DIFFERENTIAL TOXICITY OF RIFAMPIN ON HEPG2 AND HEP2 CELLS USING MTT TEST AND ELECTRON MICROSCOPE

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Summary

Background: Rifampin is an antibiotic widely used for the treatment of mycobacterial infections such as tuberculosis and leprosy. The drug produces hepatic, renal and bone marrow toxicity in patients. In this study, toxic effects of rifampin on cell proliferation and cellular organelles were investigated using cells with different metabolic activities.
Results: Human hepatoma cells (HepG2) and human laryngeal carcinoma cells (Hep2) were cultured in 96-well plated and were exposed to 5, 10, 20, 50 and 100 μM of rifampin. Toxicity of the drug was assessed by MTT assay. Toxicity was evident from 10 mg/ml upward on HepG2 cells with direct relationship with concentration. Electron microscopic survey showed broad disruption in the membranes of cell organelles including the nucleus. Hep2 cells were unaffected by the drug in all concentrations in MTT assay and electron microscopy survey.
Conclusion: Rifampin is toxic to hepatic cells in cell culture even in the concentration that is very close to its C_{max} in clinical settings when it is used in usual doses in the treatment of various infectious diseases. Considering the result of the drug's exposure to Hep2 cells, it seems that hepatic metabolism is the main determinant of its toxicity. More studies using protective measures will clarify the mechanism of rifampin toxicity.

Key words: rifampin, HepG2, Hep2, cell toxicity, MTT test, electron microscope

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Introduction

Cell viability assays measure the number of live cells in culture. These cell-based assays are frequently used for drug discovery using high-throughput screening (1), environmental assessment of chemicals (2) and biosensors for monitoring cellular behavior (3). Some biochemical methods, such as the MTT assay are widely used in toxicity screening assays (4,5).

Rifampin is an important drug in the treatment of human mycobacterial and other infections. It is widely used as an essential drug in the treatment of tuberculosis and leprosy, in combination with other drugs (6). The drug has been shown to produce hepatic toxicity in animal studies (7,8) and clinical settings (9,10). Tubulo-interstitial nephritis (11) and acute renal failure (10) is another remarkable adverse effect of the drug. The drug is also known to inhibit protein synthesis (12) and induces chromosomal abberation (13,14). Toxic effects of the drug were shown in in vitro experiments on some cultured cells (15).

In this study, we investigated the effect of rifampin on two different cell cultures, one with high metabolic capacity (human hepatoma cell line, HepG2) and one without this capacity (human larynx carcinoma cell line, Hep2), compared to the former one. In fact, we developed a procedure to distinguish between the direct toxic effects of rifampin and toxic effects of its metabolites produced by liver cells. We also investigated the effects of rifampin in both cell lines by electron microscopy to identify the main organelles that are affected by the drug. In cell culture experiments, we can study pure toxic effects of a substance, independent of other organs or the whole organism.

Materials and methods

Materials

Rifampin was kindly provided by Hakim Pharmaceutical Company (Tehran, Iran). The substance was dissolved in DMSO. DMSO, MTT and DMEM powder were purchased from Merck Company (Tehran, Iran). Gentamicin was purchased as injectable ampoules from Daru Pakhsh Pharmaceutical Company (Tehran, Iran).

Cell cultures and treatments

HepG2 and Hep2 cells were obtained from Pasteur Institute Collection of Cell Cultures, Tehran, Iran (ECACC No. 86121112) and were cultured in 25 cm^2 DMEM supplemented with 10% FBS and gentamicin (with a final concentration of 100 mg/l) under standard conditions and subcultured in the ratio 1:3 twice per week. Passages 1–15 were used for experiments. Cells were seeded at a density of 1000 cells/well in 96-well plates (Greiner, UK) and incubations with various concentrations of rifampin were started 24 h after seeding and continued for 48 hours.

Cytotoxicity assay

HepG2 cells were cultured in 96 wells plates at a concentration of 1×10^3 cells/well, and incubated at 37 ºC, in a 5% CO_2 incubator. After 24 h the culture supernatant was changed and different amounts of rifampin were added to produce final concentrations of 5, 10, 20, 50 and 100 μM in culture medium. The plates were incubated for 48 h. Then, 20 μl of MTT (methyl-tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide]) (Sigma, USA) at a concentration of 5 mg/ml was added to each well. The plates were incubated for 4 h at 37 ºC in a 5% CO_2

incubator. The growth medium was removed; 200 µl of DMSO and 20 µl of glycine buffer were added and incubated at room temperature for 30 min.
The absorbance of each well was measured by an ELISA reader (Microplate reader MR 600, Dynatech, USA) at a wavelength of 570 nm. Determination of percent of growth inhibition was carried out using the following formula:

$$\text{Growth inhibition (\%)} = [(C - T)/C] \times 100$$

Where C is the mean absorbance of control group and T is the mean absorbance of test group.

Transmission electron microscopy (TEM)

HepG2 and Hep2 cells were grown in 150 cm2 tissue culture flask. When cells reached to near 100% confluency, they were incubated with different concentration of rifampin for 24 hours. Then cells were detached and washed twice with DMEM medium without serum in order to remove the proteins of serum. The cells were fixed for overnight in 2.5% glutaraldehyde in phosphate buffer (pH 7.3) at 4 ºC. The cells were washed three times in phosphate buffer (10 min each times, 4 ºC), then postfixed in 2% OsO_4 in phosphate buffer for 1 hour at at 4 ºC. The cells were washed twice in water and dehydrated using ethanol. Then the cells were infiltrated and embedded in low-viscosity epoxy resin (Spurr resin). Thin sections of about 70-100 nm were cut on a LKB Ultramicrotome (Type 4801A, Sweden)) with a diamond knife (Diatome, Switzerland). Sections were picked up on 300 nm mesh copper grids and stained with 2% aqueous solution uranyl acetate for 3 min and Reynold's lead citrate for 5-10 min. Then the cells were viewed and photographed with a LEO-912 AB (Germany) transmission electron microscope. Control sections were prepared with cells incubated with the solvent (DMSO).

Statistical evaluations

The data were expressed as mean ± standard deviation (SD) of 8 independent experiments. Wherever appropriate, the data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons. A value of $p<0.05$ was considered significant. Instat for Windows version 3 software was used for the statistical analysis and excel 2003 was used for producing diagrams.

Results

Cytotoxicity assay

HepG2 cells- Figure 1 shows the results of MTT assay in HepG2 cells cultured for 48 hours in DMEM containing different concentrations of rifampin (see above). Rifampin did not produce a significant reduction in cell number in 5 µM concentration ($p<0.05$), but produced significant dose-dependent reduction in cell number in 10 ($p<0.01$), 20, 50 and 100 ($p<0.001$) µM concentrations. The drug reduced the number of cells by 12.32%, 37.58%, 55.63% and 76.42% in 10, 20, 50 and 100 µM respectively. DMSO did not reduce the number of cells to a significant amount and was not toxic to cells.

Hep2 cells- As can be seen in Figure 2, rifampin did not produce a significant reduction in cell number in all concentrations ($p<0.05$) in this cell line. In the other words, rifampin is not toxic to Hep2 cells.

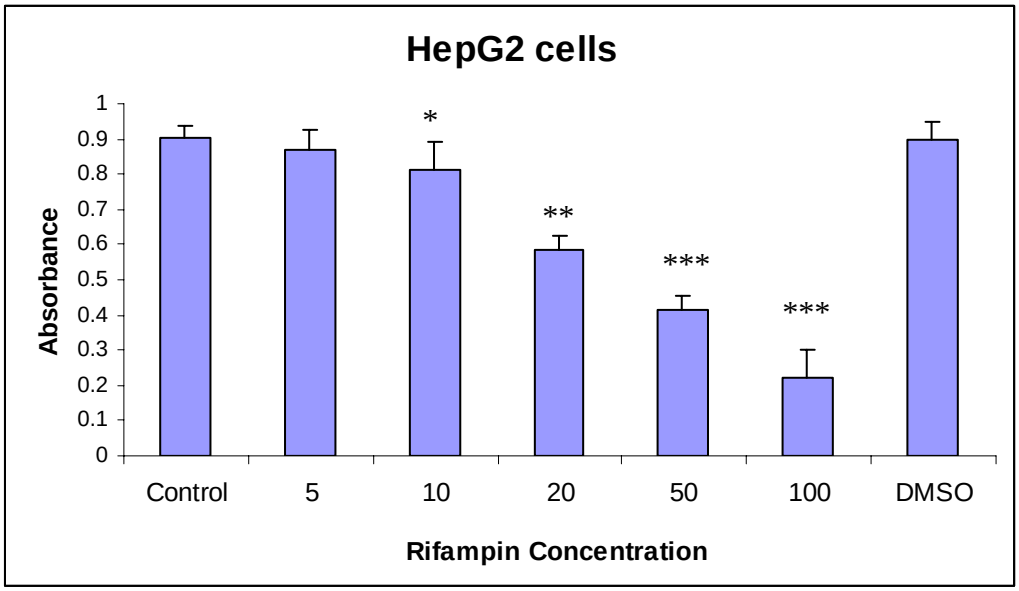

Figure 1- The effects of Rifampin on HepG2 cell growth in MTT assay.
HepG2 cells (1000 cells per well) were grown in MEM for 24 h and exposed to Rifampin for an additional 24 h.
Rifampin concentrations are in μM.
* $p<0.05$
** $p<0.01$
*** $p>0.001$

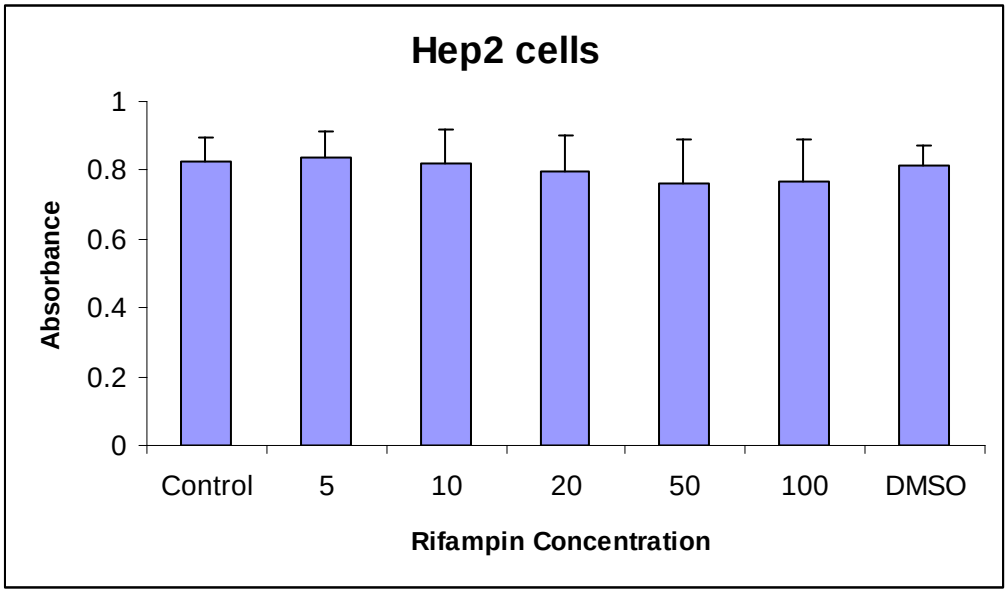

Figure 2- The effects of Rifampin on Hep2 cell growth in MTT assay.
Hep2 cells (1000 cells per well) were grown in MEM for 24 h and exposed to Rifampin for an additional 24 h.
Rifampin concentrations are in μM.

TEM observation

HepG2 cells- Figure 3 shows a control HepG2 cell with clear mitochondria and endoplasmic reticulum. Lipid bilayers of organelles are distinct from cytoplasmic media without any signs of damage. Figure 4 shows a HepG2 cell exposed to 50 μM of rifampin. Damage to cellular organelles and elongation of them with abnormal shape and without normal structure is obvious. Inflammation of rough endoplasmic reticulum is also evident. Nucleus deformation and chromosomal condensation can be clearly observed in Figure 5. No obvious damage to cell was observed in cells exposed to 5 μM of rifampin (Figure not shown).

Hep2 cells- These cells were not affected in microscopic evaluation, as they were not affected in MTT cytotoxicity assay (Figure not shown).

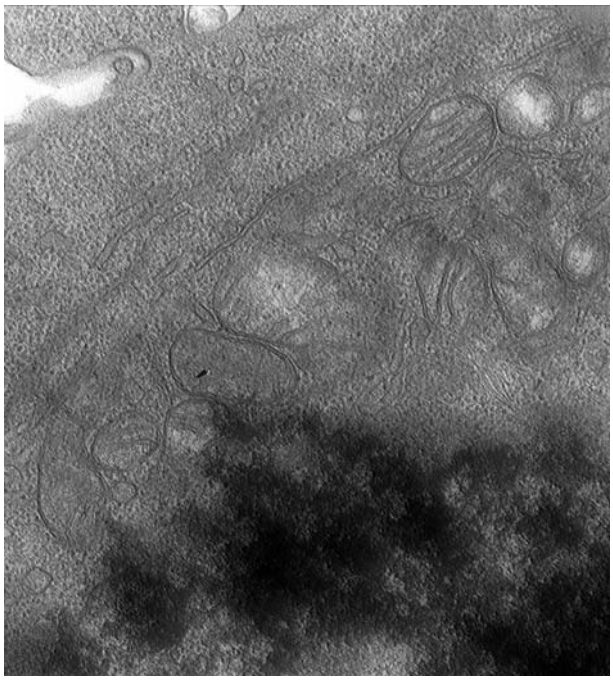

Figure 3- Electrone microscopic photo of a control HepG2 cell with clear and normal organelles (Magnification ×50000).

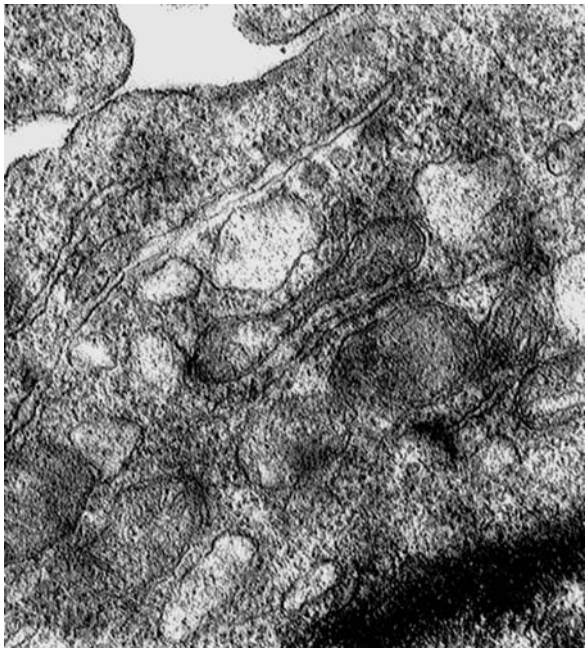

Figure 4- Electrone microscopic photo of a HepG2 cell exposed to 50 mM rifampin for 48 hours. Damage to cellular organelles with abnormal shape and structure is obvious. Inflammation of rough endoplasmic reticulum is also evident. (Magnification ×80000).

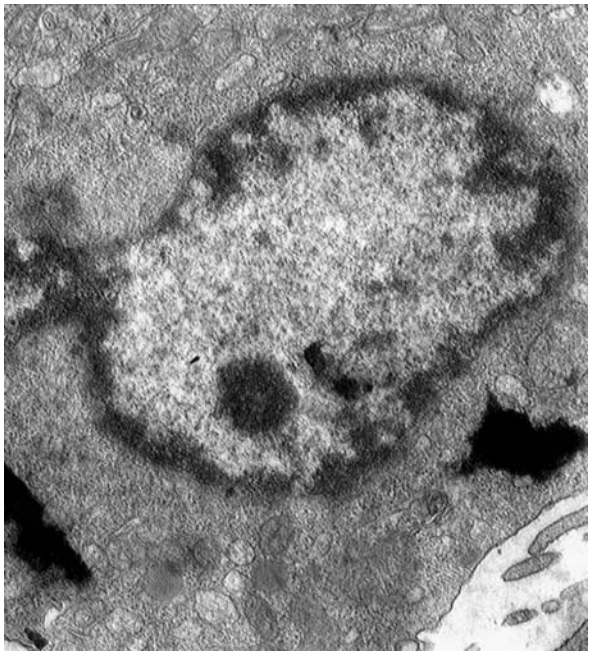

Figure 5- Electrone microscopic photo of a HepG2 cell exposed to 50 mM rifampin for 48 hours. Nucleus deformation and chromosomal condensation can be clearly observed. (Magnification ×31500).

Discussion

In this study, the effects of rifampin on two different cell lines with different metabolic capacity were investigated. Results of MTT assay with HepG2 cells show that the drug have significant toxicity on these cells. Toxic effect was evident from the concentration of 10 μM (8.23 μg/ml) and increased in a concentration-dependent manner. On the other hand, maximum therapeutic plasma concentrations of rifampin are 7-10 μg/ml (16) that overlaps the lowest toxic concentration in our experiments. Isefuku et al (15) reported that rifampin can inhibit the proliferation of osteoblast-like cells in vitro at concentrations of 10 μg/ml and above. In clinical setting, hepatic toxicity is a rare incident (16) because in vivo environment produce a better and more effective protection against toxic agents. HepG2 cells may also be more sensitive than normal hepatic cells against toxic damage due to rifampin.

Electron microscopic examination of HepG2 cells additively confirmed the results of MTT cytotoxicity test. The drug damaged cellular organelles including endoplasmic reticulum and mitochondria. Similar TEM data was reported in an in vivo animal experiment (7) and our results are in agreement with them.

Rifampin did not adversely affect Hep2 cells in all concentrations tested. To our knowledge, there is not any clinical report of rifampin in laryngeal tissue. These cells are originated from larynx carcinoma cells that possess lower metabolic activity against foreign substances and they may not have significant expression of phase I metabolic enzymes. This may explain why results do not agree with each other in the two cell lines.

As reviewed by Knasmuller et al. (17), HepG2 cells express a wide range of phase I enzymes such as cytochrome P450 (CYP) 1A1, 1A2, 2B, 2C, 3A and 2E1, arylhydrocarbon hydrolase, nitroreductase, *N*-demethylase, catalase, peroxidase, NAD(P)H:cytochrome *c* reductase, cytochrome P450 reductase, and NAD(P)H, Quinone oxidoreductase and phase II enzymes such as epoxide hydrolase, sulfotransferase, glutathione *S*-transferase (GST), uridine glucuronosyl transferase, and *N*-acetyl transferase. Some of these enzymes present in higher concentrations in growing than in confluent cells (18). Rifampin is metabolized by hepatic microsomes to various active and inactive metabolites. The drug also induces the most important cytochrome P450 enzyme responsible for the metabolism of foreign compounds (CYP3A4), including itself (19). The drug produces renal damage as well, as kidney is the second organ rich in metabolizing enzymes. It appears that phase I metabolism is responsible for the production of reactive metabolites that damage cellular macromolecules and produce lipid peroxidation. Electron microscopic examination of HepG2 cells exposed to 50 mM rifampin also shows disruption of membranes of cellular organelles including mitochondria (Figure 4). Meanwhile, DNA condensation (Figure 5) confirms DNA damage possibly due to reactive metabolites of the drug. HepG2 cells have been found to be a suitable tool for genotoxicity testing (20). There is argument on the value of in vitro assays for detecting hepatotoxicity, but drugs which tested positive in these assays (e.g. greater than 50% effect at 30 μM in HepG2 cells), there was high probability of human toxicity (i.e. they had high specificity) (21).

In conclusion, we designed an experiment distinguishing direct toxicity of a substance and indirect toxicity due to active metabolites of it resulting from metabolic activation. We are going to develop our experiment using antioxidants and free radical scavengers that theoretically may significantly reduce these effects in hepatic and other cell lines.

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References

1- White RE. High-throughput screening in drug metabolism and pharmacokinetic support of drug discovery. Annu Rev Pharmacol Toxicol 2000; 40: 133-157.
2- Ni Shuilleabhain S, Mothersill C, Sheehan D, O'Brien NM, O' Halloran J, Van Pelt FN, Davoren M. In vitro cytotoxicity testing of three zinc metal salts using established fish cell lines. Toxicol In Vitro 2004; 18: 365-376.
3- Ehret R, Baumann W, Brischwein M, Schwinde A, Stegbauer K, Wolf B. Monitoring of cellular behaviour by impedance measurements on interdigitated electrode structures. Biosens Bioelectron 1997; 12: 29-41.
4- Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, Silber PM. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. Chem Biol Interact 1999; 121: 17-35.
5- Heussner AH, Dietrich DR, O' Brien E. (2005). In vitro investigation of individual and combined cytotoxic effects of ochratoxin A and other selected mycotoxins on renal cells. Toxicol. In Vitro 2005; 20: 332-341.
6- William A, Petri J. Chemotherapy of tuberculosis, *mycobacterium a vium* complex disease, and leprosy. In: Brunton LL, ed. Goodman and Gilman's the Pharmacological Basis of Therapeutics, PA: McGraw-Hill Companies, Inc., 2006: 1203-1223.
7- Piriou A, Maissiat R, Jacqueson A, Warnet JM, Claude JR. Ultrastructural changes in the parenchymal liver cells of rats treated with high doses of rifampicin. Br J Exp Pathol 1987; 68: 201-207.
8- Sodhi CP, Rana SV, Mehta SK, Vaiphei K, Attari S, Mehta S. Study of oxidative-stress in isoniazid-rifampicin induced hepatic injury in young rats. Drug Chem Toxicol 1997; 20: 255-269.
9- Dutt AK, Moers D, Stead WW. Undesirable side effects of isoniazid and rifampin in largely twice-weekly short-course chemotherapy for tuberculosis. Am Rev Respir Dis 1983; 128: 419-424.
10- Rajan MA, Soundararajan R, Krishnamurthy V, Ramu G. Acute renal failure following rifampicin. Indian J Lepr 1987; 59: 286-292.
11- Gallieni M, Braidotti P, Cozzolino M, Romagnoli S, Carpani P. Acute tubulo-interstitial nephritis requiring dialysis associated with intermittent rifampicin use: case report. Int J Artif Organs 1999; 22: 477-481.
12- Buss WC, Morgan R, Guttmann J, Barela T, Stalter K. Rifampicin inhibition of protein synthesis in mammalian cells. Science 1978; 200(4340): 432-434.
13- Aboul-Ela EI. Cytogenetic effects of rifampicin in somatic and germinal cells of the mouse. J. Appl. Toxicol. 1995; 15: 325-326.

14- Aly FA, Donya SM. In vivo antimutagenic effect of vitamins C and E against rifampicin-induced chromosome aberrations in mouse bone-marrow cells. Mutat Res 2002; 518: 1-7.
15- Isefuku S, Joyner CJ, Simpson AH. Toxic effect of rifampicin on human osteoblast-like cells. J Orthop Res 2001; 19: 950-954.
16- United States Pharmacopeial Convention Inc. USPDI (volume 1, Drug information for the health care professional). 2001: 2597, Micromedex, Englewood, USA.
17- Knasmüller S, Parzefall W, Sanyal R, Ecker S, Schwab C, Uhl M, Mersch-Sundermann V, Williamson G, Hietsch G, Langer T, Darroudi F, Natarajan AT. Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. Mutat Res 1998; 402:185–202.
18- Alexandre, E., David, P., Viollon, C., Wolf, P., Jaeck, D, Azimzadeh, A., Nicod L, Boudjema K, Richert L. Expression of cytochromes P-450 2E1, 3A4 and 1A1/1A2 in growing and confluent human HepG2 hepatoma cells-effect of ethanol. Toxicol In Vitro 1999; 13: 427-435.
19- Strolin Benedetti M, Dostert P. Induction and autoinduction properties of rifamycin derivatives: a review of animal and human studies. Environ Health Perspect, 102 Suppl 1994; 9: 101-105.
20- Knasmuller S, Mersch-Sundermann V, Kevekordes S, Darroudi F, Huber WW, Hoelzl C, Bichler J, Majer BJ. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge. Toxicology 2004; 198: 315-328.
21- Xu JJ, Diaz D, O'Brian PJ. Applications of cytotoxicity assays and pre-lethal mechanistic assays for assessment of human hepatotoxicity potential. Chem Biol Interact 2004; 150: 115-128.