 h [38].

In order to determine the potential of these drugs in the treatment of lung cancer cases, the cytotoxic effects of these drugs on A549 cell line were analysed and the results were presented in Figure 3.

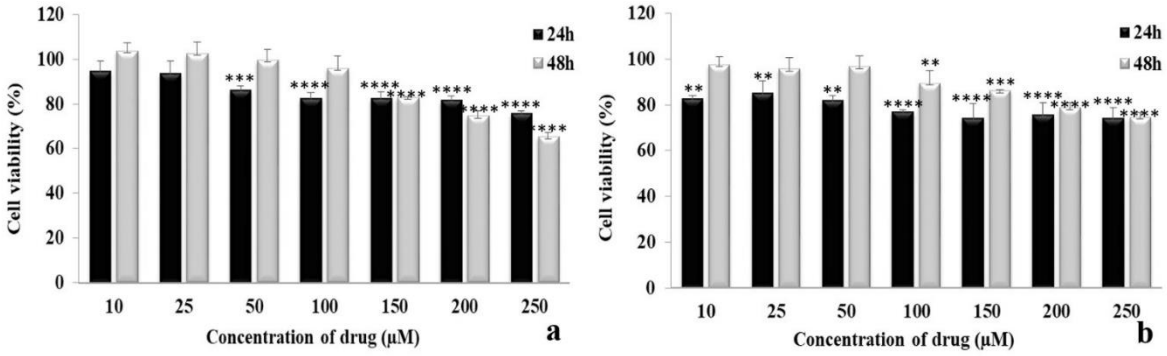


***Figure 3.*** *The effects of itraconazole (a) and fluconazole (b) on cell viability of human lung cancer cell line A549. The percentages of viability were calculated compared with the control group. The results were presented as mean ± SD of three independent experiments. *=p<0.05; **=p<0.01; ***=p<0.001; ****=p<0.0001 indicate significant differences between control and other studied groups by Tukey's multiple range tests.*

The cytotoxic effects of both drugs on the A549 cell line were very limited, and the maximum inhibition values for proliferation were determined as 34% and 25% for ICZ and FCZ, respectively. Interestingly, unlike the results obtained for breast cancer cell lines, the anti-proliferative effect of

drugs used on the A549 cell line generally decreased as the drug exposure time increased. When the effectiveness of the drugs was compared within each concentration value, the cytotoxic effect of 10 μM FCZ for 24 h drug exposure was significantly greater than 10 μM ICZ and the cytotoxic effect of 250 μM ICZ for 48 h drug exposure was statistically significantly greater than that of FCZ at the same concentration ($p<0.05$). Although we determined that the ICZ was not very effective in preventing the proliferation of A549 cells, it was shown to inhibit angiogenesis in non-small cell lung cancer and have antitumor activity in prostate cancer patients in some previous studies [18,39]. In fact, these results are compatible with the finding of approximately 68% reduction in the formation of new vessels observed as a result of ICZ treatment in matrigel pretreated mice, in other words, ICZ has the power to suppress angiogenesis *in vivo* [40]. Rudin et al. (2013) evaluated the potential of ICZ usage for the treatment of metastatic non-squamous non-small cell lung cancer in pre-clinical investigations. The knowledge that tumor-associated angiogenesis plays an important role in tumor growth and progression was the mainstay of the study hypothesis. The authors found that ICZ selectively inhibited endothelial cell proliferation with an $IC_{50}$ value of 0.16 M, despite that no inhibitory effect was determined in multiple nonendothelial controls ($IC_{50}$>100 M). They also found that ICZ inhibited endothelial cell proliferation such as vascular endothelial growth factor and fibroblast growth factor in a dose-dependent manner in pre-clinical studies. It was reported that oral administration of ICZ to animals with non-small cell lung cancer significantly inhibited tumor growth, similar to cisplatin, which is widely used for this purpose, and even more significant growth suppression was observed when these two drugs were administered together. The reduced tumor microvessel density, in other words, the antiangiogenic effect of the drug, probably plays an important role in suppressing tumor growth. Beside this, the potential anticancer activities of standard chemotherapy drugs pemetrexed and the combination of pemetrexed and ICZ were evaluated clinically in lung cancer patients. The results of the study clearly revealed that the overall survival time of patients who used the combination of pemetrexed and ICZ increased from 8 months to 32 months compared to the control group using only pemetrexed [41].

In summary, although both ICZ and FCZ are not very effective in preventing the proliferation of lung cancer cells, both drugs have a moderate cytotoxic effect on MDA-MB-231 and especially MCF-7 breast cancer cells. In addition to these studies, MRC-5 fibroblast cell line was used in order to determine the effect of used drugs on healthy cells. It is a great chance that the both of these drugs did not show any cytotoxic effects on MRC-5 fibroblast cells even after 48 h of exposure (data not shown). Therefore, ICZ might be used in the alternative treatment of breast cancer cases but further studies are needed to identify the mechanism of action.

# IV. CONCLUSION

The cytotoxicity of the azole compounds is mainly based on the inhibitory effects of these compunds on the cytochrome P450 enzyme system. Triazole compounds constitute an important group in repurposing studies since they are less toxic than imidazole compounds as a result of lower affinity for cytochrome P450 enzymes. In the light of aforementioned information, it is inevitable that triazole compounds are involved in repurposing studies for cancer treatment.

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