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COMPARISON OF CYTOPROTECTIVE ANALYSIS OF AZOLES TO HEPG2 CELLS

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ABSTRACT

Human liver cell line is considered the most suitable toxicology as well pharmaceutical experiments for drugs development. HepG2 cells and primary hepatocytes have proven useful in in-vitro studies. HepG2 cells as liver cells are used in this study, some azoles have proliferative capability on liver cells proliferation activity of different azoles observed in this study. This study has shown proliferation activity of fluconazole, omeprazole, voriconazole and itraconazole out of all itraconazole showed more proliferation of liver cells compared with untreated cells as well rest of three treated group estimated via MTT and antioxidants also act as antiapoptotic agents to reduce apoptosiswith antioxidant after treatment cells showed enhanced oxidative level proliferation was estimated via VEGF ELISA and apoptosis via p53 ELISA.

Keywords: Liver cell line, azoles (fluconazole, omeprazole, voriconazole and itraconazole), angiogenesis, anti-apoptosis and proliferation

INTRODUCTION

Human liver cell line is considered the most suitable toxicology as well pharmaceutical experiments for drugs development. In in-vitro studies, HepG2 cells and primary hepatocytes have proven useful. Compare to primary hepatocytes, HepG2 cells are easy to handle and have higher proliferation capacity than primary

hepatocytes **[1]**. Voriconazole is a secondary triazole antifungal drug **[2-4]** and is derivative of fluconazole **[5]** with its improved activity against invasive aspergillosis (a serious fungal infection caused in the lungs of immunocompromised patients) and is also used in the treatment of other dangerous fungal infections **[2, 6-8]**. Omeprazole is a member of heterocyclic aromatic organic compounds (benzimidiazoles) **[9]** also known as proton pump inhibitor, is commonly used as effective treatment for all upper gastrointestinal ailments such as Gastroesophageal reflux disease (GERD) as well as in peptic ulcers **[10, 11]**. Omeprazole is also effective to eradicate *H. pylori* infection, in combination with some antibiotics **[12]**. Omeprazole as a PPL also recommended as a first line pharmacotherapy for dyspepsia **[13]**. Fluconazole, was introduced 40 years ago and still a first-lineantifungal agent for the treatment and prophylaxis of various fungal problems such as invasivecandidiasis in children and infants **[14-16]**. Fluconazole lacks major side effects, and has broad efficacy against most pathogenic yeasts, including *C. albicans*due to this fluconazole is also available in oral and intravenous formulations and has no adverse effects in the production of steroid hormones **[17-20]**. Itraconazole belongs to azole family and is approved by Food and Drug Administration **[21]**. It was introduced in 1992 at clinical level. Since then, it is used as antifungal therapy. Itraconazole is currently available in capsule as well as oral solution complex, but it can also be administered intravenously with cyclodextrin. It has 55% absorption rate (less than fluconazole) in capsule form and ~80% in oral solution form. . Drug may persist in tissues for longer times and is highly metobolized in liver. 35% is released with urine as metabolites and 54% is excreted in feces. However, metabolism is somewhat reduced in liver impaired patients **[22]**. A global anti-fungal surveillance study was conducted from 1997 to 2007 which showed increasing resistant concentrations of azoles **[23, 24]**. Furthermore, a small study conducted in liver transplant patients facing invasive fungal infection of *C. parasilosis* were found resistant to fluconazole. A number of studies have confirmed that even in commensal yeast microfloras of both immunocompromised and immunocompetent people resistance against fluconazole can be acquired. In pathogenic yeast *C.albicans* obtained from HIV's hosts, genetic clusters of fluconazole resistance were identified. This resistance could persist and has the ability to spread clonally in humans. Other than this subtitutional changes between amino acids can also cause resistance of fluconazole

such as Isolate S2 was shown to have a G-to-S amino acid substitution at position 464 (G464S) in Upc2p, resulting in the upregulation of *ERG11* and increased fluconazole resistance **[25, 26]**. Synergistic drug interactions were rationalized by global genetic interaction.The synergistic combinations were active against in vivo model of Cryptococcus infection and fluconazole-tolerant clinical isolates **[17, 27]**. The combined effect of fluconazole with doxycycline and tetracycline is effective against resistance strain of *C. albicans* athigher doses **[28]** doxycycline works in a way that it converts the effect of fluconazole in fungicide manners to prevent the rise of drug resistance again in future **[28]**.

MATERIALS AND METHODS

HepG2 Cell Line Sampling:

HepG2 cell line was obtained from cell and tissue culhe stem cell's laboratory of The University of Lahore. The cell lines were kept in liquid nitrogen and then resuscitate from cryo vials when there is need of culturing.

Culturing of HepG2 Cell Lines

HepG2 cells were cultured in Cell & Tissue Culture Lab, Centre for Research in Molecular Medicine (Crimm), University of Lahore. Cells were cultured in T75 flasks in Dulbecco's Modified Eagle's Medium (DMEM) (high glucose) (Caisson's Lab, USA), supplemented with 100 µg/ml streptomycin (Caisson's Lab, USA), 100 units/ml penicillin (Caisson's Lab, USA) and 10% fetal bovine serum (Sigma Aldrich, USA) in a humidified incubator at 5% CO_2 and 37°C temperature. Medium was replaced every 2-3 days.

Treatment Of Hepg2 Cells With Different Doses Of Fluconazole, Omeprazole, Voriconazole And Itraconazole

HepG2 cells as an alterantive of hepatocytes were cultured onto 96-well plate for measurement of cell viability, cell proliferation apoptosis and for evaluation of antioxidants. Divide into five groups, One group was un-treated (UT). Remaining four groups were named as, HepG2 cells treated with Fluconazole (F), HepG2 cells treated with Omeprazole (O), HepG2 cells treated with Voriconazole (V), and fifth group was as Itraconazole (I), in case of MTT with dose range of 5mg/ml-200mg/ml while 100mg/ml was observed as best dose in case of azoles all were showing more proliferation compared with others. While the cultured medium that is harvested from these post treated groups also called as secretome was used for ELISA of VEGF and p53 and antioxidants.

MTT Assay

Cell viability was analyzed through MTT assay according to manufacturer's protocol (Sigma Aldrich, USA). Assay was run in triplicate for each experimental group. For the evaluation of the

proliferative potential of HepG2 cells after treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA) assay was performed using a 96-well plate. A monolayer of cells was washed with phosphate buffer saline (PBS) and incubated in 100µl serum free DMEM medium and 25 µl MTT solution (5mg/ml) for 2 hours. The purple color formazan crystals appeared were then solubilized with 10% sodium dodecyl sulfate (SDS) and absorbance was taken at 570 nm. Percentage viability was calculated by previous method, mentioned by Irshad *et al*., **[29]**.

% Cell viability = Experimental (OD570)/Control (OD570) × 100

Enzyme Linked Immuno Sorbant Assay (ELISA)

Solid phase sandwich ELISA was performed for VEGF (Santa Cruz Biotechnology, USA) in a 96-well plate (Corning, USA). Capture antibody i.e. rabbit polyclonal anti VEGF antibody (Santa Cruz biotechnology, USA) was diluted in a coating buffer to a concentration of 2-10 µg/ml. 100 µl of this diluted antibody was then transferred to each well of the plate and incubated for 120 minutes. After the incubation the capture antibody was discarded from each well and then washed. Each well was washed three times with 1X TBS-T (washing solution) for five minutes. The solution was removed by flicking the plate over a sink. Then, 200µl of blocking solution (BSA) was added in each well of the plate and incubated for 30 minutes. Following incubation, blocking solution was removed. Add 200µl of culturing medium harvested from different experimental groups to each well and after 30 minutes medium was removed and each well was washed three times for 5 minutes. After washing, 100µl of secondary antibody HRP conjugated donkey anti rabbit secondary antibody (Santa Cruz biotechnology, USA) was added to each well and the plate was incubated for 60 minutes. After incubation, secondary antibody was removed and each well was washed three times for 5 minutes. For HRP detection, Tetramethylbenzidine (TMB) (Invitrogen Inc., USA) is the most popular chromogenic substrate. 100µl of TMB were added (chromogenic substrate) to each well then the plate was incubated for 20 minutes. After incubation, 100µl of stop solution 0.18 M sulphuric acid (H_2SO_4) were added to stop the TMB reaction, absorbance was taken at 450 nm by using the microtiter plate.

Antioxidant Estimation

Antioxidant assays (GSH & SOD) were analyzed to check oxidative stress after treating cultured cells in 6-well plates. Glutathione Reductase (GSH) assay was performed in a 96-well plate with a reaction

mixture of 200 µl in each well. A reaction mixture was preparedby mixing 20 mM KH_2PO_4 buffer (pH 7.5), 40 mM EDTA, and 10 mM oxidized glutathione. Medium obtained from different experimental groups were added in the reaction mixture. At the end, 20 mM NADPH was added and absorbance was taken using spectrophotometer. Its absorbance was taken at 340 nm and was plotted in the graph. Superoxide dismutase (SOD) activity was also estimated by method of **[30]** in 96-well plate. Reaction mixture was prepared for this assay in which secretome of different experimental groups of post treatment on BMSCs and HepG2 cells were mixed with 100mM KH2PO4 buffer (pH 7.8), 0.1mM EDTA, 13mM methionine, 2.25mM nitro-blue tetrazolium chloride (NBT), 60µM riboflavin. Its Optical density was measured at 560 nm by spectrophotometer.

RESULTS

Increased Proliferative Activity of Azoles

HepG2 cell as an alternative for Hepatocytes were used and treated with the all azoles at doses 5-200 mg/ml. cells were analyzed by using MTT assay which is most reliable assay for analysis of cell viability, cytotoxicity or cytoprotectivity.

Figure 1 showing cytoprotective values plot of absorbance, **Table 1** showing the standard error of mean.

Figure 1, Proliferation rates of HepG2 cells after treating with Fluconazole, Omeprazole, Voriconazole and Itraconazole between untreated or treated groups where HepG2 cells are treated with azoles at different amount of doses where **Figure 2** showing apoptosis values plot of absorbance, **Table 2** showing the standard error of mean.

Enzyme Linked Immuno Sorbent Assay (ELISA) by Azoles for Liver Cells

Levels of Apoptosis in Treated Cells by Azoles via ELISA

ELISA was performed for evaluation of the apoptosis level by using p53 antibody. As p53 is the principal factors for apoptosis, HepG2 cell lines when treated with azoles showed low levels of apoptosis as compared to pre-treated cells **Table 2** showing the and **Figure 2** below.

Assessment of Angiogenesis in Treated HepG2 Cells with Azoles via ELISA

After treating HepG2 cells with azoles, comparatively high level of angiogenesis was seen via ELISA. So, according to this study treatment of cell line by azoles increase the level of angiogenesis. As VEGF is the major factor of angiogenesis, its level was increased in treated cells when treated with azoles as compared to untreated cells as shown in the **Table 3** and **Figure 3**.

Evaluation of Antioxidative Enzymes by Azoles

Glutathione Reductase (GSH) Estimation by Azoles

After treating cells with azoles the post cultured medium (secrotome) collected was analyzed for GSH activity separately. GSH activity was increased in treated groups compared with untreated group which indicates that azoles showing low levels oxidative stress after the treatment shown in the **Table 4** and **Figure 4**.

Superoxide Dismutase (SOD) Estimation by Azoles

After treating cells with azoles and the medium collected from a treatment was analyzed for SOD activity separately. SOD activity was decreased in the presence of azoles indicating low levels of reactive oxidants species and stress after the treatment as shown in the **Table 5** and **Figure 5** below.

Table 1: The Cell Cytoprotective Values of Untreated and Treated HEPG2 Cells

Azoles	Untreated	5 mg/ml	50 mg/ml	100 mg/ml	200 mg/ml
Fluconazole	1.243 ±0.02309	1.510 ±0.04000	2.667 ±0.4041	2.940 ±0.06083	2.650 ±0.5462
Omeprazole	1.243 ±0.02309	1.543 ±0.04163	2.743 ±0.05508	2.873 ±0.01528	2.940 ±0.03000
Voriconazole	1.243 ±0.02309	1.287 ±0.2517	1.433 ±0.02517	2.127 ±0.04509	2.227 ±0.04726
Itraconazole	1.243 ±0.02309	1.437 ±0.02887	2.683 ±0.05508	3.827 ±0.1387	3.757 ±0.1222

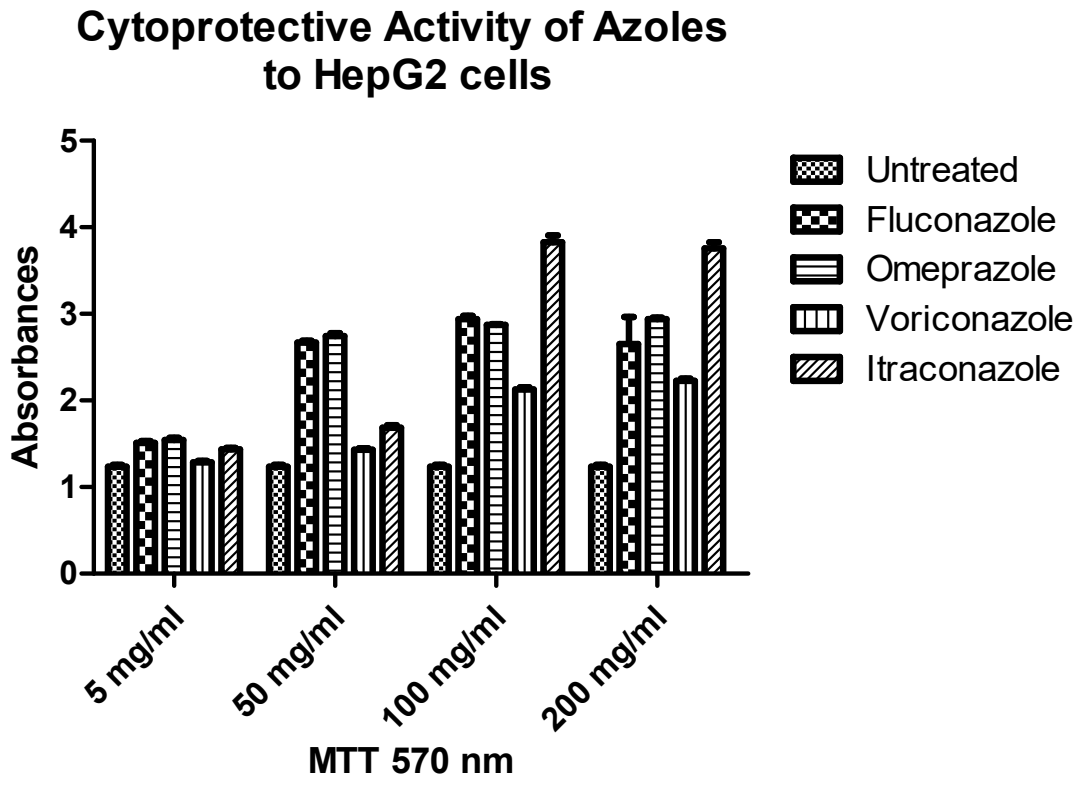

Figure 1: Proliferation rates of HepG2 cells after treating with Fluconazole, Omeprazole, Voriconazole and Itraconazole between untreated or treated groups where HepG2 cells

Table 2: apoptosis values of untreated and treated hepg2 cells

Groups	Values(±sem)
Untreated	0.2483±0.002517
Treated with Fluconazole (100 mg/ml)	0.1599±0.001550
Treated with Omeprazole (100 mg/ml)	0.1077±0.002517
Treated with Voriconazole (100 mg/ml)	0.1483±0.003512
Treated with Itraconazole (100 mg/ml)	0.09057±0.0006028

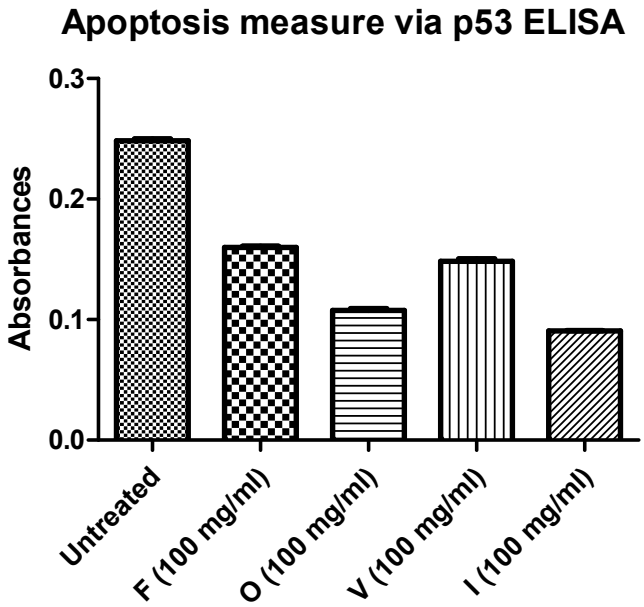

Figure 2: Where F is HepG2 cells treated with fluconazole O is HepG2 cells treated with omeprazole V is HepG2 cells treated with voriconazole and I is HepG2 cells treated with itraconazole with secrotome of 100mg/ml

Table 3: Angiogenesis Values of Untreated and Treated HEPG2 Cells

Groups & Doses	Values(±SEM)
Untreated	0.2333±0.003512
Treated with Fluconazole (100 mg/ml)	0.2643±0.004163
Treated with Omeprazoel (100 mg/ml)	0.2357±0.003055
Treated with Voriconazole (100 mg/ml)	0.2887±0.003215
Treated with Itraconazole (100 mg/ml)	0.3113±0.01206

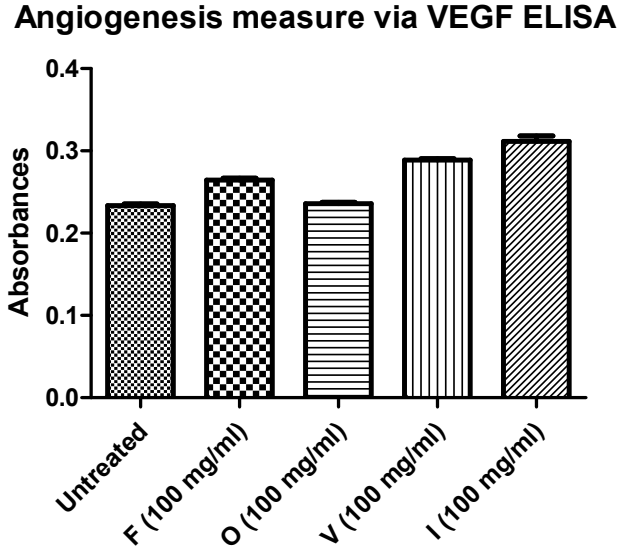

Figure 3: Where F is HepG2 cells treated with fluconazole, O is HepG2 cells treated with omeprazole, V is HepG2 cells treated with voriconazole, and I is HepG2 cells treated with itraconazole with secrotome of 100mg/ml

Table 4: Antioxidant GSH Values of Untreated and Treated HEPG2 Cells

Groups & Doses	Values(±SEM)
Untreated	0.2313±0.003215
Treated with Fluconazole (100 mg/ml)	3.397±0.03215
Treated with Omeprazoel (100 mg/ml)	1.063±0.01528
Treated with Voriconazole (100 mg/ml)	0.3633±0.03402
Treated with Itraconazole (100 mg/ml)	3.953±0.05508

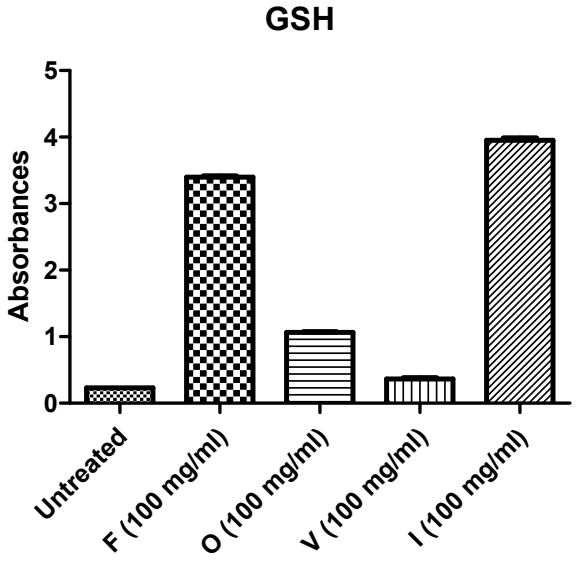

Figure 4: Where F is HepG2 cells treated with fluconazole O is HepG2 cells treated with omeprazole V is HepG2 cells treated with voriconazole and I is HepG2 cells treated with itraconazole with secrotome of 100mg/ml where itraconazole showing more GSH release as compared with untreated and other three groups

Table 5: Antioxidant SOD Values of Untreated and Treated HEPG2 Cells

Groups & Doses	Values(±SEM)
Untreated	0.3180±0.02696
Treated with Fluconazole (100 mg/ml)	0.2283±0.02108
Treated with Omeprazoel (100 mg/ml)	0.3887±0.007095
Treated with Voriconazole (100 mg/ml)	0.2919±0.002324
Treated with Itraconazole (100 mg/ml)	0.1977±0.002517

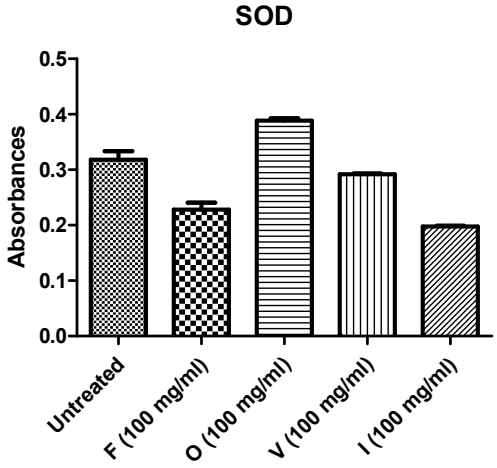

Figure 5: Where F is HepG2 cells treated with fluconazole O is HepG2 cells treated with omeprazole V is HepG2 cells treated with voriconazole and I is HepG2 cells treated with itraconazole with secrotome of 100mg/ml where itraconazole showing less release of SOD as compared with untreated and other three groups

DISCUSSION

Outcome of this study shows azoles especially itraconazole is effective as having Proliferative properties of liver cell lines. Hence can give rise to cancer. Therefore, it cannot be given to patients with cancer. Azoles are famous for their different therapeutic activities all over the globe. In the current study, four azoles have been evaluated for their cytoprotective capability. All azoles displayed proliferative effect in liver cells as evidenced bydifferent assays which were performed to check their effect for 24 hours treatment.MTT assay was performed to evaluate cytoprotective activity of azoles at different doses. More proliferation was observed at 100 mg/ml dose in case of all four azoles. These azoles showed reduced cell death in MTT assay and can act as hepatoprotective agent in liver problems such as many compounds have been reported **[31]**. Sandwich ELISA was performed to evaluate apoptosis and angiogenesis, apoptosis is the cell death characterized by increased level of p53 and angiogenesis level is measured by vascular endothelial growth factor (VEFG) in different studies **[32]**. Cells make VEGF to initiate blood vessel formation or stimulate further formation on existing vessels **[33]**. p53 antibody was used to evaluate cellular apoptosis while less apoptosis was observed with treated groups as compared with untreated group wand ELISA with VEGF antibody was performed to estimate angiogenesis level more level of VEGF release was observed in treated groups compared with untreated group which indicates there is more angiogenesis and less apoptosis it was more better in case of itraconazole group.ELISA results showed low level of p53 and high level of VEGF in treated cells as compared to untreated group.Hence, all azoles have the potential to increase angiogenic activity of treated cells. Among all azoles, itraconazole displayed least apoptosis and maximum angiogenesis at same dose.Presence of free harmful radicals in body showed damage at cellular level during oxidative stress. To combat these radicals, antioxidants are considered the first line defence **[34-40]**. In this study, glutathione reductase and superoxide dismutase enzymes were used to observe the oxidative stress in cells. Both reduced the level of oxidative stress.

CONCLUSION

All the assays evaluated, have shown that these azoles thatwere tested, have proliferative effects on liver cell whichindicate that these drugs can be useful for liver cellproliferation or injury protection in future.

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