IN VITRO HEPATOTOXICITY EVALUATION OF ATYPICAL ANTIPSCYHOTICS

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Abstract - Liver is the most important target of drugs so that it is very important to determine the hepatotoxicity. HepG2 cells, which are well characterized human hepatoma cells, are the most commonly preferred model for hepatotoxicity evaluation in vitro. It is known that Quetiapine, Olanzapine, Risperidone and Aripiprazole treatment, which are administrated as the top prescribed medications all over the world, has rarely been associated with hepatotoxicity, however some cases of acute liver injury secondary to the use of these antipsychotics were described. Therefore, in this study, we aimed to evaluate hepatotoxicity associated with atypical antipsychotics by using HepG2, human hepatocarcinoma cell line, via cytotoxicity testing and since it is now known that apoptosis in the liver plays a role in the hepatotoxicity of chemicals, flow cytometry was used to detect apoptosis. According to our results, Aripiprazole was found to be the most hepatotoxic drug among the others, and all of the drugs tested were shown to induce cell death by triggering apoptosis.

Index Terms - Aripiprazole, in vitro hepatotoxicity testing, flow cytometry, HepG2 cells, olanzapine, risperidone, quetiapine.

I. INTRODUCTION

Drug induced liver injury is the major problem limiting drug discovery process and leading to drug withdrawals. Therefore in vitro hepatotoxicity screening gains importance in early drug discovery process. HepG2, which are well characterized human hepatoma cells, are the most commonly preferred model for hepatotoxicity evaluation in vitro. They have advantages like immortality, stability and easy to use phenotype. They are widely used in safety screening of novel drugs with single or multiple points such as cell viability and the functional metabolic states [1, 2, 3]. Quetiapine (QET) is an atypical antipsychotic drug that is frequently used for the treatment of schizophrenia. It is one of the most prescribed drugs and there is a remarkable increase in the use of quetiapine among other antipsychotic drugs [4, 5]. Olanzapine (OLA) and risperidone (RIS), the other most commonly used atypical antipsychotics, also have been shown to be effective and safe. Aripiprazole (ARI) is a novel drug which have a different pharmacological profile than the other antipsychotics. It is a partial agonist of serotonergic 5-HT_{1A}, 5-HT_{2A} and dopaminergic D_2 receptors and it has been shown to be the one of the mostly prescribed psychiatric drugs and generally well tolerated [6]. However testicular alterations, haemotoxicity, hepatoxicity, cardiotoxicity may be observed with antipsychotic treatment [7, 8]. Although, it is known that QET, OA, RIS and ARI treatment has rarely been associated with hepatotoxicity, cases of acute liver injury secondary to the use of these antipsychotics were described [9, 10, 11].

In other studies, limited hepatotoxicity risks related to these drugs were reported, however hepatotoxic effects of them were not adequately investigated. At this point, it is emphasized that it is required to perform experimental studies regarding the effects of antipsychotic treatment primarily in vitro. Generally, low dose chemical exposure induces cell proliferation whereas they inhibit DNA synthesis at higher doses and lead to necrosis or apoptosis and show toxicity [12]. Therefore, unlike the other studies performed previously, in this study, we aimed to evaluate hepatotoxicity associated with QET, OA, RIS and ARI by using HepG2, human hepatocarcinoma cell line which shares many genotypic and phenotypic characteristics of normal liver cells and also the liver enzymes, via cytotoxicity testing and since it is now known that apoptosis in the liver plays a role in the hepatotoxicity of chemicals, flow cytometry was used to detect apoptosis.

II. MATERIALS AND METHODS

A. Chemicals

All of the chemicals used in the study were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, USA) and Merck (Merck KGaA, Darmstadt, Germany), and used without further chemical or biological purification.

B. MTT Test

The tetrazolium salt MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide can be used to measure the metabolic activity of viable cells. Tetrazolium salts are reduced to formazan by mitochondrial succinate dehydrogenase, an enzyme which is only active in cells with an intact.

The formazan is quantified spectrophotometrically and correlates with the number of viable cells [13]. HepG2 human liver hepatocellular carcinoma (ATCC® HB-8065™) cells were used to test hepatotoxicity of the compounds. HepG2 cells were incubated according to the instructions of the supplier at 37 °C in a humidified atmosphere of 95% air and 5% CO_2T. After 24 hours of incubating period, the culture mediums were removed and compounds was added to culture medium between 0.000316-1 mM concentrations. After 24 hours of incubation, cytotoxicity test was performed using the MTT assay [14]. MTT solution (5 mg/mL MTT powder in PBS) was prepared and then diluted to a final concentration of 0.5 mg/mL with the culture medium. 100 µL of this mixture was added to each cell. After 3 h incubation period at 37°C, 5% CO_2, the contents of the wells were removed and DMSO (100 µL) was added to each well. Then, OD of the plate was read at 540 nm. Inhibition % was calculated for each concentration according to the formula below and IC50 values were determined by plotting a dose response curve of inhibition % versus compound concentrations tested:

$$\text{Inhibition\%}=100-[(OD_{testcompound}-OD_{blank})/(OD_{solventcontrol}-OD_{blank}) \times 100]$$

C. Flow Cytometric analysis of apoptosis

Death pathway of the carcinogenic cell lines was detected by Annexin V-FITC Apoptosis Detection Kit (BD, Pharmingen) as reported in the manufacturer's instructions. After MTT test, drugs were used at their IC50/2, IC50, and 2 x IC50 concentrations. After 24 h incubation period, cells were harvested by centrifugation at 1200 rpm for 5 min. at room temperature, rinsed with cold water twice, and then suspended at 1×10^6 cells/mL concentration in Annexin V-FITC binding buffer. Propidium iodide (5 mL) and annexin V-FITC (5 mL) were added for staining the cells, and the fluorescence measurements were performed using a flow cytometer. FCSExpress software was used to display the percent of normal and apoptotic cells at different stages. In the diagrams, Q1, Q2, Q3, and Q4 demonstrate the necrotic cells (positive for PI and negative for annexin/ FITC), late apoptotic or necrotic cells (positive for annexin and PI), live cells (negative for annexin and PI), and apoptotic cells (negative for PI and positive for annexin), respectively. The experiments were carried out in triplicates.

III. RESULTS AND DISCUSSION

A. MTT Test

MTT assay, based on the capability of metabolically active cells that change the yellow MTT colour to a blue formazan salt, is one of the most preferred cytotoxicity assays [15]. We assessed the hepatotoxicity of QET, RIS, OLA, and ARI with various concentrations (1mM, 0.316 mM, 0.1 mM, 0.0316 mM, 0.01 mM, 0.00316 mM, 0.001 mM, 0.000316 mM) on HepG2 cells. IC50 values of these drugs are presented in Table 1. Liver is the most important target of drugs due to its role in metabolism [16, 17]. As it is known that hepatotoxicity is an important reason of drug withdrawal [18, 19] it is very important to determine the hepatotoxicity of drugs in the early stages of drug discovery [20].

Therefore, investigating drug-induced hepatotoxic effects in vitro is considered as an important field of research. HepG2 cells serve as an important model for in vitro hepatotoxicity testing because of their similarities with normal liver cells [21, 22]. According to our results, ARI was found to be the most hepatotoxic drug whereas QET, OLA and RIS come next respectively. Liver dysfunction was reported in particular for OLA, QET and RIS whereas hepatitis was not induced with atypical antipsychotics except clozapine, risperidone and olanzapine. However, all of these toxic effects were reversible after dose reduction or discontinuation of the drug [23]. To our knowledge, previous reports only informed that ARI when used chronically for psychosis in combination with CYP3A4 affecting drugs may lead to loss of activity or hepatotoxicity [24]. Also there has been only one case report related to ARI-induced hepatotoxicity [23]. RIS was shown to cause hepatic enzyme elevations and hepatitis. OLA was shown to be associated with liver enzyme elevations and liver injury whereas QET induced liver damage and acute liver injury [25]. However, all of the reported cases were limited and only weak evidence has been identified in support of a causal link between most antipsychotics and hepatotoxicity. Our findings are supported by the previous studies showing hepatotoxicity risk related to these drugs as shown above and indicate this test as a primary hepatotoxicity screening test.

B. Flow Cytometry Test of Apoptosis

Although toxic cell death was primarily associated with necrosis, chemical agents were shown to exert toxicity via induction of apoptosis, generally in low doses [12]. Annexin V-FITC is a reagent that can detect and measure quantity of apoptotic cells by flow cytometry. PI and Annexin V-FITC coloring show live, apoptotic, dead, and late apoptotic or necrotic cells [26]. Thus, this method provides an evidence of the cell death pathway.

Table 1. IC50(µM) values of RIS, OLA, ARI and QET

Compound	IC50(µM)
Risperidone	474.25
Olanzapine	221.11
Aripiprazole	9.46
Quetiapine	60.81

Table 2 presents the percentages of the drugs inducing Q1, Q2, Q3, and Q4 (necrotic cells (positive for PI and

negative for annexin/ FITC), late apoptotic or necrotic cells (positive for annexin and PI), live cells (negative for annexin and PI), and apoptotic cells (negative for PI and positive for annexin), respectively.

Table 2. Data of Flow Cytometric Analysis

	Risperidone			Olanzapine			Aripiprazole			Quetiapine		
	IC50/2	IC50	2*IC50	IC50/2	IC50	2*IC50	IC50/2	IC50	2*IC50	IC50/2	IC50	2*IC50
Q1	0.3	0.2	1.8	0.2	1.3	0.3	15.6	1.2	9.7	0.3	13.2	0.3
Q2	16.4	23.1	29.4	22.9	32.5	27.3	3.8	37.7	36.0	18.4	15.4	24.1
Q3	64.4	50.4	17.8	62.5	60.5	4.1	80.4	53.8	47.3	73.0	66.0	58.9
Q4	18.9	26.3	51.0	14.4	5.7	68.3	0.2	7.3	7.0	8.3	5.3	16.8

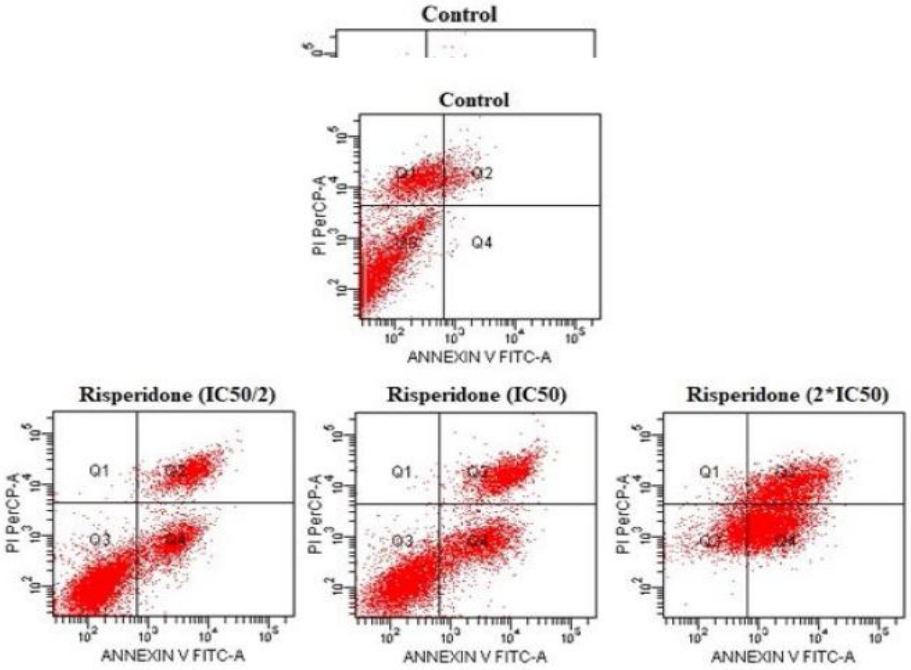


Figure 1. Flow cytometric analysis diagram of Aripiprazole

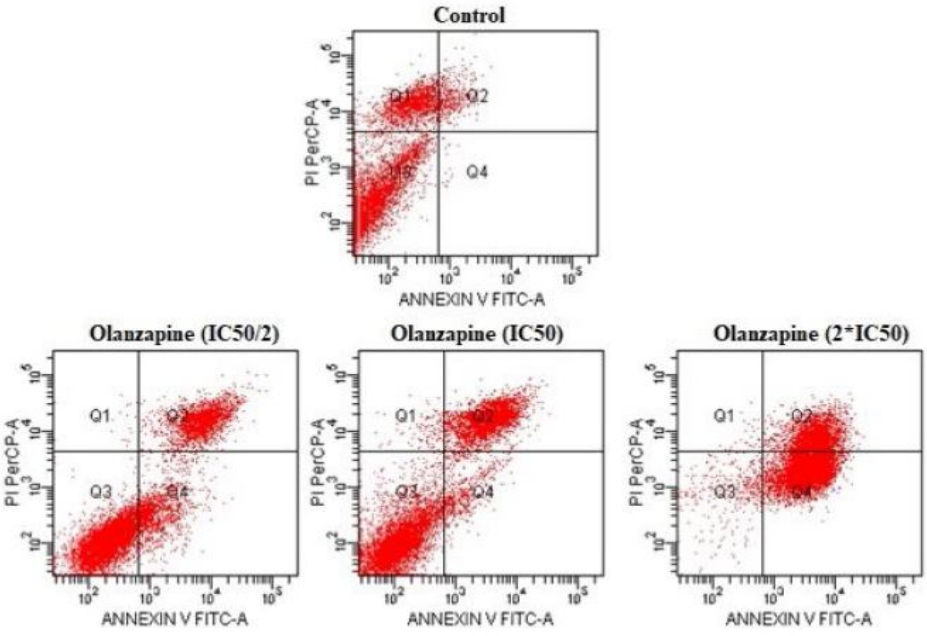


Figure 2. Flow cytometric analysis diagram of Olanzapine

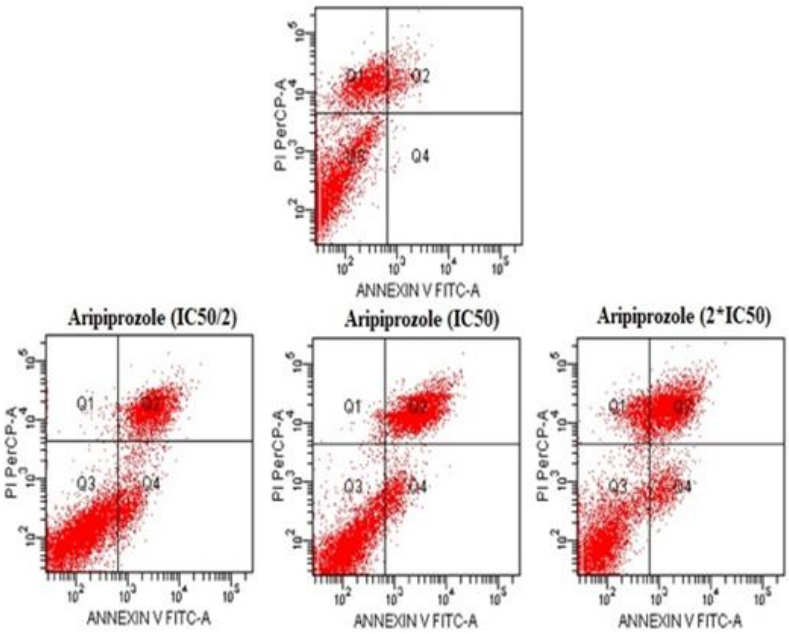


Figure 3. Flow cytometric analysis diagram of Quetiapine

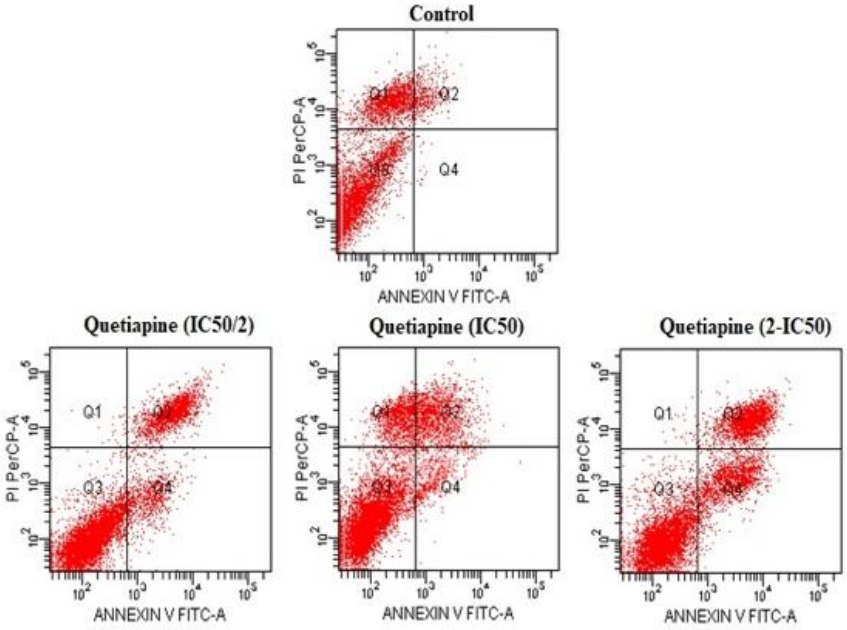


Figure 4. Flow cytometric analysis diagram of Risperidone

ARI which was found to be the most hepatotoxic among the compounds. Chemicals have been shown to induce apoptosis at low doses and only very high doses lead to necrosis [12]. However, RIS, OLA, QET and ARI at their IC50 and IC50x2 concentrations induced apoptotic cell death. It is noteworthy that at IC50/2 concentrations, RIS, OLA and QET induced apoptotic cell death whereas ARI induced necrotic cell death (Table 3, Figure 1, 2, 3 and 4). Mitochondrial pathway of apoptosis causes the permeabilization of the outer mitochondrial membrane and as a result, mitochondrial proteins, which lead apoptosis in other compartments, are released [12]. Thus, apoptosis mostly related to mitochondrial dysfunction, these agents may be capable of inducing mitochondrial toxicity. Also, in a recent paper they were shown to impair the mitochondrial dynamics [8].

CONCLUSION

In conclusion, QET, OLA, RIS and API may induce hepatotoxicity, by inducing apoptotic cell death. However, nuclear morphology, plasma membrane integrity, mitochondrial function, intracellular calcium concentration, and oxidative stress are recommended to be investigated to explore the possible toxicity mechanism of QET, OLA, RIS and API. Our study appears to be important in terms of

early screening of atypical antipsychotic hepatotoxicity and provide a basis for preclinical research, which was not carried out adequately before and also suggests possible mechanisms implicated in the toxicity of these drugs.

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