



## Carlinside reduces hepatic bilirubin accumulation by stimulating bilirubin-UGT activity through Nrf2 gene expression

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### ABSTRACT

Accumulation of bilirubin, primarily because of its insolubility, has been found to be associated with liver diseases including jaundice. Free bilirubin is insoluble; its glucuronidation by bilirubin-UGT enzyme (UGT1A1) makes it soluble and eliminates it through urine and faeces. Taking CCl<sub>4</sub> induced rat liver dysfunction model, we demonstrated that suppression of UGT1A1 activity in rat liver increased serum bilirubin level which could be reversed by carlinside (Cln), a flavone glycoside. Although Cln is a flavone compound, it escaped self-glucuronidation in the intestine and readily absorbed. Kinetic study of microsomal UGT1A1 from HepG2 cells suggested that Cln enhanced enzyme activity by increasing  $V_{max}$  without altering  $K_m$ . This altered  $V_{max}$  was found to be due to UGT1A1 overexpression by Cln which was observed in both HepG2 and rat primary hepatocytes. Since Nrf2 is the transcription factor of UGT1A1, we examined whether Cln effect on UGT1A1 overexpression is mediated through Nrf2. In Nrf2 knock-out cells, Cln could not elevate UGT1A1 activity indicating Nrf2 to be its target. Cln significantly increased Nrf2 gene expression in HepG2 cells which was subsequently localized in nuclear region. Results from ChIP assay showed that Cln markedly augmented Nrf2 binding to UGT1A1 promoter that consequently enhanced reporter activity. Our findings therefore show that Cln upregulated Nrf2 gene expression, increased its nuclear translocation and stimulated UGT1A1 promoter activity. Total outcome of these events brought about a significant increase of bilirubin glucuronidation. Cln therefore could be a worthy choice to intervene hyperbilirubinemia due to liver dysfunction.

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## 1. Introduction

In mammals, bilirubin is the most toxic metabolites that require to undergo detoxification in the liver to prevent its increased accumulation. Bilirubin is produced in large quantities from the normal turnover of hemoglobin and other hemoproteins, however during normal condition human body has evolved an efficient detoxification mechanism to eliminate it. Bilirubin behaves like a lipophilic compound; this imposes its insolubility that adversely affects its excretion [1]. Glucuronidation of bilirubin makes it soluble and renders its excretion through bile and urine [2,3]. Bilirubin conjugation reaction with glucuronic

acid is catalyzed by Bilirubin-UDP-glucuronosyltransferase 1A1 (UGT1A1) or Bilirubin-UGT [4], one of the 19 members of the UGT gene family [5]. UGT gene expression is regulated by an antioxidant response element (ARE) in their promoter region [6]. A transcription factor Nrf2, one of the members of NF-E2 family of nuclear basic leucine zipper transcription factors [7], which binds to ARE region and regulates UGTs transcription. In Nrf2<sup>-/-</sup> mice UGTs expression is greatly impaired in liver and gastrointestinal tract [8,9] that leads to accumulation of bilirubin [10]. Since persistent hyperbilirubinemia may cause serious health problems including death, glucuronidation of bilirubin by UGT1A1 is a vital requirement. Therefore, a search for UGT1A1 inducer becomes extremely important.

At one time only choice to deal with the accumulation of insoluble bilirubin was to treat with phenobarbital barbiturate. Earlier reports with phenobarbital have demonstrated that it stimulates UGT1A1 activity thereby enhanced rapid glucuronidation reaction thus converting bilirubin to its readily excretable glucuronidated form [11]. Phenobarbital has considerable side

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effects, better alternative to it includes, dietary flavones which do not have problem of side effects and are considered suitable to treat hyperbilirubinemia [12,13]. One of the important flavonoids belonging to this class is chrysin (5,7-dihydroxyflavone); chrysin has been shown to induce UGT1A1 expression in liver cells and greatly enhanced bilirubin glucuronidation in HepG2 cells [14,15]. Moreover, there are convincing evidences on chrysin's ability to induce UGT1A1 expression in tissue culture models [16]. However, transgenic mice that expresses human UGT1 locus, when orally treated with chrysin showed no effect on liver UGT1A1 expression because of the problem of bioavailability [17,18]. Majority of the beneficial flavonoids are found to be the substrates of the intestinal UGTs as in the case of chrysin and that created problems for their bioavailability [13,17]. This has restricted their use to augment important phase 2 antioxidant enzyme families like UGTs. Hence, there is a crucial need to have a safe, effective and bioavailable compound that can deal hyperbilirubinemia problem.

In this report we describe that Carlinoside (Cln), a dietary flavone glycan, readily eliminates insoluble bilirubin through the process of glucuronidation by augmenting UGT1A1 activity in CCl<sub>4</sub> induced hyperbilirubinemic rat model. By taking human hepatoma cell line HepG2 and rat primary hepatocyte (PH) we have shown that Cln overexpresses UGT1A1 by inducing Nrf2 gene, this causes a significant increase in its activity thereby converting insoluble bilirubin to its soluble form. Cln therefore could be an attractive drug candidate to deal hyperbilirubinemia.

## 2. Materials and methods

### 2.1. Chemicals and reagents

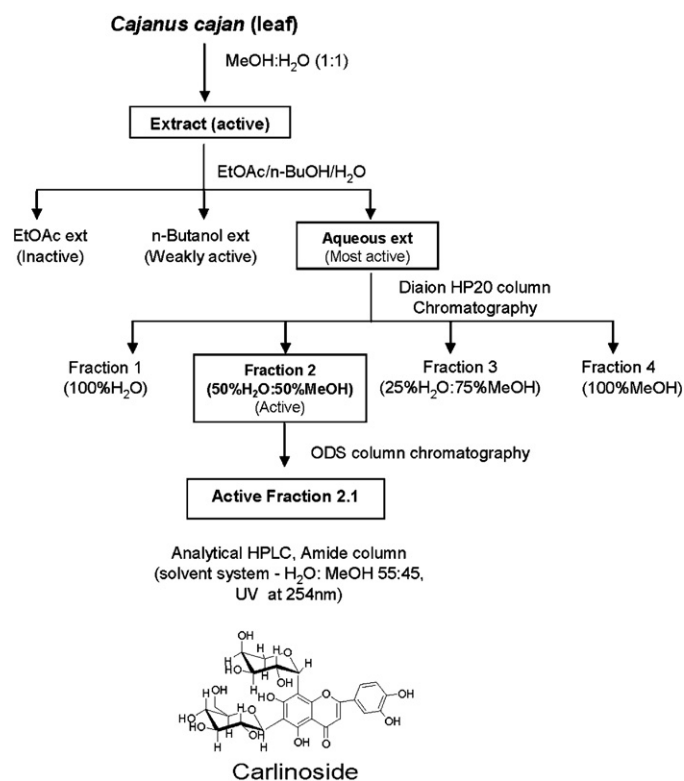
Uridine 5'-diphosphoglucuronic acid triammonium salt, hemin, isocitrate dehydrogenase and PCR primers for UGT1A1, Nrf2 and HO-1 were purchased from Sigma Chemical Co. (St Louis, MO, USA). [<sup>3</sup>H]-Leucine (specific activity 1000 Ci/mmol) was obtained from GE Healthcare Biosciences Ltd., Kowloon, Hong Kong. Assay kits for bilirubin, GPT and GOT were procured from Span Diagnostics, India. Carbon tetrachloride (CCl<sub>4</sub>), n-Butyl acetate, Ethyl anthranilate, Pentane 2-one, isocitrate and NADPH were purchased from E. Merck, Darmstadt, Germany. All tissue culture materials were obtained from Gibco BRL, Life Technologies Inc., Gaithersburg, USA. Primary antibodies for anti-UGT1A1 (anti-goat), anti-Nrf2 (anti-rabbit), anti-Keap1 (anti-goat), anti-HO-1 (anti-goat) and alkaline phosphatase conjugated anti-goat, anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology Inc., CA, USA. Control (Cat. No. sc-37007) and Nrf2 siRNA (Cat. No. sc-37030) were also purchased from Santa Cruz Biotechnology Inc., CA, USA. All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

### 2.2. Isolation of carlinoside from *Cajanus cajan* leaf

The fresh mature leaves of *C. cajan* were pulverized in a motor driven grinder with methanol-water (MeOH:H<sub>2</sub>O, 1:1) followed by our earlier report [19] and then fractionated through Diaion HP20 and ODS column chromatography. The eluent was loaded into an analytical HPLC amide column which gave a single peak in analytical Symmetry C18 column with water:methanol (55:45) as solvent and detection at 254 nm. The peak was identified as Carlinoside, by 1D, 2D NMR and Q-TOF-MS and making a similar derivative (Fig. 1).

### 2.3. Animals and treatment

Adult male albino rats of the Sprague-Dawley strain weighing 180–220 g were maintained under standard laboratory conditions



**Fig. 1.** Flow chart of carlinoside (Cln) isolation. Methanol extract of *C. cajan* leaves was chromatographed through HP-20 Diaion and ODS column followed by HPLC in Symmetry C-18 column which was eluted with water:methanol (55:45) solvent. Structure of Cln is provided in the lower panel.

in clean polypropylene cages (2–3 animals/cage) with food and water made available *ad libitum*, under 12 h light/dark cycle at  $25 \pm 2$  °C. Animals were acclimated about one week prior to use. For analysis of Cln effect on CCl<sub>4</sub> induced liver damage, 5 rats per group were administered CCl<sub>4</sub> at 0.25 ml/kg bw with corn oil (1:1, v/v) i.p. every alternate day for 28 days and observation was continued till 42 days without further administration of CCl<sub>4</sub>. At day 28, Cln was administered at a dose of 25 mg/kg bw to one group of CCl<sub>4</sub> treated rats through oral gavage. On termination of the experiments rats were sacrificed and blood samples were collected for estimation of serum bilirubin and liver function marker enzymes, GPT and GOT. Liver was perfused with ice-cold 1.15% KCl and immediately frozen on dry ice and stored at  $-80$  °C for further studies. Liver tissues were also kept for histological analysis. To determine bioavailability, 5 rats were administered with a single oral dose of 50 mg/kg Cln. Blood was collected and liver was taken out after 4 h to determine the amount of Cln through HPLC analysis. All experiments were performed following the guidelines prescribed by the Animal Ethics Committee at Visva-Bharati University, Santiniketan, India.

### 2.4. Cell culture and treatment

The human hepatoma HepG2 cell line was a kind gift from National Centre for Cell Science, Pune, India and Dr. Partha P. Banerjee, Georgetown University Medical Centre, Washington DC, USA. HepG2 cells were cultured in MEM (minimum essential medium) containing Earle's salts and non-essential amino acids supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere at 37 °C [20]. For treatments, CCl<sub>4</sub> was dissolved in 10% DMSO and used at concentrations of 6 mM in HepG2 incubations along with 10 µM

Cln for 24 h or as per the design of the experiment. In all the cases Cln was used 1 h prior to CCl<sub>4</sub> treatment.

Primary culture of hepatocytes was performed by isolating the cells from male Sprague-Dawley rat liver using collagenase perfusion as described previously [21]. The initial hepatocyte viability measured by trypan blue exclusion was always >90%. Cells were cultured in MEM containing 10% FBS and treated with 10  $\mu$ M Cln. Control and treated cells were lysed in lysis buffer (1% NP-40, 20 mM HEPES (pH 7.4), 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml pepstatin and 1 mM PMSF) and centrifuged for 10 min at 10,000  $\times$ g. Nuclear extract was prepared following a previously described method [22]. Amount of proteins in the samples were determined by the method of Lowry et al. [23].

## 2.5. Measurement of bilirubin-UGT activity

Microsomes were prepared from rat liver and HepG2 cells following a method described previously [24]. Microsomal protein was resuspended in MOPS buffer, pH 7.2 (20 mM MOPS, 100 mM KCl, 20 mM NaCl, 3 mM MgCl<sub>2</sub>) at a concentration of 10–20 mg protein/ml and frozen under liquid N<sub>2</sub> until required. Bilirubin-UGT activity was therefore determined using microsomal protein by the method described by Seppen et al. [25], with modifications. Briefly, bilirubin dissolved in 0.2 N NaOH and bound to BSA (final concentration: 0.34 mM bilirubin, 8 mg/ml BSA) was incubated in a mixture containing 20 mM Tris-Cl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM saccharolactone, 0.1% Triton X-100 and 1 mg of microsomal protein/ml in a final volume of 200  $\mu$ l. The reaction was initiated by addition of UDP-glucuronic acid with 5 mM final concentration and incubated for 30 min at 37 °C. The reaction was stopped by addition of 300  $\mu$ l glycine-HCl buffer (0.4 M HCl, pH adjusted to 2.7 with glycine) and the samples were diazotized and extracted following the method of Heirwegh et al. [26]. 1.5 volumes of diazo-reagent (0.1 ml of ethyl anthranilate, 0.3 ml of 70 mM NaNO<sub>2</sub>, and 0.1 ml 88 mM ammonium sulfamate in 10 ml of 150 mM HCl) was added to the mixture for 30 min at room temperature. The diazo reaction was stopped by adding 1 volume of freshly prepared ascorbic acid solution (570 mM). The bile pigment formed was extracted by vigorous shaking with 3 volumes of methylpropylketone:n-butyl-1-acetate (17:3, v/v) and the absorbance of the organic phases were measured at 530 nm. The amount of bilirubin glucuronides was determined from the  $E_{530}$  value,  $E_{530} = 44,400 \text{ M}^{-1} \text{ cm}^{-1}$ .

## 2.6. Measurement of heme oxygenase activity

Heme oxygenase (HO) activity was determined according to a previously described method [24] with little modification. Briefly, the reaction mixture (6.0 ml) contained microsomes (10 mg protein); 105,000  $\times$  g supernatant (6 mg of protein); 17  $\mu$ M hemin; 180  $\mu$ M NADPH; 0.85 mM isocitrate, 0.2 mg/ml of isocitrate dehydrogenase and 0.1 M potassium phosphate buffer, pH = 7.4. The reaction mixture was divided equally and added to two cuvettes, incubated for 5 min at 37 °C, reaction was initiated by adding NADPH (in 0.4 mM NaHCO<sub>3</sub> final concentration) to the sample cuvette and equal amount of NaHCO<sub>3</sub> in the reference cuvette. HO activity was estimated on the basis of bilirubin formation at 468 nm and HO activity was calculated from the millimolar extinction coefficient,  $E_{468} = 57 \text{ mM}^{-1}$ .

## 2.7. Electrophoresis and immunoblotting

60  $\mu$ g protein from the lysates of control and treated HepG2 cells or rat PH was resolved on 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA) with the help of semi-dry trans

blot apparatus (Bio-Rad Trans-Blot<sup>®</sup> SD Cell, USA) using transfer buffer (25 mM Tris, 193 mM glycine, 20% methanol, pH 8.5). The membranes were first incubated with primary antibodies of UGT1A1 (goat polyclonal, 1:1000), Nrf2 (rabbit polyclonal, 1:1000), Keap1 (goat polyclonal, 1:1000) and HO-1 (goat polyclonal, 1:1000) followed by corresponding ALP-conjugated secondary antibodies at 1:1000 dilution using SNAP i.d. (Millipore, Bedford, MA). The protein bands were detected by using 5-bromo 4-chloro 3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

## 2.8. Reverse transcription PCR and real-time quantitative PCR

Total RNA was extracted from control and treated HepG2 cells or rat PH using the TRI Reagent. RT-PCR was performed using First Strand cDNA Synthesis Kit, Fermentas Life Sciences, Revert Aid<sup>™</sup>, Hanover, MD, USA, following a previous description from this laboratory [20,27]. Alteration in UGT1A1 and Nrf2 gene expression was observed using their specific primers (human UGT1A1 forward: 5'-AACAAGGAGCTCATGGCTCC-3'; reverse: 5'-GTTCGCAAGATTC-GATGGTCG-3'; human Nrf2 forward: 5'-TGCCCCCTGGAAGTGT-CAAACA-3'; reverse: 5'-CAACAGGGAGCTTAATGATTT-3' [28]; human HO-1 forward: 5'-AACTTTCAGAAGGGCCAGGT-3'; reverse: 5'-CTGGGCTCTCTTGTTC-3'; rat UGT1A1 forward 5'-CCACC-CAATGCCCTGCT-3', reverse: 5'-CACCATTGGAACCCATTGC-3'; rat HO-1 forward: 5'-CACCAGCCACACAGCACTAC-3', reverse: 5'-CACC-CACCCCTCAAAAGACA-3'; *gapdh* forward: 5'-GCCATCAAC-GACCCCTTC-3'; reverse: 5'-AGCCCCAGCCTTCTCCA-3'). Alteration in gene expression was confirmed further by real-time PCR using Power SYBR green PCR master mix (Applied Biosystems, CA, USA). 3  $\mu$ g of total RNA from each sample was reverse transcribed and PCR was performed with gene specific primers in a total volume of 20  $\mu$ l in the following real-time PCR conditions: initial activation step (95 °C, 15 min), cycling step (denaturation 95 °C, 30 s, annealing at 55 °C, 30 s, and finally extension for 72 °C, 30 s, 40 cycles) followed by their melt curve analysis (55–60 °C, 15 s, 40 cycles). A house keeping gene, *gapdh*, was simultaneously amplified in separate reactions and acted as an internal control. The Ct value was corrected using corresponding Ct value of *gapdh* controls. Data from 3 determinations (means  $\pm$  SEM) are expressed as relative expression level.

## 2.9. Nrf2 siRNA interference assay

HepG2 cells ( $1 \times 10^6$  cell/well) were transfected with 80 pmoles of control or Nrf2 siRNA (Santa Cruz Biotechnology Inc., CA, USA) using lipofectamine<sup>™</sup>2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 48 h of transfection, cells were incubated with 10  $\mu$ M of Cln. At the end of incubation cells were harvested and analyzed for Western blot and RT/qPCR analysis.

## 2.10. [<sup>3</sup>H]-Leucine incorporation study

HepG2 cells were serum starved in Krebs's Ringer Phosphate (KRP) buffer supplemented with 0.2% bovine serum albumin. To determine the amount of UGT1A1 protein synthesis, untransfected or Nrf2 siRNA transfected HepG2 cells were incubated without or with Cln in the presence of 10  $\mu$ Ci/ml [<sup>3</sup>H]-leucine for 0–12 h time period. Cells were lysed and supernatant was pulled down by using anti-UGT1A1 antibody following our previous description [20]. Radioactive count was measured in liquid scintillation counter (Perkin Elmer, Tri-Carb 2800TR).

## 2.11. Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to our previous description [29] using a ChIP assay kit (Upstate, Temecula, CA, USA). Briefly, 70%

confluent HepG2 cells ( $1 \times 10^6$  cells/well) were incubated with  $10 \mu\text{M}$  Cln for 8 h, and then fixed in 1% formaldehyde for 10 min at  $37^\circ\text{C}$ . Cells were lysed, nuclei isolated by centrifugation and chromatin was sheared using a sonicator. Chromatin was immunoprecipitated with  $2 \mu\text{g}$  of anti-Nrf2 or control IgG antibody (Santa Cruz Biotechnology Inc., CA, USA). The primers for human *UGT1A1*-ARE promoter [30] sequence (*UGT1A1* forward: 5'-GTACTTGCTGTGGTACCTCCAGAAT-3' and reverse: 5'-GGCGCCTTGCTCCTGCTCGAGGTTC-3') was used for amplification of immunoprecipitated DNA. The PCR products were resolved on ethidium bromide stained 1.5% agarose gel and image was captured by Bio-Rad gel documentation system using Image Lab software.

## 2.12. Reporter assay

*UGT1A1* promoter-luciferase reporter construct was the kind gift provided by Prof. Chantal Guillemette of Laval University, Quebec, Canada and pNFE2-luc construct containing the 930 bp promoter region (Chr. 12q13:52980872-52981801) of the human Nrf2 gene (Gene ID: 4778) was procured from Switch Gear Genomics, Menlo Park, CA, USA. HepG2 cells were seeded in a 24 well plate ( $1 \times 10^5$  cells/well) and were grown overnight to 50% confluence one day before transfection. Cells were then transfected with 750 ng of the reporter constructs along with 5 ng of Renilla luciferase plasmid using Lipofactamine™ 2000. Following 48 h of transfection, cells were treated with Cln ( $10 \mu\text{M}$ ). Luciferase

activity was determined using Promega Dual-Luciferase Reporter Assay System.

## 2.13. Immunofluorescence assay

Control or treated HepG2 cells were incubated with anti-rabbit Nrf2 antibody (1:100) for 2 h followed by immunostaining with FITC-conjugated goat anti-rabbit antibody (1:1000) for 1 h. Nucleus was stained with propidium iodide (Invitrogen, Carlsbad, CA, USA) following manufacturer instructions. Coverslips were mounted in glycerol/PBS and images were captured in fluorescence microscope (Carl Zeiss, USA) using Jenoptik software (ProgRes® CapturePro 2.7).

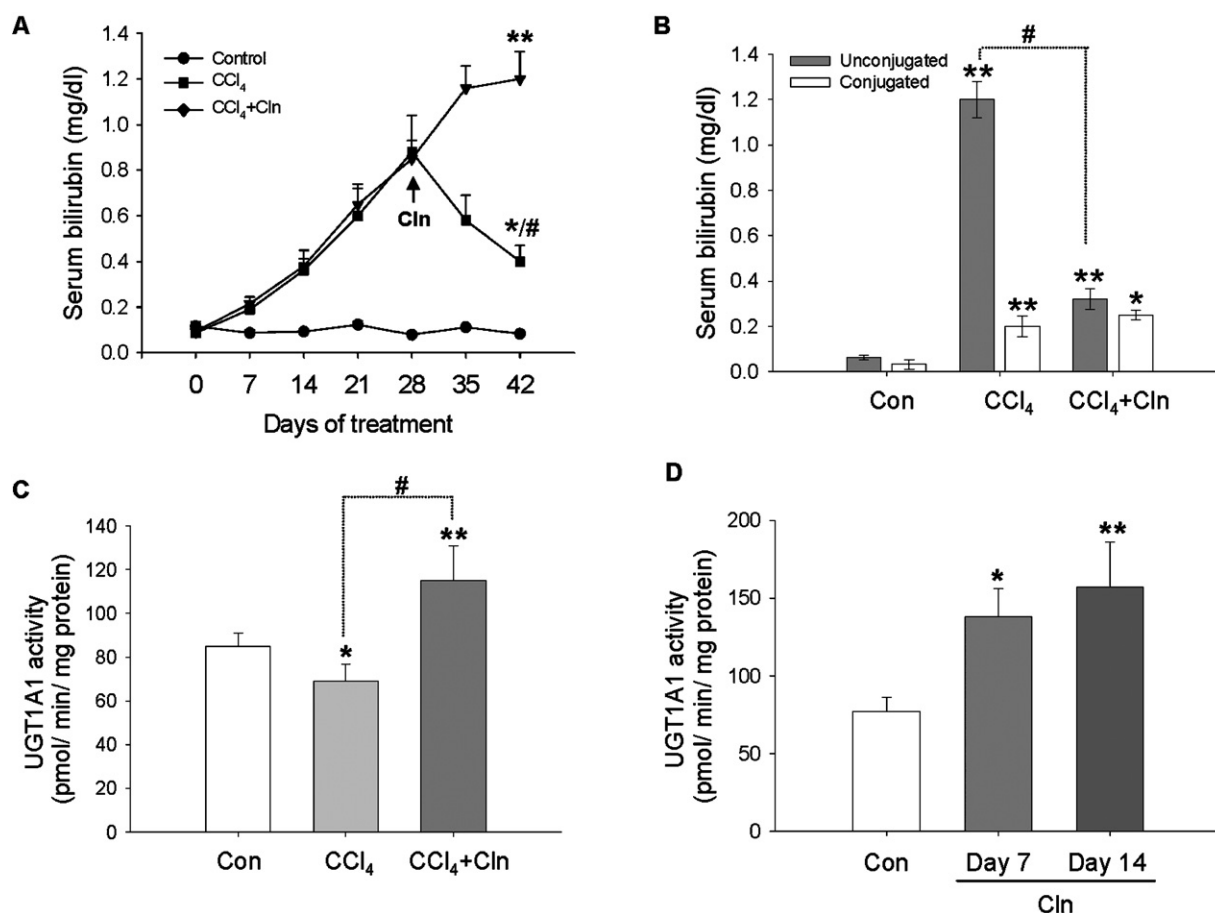
## 2.14. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) where the F value indicated significance, means were compared by a post hoc multiple range test. All values were means  $\pm$  SEM.

## 3. Results

### 3.1. Cln reverses $\text{CCl}_4$ induced liver damage by stimulating bilirubin-UGT activity

$\text{CCl}_4$  is a well-established hepatotoxin and used in animal models to induce liver damage [31], for this reason we have



**Fig. 2.** Cln waives  $\text{CCl}_4$  induced accumulation of bilirubin and stimulates UGT1A1 activity in the liver. (A) Rats were administered  $\text{CCl}_4$  for 28 days and then treated with Cln by oral gavage up to 42 days. Blood was collected from control and treated rats on the days mentioned and serum samples were subjected for the estimation of total bilirubin. (B) Conjugated and unconjugated bilirubin was determined in serum of 42 days treated rats to see the extent of bilirubin glucuronidation. (C) Bilirubin-UGT activity was measured in microsomal samples from treated and untreated rat liver after 42 days of treatment. (D) Rats were treated orally with 25 mg/kg bw Cln and induction of UGT1A1 activity was determined using liver microsomal preparation on the days mentioned. Each experiment was performed in triplicate. Values are expressed as mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.05$  and \*\* $p < 0.001$  (vs. control), # $p < 0.001$  (vs.  $\text{CCl}_4$ ).



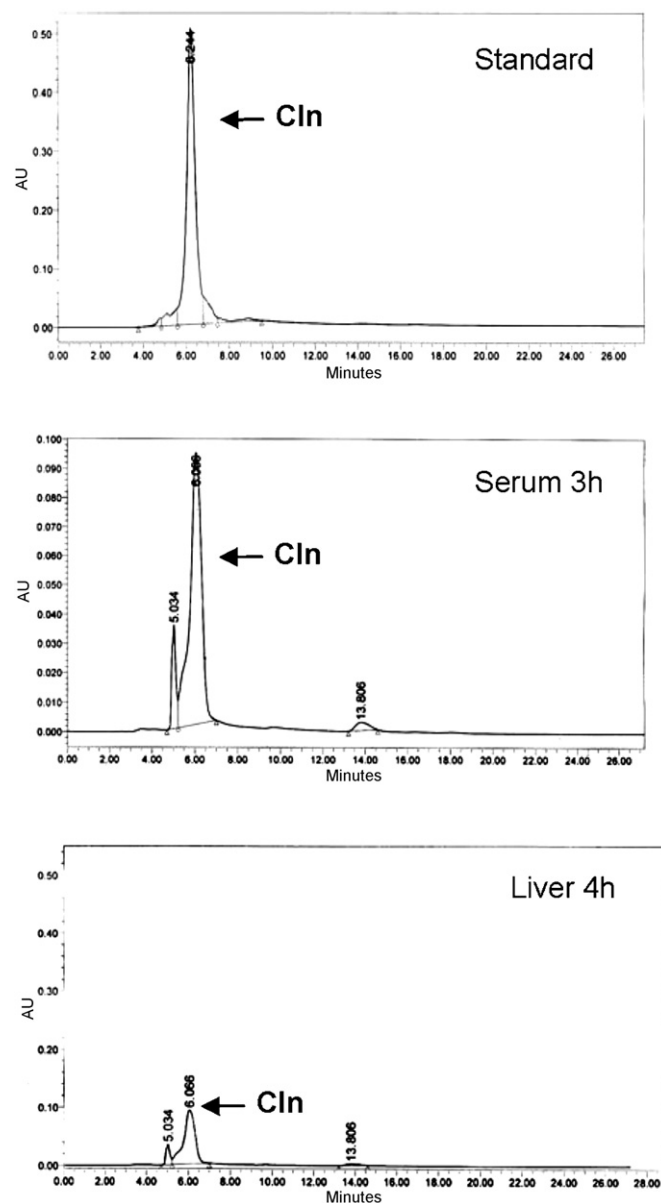
selected CCl<sub>4</sub> for our present study. Chronic administration of CCl<sub>4</sub> for 28 days resulted considerable damage to the liver. Liver histology due to CCl<sub>4</sub> treatment showed sinusoidal congestion, infiltration of inflammatory cells, perinuclear vacuolization in the hepatocytes and disintegrated hepatocytes, all these indicate serious damage to the liver. This was also reflected from the sharp increase of serum GPT and GOT activity (Fig. S1A–C). Administration of Cln to one group of CCl<sub>4</sub> treated rats on day 28 considerably improved liver histology as observed at day 42 and serum GPT and GOT activity within a week or two. These results suggest that Cln treatment effectively waived adverse effects of CCl<sub>4</sub> on liver. Chronic treatment of this dose of CCl<sub>4</sub> elevated serum bilirubin to a very high level till day 42 in a linear fashion while administration of Cln with one group of CCl<sub>4</sub> treated rats at day 28 drastically reduced serum bilirubin level on day 42 suggesting that Cln is efficiently removing bilirubin and most likely through solubilization (Fig. 2A). Since results with Cln implicate greater solubilization of bilirubin we determined unconjugated and conjugated levels of serum bilirubin with CCl<sub>4</sub> or Cln treated rat. CCl<sub>4</sub> greatly increased unconjugated bilirubin which was remarkably dropped in the presence of Cln ( $p < 0.001$ ) at 42 days of treatment (Fig. 2B). This effect of Cln was obtained within two weeks of administration because it was administered on day 28 on treated rats and effect was observed at 42 day.

Such a marked alteration from insoluble to soluble form of bilirubin due to Cln suggests a massive increase in glucuronidation reaction catalyzed by bilirubin-UGT enzyme (UGT1A1). We therefore determined Cln effect on UGT1A1 (bilirubin-UGT) enzyme activity with liver microsomal preparation from CCl<sub>4</sub> treated rats. CCl<sub>4</sub> significantly reduced UGT1A1 activity ( $p < 0.05$ ) while oral administration of Cln at day 28 greatly enhanced its activity when observed at 42 day (Fig. 2C). Such a robust increase in UGT1A1 activity by Cln in CCl<sub>4</sub> treated rats prompted us to investigate further to observe whether Cln alone could augment UGT1A1 activity. Fig. 2D demonstrates that 14 days Cln treatment stimulated UGT1A1 activity to more than 2 fold as compared to control. This appears to be intriguing but question remained on the mechanism through which Cln manifested UGT1A1 upregulation. This question we addressed through *in vitro* experiments with hepatocytes; prior to that we examined Cln bioavailability, as flavone compounds have a history of poor absorption through the intestine.

To observe its absorption through gastrointestinal tract, Cln was administered through oral route at a dose of 50 mg/kg bw. Fig. 3 shows that considerable amount of Cln could be detected in the serum at 3 h of oral administration and an appreciable quantity of it could be detected in the liver extract at 4 h. This shows that bioavailability would not be a problem with Cln as faced by other bioactive flavone compounds [18,32].

### 3.2. Stimulation of bilirubin UGT activity by Cln is due to its overexpression

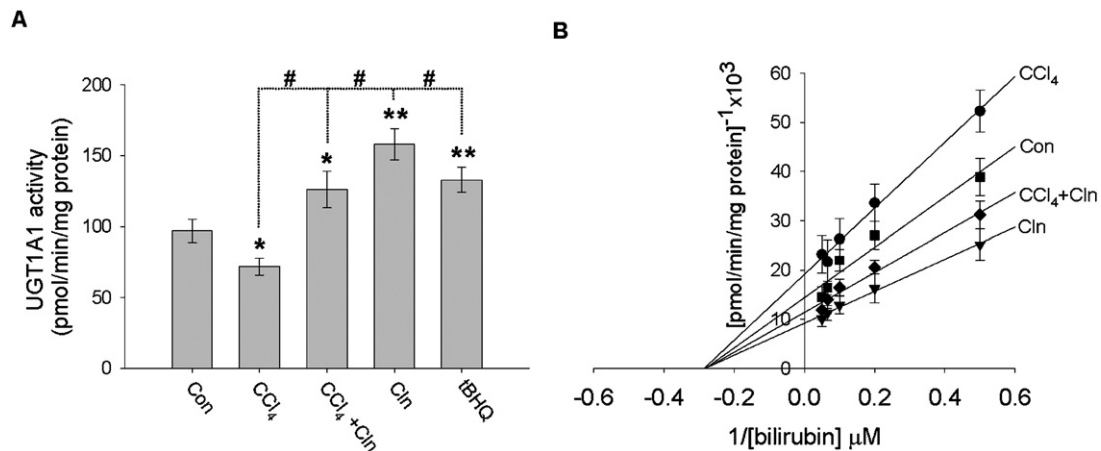
To understand the mechanism of Cln regulation of UGT1A1 activity, we performed experiments with human liver cell line, HepG2 and rat primary hepatocyte (PH). UGT1A1 activity was determined by using microsomal preparation from HepG2 cells incubated with CCl<sub>4</sub>, Cln or tBHQ. CCl<sub>4</sub> inhibition of UGT1A1 activity was not only suppressed by Cln, it further enhanced the activity significantly ( $p < 0.001$ ) (Fig. 4A). Similar trend of result was obtained with PH cells (Fig. S2A), indicating that Cln does not contradict CCl<sub>4</sub> but its induction for UGT1A1 expression overcomes CCl<sub>4</sub> inhibitory effect. We used tBHQ because of its known UGT inducing activity and compared with Cln to evaluate Cln efficiency for inducing UGT activity in HepG2 cells. It would be evident from Fig. 4A that Cln and tBHQ both effected about 1.5 fold increase in



**Fig. 3.** Cln efficiently absorbed in the serum and liver on oral administration. Rats ( $n = 5$ ) were administered with a single oral dose of 50 mg/kg bw Cln. Serum was collected and liver was taken out to analyze the presence of Cln through HPLC against a standard Cln peak. Figures are representative of one of three independent experiments.

UGT1A1 activity over the control, Cln effect was more prominent and significantly higher than tBHQ ( $p < 0.05$ ). A kinetic study on UGT1A1 demonstrated that  $K_m$  remained unaltered by CCl<sub>4</sub> or Cln, whereas  $V_{max}$  was decreased by CCl<sub>4</sub> ( $p < 0.01$ ) and considerably increased by Cln ( $p < 0.001$ ) over the control (Fig. 4B). These results led us to presume that Cln opposing effect of CCl<sub>4</sub> could be mediated through UGT1A1 expression. To examine this, we determined UGT1A1 protein expression.

Fig. 5A demonstrates that Cln overexpresses UGT1A1 protein while CCl<sub>4</sub> downregulates the same. Cln could effectively suppressed CCl<sub>4</sub> inhibitory effect on UGT1A1 expression. We performed immunoblot with HepG2 and PH cells incubated with CCl<sub>4</sub> or Cln or both. There was a significant increase of UGT1A1 expression from 5  $\mu$ M to 10  $\mu$ M, whereas 20  $\mu$ M of Cln did not produce considerable higher effect over 10  $\mu$ M (Fig. 5B). PH cells showed similar trend (Fig. S2B). HepG2 cells incubated with Cln

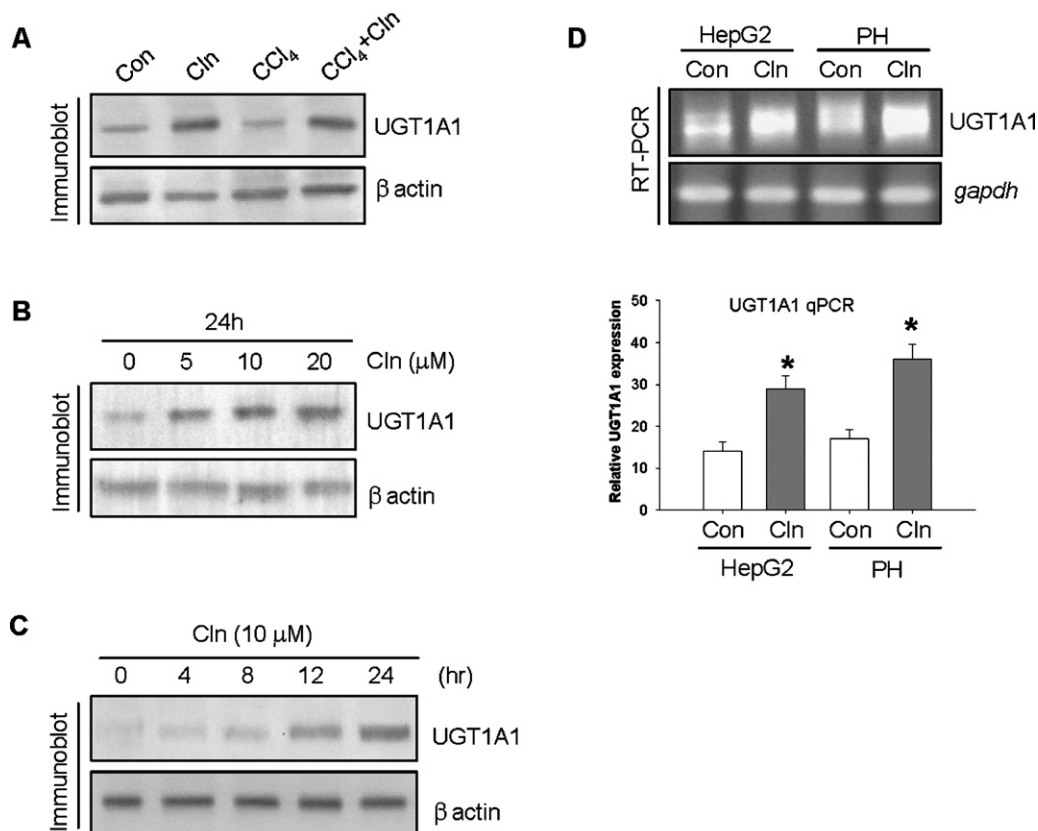


**Fig. 4.** Cln induces UGT activity by increasing  $V_{max}$ . (A) HepG2 cells were treated with or without CCl<sub>4</sub> (6 mM) or Cln (10 μM) or tBHQ (10 μM) for 24 h. Microsomal samples were prepared to determine UGT activity towards bilirubin. (B) Kinetic analysis of bilirubin-UGT activity was performed. Microsomal samples from HepG2 cells were prepared from the untreated (■) or CCl<sub>4</sub> (6 mM, ●) or Cln (10 μM, ▼) or CCl<sub>4</sub> + Cln (◆) treated incubations and used with varied concentrations of bilirubin (0–20 μM) in the presence of 5 mM UDPGA for each reaction. On termination of reactions amount of glucuronidated bilirubin was plotted using double reciprocal plot to analyze  $K_m$  and  $V_{max}$  values. Each value is the mean ± SEM of three independent experiments, \* $p$  < 0.05 and \*\* $p$  < 0.001 (vs. control), # $p$  < 0.001 (vs. CCl<sub>4</sub>).

for different time periods (from 0 to 24 h) and subsequent immunoblotting demonstrated a time dependent increase in the UGT1A1 expression (Fig. 5C). To examine whether this enhanced UGT1A1 protein expression by Cln was due to its induction of UGT1A1 gene, we performed RT-PCR and qPCR of UGT1A1 from Cln incubated HepG2 and PH cells. Cln greatly enhanced UGT1A1 gene expression ( $p$  < 0.01) in both the cases (Fig. 5D).

### 3.3. Cln augmentory effect on UGT1A1 is mediated through Nrf2

Overexpression of UGT1A1 may occur through Ah receptor or Nrf2. Since majority of reports indicated Nrf2 as the regulator of UGT1A1 gene, we checked this possibility through siRNA driven Nrf2 knockout HepG2 cells. Cln could not elevate UGT1A1 protein and gene expression in these cells while in untransfected or mock



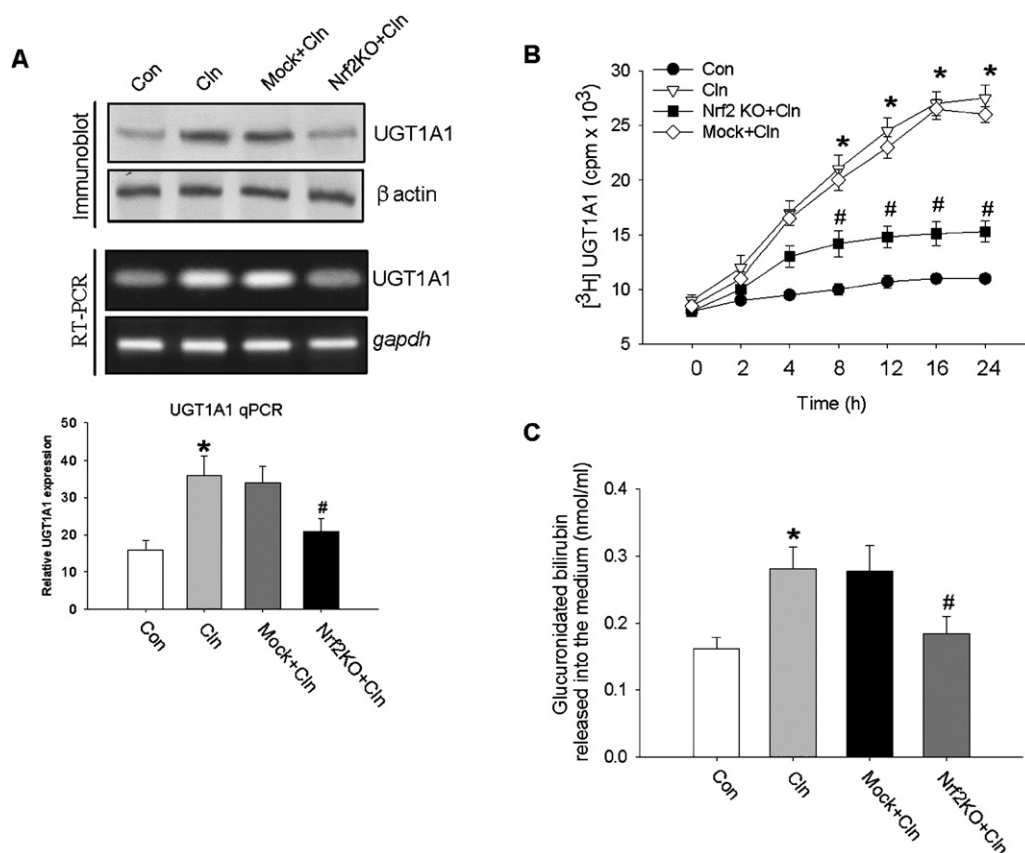
**Fig. 5.** Dose and time dependent increase of UGT1A1 expression by Cln. (A) HepG2 cells were incubated with or without CCl<sub>4</sub> (6 mM) or Cln (10 μM) or CCl<sub>4</sub> + Cln for 24 h. Microsomal sample was subjected to immunoblot analysis using anti-UGT1A1 antibody. (B) HepG2 cells were treated with or without Cln at different doses (5, 10 and 20 μM) for 24 h. Microsomal sample was immunoblotted with anti-UGT1A1 antibody. (C) UGT1A1 expression was observed for indicated time periods. β-Actin served as loading control. (D) HepG2 cells and primary hepatocytes (PH) were treated with or without Cln (10 μM) for 24 h. On termination of incubation, cells were lysed and total RNA was extracted for RT-PCR and real time PCR analysis of UGT1A1. GAPDH served as internal control. Figures are representative of one of three independent experiments. Mean ± SEM were calculated from 5 individual experiments. \* $p$  < 0.001 (vs. control).

siRNA transfected cells Cln effected massive increase of UGT1A1 expression (Fig. 6A). These results have following indications, (i) Cln stimulation of UGT1A1 expression is mediated through Nrf2, (ii) Cln has no direct effect on UGT1A1 expression and (iii) Cln effect possibly does not include Ah receptor pathway. Further work by monitoring UGT1A1 protein synthesis in Cln incubated HepG2 cells was studied in the presence of [ $^3$ H]-leucine and at each time point, cells from control or Cln incubations were lysed and immunoprecipitated using anti-UGT1A1 antibody attached to sepharose 4B beads. The precipitated protein was monitored for [ $^3$ H]-leucine incorporation in a time dependent manner as indicated. A linear increase in the Cln induced UGT1A1 protein synthesis could be observed till 12 h of Cln incubation which was considerably decreased in Nrf2 knockout cells (Fig. 6B). This shows that UGT1A1 protein synthesis is dependent on Nrf2. This was further evident from the increased bilirubin glucuronidation due to Cln which was sufficiently depressed in Nrf2 knockout cells (Fig. 6C).

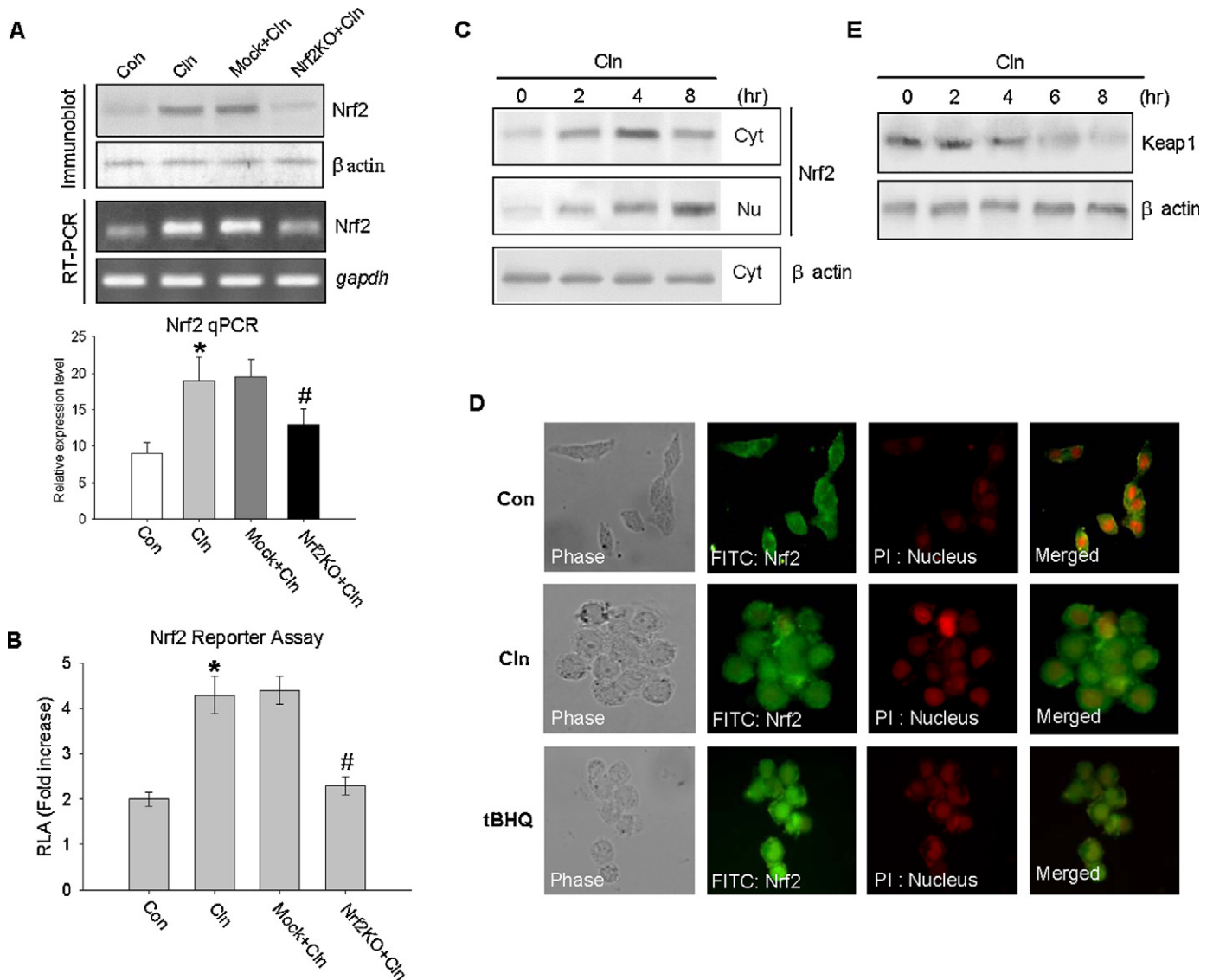
### 3.4. Overexpression of Nrf2 by Cln is associated with increased UGT1A1 promoter activation

To investigate Cln effect on Nrf2, we determined Nrf2 protein and gene expression in Cln incubated HepG2 cells and found Cln significantly enhanced its protein and gene expression. As anticipated, this effect of Cln was not seen in Nrf2 knockout cells (Fig. 7A). To provide additional evidence of Cln effect on Nrf2, we

performed Nrf2 reporter assay and found that promoter activity was considerably increased ( $p < 0.001$ ) due to Cln as compared to the control cells (Fig. 7B). Time dependent increase in the Cln induced Nrf2 protein expression and its distribution to the cytosolic or nuclear region was also analyzed in HepG2 cells. It would be evident from Fig. 7C that Nrf2 protein could be detected in the nuclear fraction at 2 h which was greatly increased at 8 h. Same results were obtained with immunofluorescence detection; massive accumulation of Nrf2 protein could be visualized in the nuclear region of Cln treated cells at 8 h whereas it was restricted to cytoplasm in control cells. Similar results were obtained with tBHQ (Fig. 7D). Excess of Nrf2 accumulation in the nuclear region could be due to enhanced Nrf2 expression and may also be for Keap1 degradation which stabilizes Nrf2 in the cytoplasm. It could be seen from Fig. 7E that Keap1 reduction in cytosol occurred between 6 and 8 h which corresponded with the higher Nrf2 in nuclear region at 8 h suggesting that enhanced Nrf2 nuclear localization is also associated with Keap1 degradation in response to Cln. Since silencing of Nrf2 blocked Cln induced UGT1A1 expression and activity, Nrf2 appears to be the target of Cln. However, heme oxygenase-1 (HO-1), another important Nrf2 target gene, is involved in the production of bilirubin from heme. Thus induction of HO-1 by Nrf2 may results in increased turn over of bilirubin, whereas Cln was found to reduce bilirubin through UGT1A1. To look into this confusion, rats were treated without (control) or with Cln and sacrificed on day 7 and 14. The results of this experiment showed an increase in HO-1 gene expression but



**Fig. 6.** Cln overexpressed UGT1A1 through Nrf2. (A) HepG2 cells were transfected with mock or Nrf2 siRNA (Nrf2 KO) followed by incubation with or without 10  $\mu$ M Cln for 24 h. Microsomal sample was subjected to immunoblot analysis using anti-UGT1A1 antibody.  $\beta$ -Actin served as loading control. Total RNA was extracted from the above incubations followed by RT-PCR and Real-time PCR (qPCR) analysis using UGT1A1 gene specific primers where GAPDH served as an internal control. (B) HepG2 cells were incubated for different time periods (0–24 h) as indicated without or with 10  $\mu$ M Cln following their transfection with control or Nrf2 siRNA (Nrf2 KO) in the presence of 10  $\mu$ Ci of [ $^3$ H]-leucine. Cells were lysed and immunoprecipitated with anti-UGT1A1 antibody and processed for radioactive counting. (C) siRNA transfected HepG2 cells (Nrf2 KO) were incubated with or without 10  $\mu$ M Cln for 24 h. Conjugation of bilirubin was studied using a saturated concentration of bilirubin (120  $\mu$ M) and amount of glucuronide formed was determined in the medium by HPLC. Mean  $\pm$  SEM was calculated from 5 individual experiments. \* $p < 0.001$  (vs. control); # $p < 0.01$  (vs. Cln).



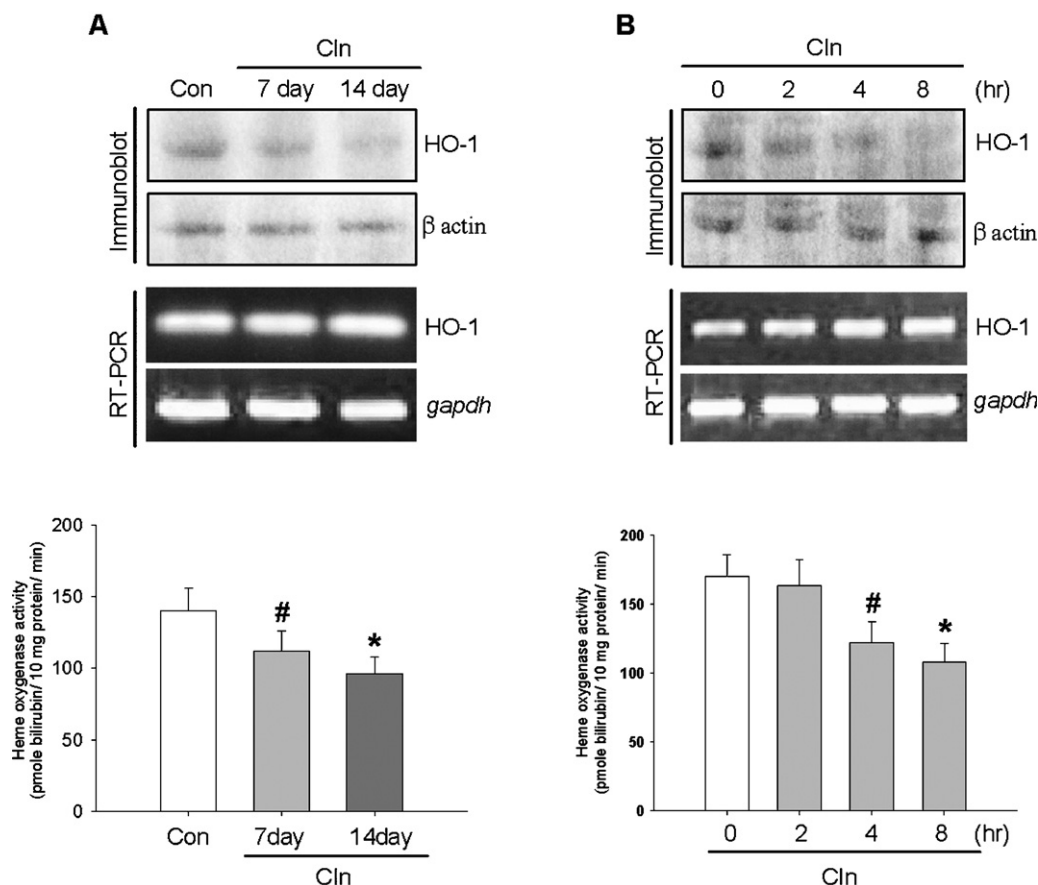
**Fig. 7.** Cln induces Nrf2 expression and its nuclear translocation. (A) siRNA transfected HepG2 cells (Nrf2 KO) were incubated with or without Cln for 6 h. On termination of incubations cells were lysed and immunoblotted with anti Nrf2 antibody.  $\beta$  actin served as an internal control. RNA extracted from the cells was subjected to RT-PCR and qPCR analysis using Nrf2 gene specific primers taking *gapdh* as an internal control. (B) pNrf2-luc reporter plasmid transfected HepG2 cells were co-transfected with or without mock or Nrf2 si-RNA (Nrf2 KO). After transfection HepG2 cells were incubated without or with 10  $\mu$ M Cln. On termination of incubation reporter activity was measured by using luciferase assay system. (C) HepG2 cells were incubated for different time periods (0–8 h) as indicated without or with 10  $\mu$ M Cln. Cytosolic and nuclear extracts were prepared and immunoblotted with anti-Nrf2 antibody.  $\beta$  actin served as an internal control. (D) HepG2 cells were incubated without or with Cln or tBHQ, on termination of incubation cells were fixed and permeabilized. Cells were then incubated with anti-Nrf2 antibody for 4 h followed by FITC conjugated goat anti-rabbit secondary antibody for 2 h. PI was used to stain the nucleus. Figures are representative of one of three independent experiments (magnification 400 $\times$ ). (E) HepG2 cells were incubated without or with 10  $\mu$ M Cln for 0–8 h as indicated and lysates from each incubation was immunoblotted with anti-Keap1 antibody.  $\beta$  actin served as an internal control. Each value is the mean  $\pm$  SEM of 5 individual experiments. \* $p$  < 0.001 (vs. control); # $p$  < 0.01 (vs. Cln).

surprisingly Western blot analysis exhibited a significant decrease in HO-1 protein expression in response to Cln (Fig. 8A). The same was observed in *in vitro* experiments with HepG2 cells (Fig. 8B). However, Cln significantly reduced heme oxygenase activity both in *in vivo* and *in vitro* experiments (Fig. 8A and B); this may be a reflection of reduced protein expression in response to Cln. Taken together, Cln enhanced UGT1A1 gene and protein expression while it increased HO-1 gene expression but decreased protein expression along with the inhibition of enzyme activity.

To demonstrate that Nrf2 binding to UGT1A1 promoter effected greater expression of UGT1A1, we performed chromatin immunoprecipitation (ChIP) assay with control and Cln treated HepG2 cells. Chromatin preparation from these cells was immunoprecipitated with anti-Nrf2 antibody. UGT1A1 promoter region was

therefore amplified using oligonucleotide primers from the DNA recovered from immunoprecipitated complex of both control and treated cells. Nrf2 binding to UGT1A1 promoter region was significantly enhanced in Cln incubated cells (Fig. 9A), suggesting greater promoter activity. To examine promoter activity, UGT1A1 promoter-luciferase reporter construct was transfected to HepG2 cells followed by incubation with Cln. There was more than two fold increase in UGT1A1 luciferase activity by Cln as compared to control which was not observed in Nrf2 knockout cells (Fig. 9B). The results so far obtained suggest that Nrf2-UGT1A1 pathway is greatly stimulated due to overexpression of both Nrf2 and UGT1A1, Cln triggers Nrf2 expression which in turn increased UGT1A1 gene and protein expression resulting greater conversion of insoluble to soluble form of bilirubin. This was checked in HepG2 cells loaded





**Fig. 8.** Cln induces HO-1 gene transcription but reduces its protein expression and activity. (A) Rats were administered without or with Cln at a dose of 25 mg/kg bw and sacrificed on the days as indicated. Total RNA was prepared from the liver samples and subjected to RT-PCR analysis using HO-1 gene specific primers where *gapdh* served as loading control. Homogenates were prepared from the liver samples and immunoblotted with anti-HO-1 antibody.  $\beta$  actin served as an internal control. Portions of the liver homogenates were subjected to microsomal preparation to determine heme oxygenase activity expressed in terms of bilirubin formation. (B) HepG2 cells were incubated without or with 10  $\mu$ M Cln for 6 h. On termination of incubations cells were lysed and total RNA was prepared for RT-PCR and cell lysate was immunoblotted using gene specific primers and antibody respectively. Each value is the mean  $\pm$  SEM ( $n = 5$ ) of 3 individual experiments. <sup>#</sup> $p < 0.02$ , <sup>\*</sup> $p < 0.001$  (vs. control).

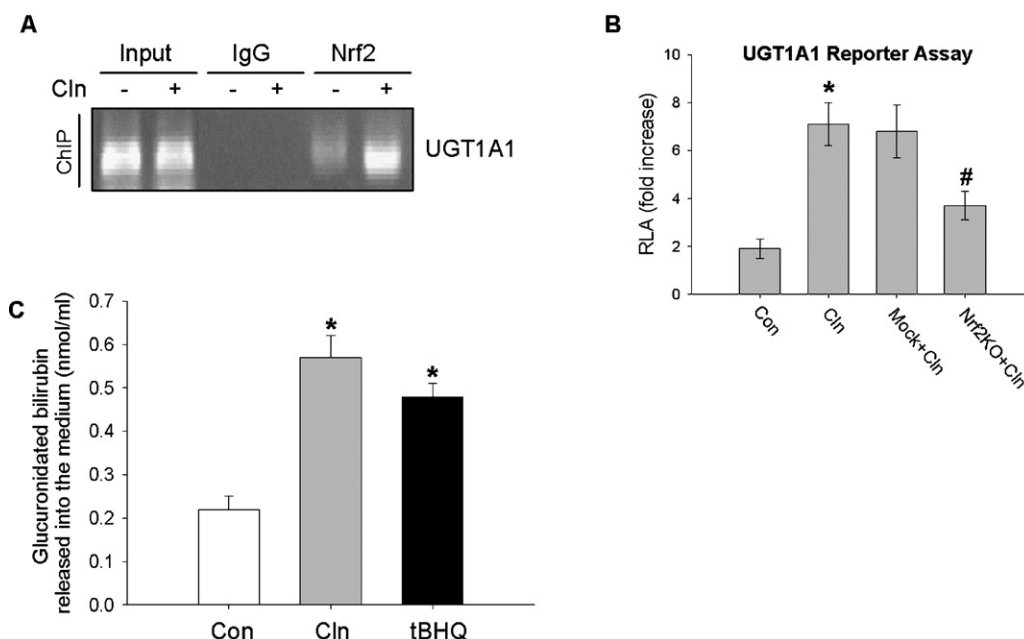
with bilirubin, thoroughly washed and then incubated without or with Cln or tBHQ for 24 h. On termination of incubation, conjugated bilirubin secreted into the medium was estimated. Fig. 9C shows that conjugated bilirubin was significantly higher in Cln incubated cells ( $p < 0.01$ ) indicating physiological relevance of Cln induced Nrf2 upregulation. In this case also, Cln demonstrated significantly greater effect ( $p < 0.05$ ) than tBHQ.

#### 4. Discussion

UDP glucuronosyltransferase 1 (UGT1A1) is one of the nine members of UGT1 sub-family encoded on UGT1 locus of chromosome 2 in human [5]. This enzyme is also known as bilirubin UGT as it catalyzes the solubilization of insoluble bilirubin through glucuronidation [2–4]. Bilirubin is highly toxic; its accumulation in different tissues and organs produces serious health problems including death. Glucuronic acid conjugation is the primary route of elimination for bilirubin where UGT1A1 serves as the major isoform of UGT1A sub-family responsible for the glucuronidation of endogenous bilirubin [2–4]. UGT1A1, therefore, plays a vital role by eliminating bilirubin. To deal with excess accumulation of bilirubin that occurs during impairment of liver and jaundice, there is a need to get an effective inducer of this enzyme. Several chemical compounds are reported to induce UGT1A1 but most of them are potentially harmful [33,34].

We initiated this investigation with *in vivo* experiments since majority of the dietary flavonoids demonstrate effective glucuronidation in *in vitro* cell culture experiments but their promise fail because of bioavailability problem. We have isolated Carlino-side (Cln), a flavone glycoside from *C. cajan* leaf, it is a dietary flavone as the pulse of this plant is a popular food and contains sufficient amount of Cln. It demonstrates a strong capability in eliminating bilirubin by converting insoluble bilirubin to soluble form through glucuronidation. CCl<sub>4</sub> is a well-established liver toxicant used in animal models and leads to higher accumulation of bilirubin. It was demonstrated in some recent reports that CCl<sub>4</sub> causes suppression of glucuronidation activity of UGTs in the liver and leads to impairment of disposition kinetics of therapeutic agents through excretion [35,36]. Moreover chronic administration of CCl<sub>4</sub> has been reported to rupture erythrocytes membrane and thus release heme in excess amount which is converted to bilirubin [37]. We have observed that chronic administration of CCl<sub>4</sub> elevated serum bilirubin level with a marked increase of unconjugated form by affecting impairment of UGT1A1 activity. Interestingly, when rats have already in hyperbilirubinemic condition at 28 day of CCl<sub>4</sub> treatment, oral administration of Cln radically improved the situation within a week or two. This suggests Cln's great potentiality in dealing hyperbilirubinemia.

One of the well studied flavonoid in relation to the induction of UGT1A1 is chrysin, also available from dietary component and at



**Fig. 9.** Cln enhanced Nrf2 binding to the *UGT1A1* promoter. (A) HepG2 cells were incubated without or with 10  $\mu$ M Cln for 24 h and fixed with 1% formaldehyde. Chromatin was immunoprecipitated with anti-Nrf2 antibody and control IgG-Nrf2. Nrf2 binding to *UGT1A1* promoter was determined by PCR with *UGT1A1* ARE specific primers. The same primers were also used to amplify *UGT1A1* ARE regions before immunoprecipitation with Nrf2 antibody to show 1% input DNA. (B) p*UGT1A1*-luc reporter plasmid transfected HepG2 cells were co-transfected with or without mock or Nrf2 siRNA. After transfection cells were incubated without or with 10  $\mu$ M Cln. On termination of incubation reporter activity was determined by using luciferase assay system. (C) HepG2 cells were pre-incubated for 1 h with a saturated concentration of bilirubin (120  $\mu$ M). The cells were thoroughly washed and then treated with or without 10  $\mu$ M Cln or tBHQ for 24 h. Conjugation of bilirubin was studied by determining the amount of glucuronide formed in the medium by HPLC. Mean  $\pm$  SEM was calculated from 3 individual experiments. \* $p < 0.001$  (vs. control); # $p < 0.001$  (vs. Cln).

one time it promised for the amelioration of liver disease. Chrysin induced UGT1A1 expression and activity has been reported in primary hepatocytes [14], the human liver cell line, HepG2 [14,15] and human intestinal cell line, Caco2 [16]. Chrysin therefore has shown a great potentiality to deal hyperbilirubinemia. However, bioavailability of chrysin has become a problem because of its rapid glucuronidation in the intestine followed by its elimination [17,18]. In contrast, oral administration of Cln to rat did not showed problem for its bioavailability, a considerable amount of it could be detected in the serum and liver. Presence of glycan moieties in this flavone probably attributed a protection against intestinal glucuronidation. This presumption may be the fact because two important dietary isoflavones, aglycone daidzein and its glycosylated sister molecule, daidzin showed a significant difference in their bioavailability. Absorption of daidzin through the intestine is far more successful than daidzein [38].

Results from *in vivo* experiments with CCl<sub>4</sub> induced hyperbilirubinemic rats also provided an indication that Cln bioavailability would not be a problem. This encouraged us to study the underlying mechanism of Cln induced stimulation of UGT1A1 activity. To investigate this, we used human liver cell line, HepG2 and rat primary hepatocyte (PH). Since passaging of cell line sometime alters cellular response, PH represented natural or normal cellular characteristics. Both of these cell types responded similarly to Cln in relation to enhanced UGT1A1 activity indicating this to be an inherent property of Cln. Kinetic studies on HepG2 microsomal preparation furnished little important information. UGT1A1 activity in control and treated cells taking bilirubin as substrate exhibited no alteration of  $K_m$  due to Cln or CCl<sub>4</sub> treatment and comparable to some current reports [39,40]; while Cln dramatically increased  $V_{max}$  ( $111.2 \pm 1.3$  pmol/min/mg protein), it falls significantly due to CCl<sub>4</sub> and revive considerably by Cln. Increase of  $V_{max}$  by Cln without altering  $K_m$  indicates a likely increase of UGT1A1 expression as observed with Chrysin [14–16]. In fact, Cln strikingly enhanced gene and protein expression of UGT1A1 in both

HepG2 and PH cells. Flavone compounds have been reported to increase expression and activity of UGT1A1 in intestinal and liver cell lines [14–16,18]. Unfortunately, bioavailability problem deter their future drug promise. In that respect Cln has promise, as its availability in serum and liver on oral administration suggests its escape from intestinal glucuronidation.

Another attractive aspect of our findings is the Cln induction on Nrf2; it does not have a direct effect on UGT1A1 expression since Nrf2 knockout cells do not respond to Cln. This transcription factor which binds to ARE region expresses number of important genes including the UGTs that regulate detoxification of several endobiotic and xenobiotic compounds, dietary, therapeutics and some environmental substances [41,42]. In normal condition Nrf2 remain bounds to a protein Keap1 in the cytosolic region. On induction, Nrf2 is released from Keap1 and then translocated to the nuclear region that leads to transcription of its target genes [42,43]. We have observed that Cln overexpresses Nrf2 along with its increased translocation to nuclear region of HepG2 cells. It's binding to the ARE region of UGT1A1 promoter has been significantly augmented by Cln that consequently enhances UGT1A1 promoter activity. There may be a confusion regarding Cln effect on bilirubin reduction as HO-1 is a target gene for Nrf2 [44,45], therefore Cln induced overexpression of Nrf2 would likely increase HO-1 expression. HO-1 converts heme to biliverdin and biliverdin reductase catalyzes formation of bilirubin from biliverdin. We have found that Cln increased HO-1 gene expression but reduced protein expression and that adversely affects its activity. However, why enhanced gene expression is not reflected into protein expression is not clear to us. It may be due to the inhibition of translation or increased proteasomal degradation of HO-1. In both *in vivo* and *in vitro* experiments Cln inhibited heme oxygenase activity and that would expectedly not permit for excess bilirubin production. However, to understand the underlying mechanism for differential effect of Cln on HO-1 requires further investigation.

However, several problems have to be solved prior to make Cln as drug for hyperbilirubinemia. It requires to be determined the equivalent amount of Cln for treating hyperbilirubinemic patients, precise amount of Cln requires to be absorbed through the gut on oral administration and to study the mechanism of its elimination from the body. One of the favorable points for Cln is that it may be administered with specific diet; such a possibility requires to be explored. In conclusion, it may be stated that Cln greatly stimulates UGT1A1 activity and expression through the induction of Nrf2 gene and therefore bears a promise to deal hyperbilirubinemia. It's a dietary flavone glycan profusely available from plant source and it successfully qualified the critical test for bioavailability which is a crucial limitation for bioactive dietary flavones. At present we really have a conspicuous dearth of non-toxic chemical compounds to convert free insoluble bilirubin to its conjugated solubilized form as that would ameliorate the problem of hyperbilirubinemia. On this background Cln may serve an attractive choice.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.07.069.

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