Fluconazole, a Fungal Cytochrome P450 Enzyme Inhibitor: The Potential Role in Augmenting Hepatotoxicity and Hyperinsulinemia Induced by Dexamethasone in Rats

Hanan Mohamed AL-Shafeiy1*, Bassant M. M. Ibrahim1, Marwa M. Safar2,3, Siham M. A. El-Shenawy1, Nemat A. Z. Yassin1, and Sanaa A. Kenawy2
1Pharmacology Department, National Research Center, 12622, Giza, Egypt.
2Pharmacology Department, Faculty of Pharmacy, Cairo University, 11562, Cairo, Egypt.
3Department of Pharmacology & Biochemistry, the British University in Egypt, Cairo, Egypt.

INSULIN resistance (IR) is a widespread health problem characterized by failure of cell responses to insulin. IR increases the risk of fungal infections. Long-term administration of dexamethasone is a therapeutic need accompanied IR and fungal infections. Fluconazole is the first-line antifungal therapy that inhibit fungal cytochrome P450 (CYP450) enzyme. It has a hepatotoxic effect and moderate inhibitory effect on multiple CYP450 enzymes that could affect the pharmacokinetics of drugs used to treat insulin resistance such as metformin. This study aims to evaluate the effect of administration of fluconazole on liver function, CYP450 concentration and level of insulin resistance induced by dexamethasone and high fat diet. Insulin resistance in rats was induced by dexamethasone (0.1mg/kg/day s.c.) (DEX) for 2 weeks, after eight weeks of high fat diet (HFD) until the end of the experiment. Oral administration of fluconazole (20, 40 mg/kg) (FLU20, 40) was started concomitantly with DEX for 2 weeks. Metformin (400mg/kg) (MET) was used as a reference treatment for IR and administered orally for 2 weeks with DEX and FLU (20, 40). Concomitant administration of FLU either 20mg/kg or 40 mg/kg with DEX showed significant increase in IR, AST and ALT compared by that caused by DEX alone or with MET. FLU showed a significant decrease in serum CYP450 that elevated by DEX. Massive fibrosis was noticed in histological assessments of liver tissues of HFD+DEX+FLU (20, 40) groups. In conclusion administration of FLU in case of IR induced by HFD+DEX resulted in deterioration of liver functions and decrease in the therapeutic efficacy of MET.

Keywords: Dexamethasone, Fluconazole, Insulin Resistance, Metformin, Cytochrome P450.
Dexamethasone increases the risk of fungal infections by modulating of T-cells proliferation [9]. DEX stimulates the activity of some CYP450 isoforms and this affect the pharmacokinetics of drugs used in treatment of diabetes and IR such as metformin [10,11].

Fluconazole is a first generation triazole antifungal medication and it is considered as the first-line antifungal therapy in many cases of immune compromised people including transplant patients [12]. It acts by inhibition of the fungal CYP450 enzyme 14a-demethylase and therefore prevents the conversion of lanosterol to ergosterol, which is the essential component of the fungal cytoplasmic membrane. Fluconazole is routinely prescribed to control vulvovaginal candidiasis, and cutaneous candidiasis in diabetic patients [13,14]. Despite fluconazole is considered as a safe antifungal drug due to its selective inhibition of fungal CYP45, its hepatotoxic effect and inhibitory effect on some mammalian CYP450 isoforms, the proteins which able to catalyze various enzymatic reactions including the metabolism of xenobiotics, were also recorded as a drawback of fluconazole[15-17].

Studies that investigated the concurrent administration of DEX and FLU were more concerned about their pharmacokinetics and their antifungal effect against resistant Candida albicans [18,19].

From previous literatures, it is concluded that combined administration of fluconazole and dexamethasone induces many side effects. This study aims to evaluate the effect of fluconazole on serum CYP450, hepatotoxicity and insulin resistance that induced by dexamethasone in rats.

Material and Methods

Animals

Adult male albino Wistar rats, weighing 200-230 g, were used. They were obtained from the animal house colony of the National Research Centre (Dokki, Giza, Egypt) and were housed in plastic cages covered with wood shavings flooring. Animals were kept for at least one week in the laboratory room prior to testing under standard housing conditions (room temperature 25°C with alternating 12 hours light and dark cycles) and were allowed free access to water and food ad libitum. They were fed a standard rat pellet diet for normal group and fat-rich diet for other rats. All animals procedures were performed in accordance to the Institutional Ethics Committee of the National Research Centre (PTNo.12127).

Drugs

Dexamethasone: (Dexamethasone®) sodium phosphate ampoules were purchased from Amriyafor Pharmaceutical Industries, Alexandria-Egypt.

Fluconazole: (Diflucan®): purchased from Pfizer Company.

Metformin: (Cidophage®): purchased from Cid Company

Chemicals and test reagent kits

Rat insulin ELISA kit was obtained from Glory Science Co., Ltd.

Rat Cytochrome P450 1A2 (CYP 1A2) ELISA kit was obtained from Cloud-Clone Corp.

Rat aminotransferase (AST) colorimetric kit was obtained from Biodiagnostic Co.

Rat alanine amino transferase (ALT) colorimetric kit was obtained from Biodiagnostic Co.

Experimental design and treatment protocol

Animals were allowed free access to HFD ad libitum for 8 weeks except for normal rats. All groups except the normal control and the high fat diet (HFD) control groups were injected dexamethasone (0.1mg/kg/day) (DEX) for two weeks starting after 8 weeks of HFD application or regimen. Dose, duration and route of administration of DEX to induce insulin resistance were chosen after pilot studies and according to Okumura et al., and Caro and Amatruda[19,20]. HFD were prepared without fructose according to method described by Basciano et al.,[21]. All treatments were administered for two weeks after eight weeks of administration of HFD that continued to the end of experiment. Metformin (MET) was selected as a reference drug used to treat IR; it was administered orally in a dose of 400mg/kg/day [22]. Doses of fluconazole (20,40mg/kg/day) (FLU 20, 40) were selected and administered orally in accordance with Fisher et al., [23].

Sixty four rats were randomly assigned into eight groups; as follows:

Normal control, HFD control group, (HFD+DEX) group, (HFD+DEX+MET) group, (HFD+DEX+FLU20) group, (HFD+DEX+FLU4) group, (HFD+DEX+FLU40) group, (HFD+DEX+FLU4) group, (HFD+DEX+FLU40) group.
group, (HFD+ DEX+ FLU20+MET) group and
(HFD+DEX+FLU40+MET) group.

Glucose tolerance test (GTT)
Glucose tolerance test (GTT) was recorded at
the end of the experiment on overnight fasted rats
that injected with glucose i.p. Blood samples were
collected before injection of glucose and fasting
blood glucose (FBG) were measured. Blood drops
were collected from the tails of rats to measure blood
glucose 30, 60, 90 and 120 min after injection using
Ok meter® glucose testing strips to be analyzed by
the commercially available glucometer (OK meter,
Biotech Co., Ltd., Taiwan) [24].

Blood biochemical parameters
Blood samples were withdrawn via the
retro-orbital plexus of all rats at the end of the
experiment. Rats were fasted for at least 12 hr
before blood collections. Blood samples were
centrifuged at 3000 rpm. Serum was separated for
measurement of insulin.

Determination of insulin resistance
Insulin resistance (IR) was calculated using
homeostasis model assessment of (HOMA- IR)
[25].
IR= fasting blood glucose (FBG) mmol/l X
serum insulin (μIU/ml) /22.5

Histological assessment studies
Liver samples of all animals were dissected
immediately after death. The specimens were then
fixed in 10 % neutral-buffered formalin saline for
72 hours at least. All the specimens were washed
in tap water for half an hour and then dehydrated
in ascending grades of alcohol, cleared in xylene
and embedded in paraffin. Serial sections of 6μm
thick were cut and stained with haematoxylin and
eosin for histological investigation[26]. Images
were examined and photographed under a digital
camera (Microscope Digital Camera DP70,
Tokyo, and processed using Adobe Photoshop
version 8.0).

Statistical analysis
Data were expressed as mean ± SE of the mean
of 8 experiment. Statistical analysis were carried
out using one-way ANOVA, followed by Tukey’s
multiple comparison tests atp<0.5.

Results
Results of glucose tolerance test (GTT) for
all groups
Blood glucose (mg/dl) level was increased in
dexamethasone treated groups in comparison with
normal and HFD control groups. There
was a significant elevation in GTT in the HDF+
DEX treated group by (18%, 144% and 196% at
30, 60 and 120 min respectively), (12%, 102% and
114% at 30,60 and 120 min respectively) for HDF+
DEX+ MET treated group,(41%,360% and
291% at 30, 60 and 120 min respectively) for
HDF+DEX+ FLU20, (19%, 268% and 288% at30,60
and 120 min respectively) for HDF+DEX+
FLU40, (19%, 268% and 288% at30,60
and 120 min respectively) for HDF+DEX+
FLU20, (26%, 300% and 309% at 30,
60 and 120 min respectively) for HDF+DEX+
FLU20, (36%, 367%and316% at 30,
60 and 120 min respectively) for HDF+DEX+
FLU20, (41%, 360% and 309% at
30, 60 and 120 min respectively) for HDF+DEX+
FLU40 at the end of 10’ week in comparison with
normal and control groups as shown in Fig. 1.

Results of insulin resistance and serum AST,
ALT and CYP450

HDF+DEX treated group
Rats that treated with HDF+DEX showed
a significant increase in serum insulin, HOMA-
IR, AST, ALT and CYP450 by approximately
100%, 119%, 44%, 140% and 104% respectively
in comparison with normal and control groups as
shown Fig. 2, 3, 4, 5 and 6, respectively.

HDF+DEX+MET treated group
Rats that treated with HDF+DEX+MET
showed a significant increase in serum insulin,
HOMA-IR, AST, ALT and CYP450 by approximately
30%, 39%, 45% and 44% respectively in
comparison with normal and control groups but
they were significantly less than the findings
in the rats that treated with only HDF+DEX as
shown Fig. 2, 3, 4, 5 and 6 respectively.

HDF+DEX+ FLU 20 treated group
Rats that treated with HDF+DEX+FLU20
showed a significant increase in serum insulin,
HOMA-IR, AST, ALT and CYP450 by approximately
90%, 141%, 296%, 455% and 47% respectively in
comparison with normal and control groups. Results of ALT and AST were
significantly higher than those of groups that
treated with HDF+DEX or HDF+DEX+MET.
The concentration level of CYP450 was lowered
significantly if compared with HDF+DEX group
as shown Fig. 2, 3, 4, 5 and 6 respectively.

HDF+DEX+ FLU 40 treated group
Rats that treated with HDF+DEX+FLU40
showed a significant increase in serum insulin,
HOMA-IR, AST, ALT and CYP450 by approximately
130%, 197%, 322%, 487% and 61% respectively in
comparison with normal and control groups. Results of insulin, ALT and AST
were significantly higher than those of groups that

Insulin resistance in rats was induced by subcutaneous injection of dexamethasone for two weeks, after eight weeks of feeding freely them HFD and continued until the end of the experiment.

Data were expressed as mean ± SEM (n = 8). The statistical comparison of difference between groups was carried out using one-way ANOVA followed by Tukey's multiple comparison tests.

At p< 0.05

@ Significantly different from normal group
*Significantly different from HFD control group
# Significantly different from DEX group
$ Significantly different from MET group
Insulin resistance in rats was induced by subcutaneous injection of dexamethasone for two weeks, after eight weeks of feeding freely them HFD continued until the end of the experiment.

Data are expressed as mean (n = 8). The statistical comparison of difference between groups was carried out using one-way ANOVA followed by Tukey's multiple comparison tests. At p< 0.05

@ Significantly different from normal group
*Significantly different from HFD control group
# Significantly different from DEX group
$ Significantly different from MET group

Fig. 3. Effect of high fat diet, dexamethasone (0.1mg/kg), metformin (400mg/kg) and fluconazole (20, 40 mg/kg) on insulin resistance using homeostasis model assessment (HOMA-IR).

Fig. 4. Effect of high fat diet, dexamethasone (0.1mg/kg), metformin (400mg/kg) and fluconazole (20, 40 mg/kg) on serum liver aspartate aminotransferase (AST) (U/L).

@ Significantly different from normal group
*Significantly different from HFD control group
# Significantly different from DEX group
$ Significantly different from MET group

Fig. 5. Effect of high fat diet, dexamethasone (0.1mg/kg), metformin (400mg/kg) and fluconazole (20, 40 mg/kg) on serum liver alanine transferase (ALT) (U/L).

Insulin resistance in rats was induced by subcutaneous injection of dexamethasone for two weeks, after eight weeks of feeding freely them HFD continued until the end of the experiment.

Data are expressed as mean ± SEM (n = 8). The statistical comparison of difference between groups was carried out using one-way ANOVA followed by Tukey’s multiple comparison tests.

At p< 0.05

@ Significantly different from normal group
*Significantly different from HFD control group
# Significantly different from DEX group
$ Significantly different from MET group

Fig. 6. Effect of high fat diet, dexamethasone (0.1mg/kg), metformin (400mg/kg) and fluconazole (20, 40 mg/kg) on serum Cytochrom p450 (cyp 1A2).

Insulin resistance in rats was induced by subcutaneous injection of dexamethasone for two weeks, after eight weeks of feeding freely them HFD continued until the end of the experiment.

Data are expressed as mean ± SEM (n = 8). The statistical comparison of difference between groups was carried out using one-way ANOVA followed by Tukey’s multiple comparison tests.

At p< 0.05

@ Significantly different from normal group
*Significantly different from HFD control group
# Significantly different from DEX group
$ Significantly different from MET group

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treated with HDF+DEX or HFD+DEX+MET. Concentration of CYP450 was lowered pronouncedly if compared with HDF+DEX group as shown Figs. 2, 3, 4, 5 and 6, respectively.

**HDF+DEX+ FLU 20+MET treated group**

Rats that treated with HDF+DEX+ FLU20+MET showed a significant increase in serum insulin, HOMA-IR, AST, ALT by approximately 75%, 114%, 306%, 472% respectively in comparison with normal and control groups. Results of ALT and AST were significantly higher than those of groups that treated with HDF+DEX or HFD+DEX+MET. Serum insulin was decreased significantly when compared with HDF+DEX group. Concentration of CYP450 gave insignificant difference in comparison with normal and control groups as shown Fig. 2, 3, 4, 5 and 6 respectively.

**HDF+DEX+ FLU 40+MET treated group**

Rats that treated with HDF+DEX+FLU40+MET showed a significant increase in serum insulin, HOMA-IR, AST, ALT by approximately 85%, 129%, 303%, 460% respectively in comparison with normal and control groups. Results of ALT and AST were significantly higher than those of groups that treated with HDF+DEX or HFD+DEX+MET. Serum insulin was decreased significantly when compared with HDF+DEX group. Concentration of CYP450 gave insignificant difference in comparison with normal and control groups as shown Fig. 2, 3, 4, 5 and 6 respectively.

**Histological assessment**

A photomicrograph of a liver tissue section from normal control rats showed normal hepatocytes arranged in cords radiating from the central vein as shown in Fig. 7a.

A photomicrograph of a liver tissue section from HDF control rats showed normal hepatocytes with large vesicular nuclei but with noticeable blood sinusoids’ dilatation as shown 7b.

A photomicrograph of a liver tissue section from rats treated with HDF+DEX showed severe dilatation and congestion of main blood vessels as shown in Fig. 7c.

A photomicrograph of a liver tissue section from rats treated with HDF+DEX+MET showed normal hepatocytes with no dilated blood sinusoids in between, but with mild thickening of central vein’s wall and fibrosis around as shown in Fig. 7d.

A photomicrograph of a liver tissue section from HDF+DEX+FLU groups showed massive fibrosis and markedly dilated central vein with vacuolar degeneration (and pyknotic nuclei as shown in Fig. 7e.

A photomicrograph of a liver tissue section from rats treated with HDF+DEXMET+FLU (20mg and 40mg) showed many hepatocytes with vascular degeneration (black arrow). mild dilatation and congestion of blood sinusoids as shown in Fig. 7f.

**Discussion**

In the present study normal diet was replaced by HFD was used instead to potentiate the effect of dexamethasone as an inducer of insulin resistance in rats. It was reported by Stojanovska et al., that administration of HFD and dexamethasone resulted in higher insulin resistance in rats compared to those treated with dexamethasone alone [22]. This finding could be correlated to the role of HFD in the stimulating of the expression of several proinflammatory cytokines and inflammatory responsive proteins in hypothalamus and this leads to impairment of the insulin- signaling pathway as described by Prada et al., [23].

The current data revealed that dexamethasone administration resulted in a significant increase in insulin resistance when compared with normal and the HFD control groups. These results were in accordance with Stojanovska et al., and Okumura et al., and Buren et al.,[5,19,27]. This could be explained by the ability of dexamethasone to prevent glucose uptake by reducing the net translocation of the glucose transporter GLUT4 as pointed in Raymond R. Russell et al.,[28]. Administration of metformin to the rats resulted in a significant decrease in insulin resistance and showed obvious protective effect for liver tissues when compared with the groups that treated with HDF+DEX+FLU (20, 40). These findings were in line with those of Thomas et al., and Li et al., [29,30]. These results could be explained by the stimulating effect of metformin for AMP-activated protein kinase (AMPK) activity in both liver and skeletal muscle and by its downstream effect on acetyl-CoA carboxylase (ACC) [31,32].

In the present study, concomitant administration of fluconazole with dexamethasone caused further more increase in insulin resistance when compared with the effect of dexamethasone alone. These results may be explained by the negative impact of fluconazole on liver functions that noticed in this study as altered liver hyperinsulinemia could be resulted due to hepatic parenchymal cell damage or due to portal-systemic...
Fig. 7. A photomicrograph of a liver tissue sections from rats in all groups (Hx & EX 200): (a); section from a normal rats showed normal hepatocytes arranged in cords radiating from the central vein, (b); section from a control HFD rats showed normal hepatocytes with large vesicular nuclei but with noticeable blood sinusoids’ dilatation, (c); section from a rats treated with HDF + DEX showed severe dilatation and congestion of main blood vessels on the left side of figure, while the right side of figure shows mild dilatation of blood sinusoids (black arrow) and some hepatocytes with pyknotic nuclei, (d); section from a rats treated with HDF + DEX + MET showed normal hepatocytes with no dilated blood sinusoids in between, but with mild thickening of central vein’s wall and fibrosis around (e); section from HDF + DEX + FLU groups showed on the left side of the figure massive fibrosis (arrow heads) around the markedly dilated central vein. On the right side of figure hepatocytes with vacuolar degeneration (green arrow) and pyknotic nuclei (black arrow) were observed. A photomicrograph of a liver tissue section from HDF + DEX + FLU groups showed on the left side of the figure massive fibrosis (arrow heads) around the markedly dilated central vein. On the right side of figure hepatocytes with vacuolar degeneration (green arrow) and pyknotic nuclei (black arrow) were observed, (f); section from rats treated with HDF + DEX + MET + FLU showed many hepatocytes with vacuolar degeneration (black arrow), mild dilatation and congestion of blood sinusoids (arrow head) is also noticed.
shunting [33,34]. These essentials were confirmed by changani et al., who stated that increase of blood glucose is due to gluconeogenesis [34].

In the current study the histological examination of liver tissues of HDF + DEX + FLU (20, 40) groups showed a massive fibrosis and degeneration of vacuolar of hepatocytes. These Findings agreed with what reported in Rodriguez and Acosta, and Khoza et al., about hepatotoxicity resulted from administration of fluconazole [35,36].

Serum levels of ALT and AST were elevated in a very pronounced way in HDF+DEX+FLU (20,40) groups in comparison with all other groups. Their levels were also increased significantly in HDF+DEX treated rats but still less than those results of groups that administered concomitantly with fluconazole. These results were in agreement with the work of Jackson et al., [31]. Studies showed that elevated levels of ALT and AST are considered as strong markers of insulin resistance where hyperinsulinemia leads to increase ALT and AST [37,38].

In the present study levels of serum Cytochrome P450 1A2 (CYP1A2) were elevated significantly in rats treated with HDF+DEX. Groups that treated with HDF + DEX + MET and HDF + DEX + FLU (20, 40) showed significant decrease in the concentration of serum CYP1A2 when compared with HDF + DEX treated rats. This decrease in the level of CYP1A2 may be attributed to the inhibitory effect of fluconazole on mammalian’s CYP450 despite that fluconazol is more selective inhibitor of fungal CYP450 in comparison with other azoles [15,39]. These results could also be due to the increase of the levels of serum insulin in HDF+DEX+FLU (20, 40) groups, as insulin has role in down regulating CYP450 expression [40]. In the present study groups which received metformin with fluconazole HDF + DEX + MET + FLU (20, 40) showed serum level of CYP1A2 returned approximately to the normal level after its pronounced elevation caused by dexamethasone alone in HDF+DEX group. These findings were in line with other studies reported that metformin has a role in down regulation of certain isoforms of CYP450 expression [41-43].

**Conclusion**

Since fluconazole is considered as the first-line treatment in many fungal infections and the incidence of these infections is positively correlated with insulin resistance and with the impact of dexamethasone on the immune system, hence the combined administration of fluconazole and dexamethasone is very likely to happen in clinical practice. Therefore the current study revealed that fluconazole in both doses (20 and 40) mg/kg augmented the hepatotoxicity and insulin resistance effects that caused by dexamethasone. Thus, it is concluded that dose adjustment and monitoring are required in case of concomitant administration of fluconazole with dexamethasone for long duration of time.

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الفلوكونازول، مثبط إنزيم السيتوكروم P450 للفطريات: الدور المحتمل في زيادة سمية الكبد ومقاومة الأنسولين المحدثة بالديكساميثازون في الجرذان

حنان محمد الشافعي 1، نعمة يس 1، سهام الشناوي 2، مروة صفر 1، بسنت محمد ابراهيم 1
قسم الفارماكولوجي - المركز القومي للبحوث - الجيزة - مصر.
قسم الفارماكولوجي و الكيمياء الحيوية - الجامعة البريطانية بالقاهرة - القاهرة - مصر.
قسم الفارماكولوجي بالكلية للصيدلة - جامعة القاهرة - القاهرة - مصر.

 مقاومة الأنسولين تعتبر مشكلة صحية واسعة الانتشار تظهر في عدم استجابة الخلايا للأنسولين وذلك يؤدي في النهاية إلى زيادة معدل السكر بالدم وأيضاً يزيد نسبة حدوث الالتهابات الفطرية. أستخدام الديكساميثازون لفترات طويلة يعتبر دواء هام للكثير من الحالات المرضية ولكنه بدوره يؤدي إلى حدوث مقاومة للأنسولين و حدوث الالتهابات الفطرية.

 يعتبر الفلوكونازول من أقوى العقاقير المستخدمة لعلاج الفطريات ويعمل عن طريق تثبيط إنزيم السيتوكروم P450 البشري ولكن له تأثير سلبي على وظائف الكبد كما أنه يرتبط بشكل جزئي بارتفاع مستويات السيتوكروم P450 في الكبد، الذي قد يثير الفطر الالتهاب المحتوم. ونتيجة لذلك، تم استخدام هذه المركبات للوقاية من الالتهابات الفطرية.

تهدف هذه الدراسة إلى تقييم تأثيرات تناول الفلوكونازول على وظائف الكبد ومستوى السيتوكروم P450 ومقاومة الأنسولين المحتمة تجريبياً في الجرذان بإعطاء الديكساميثازون مع إعطاء نظام غذائي عالي الدهون.

تم إعطاء الجرذان غذاء عالي الدهون لمدة ثمانية أسابيع ثم تم إحداث مقاومة الأنسولين للجرذان عن طريق حقن ديكساميثازون تحت الجلد (0.1 مجم / كجم) لمدة أسبوعين و استمر الغذاء مع الديكساميثازون حتى نهاية التجربة (عشر أسابيع).

تم إعطاء الفلوكونازول عن طريق الفم (0.2 و 0.4 مجم / كجم) بالإضافة إلى الديكساميثازون لمدة أسبوعين.

كما تم إعطاء الميتفورمين (علاج مقاومة الأنسولين) عبر الفم أيضاً لمدة أسبوعين بالتزامن مع ديكسميثازون بالإضافة إلى الفلوكونازول بجرعة (0.04 مجم / كجم).

مجمعت الجرذان التي تم أعطاؤها الفلوكونازول بجرعة 0.4 مجم / كجم بالإضافة إلى الديكساميثازون أظهرت زيادة واضحة في مقاومة الأنسولين و ارتفاع مستويات إنزيمات السكريات بالتساوي مع إعطاء الديكساميثازون. كما أظهرت نفس المجموعات انخفاضاً طفيفاً في مستويات الفلوكونازول و ذلك مع إعطاء الميتفورمين مضخداً والذي أدى بدوره لارتفاع إنزيمات السيتوكروم P450 ارتفاعاً واضحاً بالمقارنة مع الجرذان الطبيعي.

كما أظهرت الدراسة الهيستوپاثولوجية حدوث تليف في أنسجة الكبد في الجرذان التي تناولت الفلوكونازول بجرعة 0.4 مجم / كجم، بجرعات تزامناً مع الديكساميثازون.

نتائج هذا البحث التي تتطلب أن تابع مقاومة الفلوكونازول للجرذان هو ما يشعر بذلك الجرذان معتاداً. نموذج الفلوكونازول مع الدهون على الديكساميثازون، يدل على زيادة في مقاومة الأنسولين و مستويات إنزيمات الكبد، واضطراب في مستويات السيتوكروم P450 مما أدى إلى تقليل الفعالية العلاجية للمتقاومين.