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ORIGINAL RESEARCH

Studies on toxicity of antitubercular drugs namely isoniazid, rifampicin, and pyrazinamide in an in vitro model of HepG2 cell line

Meenakshi Singh · Preetha Sasi · Gaurav Rai · Vinod H. Gupta · Deepak Amarapurkar · Pramod P. Wangikar

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Abstract Antitubercular drugs (ATT) are known to be majorly metabolized and detoxified in liver by both Phase I and Phase II group of drug metabolizing enzymes. These drugs as well as their metabolites are toxic and during this process cause injury to liver. In this study, we have investigated the in vitro hepatotoxic potential of both individual as well as combination ATT drugs using an in vitro model of human hepatocellular carcinoma cell line (HepG2). The cells were treated with varied concentrations of ATT drugs namely isoniazid (INH), rifampicin (RIF), and pyrazinamide (PYZ) for different durations. Cytotoxicity assay using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) as well as morphological analysis using phase contrast microscopy have shown that concentrations used were not cytotoxic. However, pre-treatment with sub-cytotoxic concentrations of INH and PYZ increased the toxicity with the same drugs. This report corroborates the clinical finding that long-term treatment as well combination drug therapy with ATT induces hepatotoxicity rather than individual drugs.

Keywords Antitubercular therapy · Human hepatocellular liver carcinoma cell line · ATT-induced drug hepatotoxicity · Drug metabolizing enzyme

Introduction

Tuberculosis is a leading cause of death among infectious diseases and is a significant socio-economic burden in India (WHO report, 2008). Currently, a combination therapy, consisting of isoniazid (INH), rifampicin (RIF), pyrazinamide (PYZ), and ethambutol (ETM) is commonly used against TB. These drugs lead to adverse reactions including hepatotoxicity. The frequency of hepatotoxicity varies from 2 to 28% in different populations and occurs even when the drug has been given at the recommended doses (Tostmann *et al*., 2008). Approximately 9.5% of Indian patients have been reported to develop antitubercular therapy (ATT)-induced hepatotoxicity (Agal *et al*., 2005). This often results in discontinuation of the most effective first-line drugs. Non-compliance not only leads to morbidity and mortality but also results in the emergence of drug-resistant strains. Majority of ATT-induced hepatotoxicity incidences are evident in the first month of treatment for a combination of RIF and INH but can occur up to the third month when PYZ is combined (Agal *et al*., 2005).

INH which has been clinically used as a first-line drug for tuberculosis treatment since 1952 is adversely associated with hepatotoxicity (Girling, 1978). INH and its metabolites have been implicated in hepatotoxicity in humans (Huang *et al*., 2003), and in an in vitro model of human hepatocellular carcinoma cell line (HepG2 cells) (Wu and Cederbaum, 1996). RIF, which is usually co-administered with INH, synergises the hepatotoxicity of INH, possibly due to its potent induction of CYP 450 enzymes thereby increasing

Meenakshi Singh and Preetha Sasi contributed equally to this study.

M. Singh · P. P. Wangikar (✉)
Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, Maharashtra, India
e-mail: pramodw@iitb.ac.in

P. Sasi · G. Rai · V. H. Gupta
Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

D. Amarapurkar
Department of Gastroenterology, Bombay Hospital and Medical Research Centre, Mumbai 400020, India

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the production of toxic metabolite hydrazine (HYD) (Yew, 2002; Yew and Leung, 2006). Several studies have also implicated the role of PYZ in the development of ATT-induced drug hepatotoxicity (Yee *et al*., 2003; Schaberg *et al*., 1996).

Recently, Tostmann *et al.* (2008) have reported in vitro interaction between the drugs INH, RIF, and PYZ. They showed that pre-treatment with a non-toxic concentration of INH or hydrazine (HYD) increased in vitro toxicity of INH. However, the effect of pre-treatment with individual drugs INH, RIF, PYZ or combination of above drugs like INH and RIF or INH and PYZ on the ATT-induced hepatoxicity is largely unknown.

Thus, in view of the above, the objective of this study is to investigate the in vitro hepatotoxic potential of both individual as well as interactions of INH, RIF, and PYZ drugs using a human liver model of HepG2 cell line.

Materials and methods

Chemicals

Isoniazid, rifampicin, and pyrazinamide were kindly donated by Lupin Pvt Ltd, India. Dimethyl sulfoxide (DMSO) was obtained from Merck (Darmstadt, Germany). MTT 3-(4,5-dimethylthiazolyl-2-yl) 2,5-diphenyl tetrazoliumbromide (MTT) was obtained from Sigma. All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were of analytical grade.

HepG2 cell culture

Human hepatocellular liver carcinoma cell line (HepG2), passage less than 20 was obtained from NCCS, Pune (India) for in vitro hepatotoxicity studies. HepG2 cells were maintained in the logarithmic phase of growth in Dulbecco's modified Eagle's medium (Himedia, India) supplemented with 2-mM glutamine, 10% fetal calf serum, 40 U/ml streptomycin, and 50 U/ml penicillin. The cells were subcultured once they reached 80–90% confluence. Cell viability was assessed by trypan blue dye exclusion method using a haemocytometer (Neubaur, USA). The cells were harvested with ethylene diamine tetra acetic acid (0.25% EDTA/Trypsin) (Himedia, India) washed and used for subsequent cytotoxicity assays.

Cytotoxicity assay [microculture tetrazolium (MTT) assay]

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells (Fotakis George, 2006). The amount of MTT reduced by cells to its blue formazan derivative was quantified spectrophotometrically at 590 nm with an ELISA reader (i MARK, Biorad, USA).

Cytotoxicity assay using MTT was performed to study the in vitro drug toxicity of antitubercular drugs INH, RIF, and PYZ. HepG2 cells were seeded at a density of 0.5×10^4 cells in a 96-well microtitre culture plate (Nalgene, Nunc, Denmark) in a 200 µl culture overnight for adherence. At day 2 the cells were treated with varied concentrations of INH, PYZ (1–200 mM) and RIF (25–100 µM). After 24-h incubation with the drugs cytotoxicity was assessed by determining cell viability using MTT assay.

The assay helped to determine the non-toxic concentration of the INH, RIF, and PYZ. The experiment results were obtained from three independent experiments each performed in triplicate. The data is represented as mean absorbance ± SD whereas the cell viability is expressed as % cell viability (of control).

Drug toxicity post pre-treatment with TB drugs

To study the in vitro interactions between INH, RIF, PYZ on HepG2 cells, cells were pre-treated with non-toxic concentration of INH (5 mM), RIF (50 µm), PYZ (5 mM) for 24 h followed by treatment with varied concentrations of INH (50–200 mM), PYZ (50–200 mM), combination drugs INH:RIF (50 µm:200 mM) and INH:PYZ (100:100 mM) for next 24 h.

Phase contrast microscopy

HepG2 cells were exposed to the different concentrations of individual and different combination ATT drugs was viewed at ×10 objective using Olympus Phase contrast microscope (Olympus IX71, Japan).

Results

In vitro drug cytotoxicity assay

The effect of in vitro drug toxicity of antitubercular drugs INH, RIF, and PYZ on the cell viability of HepG2 cells is depicted in Table 1 and Fig. 1. HepG2 cells were treated with varied concentrations of INH, PYZ (1–200 mM), and RIF (25–100 µM). The assay helped determine the non-toxic concentration of the INH, RIF, and PYZ.

Table 1 Effect of in vitro drug toxicity of treatment with antitubercular drugs namely isoniazid (INH), rifampicin (RIF), and pyrazinamide (PYZ) on the cell viability (% of control) of HepG2 cells

Drug	Concentration of drug	Absorbance Mean ± SD	% Cell viability
Without pre-treatment			
INH	1 mM	0.37 ± 0.07	125.47
	5 mM	0.29 ± 0.06	99.73
	10 mM	0.37 ± 0.02	128.56
	50 mM	0.27 ± 0.02	91.31
PYZ	1 mM	0.26 ± 0.01	87.71
	5 mM	0.30 ± 0.08	102.13
	10 mM	0.30 ± 0.07	104.19
	50 mM	0.30 ± 0.08	102.13
RIF	25 μM	0.33 ± 0.02	114.66
	50 μM	0.34 ± 0.03	116.03
	100 μM	0.18 ± 0.05	192.41
With pre-treatment INH (5 mM)			
PYZ	200 mM	0.18 ± 0.01	60.08
RIF and PYZ	50 μM and 200 mM	0.22 ± 0.02	58.08
INH and PYZ	100 mM and 100 mM	0.18 ± 0.05	72.21
With pre-treatment PYZ (5 mM)			
INH	200 mM	0.16 ± 0.01	56.13
RIF and PYZ	50 μM and 200 mM	0.19 ± 0.01	63.51
INH and PYZ	100 mM and 100 mM	0.23 ± 0.01	77.24

Cell viability is measured as absorbance at 590 nm of the blue crystals that are the metabolized product of MTT Data is represented as Mean ± SD absorbance and % cell viability (of control)

After 24-h incubation with the drugs we did not observe any significant cytotoxicity with INH (1–50 mM), RIF (25–100 μm), and PYZ (1–50 mM) (Table 1).

Drug toxicity post pre-treatment with INH, RIF, and PYZ

In order to mimic the long-term effect of ATT treatment, we pre-treated the HepG2 cells for a 24-h duration with non-toxic concentrations of INH (5 mM), RIF (50 μM), and PYZ (5 mM) followed by another 24-h treatment with varied increased concentrations of INH (50–200 mM) (Fig. 1a), PYZ (50–200 mM) (Fig. 1b), RIF:PYZ (50 μM and 200 mM), INH:PYZ (100 mM and 100 mM).

The data suggests that pre-treatment with INH or PYZ followed by treatment with INH, PYZ decreased the cell viability by approximately 40 and 30%, respectively (Fig. 1). Also pre-treatment with INH and subsequent treatment with combination drug RIF and PYZ, INH and PYZ showed cytotoxicity by up to 40 and 30%, respectively (Table 1). Similarly, PYZ pretreatment followed by RIF and PYZ, INH and PYZ also exhibited a similar trend that is approximately 36 and 23% decrease in cell viability.

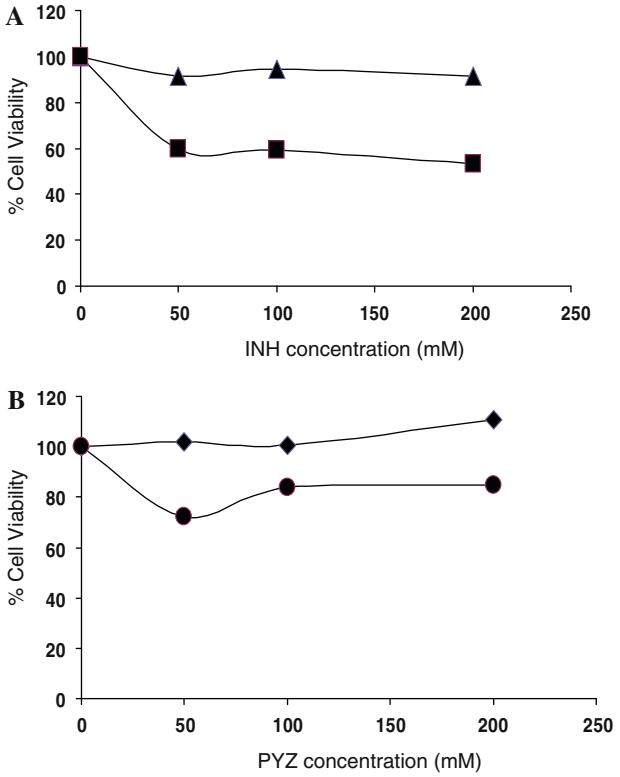

Fig. 1 **a** Change of HepG2 cell viability after treatment by *filled triangle* isoniazid (INH) at various concentrations (24-h treatment period). The effect of drug toxicity was studied in vitro by exposing HepG2 cells to non-toxic concentration of *filled square* isoniazid (INH 5 mM) for 24 h and then subsequent treatment of cells with various increasing concentrations of Isoniazid (INH 50–200 mM). **b** *Filled diamond* pyrazinamide (PYZ) at various concentrations (24-h treatment period). *Filled circle* pyrazinamide (PYZ 5 mM) for 24 h and then subsequent treatment of cells with various increasing concentrations of pyrazinamide (PYZ 50–200 mM). Data points representing mean cell viability % of control

Morphology observation

The HepG2 cells treated with varying amounts of ATT drugs were observed with phase contrast inverted microscope (Fig. 2). Only the representative results are shown here. Morphological examination of HepG2 cells revealed striking changes.

Untreated HepG2 cells show rather an epithelial-like morphology (Fig. 2a). At low concentrations of both INH and PYZ (Fig. 2b–g), the classical morphology of cells was maintained. However, upon pretreatment with INH (5 mM) or PYZ (5 mM) for 24 h and subsequent treatment with INH (200 mM)/PYZ (200 mM) cells displayed typical cubic to round cell shape (Fig. 2h–k). Some cells sloughed off the culture plate. Other cells showed swollen morphology with membrane blebbing and finally necrotised. This typical change in morphology reflects the drug toxicity.

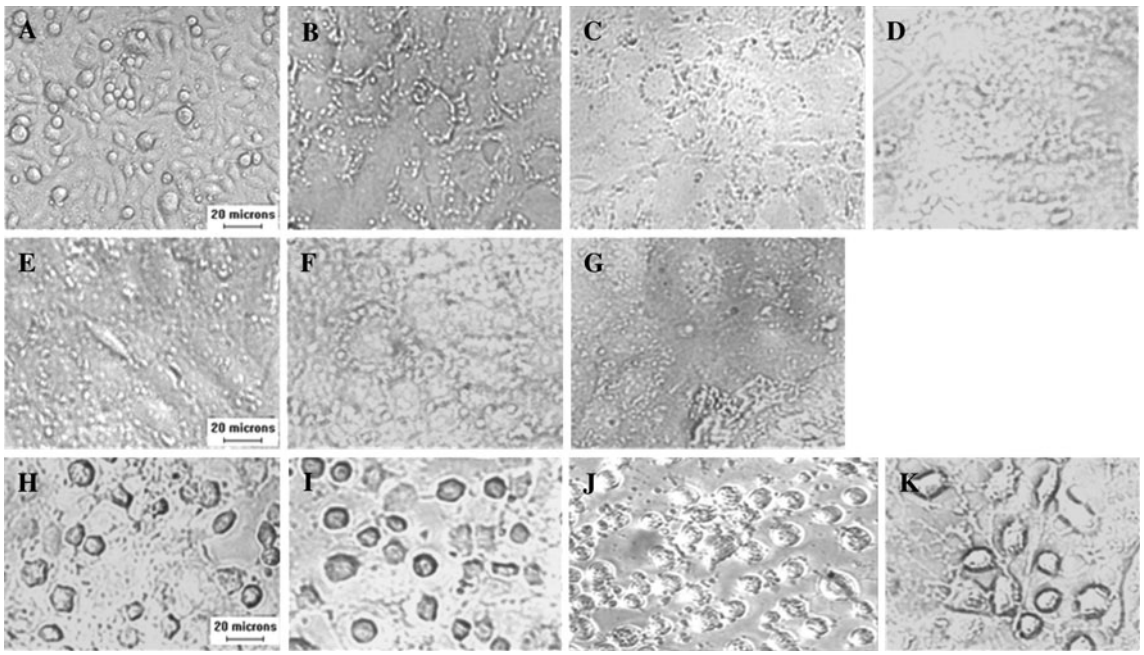

Fig. 2 Phase-contrast micrographs (×100 magnification) depicting morphological changes in HepG2 cells in untreated (control) condition (**a**) as well as in response to various concentrations of isoniazid (INH) (1, 5, and 10 mM) (**b–d**) pyrazinamide (PYZ) (1, 5, 10 mM) (**e–g**), pre-treatment with INH (5 mM) (**h**, **i**), and PYZ (5 mM) (**j**, **k**) for 24 h followed by consequent treatment with INH (200 mM)/ PYZ (200 mM) for next 24 h

Discussion

Our results suggest that pre-treatment of Hep G2 cells with INH, PYZ followed by treatment with higher concentration of the same increased the toxicity with both INH and PYZ. This corroborates the clinical findings that patients on multiple drug therapy over a long duration of time experience significant hepatotoxicity than on individuals on single drug. These results are similar to earlier published reports where it was found that Acetaminophen (APAP) induced a concentration-dependent cytotoxic effect in HepG2 cells which was significantly increased with pre-treatment with RIF or INH (Nicod *et al*., 1997).

INH, RIF, and PYZ are potentially hepatotoxic drugs with INH being the main culprit. ATT-induced hepatotoxicity is largely due to the above toxic drugs or its metabolites. Clearance of the ATT drugs depends on the activities of several enzymes, such as *N*-acetyl transferase 2 (NAT2), cytochrome P450 oxidase (CYP), and glutathione *S*-transferase (GSTM1). Cytochrome P450s (CYPs) are responsible for the biotransformation of xenobiotics and endogenous compounds. The regulation of CYPs is the most common mechanism that can lead to drug–drug interactions due to drug and/or metabolite buildup in that individual (Kalgutkar *et al*., 2007). The mechanism of action of these drugs is largely due induction or inhibition of the drug metabolizing enzymes.

Isoniazid and its metabolite HYD was the first among ATT drugs reported to cause hepatotoxicity. Recent report suggests that INH induces oxidative stress, mitochondria dysfunction, and apoptosis in HepG2 cells (Bhadauria *et al*., 2007). On the other hand, RIF is a potent inducer of Cytochrome P450 2E1 (CYP2E1) and thus regulates the production of hepatotoxic agents (Walubo *et al*., 2005). CYP2E1 produces free radicals independent of a ligand, which can cause cell damage from lipid peroxidation and DNA strand breaks (Caro and Cederbaum, 2004). Previous studies suggest that hepatic CYP2E1 plays an essential role in isoniazid-induced hepatotoxicity through generation of free radicals (Huang *et al*., 2003; Yue *et al*., 2004; Shen *et al*., 2006). This could be one of the possible mechanisms by which RIF enhances toxicity of INH. Shen *et al.* (2008) provided interesting findings that rifampicin exacerbated isoniazid toxicity in human hepatocytes but not in rat hepatocytes. Our current finding also suggests that RIF increases or synergises with the INH toxicity by the possible mechanism of increased production of hydrazine.

PYZ is another standard ATT drug. Though hepatotoxic, little is known about the mechanism of PYZ toxicity and its interaction with other ATT drugs. Reported cases of severe hepatotoxicity with PYZ/RIF regimen in TB patients without HIV infection have raised huge concern over its safety in patients (CDC update, 2001). Also a recent report suggested that the risk of hepatitis in patients receiving

PYZ/RIF for prevention of latent tuberculosis increased 3-fold as compared to patients receiving INH alone (McNeill *et al*., 2003) Inhibitory effect of PYZ on CYP isoenzyme activities in rats has been elucidated (Facino and Carini, 1980), though in human liver microsomes no such inhibition in the activity of CYP1A2, 2C9, 2C19, 2D6, 2E1, and CYP3A was observed (Nishimura *et al*., 2004). With the limited data available on PYZ-induced hepatotoxicity little can be commented on its mechanism of toxicity. More studies need to be attempted to answer the above question.

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