BASIC STUDIES

Identification of paraxanthine as the most potent caffeine-derived inhibitor of connective tissue growth factor expression in liver parenchymal cells

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Keywords

Abbreviations
15-PGJ2, 15-deoxy-(12,14)-prostaglandin J2; cAMP, cyclic adenosine monophosphate; cAMP-PDE, cAMP-specific phosphodiesterase; CTGF, connective tissue growth factor; HMIS, hazardous materials identification system; HSC, hepatic stellate cells; hydrate (Rp-diastereomer of adenosine-3’,5’-cyclic monophosphothioate); PC, hepatocytes (parenchymal cells); PPARγ, peroxisome proliferator-activated receptor γ; Rp-cAMP, Rp-adenosine 3’,5’-cyclic monophosphorothioate triethylammonium salt; siRNA, small interfering RNA; TGF-β, transforming growth factor β.

Abstract

Background: Recently, we identified hepatocytes as the major cellular source of profibrogenic connective tissue growth factor (CTGF/CCN2) in the liver. Based on reports of a hepatoprotective effect of coffee consumption, we were the first to provide evidence that caffeine suppresses transforming growth factor (TGF)-β dependent and -independent CTGF expression in hepatocytes in vitro and in vivo, thus suggesting this xanthine-alkaloid as a potential therapeutic agent. Aim: This study aims at comparing the inhibitory capacities of caffeine and its three demethylated derivates paraxanthine, theophylline and theobromine on CTGF expression in hepatocytes and hepatic stellate cells (HSC). Results: Our data suggest paraxanthine as the most important pharmacological repressor of hepatocellular CTGF expression among the caffeine-derived metabolic methylxanthines with an inhibitory dosage (ID)50 of 1.15 mM, i.e. 3.84-fold lower than what is observed for caffeine. In addition, paraxanthine displayed the least cell toxicity as proven by the water-soluble tetrazolium-1 cell vitality assay. However, caffeine or any of the metabolites did not inhibit CTGF expression in HSC. At the toxicological threshold concentration of 1 mM for paraxanthine, we observed an inhibition of hepatocellular CTGF synthesis by 44%, which was strongly reverted in the presence of the specific competitive cyclic adenosine monophosphate inhibitor Rp-adenosine 3’,5’-cyclic monophosphorothioate triethylammonium salt. Furthermore, CTGF protein expression induced by various concentrations of TGF-β (0.13–1 ng/ml) is still reduced by, on average, 27%/45% in the presence of paraxanthine (1.25 mM/2.5 mM).

Conclusion: Our data provide an evidence-based suggestion of the caffeine-derived primary metabolite paraxanthine as a potentially powerful antifibrotic drug by its inhibitory effect on (hepatocellular) CTGF synthesis.

Caffeine, i.e. 1,3,7-trimethylxanthine, is the most widely consumed pharmacologically active substance in the world. In North America, 90% of adults consume caffeine daily (1). Caffeine is metabolized in the liver by the cytochrome P450 oxidase enzyme system (CYP1A2) into the three metabolic dimethylxanthines, i.e. paraxanthine (1,7-dimethylxanthine; 84%), theobromine (3,7-dimethylxanthine; 12%) and theophylline (1,3-dimethylxanthine; 4%) (1–4). Further, demethylation and oxidation forms urates and uracil derivatives. About a dozen caffeine metabolites can be recovered in the urine, but they comprise < 3% of the ingested caffeine (1, 3).
with chronic liver diseases is associated with a milder course of fibrosis (5, 6), especially in alcoholic cirrhosis (6–8), and with a decrease in alanine aminotransferase (7, 8) and γ-glutamyltransferase (8) activities. However, the underlying pathophysiological mechanisms remained obscure.

Connective tissue growth factor (CTGF/CCN2) is widely regarded as a central modulator of the activities of the profibrogenic master cytokine transforming growth factor (TGF)-β. Previously, we could show that hepatocytes [parenchymal cells (PC)] are the major cellular source of CTGF in the liver, that CTGF is spontaneously upregulated in the hepatocyte under conditions of cellular stress and that it strongly responds to TGF-β stimulation (9, 10). Even though CTGF is found to be overexpressed in fibrotic lesions, and in vivo intoxication of the liver in the rat leads to an increase in hepatocellular CTGF in immunohistochemical staining (9), its exact pathophysiological role in regard to fibrogenic processes of the liver still needs to be defined (11).

However, it was shown that silencing of CTGF by small interfering RNA (siRNA) almost entirely inhibits fibrotic remodelling of livers in mice previously subjected to hepatotoxic agents (12, 13).

In a recent study, we provided evidence that caffeine suppresses TGF-β-dependent and -independent CTGF expression in hepatocytes in vitro and in livers subjected to d-galactosamine toxicity in vivo via a mechanism that involves upregulation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ and thus sensitization towards the natural PPARγ ligand 15-PGJ2, as well as the enhanced degradation and inhibition of phosphorylation of the TGF-β effector Smads 2 and 3 (14, 15).

Based on these findings, our current study aims at comparing the inhibitory capacities of caffeine and its three hypomethylated derivatives paraxanthine, theophylline and theobromine on CTGF expression in hepatocytes and hepatic stellate cells (HSC). The results may help initiate pioneer future investigations for the evaluation of a caffeine-derived therapeutic agent in the treatment of fibrotic liver diseases.

Materials and methods

Materials

Caffeine (1,3,7-trimethylxanthine; #27600), paraxanthine (1,7-dimethylxanthine; #41761), theobromine (3,7-dimethylxanthine; #88304), theophylline (1,3-dimethylxanthine; #88308) and Rp-CAMP (Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate; #A165) were from Sigma-Aldrich (St Louis, MO, USA). The three-dimensional shapes and structures of caffeine and its hypomethylated metabolites used in this study are shown in Figure 1. Recombinant human TGFβ-1 (240-B) was from R&D Systems (Minneapolis, MN, USA).

A goat polyclonal anti-CTGF/CCN2 (L-20, sc-14939; Santa Cruz, Santa Cruz, CA, USA), epitope mapping within an internal region of CTGF of human origin, was used for Western blotting analysis.

Animals

Adult Sprague–Dawley rats (body weight 200–250 g) had free access to a standard laboratory chow diet and normal tap water throughout the experimental period. All animals received care and treatment in compliance with the German Animal Protection Act, which is in accordance with the German Research Council’s criteria.

Isolation and culture of primary rat hepatic stellate cells

Hepatic stellate cells were isolated by the pronase–collagenase perfusion technique (16), purified by a single-step density gradient centrifugation with Nycodenz (Nygaaard, Oslo, Norway) as described (17) and characterized by their typical light microscopic appearance, immunostaining for desmin and vimentin and vitamin A-specific autofluorescence. Cell viability checked by the trypsin blue exclusion test was normally > 95%, and the mean purity was 90 ± 5%. The cells were cultured for 4 days in serum-free Dulbecco’s modified Eagle medium (DMEM; BioWhittaker Europe, Verviers, Belgium), supplemented with 4 mM L-glutamine (PAA Laboratories, Coelbe, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories) under a humidified atmosphere of 5% CO2/95% O2.

Cell culture and preparation of rat hepatocytes

Parenchymal cells were isolated from male Sprague–Dawley rats (180–320 g body weight) by the two-step collagenase method of Seglen (16) modified as described before (18). Cells were isolated and cultured in the complete absence of (TGF-β-containing) foetal calf serum (FCS). The viability of the final parenchymal cell suspension, checked by trypsin blue exclusion, was around 85%, and cell recovery was 20 × 50 × 10⁶ cells/liver. Contamination with other non-parenchymal cells was < 2%.

Cells were seeded in 2 ml Gibco™ HepatoZYMESFM™ (Invitrogen, Carlsbad, CA, USA) without FCS on type I collagen (BD Bioscience, Clontech, Palo Alto, CA, USA)-coated plastic dishes (Becton Dickinson, Franklin Lakes, NJ, USA) with a density of 5.4 × 10⁶ cells/cm². They were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air supplemented with 4 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (100 mg/ml) (all from Cambrex, East Rutherford, NJ, USA). The first change of the medium was 1 h after seeding and unattached PC were washed off with DMEM (Cambrex). PCs were then cultured for various times in Gibco™ HepatoZYMESFM™, supplemented as described above.
Sodium dodecyl sulphate – polyacrylamide gel electrophoresis and Western blotting
Preparations of cytoplasmatic cell extracts, determination of protein concentrations and Western blot analysis were performed as described previously (10, 19). Densitometric quantification of the blot results was performed using the Lumi-Imager System (Roche Diagnostics, Mannheim, Germany) and the LUMIANALYST 3.0 software (Roche Diagnostics).

Reverse transcriptase-polymerase chain reaction for rat connective tissue growth factor
Total cellular RNA was extracted with the Qiagen RNAeasy purification kit (Qiagen, Hilden, Germany). cDNA was reverse transcribed using the First-Strand cDNA synthesis kit (Invitrogen). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using the Biometra T3000 Thermocycler PCR System (Biometra, Göttingen, Germany) and the following primers: rCTGF

Fig. 1. Two-dimensional (2D) and 3D shapes and structures of caffeine and its derived metabolic methylxanthines used in this study. In 3D shapes, atoms are colour coded: C, grey; N, dark blue; O, red; H, white.
Generation of recombinant connective tissue growth factor reporter adenovirus (Ad-hCTGF-Luc)

For generation of the reporter adenovirus Ad-hCTGF-Luc, the ~2.5-kbp ClaI fragment of vector pGL3-Basic-hCTGF-Luc (10) harbouring a fusion of human CTGF gene promoter and the luciferase reporter gene was cloned into the ClaI site of vector pAE1sp1A (20), resulting in the generation of pAE1sp1A-hCTGF-Luc. The integrity of cloning boundaries was verified by restriction analysis and sequencing with the flanking primers 5′-GGC TAA CCG AGT AAG AAT TTG-3′ and 5′-GAC CAT CAA TGC TGG AG-3′ that were obtained from MWG-Biotech AG.

The integration of the reporter cassette from pAE1sp1A-hCTGF-Luc into the adenoviral backbone vector pJM17 (21) was performed by in vitro homologous recombination in the human embryo kidney cell line 293 using a protocol described before (22). Successful generation of recombinant viral particles was visualized by viral foci formation. After total infection, the viral particles were released from cells by three rounds of a freeze–thaw cycle and separated from cell debris by centrifugation at 600 × g for 10 min. To generate high-titre viral stocks, 293 cells were reinfected at a multiplicity of infection of 1 and grown for 3–4 days. The amplified viruses were harvested, concentrated through standard CsCl gradient centrifugation and subsequently purified using the BD Adeno-X™ Purification Filter system (BD Biosciences) according to the manufacturer’s instructions.

Luciferase gene reporter assay

Cells were cultured in black 96-well plates and infected with 1 × 10⁸ virons/ml of Ad-hCTGF-Luc reporter virus. After specific treatment, the Luciferase activity was measured as described previously (1).

Water-soluble tetrazolium-1 cell vitality assay

To quantify cell proliferation, the water-soluble tetrazolium (WST)-1 assay using a sulphonated tetrazolium salt, 4-(3-[4-ioophenyl]-2-[phenyl-2H-5-tetrazolio-1,3-benzene disulphonate]) (Roche Diagnostics), was performed as recommended by the manufacturer.

Analysis of data

Where applicable, spss 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis, applying two-tailed unpaired Student’s t tests for comparison of means with a P-value for significance set at least at 0.05. Correlations between variables were analysed with the Pearson correlation test. Again, values of P < 0.05 were considered statistically significant. Furthermore, we performed regression analysis to assess associations between CTGF protein expression, promoter activation or cAMP values, and methylxanthine or TGF-β concentrations.

Molecular visualization of caffeine and its metabolic dimethylxanthine derivatives was performed using the molecular visualization system ChemDraw Ultra 8.0 and Chem3D Ultra 8.0 (CambridgeSoft, Cambridge, MA, USA).

Results

Dose-dependent effects of caffeine and its derived metabolites paraxanthine, theophylline and theobromine on connective tissue growth factor protein expression and on the transcriptional activation of the connective tissue growth factor promotor in rat hepatocytes and hepatic stellate cells

Treatment of primary rat PC with caffeine confirmed the previously observed inhibitory effect of the CTGF promotor and CTGF protein accumulation on transcriptional activation (Fig. 2) (14). The estimated inhibitory dosage (ID₅₀) was set at 6.40 mM for transcriptional activation (Fig. 2A) and at 4.42 mM for protein expression (Fig. 2B).

All primary metabolic caffeine derivatives, paraxanthine, theophylline and theobromine, also exerted an inhibitory effect on CTGF protein expression (Fig. 3A) and promoter activity (Fig. 3B), although dose dependency and degree of inhibition differed among them. The calculated ID₅₀ was set at 1.15 mM for paraxanthine, 2.46 mM for theophylline and 1.48 mM for theobromine for CTGF protein expression (Fig. 3A) and at 0.82 mM for paraxanthine, 4.72 mM for theophylline and 1.95 mM for theobromine for CTGF promotor activity (Fig. 3B).

Using Pearson’s correlation and simple regression analysis for the association of mean CTGF protein expression and dosage of each methylxanthine, we calculated correlation coefficients of −0.78 for caffeine, −0.62 for paraxanthine, −0.77 for theophylline and −0.87 for theobromine. Coefficients of determination (R²) were 0.61 for caffeine, 0.39 for paraxanthine, 0.59 for theophylline and 0.76 for theobromine. In general, the metabolic derivatives proved to be more powerful repressors of hepatocellular CTGF expression than caffeine itself. Paraxanthine, in particular, exerted a highly
suppressive effect on CTGF synthesis. Table 1 summarizes the concentrations of the compounds necessary to inhibit CTGF protein expression and transcriptional activity by 25% (ID25), 50% (ID50) and 75% (ID75). In order to exclude hepatocyte toxicity as a possible cause of the observed reduction in hepatocellular CTGF expression in the presence of caffeine or its metabolites, a WST-1 assay was performed (Fig. 3C), and the
morphological development of PC was controlled under the microscope (Fig. 3D). Of all the metabolites tested (including caffeine), paraxanthine displayed the least toxicity towards cultured PC.

Next, we tested the possibility of transferring the results of a repressive effect of caffeine on CTGF synthesis obtained in PC to a primary rat HSC. However, caffeine did not inhibit CTGF expression in HSC, either at the transcriptional level, as demonstrated by RT-PCR (Fig. 2C, right), or at the protein level (Fig. 2C, left). Similar results were found for the primary metabolites of caffeine (data not shown). These findings are not surprising as we could show previously that caffeine exerts its inhibitory effect on CTGF expression primarily through an interruption of TGF-β-induced Smad2/3 signalling to the CTGF promoter (14), whereas the regulation of CTGF expression in HSC is largely TGF-β independent (10).

Definition of the inhibitory potential of caffeine, paraxanthine, theophylline and theobromine on connective tissue growth factor expression induced by various concentrations of transforming growth factor-β

Transforming growth factor-β is widely regarded as the profibrogenic master cytokine and major inducer of hepatocellular CTGF expression. We therefore investigated the effect of caffeine, paraxanthine, theophylline and theobromine on CTGF expression induced by various concentrations of recombinant TGF-β (Fig. 4A–D). On average, 1 ng/ml TGF-β increased CTGF expression by about 2.3-fold. Overall, the means of CTGF protein expression induced by various concentrations of TGF-β (0.13–1 ng/ml) were reduced, on average, by 30%/46% in the presence of caffeine (2.5 mM/5 mM) (Fig. 4A), by 27%/45% in the presence of paraxanthine (1.25 mM/2.5 mM) (Fig. 4B), by 11%/36% in the presence of theophylline (1.25 mM/2.5 mM) (Fig. 4C) and by 16%/30% in the presence of theobromine (1.25 mM/2.5 mM) (Fig. 4D). Therefore, unmetabolized caffeine was identified as the most potent methylxanthine in terms of inhibiting TGF-β-dependent CTGF expression, closely followed by paraxanthine. The reducing effect of theophylline or theobromine on TGF-β-induced stimulation of CTGF expression in hepatocytes was not considered significant, as the relative magnitude of increase caused by TGF-β remained unchanged between untreated cells and those treated with theophylline or theobromine.

Efficiency of the specific protein kinase A inhibitor, Rp-adenosine 3’,5-cyclic monophosphorothioate triethylammonium salt, in reverting the repressive effects of caffeine, paraxanthine, theophylline and theobromine on connective tissue growth factor synthesis

As described above and shown in Figures 2–4, all three metabolites and caffeine itself reduced hepatocellular CTGF production with varying efficacies despite their similar mechanisms of action. This suggests that competitive inhibition of the cAMP-PDE may be unequally involved in mediating the repressive signals of these methylxanthines to the CTGF promoter.

Therefore, it was tempting to define the role of cAMP in mediating the metabolite-specific inhibition of hepatocellular CTGF expression by using the specific competitive protein kinase A inhibitor Rp-cAMP. Rp-cAMP had no significant effect on the basal level of CTGF synthesis (Fig. 5A). However, rat PC subjected to caffeine displayed a striking and highly significant reversal of caffeine effects on CTGF expression when previously treated with Rp-cAMP (P < 0.0001; average 140% inhibition of caffeine effects). In paraxanthine-treated cells, Rp-cAMP exerted its inhibitory effect even more profoundly (P < 0.0001; 229%). In theophylline-treated cells, Rp-cAMP caused an average 108% inhibition of the maximal inhibitory effect of theophylline (P < 0.0001), whereas the Rp-cAMP effect was again much more pronounced in theobromine-treated PC, with an average 207% inhibition of theobromine-repressed CTGF synthesis (P < 0.0001). Results were confirmed by the CTGF luciferase reporter assay, suggesting that the cAMP pathway acts at the transcriptional level (Fig. 5B).

Effects of caffeine, theophylline and theobromine on paraxanthine-induced repression of hepatocellular connective tissue growth factor expression

Comparative analysis of caffeine and its derived metabolites identified paraxanthine as the most potent inhibitor of CTGF expression in PCs (Figs 2 and 3).

In order to enhance the efficiency of this methylxanthine, we looked for a possible synergism between paraxanthine and the other metabolites as well as caffeine in terms of CTGF repression at concentrations close to the respective ID50 determined previously. As seen in Figure 6, the effects of paraxanthine and the other methylxanthines on CTGF production were additive, and additional inhibition was calculated at, on average, 37% for caffeine, 41% for theophylline and 39% for theobromine. Thus, even though the combination of paraxanthine and theophylline exerted a highly suppressive effect on CTGF synthesis, the degree of inhibition did not differ significantly and to a pathobiochemically relevant amount from the other combinations.

Discussion

The present investigation is based on our previous finding that caffeine, similar to 8-Br-cAMP, strongly downmodulates TGF-β-induced CTGF expression in hepatocytes in vitro and in livers subjected to D-galactosamine toxicity in vivo by stimulation of degradation of the TGF-β effector Smad2, inhibition of Smad3 phosphorylation and upregulation of the PPARγ-receptor (14, 15). This is of note, as experimental silencing of
CTGF by siRNA was shown to inhibit experimental liver fibrosis in mice (12, 13). Here, we provide evidence that not just caffeine itself but also its metabolic methylxanthines suppress CTGF expression, and performed intensive studies for comparing caffeine and its metabolites in terms of their pharmacological potency and mechanisms of action leading to CTGF suppression.

However, when discussing the data demonstrated in this study, it should be considered that caffeine applied at higher concentrations displays significant cell toxicity, so that the observed effect of caffeine on hepatocellular CTGF expression might, at least in part, be attributed to this cytotoxic effect. This also holds for theophylline.

More interestingly, our data suggest that paraxanthine is the most powerful pharmacological repressor of hepatocellular CTGF expression among the drug family of methylxanthine derivatives that does not display significant cytotoxicity at the tested concentrations.

The members of this caffeine-derived family of xanthine alkaloids exert their effects primarily through
two distinct mechanisms, i.e. (i) inhibition of the cAMP-PDE and/or (ii) competitive antagonism of adenosine receptors (particularly subtypes 1 and 2A) (23). A strong inhibition of cAMP-PDE has been proven for all metabolites, apart from theophylline, which in contrast, compared with its family members, displays a higher affinity to adenosine receptors (24–33).

Because methylxanthine derivatives are antagonists, not agonists, at adenosine receptors, one would expect no pharmacological effects unless the receptor is being activated by endogenous adenosine. It was reported that endogenous adenosine release from damaged liver cells plays an important role in the pathogenesis of toxic liver fibrosis, but the intracellular events responsible were not identified (34). Other reports, however, provided evidence that adenosine is able to reverse a pre-established CCL4-induced micronodal cirrhosis in rats (35). This diverse effect of adenosine on hepatic fibrogenesis may in part be explained by the differential effect of activated adenosine receptors on intracellular cAMP levels. Studies in isolated rat hepatocytes demonstrated that the concentration of cAMP increased in response to stimulation of type 2A adenosine receptors, while it decreased in response to stimulation of type 1 receptors (36, 37). This is explained by the fact that the type 2A adenosine receptor is linked to a Gs-protein, which stimulates adenylatecyclase activity whereas the type 1 adenosine receptor is coupled to a Gi-protein, which inhibits it (36, 37). Therefore, binding of caffeine to the type 2A receptor may result in a negative effect of this methylxanthine on cAMP levels by stabilizing an ‘antagonist conformation’ of adenosine receptors (28). As may be deducted from this functional diversity, blockade of type 1 and 2A receptors appears to be the less likely mechanism for the CTGF inhibitory action of methylxanthines, compared with competitive inhibition of the CAMP-PDE. This is also supported by the fact that theophylline, a pan-specific adenosine receptor antagonist, but only a slight inhibitor of CAMP-PDE (38), has the weakest effects on CTGF expression in rat PC, which are furthermore hardly reverted by Rp-cAMP.

The apparent beneficial effect of methylxanthines in preventing fibrotic remodelling of the liver should be considered as a ray of hope in terms of developing drug-based antifibrotic therapy approaches. After all, the ID50 value for caffeine was 4.42 mM. Initially, this finding suggests that habitual coffee consumption might be sufficient in order to gain a healthful effect. However, one cup of coffee at 120 mg caffeine results in blood concentrations of 0.015 mM (1). Therefore, large amounts of coffee would be required to be consumed in order to achieve blood concentrations of 4.42 mM.

Alternatively, the lethal dose of caffeine for humans, when applied intravenously, is set at about 13 g (27). With a volume of distribution in humans of 42 L, this results in a blood concentration of 1.6 mM; therefore, a
concentration of 4.42 mM would be a lethal dose. However, caffeine is efficiently metabolized in PCs in vivo. Thus, absorbed caffeine is concentrated in the liver, in particular in PCs, resulting in increased liver-specific concentrations that do not necessarily reflect those levels observed in the circulation. Still, the lower the ID_{50} needed for an inhibition of hepatocellular CTGF synthesis, the better.

**Fig. 4.** Inhibitory potential of caffeine, paraxanthine, theophylline and theobromine on connective tissue growth factor (CTGF) expression induced by various concentrations of transforming growth factor (TGF)-β. Rat parenchymal cells were treated for 24 h with recombinant human TGF-β1 at the indicated doses added to the culture medium in the absence or the presence of 2.5 mM/5 mM caffeine (A), 1.5 mM/2.5 mM paraxanthine (B), 1.5 mM/2.5 mM theophylline (C) or 1.5 mM/2.5 mM theobromine (D), respectively, and then harvested, and Western blot analysis was performed as described in “Materials and methods”. Blots were quantified relative to β-actin using the Lumi Imager System. The diagrams display mean CTGF protein expression [CTGF/β-actin (BLU)] of triplicate determinations from four different cultures and are described as a fraction of the untreated control (%)/C6 standard deviation. A representative blot for each is demonstrated. CTGF protein expression is plotted against TGF-β concentrations. The continuous line indicates TGF-β-treated controls, while the dashed line indicates TGF-β-stimulated cells treated with 2.5 mM caffeine or 1.5 mM paraxanthine, theophylline or theobromine respectively. The dotted line indicates TGF-β-stimulated cells treated with 5 mM caffeine or 2.5 mM paraxanthine, theophylline or theobromine respectively.
Paraxanthine, which with an ID$_{50}$ of only 1.15 mM, was identified as the most potent inhibitor of CTGF synthesis in liver parenchymal cells, is currently believed to exhibit a lower toxicity than caffeine, and blood levels commensurating with average intake appear to be fairly innocuous. The health hazard class according to the US Hazardous Materials Information System (HMIS) (39) and US National Fire Protection Association classification (40) is set at 1 for paraxanthine and theobromine whereas theophylline and caffeine are classified as 2. However, among paraxanthine and theobromine, the first displays much more attenuated symptoms following exposure. Chronic exposure to paraxanthine at concentrations of 1–4 mM has so far been only weakly associated with increased mutagenicity in humans and teratogenicity in mice (39–41), whereas chronic exposure to the same concentrations of theobromine led to much stronger mutagenicity in humans, mice and hamsters, to reproductive disabilities in rats and mice as well as to teratogenicity in mice following both oral and intraperitoneal application (39, 40). Also, theobromine is rated as a Group 3 carcinogen of the International Agency for Research of Cancer classification (42). Of note, our studies also confirmed paraxanthine as the least harmful caffeine-derived metabolite in terms of hepatocyte toxicity in vitro.

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Finally, considering the above-mentioned toxicological threshold concentration of 1 mM for these...
metabolites, we observe a 44% inhibition of hepatocellular CTGF synthesis for paraxanthine, whereas theobromine achieves only 38% inhibition.

In conclusion, our data are the first to provide an evidence-based suggestion of the caffeine-derived metabolite paraxanthine as a potentially powerful antifibrotic drug by its inhibitory effect on (hepatocellular) CTGF synthesis. This proposal is based on earlier reports that demonstrate that silencing of CTGF by siRNA prevents fibrotic remodelling of murine livers previously subjected to hepatotoxic agents (12, 13). Therefore, future studies are urgently recommended to evaluate the significance of a therapeutic paraxanthine application in patients with chronic liver diseases and hepatic fibrogenesis.

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