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Identification of toxicological biomarkers of paroxetine in hepatocytes using proteomic analysis

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Abstract :
Purpose: Paroxetine is antidepressant drug used as first line treatment in various depression disorders. With few reports of acute liver failure the effect of paroxetine on liver is very less studied. To understand the effect on paroxetine on liver cells we studied the effect on protein expression in cultured hepatocytes.
Methods: Hepatocyte cells were treated with paroxetine and its effect on protein expression of hepatocytes determined by gel electrophoresis (SDS and 2-D). Cytotoxicity was assessed using the MTT assay. ANOVA method with Tukey's test to identify differences between the exposure and control groups.
Results: Hepatoglobin, serotransferrin, Apolipoprotein level was increased while levels of hemopexin, Alpha-2-HS-glycoprotein decresed. Using String db tool role/function of identified proteins was identified. These identified proteins can be investigated further for the complete understanding the mechanism or biomarker development for the paroxetine induced liver injury.
Keywords: Animal proteomics, Biomarkers, Hepatocytes, Comet assay, PAGE, 2D-PAGE.

Introduction:

The liver is prone to drug-induced injury due to its anatomical location (hepatic portal system) and physiological role in body (central role in detoxification). Drug-induced hepatotoxicity contributes to more than half of the cases of acute liver failure. More than 1000 drugs have been associated with idiosyncratic hepatotoxicity and drug-induced liver injury (DILI) is the main reason for removing approved medications from the market. This list includes NSAID's (Paracetmaol, Ibuprofen, Diclofenac), Antibiotics (Amoxicillin, Isoniazid, Rifampicin), Immunosuppressants (Azathioprine, Cyclophosphamide), Anti-epileptics (Phenytoin, Valproic acid) and Psychiatric drugs (Chlorpromazine, Paroxetine) [1, 2].

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Paroxetine is a drug belonging to selective serotonin reuptake inhibitors (SSRIs) class of first line antidepressants used in the primary care and psychiatric practices. It is mainly used to treat depression, obsessive-compulsive disorder, anxiety disorders, post-traumatic stress disorders (PTSD), and premenstrual dysphoric disorder (PMDD). It is associated with reports of acute failure when used for therapy. The mechanism of the PXT induced toxicity is still very less understood. It is considered that it causes the idiosyncratic toxicity. Drugs that have the potential to cause idiosyncratic drug toxicity may regulate common physiological or biochemical processes. Understanding the response to such drugs at the molecular level has potential both to elucidate the mechanisms of toxicity and to predict idiosyncratic toxicity. Recently many studies have been conducted to study and analyze gene expression, protein expressions altered by toxicants so that the mechanism behind their toxicity could be studied. However, very few studies of paroxetine have been conducted [3–5].

Biomarkers used for identification of hepatotoxicity are not much specific. Hence there is a strong need for new biomarkers that can identify potential hepatotoxicity prior to the development of clinical signs of DILI, which typically develop only after significant injury has occurred. Furthermore, there is a need to find biomarkers that predict a person's potential sensitivity to liver injury and ones that are prognostic about the course of injury and whether the person will adapt to the insult or exhibit liver failure [6, 7].

In the present proteomic study, we evaluated the different concentrations of PXT to identify the cytotoxic concentration so that a lower concentration can be used for drug exposure of hepatocytes. Hepatocytes were exposed to different concentration of drug with different time period. Total protein was isolated from the cultured cells and protein profile was determined using gel electrophoresis and with the goal of identifying toxicological monitoring markers in hepatocytes exposed to PXT. The identified markers may have the potential for use as biomarkers of drug induced toxicity.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FCS), collagenase type IV, penicillin/streptomycin, Urea, thiourea, CHAPS, DTT, acrylamide, N, N-methylene bisacrylamide, and iodoacetamide were purchased for HIMEDIA Chemicals. Protease Inhibitor Cocktail was purchased for Sigma Chemicals. Trypsin, HPLC grade solvents Acetonitrile, Methanol, Formic acid were purchased from Merck. Paroxtine was received as a gift sample from INTAS Pharmaceuticals. Trypan blue was purchased from Life Technologies.

Cell culture

Hepatocyte isolation

Permission for animal studies was obtained from the Institutional Animal Ethical Committee. Adult either sex *swiss albino* mice, weighing 20-25 g, were obtained from animal house of institute. This mouse strain was chosen because it is frequently used in toxicological and pharmacological investigations. The animals were housed in polypropylene cages with rice husk as bedding at 25°C and 50-60% humidity. The light cycle was 14 h light/10 h dark. Feed and tap water were available *ad libitum*[8, 9].

Isolation of hepatocytes

Hepatocytes were isolated from adult *swiss albino* mice by a two-step collagenase perfusion method according to Seglen and Casciano with some modifications. The liver was perfused after cannulation of the hepatic portal vein. The thoracic inferior vena cava was cut through. The organ was washed with Hanks' calcium- and magnesium-free buffer for till the buffer flowing out become colour less.After the liver had been freed of blood the calcium-free buffer was replaced by a collagenase buffer (0.5 mg/ml) for 7-10 min. A perfusion rate of 5ml/min and a temperature around 37-39°C was maintained for both perfusates during the entire procedure [8, 9].

After the perfusion had been terminated, the liver was rapidly excised from the body cavity and transferred to a sterile Petri dish. The gall bladder and remnants of surrounding tissues were removed. Cells were released by disrupting the liver capsule mechanically and by shaking the cells into attachment medium. The cells were separated from undigested tissue with a sterile 50-μm mesh nylon filter. After washing by low-speed centrifugation at 50 g for 3 min at 4°C several times, cell viability and yield were determined by trypan blue exclusion.

Cells from three independent biological replicates with viability >85% were used. Primary cultures of mouse hepatocytes were cultured at 37°C in a humidified CO_2 with 95%/5% air/CO_2 in DMEM supplemented with 10% FBS, and 2% penicillin/streptomycin.

MTT assay

Viability of cells was assessed by measuring the formation of a formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) spectrophotometrically after some modified from Mosmann et al. Hepatocytes were incubated with 0.7mg/ml MTT for 30 min at 37°C at the end of the experiment. After washing with PBS the blue formazan was extracted from cells with isopropanol/formic acid (95:5) and was photometrically determined at 560nm[10].

SDS PAGE:

Protein samples were isolated from treated and control cells. Three separate cell pellets from sub confluent cultures were lysed with a buffer consisting of 0.1 M Tris-HCl, pH7.5, 0.1 M dithiothreitol and 4%SDS, and incubated at 95°C for 5min. Lysates were clarified by centrifugationat16,100x g for10min. These protein samples were separated using one dimensional gel electrophoresis. Protein samples were dilutes with Laemmli sample buffer in a ration 1:1 which was prepared by adding 25μL β-mercaptoethanol to 475μL Laemmli sample buffer. This mixture was vortexed briefly and heated for 5 minutes at 95°C. 20 μL of this mixture were loaded on 10% casted acrylamide gel. The gel was run at constant 200V and 20mA using Tris-Glycine SDS tank buffer for 35 min. Then gel was pulled of the cassette and rinsed with double distilled water twice. The gel was stained overnight using 0.2% Coomassie stain after fixing. After carrying out destaining of gels gel documentation was done[11, 12].

In Gel Tryptic digestion:

Gel bands were excised from control and treatment groups with the help of sterile scalpel blade. Gel bands were cut into small pieces (approx. 1mm^3) destained with 100mM NH_4HCO_3 in 50% acetonitrile (ACN) at room temperature. The proteins were reduced with 10mM dithiothreitol (DDT) (56°C; 30 min) and alkylated with 50mM iodoacetamide in 100mM NH_4HCO_3 (dark, room temperature, 30 min). The gel pieces that contained proteins were dried and then incubated in the digestion solution (40mM NH4HCO3, 9% ACN, and 20 μg/mL trypsin; 18 h, 37◦C). The tryptic peptides were extracted with 50% ACN/2.5% TFA and lyophilized for storage till further analysis via Mass Spectroscopy [13].

2-D PAGE:

Sample preparation:

Protein samples were isolated from treated and control cells and total protein was isolated. Three separate cell pellets from sub confluent cultures were lysed with a lysis buffer1 containing 10mM Tris, pH7.5, 1mM EDTA, and 5mL Protease Inhibitor Cocktail. The resulting suspension was pipette 30 times up and down. Afterwards, the homogenate was mixed with lysis buffer 2, containing 7 M urea, 2 M thiourea, and 4% CHAPS, and 40mM DTT. The protein concentration was measured according to the folin lowry method [14].

IEF and SDS-PAGE

Commercial pH 3 to 10 immobilized pH strips (Biorad) were used for this using PROTEAN® i12™ IEF system. Lyophilized isolated proteins (250 μg) was thawed and diluted in IPG sample buffer containing 8M urea, 2%CHAPS, 50 mM dithiothreitol(DTT), and 0.2% (w/v) Bio-Lyte® 3/10 ampholytes, and Bromophenol

Blue (trace) as per the instruction manual of ReadyPrep™ 2-D Starter Kit. Running conditions for IEF were the following: 250V, 20min; 4000V 2 hr; 4000Vat a rate of 10,000 V-hr for 2.5 hr. After electrophoresis, IPG strips were stored at –80°C.

IPG strips were thawed for 15 min at room temperature and then equilibrated with equilibration buffer I containing 0.375 M Tris-HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, and 2% (w/v) DTT for 10 min for incubation in shaker. Equilibration buffer I replaced with the equilibration buffer II containing 0.375 M Tris-HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, and 2.5% (w/v) iodaacetamide and kept for incubation in shaker for 10 min.

SDS-PAGE gels used were 1mm thick, 12% homogeneous lab cast gels in Mini-PROTEAN® Tetra Cell as par the instruction manual. Individual strips were placed over the casted gels and then overlayed with the low melting agarose. Running conditions for the PAGE was kept at constant voltage at 200 V at with approximate run time was 40 min[15, 16].

Visualization and image analysis: Gels were stained with coomassie brilliant blue and visualized in Biorad Gel Doc X1R and image analysis was done using FLICKER.

Protein identification and data analysis: MS data were converted to suitable format and the list of masses containing all the fragment information was submitted to Mascot (Matrix Science version 2.1) in order to identify the proteins using the International Protein Index (IPI) database for human proteins (IPI human, version 3.38), plus common contaminants such as trypsin and BSA. The search was performed using the following parameters: maximum of three missed trypsin cleavages, carbamidomethylation (Cys) as fixed variation, oxidation (Met) and acetylation (N-terminal of the protein) as variable modifications, and mass accuracy of 0.2 Da. Peptides with a minimum Mascot score of 38 indicate identifications with an error of less than 5% ($p < 0.05$). Proteins matching at least two peptides by Mascot were accepted automatically while identifications on the basis of only one peptide were accepted if the score was at least twice the threshold value for acceptance of MS/MS sequenced peptides and using an MS/MS fragment of at least 7 amino acids, and after manual validation. Spectra and protein validation were performed using open source software called MS Quant, extensively used for MS data analysis [17].

Statistical analysis:

All statistical analyses were performed using GraphPad Prism. We used the ANOVA method with Duncan's and Tukey's test to identify differences between the exposure and control groups. A $p<0.05$ was considered to indicate statistical significance in all cases.

Results:

Cells were incubated with different concentrations of PXT (30, 40, 50 and 60 mM) for 24h and 48h. MTT assays were then conducted to investigate the cytotoxicity (Fig.1). The MTT assay of cells exposed to concentrations of PXT for 24 and 48 h revealed no significant difference in cell growth ($p>0.05$). However, PXT concentrations greater than 50 mM led to a reduction in the cell population by half ($p>0.05$). Based on the MTT assays, 50mM PXT was used for further experiments.

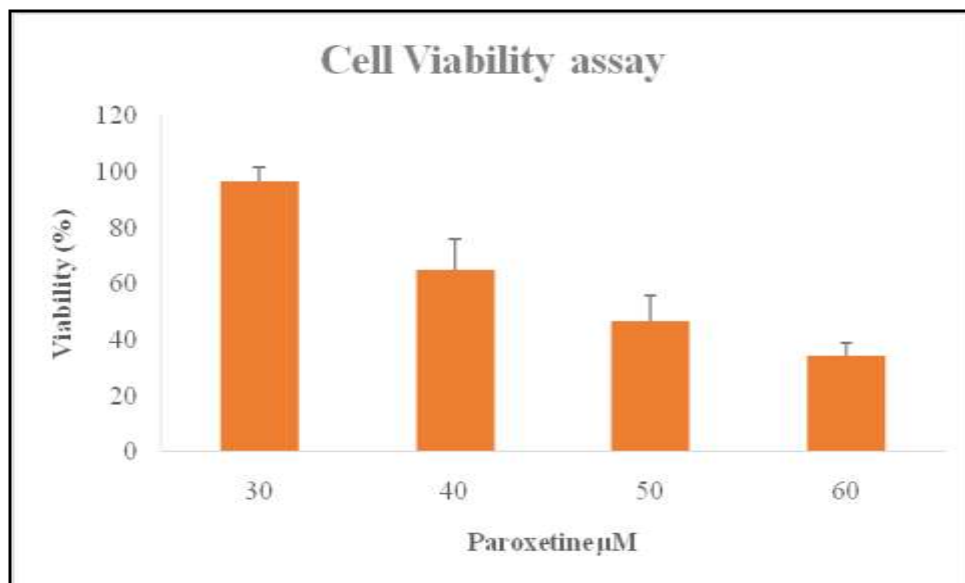

Figure 1 MTT assay

27 down-regulated and 30 up-regulated proteins which may serve as specific biomarkers of hepatotoxicity. Alpha-2-HS-glycoprotien expression was significantly decreased during Paroxtine treatment (all groups of treatment) in contrast to control group ($P<0.05$). This protein is a known carrier exclusively produced by the liver and absence of this protein leads to spontaneous tissue calcification.

Hemopexin expression showed a modest, though significant, decline ($P<0.05$) and was also significantly decrease during treatment in contrast to control group. Hemopexin forms the second line defence in case of haemolysis. Hemopexin shows high-affinity for free-circulating heme-groups and transports them to the liver for iron re-uptake.

Serotransferrin expression was significantly increased during Paroxtine treatment (all groups of treatment) in contrast to control group ($P<0.05$). Serotransferrin (transferrin) is an abundant blood plasma glycoprotein which is made in the liver. Its main function is to bind and transport iron throughout the body. Table 1 enlist all the proteins with change in expression.

Table 1 differentially expressed proteins in hepatocytes after paroxetine exposure

Uniprot Accession No	Protein description
P14847	C-reactive protein
Q61646	Haptoglobin
P07724	Serum albumin
Q91X72	Hemopexin
Q00897	Alpha-1-antitrypsin 1-4
P29699	Alpha-2-HS-glycoprotein
Q61147	Ceruloplasmin Complement C3
P01027	Complement C3 precursor
P32261	Antithrombin-III
O88947	Coagulation factor X
Q71KU9	Fibrinogen-like protein
Q9R182	Angiopoietin-related
Q00623	Apolipoprotein A1
P06728	Apolipoprotein A4 precursor

P08226	Apolipoprotein E precursor
P60710	Actin, cytoplasmic 1
Q8C196	Carbamoyl-phosphate
Q8R0Y6	formyltetrahydrofolate dehydrogenase
Q9DBE0	Cysteine sulfinic acid decarboxylase
P17182	Alpha-enolase
P29391	Ferritin light chain 1
P56395	Cytochrome b5
Q9QXD6	Fructose-1,6 bisphosphatase
P47876	Insulin-like growth factor binding protein
P27773	Protein disulfide isomerase precursor
Q03734	Serine protease inhibitor A3M
Q9DBB8	Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase
Q9JLJ2	4-trimethylaminobutyraldehyde dehydrogenase
Q99KI0	Aconitate hydratase, mitochondrial precursor
Q9Z2I9	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial
P16332	Methylmalonyl-Coenzyme A mutase
Q8BWY3	Eukaryotic peptide chain release factor subunit 1
P49722	Proteasome subunit alpha type-2
O88685	26S protease regulatory subunit 6A
P24472	Glutathione S-transferase 5.7
P15626	Glutathione S-transferase Mu 2
O35660	Glutathione S-transferase Mu 6
P19157	Glutathione S-transferase P 1
Q9WVL0	Maleylacetoacetate isomerase
P08228	Superoxide dismutase [Cu-Zn]
P09671	Superoxide dismutase
Q9QXF8	Glycine N-methyltransferase
Q00896	Serpina1c Alpha-1-antitrypsin 1-3
Q00897	Serpina1d Alpha-1-antitrypsin 1-4 precursor
P00920	Carbonic anhydrase 2
Q01768	Nucleoside diphosphate kinase B
Q63836	Selenbp2 Selenium-binding protein 2
Q9QYG0	Isoform 1 of Protein NDRG2

Discussion:

Paroxetine is a selective serotonin reuptake inhibitor (SSRI) used in the therapy of depression, anxiety disorders and obsessive-compulsive disorder is known to cause transient elevations in serum ALT levels and linked to acute liver injury. Paroxtine cause severe hemotoxicity and damage in endothelial cells. But its affect on liver cells was very less studied, we exposed animals to dose equivalent to clinical dose and tried to observe the changes in protein expression in liver. Elevated levels of AST and ALT are indications of hepatocellular injury (Yue). In the present study Hemopexin was identified as a possible marker following 1-D SDS PAGE MS analysis and 2D Gel electrophoresis. This enzyme serves scavenger for the heme/haemoglobin. While heme easily enters into endothelial cells when bound to albumin, this

translocation is completely blocked in the presence of Hx. Heme sequestration within the Hx complex further ensures protection against heme-driven oxidative processes in the extracellular space and it prevents heme-triggered inflammation and adhesion molecule expression.

Hb toxicity largely depends on the rate of hemolysis, tissue oxidant status and clearance capacity. In this network, Heptoglobin acts in concert with the plasma's small molecular reducing agents that maintain Hb in a reduced, less reactive ferrous (Fe^{2+}) oxidation state.

Objective of this study was to study the changes in protein profile of the liver when animals were exposed to paroxetine. This type of studies will help in understanding the pathways by which the paroxetine cause liver damage and can help in development of markers for early identification of toxicity, so that preventive steps can be taken to reduce the damage and continue the therapy with alternative drugs. The widely available markers such as AST, ALT intend to show the cell damage which is not limited to hepatic cells. An extensive literature search was carried out for evidence in support of the protein expression changes observed. However, some of the protein changes offer less obvious rationales, but they may lead to new insights into the mechanism of hepatotoxicity. Due to the technical limitations of the approach used, at present, in terms of basic experimentation, instrument availability and sensitivity, it was not possible to examine the entire proteome of the mice liver. It is worth noting that it was not possible to identify all the proteins with the staining methods used.

Nevertheless the identification of biomarkers, which may be of use in diagnostic high throughput assays, may be possible.

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