**In-vitro and in-silico analysis of the effect of fluconazole, an antifungal drug, on DNA**

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***Abstract:*** Fluconazole is an important antifungal drug used worldwide for the treatment of peritonitis and cryptococcal meningitis, urinary tract infections, esophageal tract infections and vaginal candidiasis. In this research, pUC19 plasmid DNA was treated with different concentrations of fluconazole in the presence of ascorbic acid, H2O2, iron, iron plus H2O2, copper and copper plus ascorbic acid, followed by agarose gel electrophoresis. Fluconazole-DNA interactions were investigated by UV-Vis spectrophotometeric titration and in-silico methods. Even in the presence of an oxidative agent and a reducing agent, the double stranded DNA was not broken with higher concentrations of fluconazole than those found in human plasma. Fluconazole concentrations ≥ 88 μM could protect 46 µM of DNA against radical hydroxyl produced by the reaction between 1.5 mM of FeSO4 and 6 mM of H2O2 while the drug concentrations ≤ 44 μM could not provide the protection. In addition, the drug could not protect DNA against the ROS originated from the reaction between copper and ascorbic acid. The binding constant of fluconazole-DNA in UV-Vis spectrophotometry analysis and docking analysis was estimated as 1.087×103 M-1 and 6.22×105 M-1, respectively.

***Keywords*:** DNA; fluconazole;antifungal; docking; interactions

RUNNING TITLE: Effect of fluconazole on DNA

**INTRODUCTION**

Fluconazole (FCZ), an antifungal agent, is used against a vast spectrum of pathogenic fungi such as [dermatophytosis](http://en.wikipedia.org/wiki/dermatophytosis), [histoplasmosis](http://en.wikipedia.org/wiki/histoplasmosis), [cryptococcosis](http://en.wikipedia.org/wiki/cryptococcosis), [coccidioidomycosis](http://en.wikipedia.org/wiki/coccidiodomycosis), [blastomycosis](http://en.wikipedia.org/wiki/blastomycosis), [candidiasis](http://en.wikipedia.org/wiki/candidiasis), *etc.*1 Fluconazole exerts its antifungal effect by inhibiting lanosterol 14α- demethylase enzyme (Erg11) which is responsible for the conversion of lanosterol to ergosterol, a very important part of the fungal plasma membrane. On the other hand, the lanosterol is potentially toxic sterol and accumulation of the sterol exert fungistatic activity.2

The study of drug-DNA interactions is very interesting because DNA (Deoxyribonucleic acid) is an important material in maintaining cell life and it is including all genetic information of cells. Interactions of drugs and chemicals with DNA and the resulting DNA damage are often associated with cancer.3 Binding of chemical substances to double-strand DNA can be by covalent, groove binding or intercalation way. Different binding modes of chemicals to DNA can expose different effects to cells, which depend on cell type and tissue.4 The minor groove of DNA is sensitive to the attacks of small drug molecules. Some of the minor groove bindings molecules (MGBs) damage DNA while some of them inhibit DNA dependent functions. Depending on the chemical structure of drugs, they show selectivity for some nucleophilic parts of DNA.5 Cooper and iron are important elements in the maintenance of chromosome structure.6 They are transition elements and show two oxidation states, oxidized states (Cu2+ and Fe3+) and reduced states (Cu+ and Fe2+). In a reduced state, they catalyze the generation of damaging reactive oxygen species (ROS) that have a toxic effect.7 There are many studies showing the correlation between iron intake and the risk of multiple cancers such as colorectal cancer, breast cancer, esophagus cancer and lung cancer.8 Also, there are several reports showing that some drugs, for instance chloramphenicol, can induce DNA breakage in the presence of iron and/or copper.9

In one research, fluconazole caused dose-dependent cytotoxicity in rat hepatocytes with high lactate dehydrogenase (LDH) as assessed by in-vitro test.10 Yuzbasioglu and his coworkers11 using in-vitro test showed that the 12.5, 25, and 50 µg/mL of fluconazole increased chromosomal aberrations (CA), sister chromatid exchange (SCE) and micronuclei frequency in human lymphocytes by dose-dependent manner while the drug was not clastogenic in in-vivo test. Correa and her coworkers12 reported that the 3, 81.6, 326.5 and 1306 µM of fluconazole increased micronuclei frequency and DNA damage index (as assessed by comet test) in African green monkey kidney (Vero) cell line. In the study conducted by Silva13, fluconazole concentrations ranging from 6 to 120 µg/mL caused the concentration-dependent increase in micronuclei frequency in cultured human peripheral blood mononuclear cells (PBMCs). Docking analysis is one of the mainstream in-silico methods that is used on a daily basis to predict different binding modes of proteins, DNA and different small molecules. Key interactions between docked molecules, binding site, binding conformation of molecules and estimated binding energy are some of the data provided by molecular docking analysis.14 Therefore, this study aimed to investigate the effect of fluconazole on DNA by treating supercoiled double-strand DNA with the drug in the presence and absence of reducing agent, oxidative agent, iron and copper. In addition in this study, the drug-DNA interaction was investigated by docking analysis and UV absorption titration method.

**MATERIAL AND METHODS**

*Reagents*

 In this research pUC19 plasmid DNA, previously purified from transformed *Escherichia coli* NEB5α strain in our lab, was used. 0.5 % of ethanol (100 %) was used to facilitate the dissolution of fluconazole. Fluconazole was purchased from Sigma-Aldrich (PHR1160-1G) and used as a test substance. The molecular weight of fluconazole (FCZ) was 306.27 g/mol and its chemical structure was as shown below:

 

Chemical structure of fluconazole

*Effect of fluconazole on DNA*

 To study the DNA-breaking effect of fluconazole, 46 µM bases of pUC19 plasmid DNA was treated with different concentrations of fluconazole (8800, 880, 88, 44 and 22 µM) at 37°C for 45 minutes. After incubation, the reaction mixtures were analyzed on 1 % agarose gel electrophoresis at 90 V for 55 minutes. The test was repeated in the presence of an oxidative agent (H2O2, 6 mM and 6 µM) and in the presence of a reducing agent (ascorbic acid, 88 µM). Untreated pUC19 plasmid DNA was also used as a control sample. All tests were performed three times in phosphate saline buffer (PBS) (pH 7.5).

*Effect of fluconazole on DNA in the presence of iron*

 The protective effect of fluconazole against hydroxyl radicals resulting from the reaction between FeSO4 and H2O2 was studied according to the method used by Ul-Haq and his coworkers.15 Briefly, 46 µM bases of pUC19 plasmid DNA was incubated with a solution including different concentrations of fluconazole (8800, 880, 88, 44 and 22 µM), 1.5 mM of FeSO4 and 6 mM of H2O2 at 37 ºC for 60 min. Sample incubated with FeSO4 (1.5 mM) plus H2O2 (6 mM) was used as positive control and untreated DNA was used as a negative control.

In order to analyze the effect of the drug on DNA in the presence of iron, the DNA was treated with the H2O2-free solution containing fluconazole (8800, 880, 88, 44 and 22 µM) and FeSO4 (1.5 mM). All tests were done in three repeats in PBS buffer (Phosphate Buffered Saline; pH 7.4).

*Effect of fluconazole on DNA in the presence of copper*

 To analyze the effect of fluconazole on copper-mediated DNA breaking, pUC19 plasmid DNA (46 mM bases) was treated with different concentrations of fluconazole (8800, 880, 88, 44 and 22 µM) and CuCl2 (60 µM) for 45 minutes at 37 ºC followed by analysis in agarose gel (1 %) electrophoresis. In addition, another test was conducted by treating the DNA with the solution containing different concentrations of fluconazole (8800, 880, 88, 44 and 22 µM) and CuCl2 (60 µM) plus ascorbic acid (60 µM). All tests were done in three repeats in PBS buffer (Phosphate Buffered Saline; pH 7.4).

*DNA binding analysis by UV absorption measurements*

 For this purpose Calf thymus DNA (ctDNA) and Fluconazole were dissolved at Tris-HCl buffer (50 mM Tris, pH 7.2). 0.5 % of ethanol (100 %) was used to facilitate the dissolution of fluconazole. The DNA solution was titrated by the fluconazole solution followed by recording their spectra at wavelengths between 220 nm and 320 nm using UV-Vis Spectrophotometer (Optizen 2120) at every 1 nm of wavelengths. The mixtures were incubated 5 minutes at room temperature before recording their spectra. Binding constant (*K*b) was calculated using the equation (1).

 (1)

 In this equation; *A*0 is the absorbance of DNA at 260 nm in the absence of ligand. *A*∞ is the final absorbance value of the saturated state of DNA and fluconazole. *A* is the absorbance value recorded at different fluconazole concentrations. *C*ligand belongs to fluconazole concentration.16

*Molecular docking analysis*

 The crystal structure of B-DNA (PDB ID: 1BNA) was downloaded from the Protein Data Bank (RCSB)17 in PDB format. The 3D structure of the drug was downloaded from the PubChem database18 in SDF format and converted to PDB format by Discovery Studio 3.0 software.19 Macromolecule (DNA) and ligand (drug) files in PDB format were prepared using AutoDock tools version 1.5.4. Molecular docking studies were calculated using Autodock Vina program.20 B-DNA was used as a rigid receptor molecule, whereas fluconazole was used as flexible ligand. The number of active torsions was set to 6. The receptor was enclosed in a script box which had 52x × 48y × 98z grid points and a grid spacing of 0.375 Å. The amount of independent docking runs wasset to 10. The lowest-energy docked conformer, considered to be the best result, is used to further analyze the molecular interactions of fluconazole with DNA. LIGPLOT (version .4.5.3) was used to plot the two dimensional (2D) schematics of the fluconazole – DNA interaction. 21

**RESULT**

*Effect of fluconazole concentrations on DNA*

 The supercoiled plasmid DNA treated with different drug concentrations (8800, 880, 88, 44, 22 μM) showed no changes in agarose gel electrophoresis compared to untreated DNA, even in the presence of ascorbic acid and H2O2 while in the presence of H2O2 plus FeSO4, the DNA was converted to linear form (Fig. 1).



Fig.1. Treatment of 46 µM bases of pUC19 plasmid DNA with fluconazole (FCZ), FCZ plus H2O2 and FCZ plus ascorbic acid (Asc). a, b, c, d and e represent 8800, 880, 88, 44 and 22 μM concentrations of FCZ, respectively. For H2O2, a and b showthe concentration of 6 mM and 6 µM, respectively and b\* represents H2O2 (6 µM) plus FeSO4 (1.5 mM). The concentration of ascorbic acid was 88 µM. Plus and minus signs represent the presence and absence of components, respectively. R, L, and S represent relaxed, linear and supercoiled form of the plasmid DNA, respectively.

*Effect of fluconazole on DNA in the presence of iron*

 When thepUC19 plasmid DNA was incubated with 8800, 880, 88, 44 and 22 µM of fluconazole plus 1.5 mM of FeSO4, no breakage of DNA occurred. Treatment of the supercoiled pUC19 plasmid DNA with FeSO4 (1.5 mM) plus 6 mM of H2O2 converted the supercoiled form of the DNA to a relaxed form and linear form because of breakages. Fluconazole concentration ≥ 88 μM could protect 46 µM of DNA against radical hydroxyl while the drug concentration ≤ 44 μM could not provide the protection (Fig. 2).

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Fig. 2: Effect of fluconazole (FCZ) on DNA in the presence of iron. pUC19 plasmid DNA was used as 46 µM. The different concentrations of fluconazole were shown as a, b, c, d and e that presented 8800, 880, 88, 44 and 22 μM, respectively. The concentrations of FeSO4 and H2O2 were 1.5 and 6 mM respectively. Plus and minus signs represent the presence and absence of components, respectively.

*Effect of fluconazole on DNA in the presence of copper*

 CuCl2 without a reductive agent did not break the supercoiled pUC19 plasmid DNA while it converted the DNA to both relaxed form and linear form in the presence of ascorbic acid as a reductive agent. Fluconazole did not induce CuCl2 to break the DNA and was unable to protect DNA from the breaking effect of CuCl2 in the presence of ascorbic acid (Fig. 3).

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Fig. 3. Effect of fluconazole (FCZ) on DNA in the presence of copper. pUC19 plasmid DNA was used as 46 µM base pairs. The concentrations of fluconazole were shown as a, b, c, d and e that represented 8800, 880, 88, 44 and 22 μM, respectively and the concentration of CuCl2 and Asc were as 60 µM. Plus and minus signs represent the presence and absence of components, respectively.

*DNA binding study of fluconazole by UV absorption titration*

 In the study conducted with the UV titration method, the absorption peak wavelength of DNA did not change while the absorbance value at 260 nm has been increased by a fluconazole concentration-dependent manner (Fig. 4A).. Fluconazole itself has an absorption peak at 261 nm, therefore the absorbance value related to fluconazole was subtracted from the obtained absorption values and observed that the absorption value of DNA was decreased by fluconazole concentration ranging from 0.000393 molars to 0.00157 molars followed by stopped the decrease in absorbance. The decrease indicated the interaction of the drug with DNA (Fig. 4B). The free binding energy and binding constant were calculated as -4.14 kcal/mol and 1.087× 103 M-1 respectively(Fig. 4C).







Fig. 4. A) Absorption spectra of ctDNA in the presence of different concentrations of fluconazole. B) Absorption of ctDNA at wavelengths of 260 nm. Initial solution (containing only 0.113 mM of ctDNA) showed 0.75 absorption value at 260 nm while the absorption value decreased to 0.502 by 0.00157, 0.00170, 0.00184 and 0.00198 molar of fluconazole at wavelengths of 260 nm. C) Plot of 1/ (*A-A*) versus 1/ Fluconazole concentration. Fluconazole and ctDNA solutions were prepared in Tris-HCl buffer (50 mM Tris, pH 7.2)

*DNA binding study of fluconazole by molecular docking method*

 Docking study showed that fluconazole (ligand) binds in the minor groove of double-helix DNA. One of the ligand conformation having lowest binding energy (-7.9 kcal/mol) made 2 hydrogen bonds including A: DG4: N2 - Flu: N7 and B:DG22: N2 - Flu: N7 with the bond length of 3.14 and 3.19 Å respectively (Fig. 5). In addition, the drug had hydrophobic bonds with the nucleotides including A: DA6 and B: BDC21.



Fig. 5. Molecular modeling of the key interactions between fluconazole and double-helix DNA. In the two-dimensional binding schematics, the bonds shown by dashed lines represent hydrogen bonds and the bonds shown by radius lines represent hydrophobic bonds. Nucleotides were shown by one letter code followed by the numbers showing their position in DNA strands, A or B within parenthesis identify DNA strands (A: CGCGAATTCGCG / B: CGCGAATTCGCG). In the three-dimensional representation of the interactions, fluconazole is shown by space fill (CPK) model in the minor groove of the double-strand DNA.

**DISCUSSION**

 In this research pUC19 plasmid DNA was used as DNA sample because the native conformation of pUC19 plasmid DNA is a supercoiled form. Any breakage in one of the double strands converts the supercoiled form to open circular (relaxed) form while breakage in the same position on both strands makes linear plasmid DNA. The migration of the linear form through agarose gel is slower than the supercoiled form and faster than the open circle form.22 Concentrations of fluconazole in human plasma have been reported as 26.9 mg/ml (87.8 µM).23 Human cells are exposed to ascorbate concentrations in the range of 10 µM to 70 µM.24 Although, regarding H2O2 concentrations in human plasma, there are various reports such as 100 mM and 5 μM.25,26 In this study, fluconazole, Asc and H2O2 concentrations were selected considering this information. Asc was used at high concentration (88 and 60 µM) found in human plasma and H2O2 was applied at two concentrations (6 µM and 6 mM) which can be considered as high concentration. In this study, even in the presence of high concentrations of an oxidative agent (H2O2, 6 µM and mM) and a reducing agent (Asc, 88 µM), the DNA was not broken by fluconazole concentrations higher than those in human plasma and higher than those used by the aforementioned investigators. These tests were not repeated in the presence of low concentrations of oxidative agent and reducing agent since high concentration of oxidative and reducing agent tests came negative. Human physiological plasma iron had been reported as 82 μM.27 Iron concentration in the human brain had been reported as 34 mM.28 Considering the mentioned information, in this study, the concentration of FeSO4 and H2O2 was selected as 1.5 afnd 6 mM as used by Li and her coworkers.29 Fluconazole concentration ≥ 88 μM could protect 46 µM of DNA against radical hydroxyl produced by the reaction between 1.5 mM of FeSO4 and 6 mM of H2O2 while the drug concentration ≤ 44 μM could not provide the protection. Therefore in our opinion, the protection was provided by binding and covering DNA by the drug. Most of the cellular copper was gathered in the nucleus and bound to DNA bases.30 The concentration of Copper (60 μM) used in this research was within the range (0.2 - 80 μM) of that in human cells.31 In the presence of ascorbate or other reducing agents, copper can lead to the production of ROS by catalyzing some reactions and damage DNA and chromatin.32 In this research, fluconazole did not induce copper to break DNA. In addition, fluconazole could not protect DNA against the ROS originated from the reaction between copper and ascorbic acid while it could protect DNA against radical hydroxyl originated from iron and H2O2. In our opinion, this result was because oxidation and DNA damaging effects of copper is 50 times faster than iron.32 In addition, copper ions bind strongly to DNA and disrupt the double-stranded structure33, thus adversely affecting drug binding to DNA. The findings of our research were not consistent with the findings of Peng and her coworkers34 who stated that fluconazole contributed to an increase of the DNA damage in vitro when complexed with iron or copper in the presence of hydrogen peroxide. In our opinion, the inconsistency was because they treated 0.1 pmol pBSSK plasmid DNA equal to 296400 µM bp of the plasmid with 50 µM of fluconazole. If they used the DNA concentration lower than fluconazole concentration, their results would be consistent with our results.

 UV-visible spectroscopy is the most common and convenient way to study the interaction between chemicals and double stranded DNA.35 The band at 260 nm of DNA arises due to the π-π\* transitions of DNA bases. “Hyperchromic” effect and “hypochromic” effect are the spectra features of DNA due to its double helical structure. The spectral change process reflects the corresponding changes in DNA in its conformation and structures after the drug bound to DNA. Hypochromism results from the contraction of DNA in the helix axis, as well as from the change in conformation on DNA; in contrast, hyperchromism derives from damage to the DNA double helix structure.36 In this research, by the addition of fluconazole, the absorbance value of the ct-DNA solution decreased at 260 nm and showed a hypochromic effect due to contraction of the double-stranded ct-DNA. Similarly, molecular docking analysis show that fluconazole can bind into DNA minor groove. A chemical substance can increase or decrease gene expression by binding to DNA and inhibit binding of proteins involved in transcription regulation.38 In line with our results, before it was reported that fluconazole concentrations caused to increase TNF-a, and decrease IL-6 and IL-10 expressions in PBMCs.13 Drug binding causes structural and conformational changes in the DNA such as DNA bending, winding double or single-strand breaks, resulting in DNA damage.39 Therefore the DNA-breaking effects (e.g. CA, SCE, and micronucleus) reported by researchers can be due to the binding of fluconazole to DNA.

**CONCLUSION**

 In conclusion, fluconazole did not break double helix DNA even in the presence of H2O2 and ascorbic acid. According to the result of the spectrophotometric and docking analysis, the drug strongly binds to double-stranded DNA. In addition, fluconazole protects DNA against radical hydroxyl originated from iron and H2O2 when its concentration is above DNA concentration.

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**CONFLICT OF INTEREST**

 The authors don’t have any conflict of interest.

И З В О Д

IN-VITRO И IN-SILICO АНАЛИЗА ЕФЕКТА ФУКОНАЗОЛА, АНТИФУНГАЛНОГ ЛЕКА, НА ДНК

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Фуконазол је важан антифунгални лек који се широко користи за лечење перитонитиса, криптококалног менингитиса, инфекција уринарног тракта и езофагуса, као и за вагиналну кандидијазу. У овом раду је ДНК плазмида pUC19 третирана различитим концентрацијама фуконазола у присуству аскорбинске киселине, H2O2, гвожђа, комбинације гвожђа и H2O2, бакра и бакра у присуству аскорбинске киселине, након чега је следила агарозна електрофореза. Фуконазол-ДНК интеракције су праћене UV-Vis спектрофотометријском титрацијом и in-silico методама. Двоструки ланац ДНК се није кидао више него у хуманој плазми ни у присуству оксидујућих и редукујућих агенаса у комбинацији са већим концентрацијама фуконазола. Фуконазол у концентрацији ≥ 88 μM је могао заштитити 46 µM ДНК од хидроксил радикала насталог у реакцији између 1,5 mM FeSO4 и 6 mM H2O2, док ту заштиту није могао пружити у концентрацији ≤ 44 μM. Лек, није могао заштитити ДНК од реактивних кисеоничних једињења насталих у реакцији бакра и аскорбинске киселине. Константа везивања фуконазол-ДНК, одређена UV-Vis спектрометријски и докинг анализом, је процењена на 1,087×103 M-1, односно 6,22×105 M-1.

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