Original Article

Oral application of 1,7-dimethylxanthine (paraxanthine) attenuates the formation of experimental cholestatic liver fibrosis

Ilidikó Klemmer,4 Shintaro Yagi2,3 and Olav A. Gressner1,4

1Wisplinghoff Medical Laboratories, Cologne, Germany; 2Department of Hepatobiliary, Pancreas and Transplant Surgery, Kyoto University Hospital, Kyoto, Japan; 3Institute for Laboratory Animal Science and Experimental Surgery, and 4Institute of Clinical Chemistry and Pathobiochemistry – Central Laboratory, RWTH- University Hospital, Aachen, Germany

Aim: Several epidemiological studies suggest a beneficial effect of coffee consumption on the formation and progression of fibrogenic diseases, particularly of the liver. Recent data now point to a modulation of transforming growth factor-β (TGF-β) signaling by paraxanthine (1,7-dimethylxanthine [1,7-DMX]), the demethylated primary metabolite of caffeine

Methods: Twenty adult Sprague–Dawley rats were bile duct ligated (BDL) or sham operated with or without concomitant oral 1,7-DMX (1 mM) application. Serum was investigated by standard biochemical analysis, in-house connective tissue growth factor (CTGF), enzyme linked immunosorbent assay (ELISA) or liquid chromatography-mass spectrometry analysis. Liver tissue was stained using hematoxylin-eosin (HE) and Sirius-red staining. Whole liver lysates, primary rat hepatocytes (PC) and hepatic stellate cells (HSC) were investigated by CTGF, and total Smad2/3 Western blot analysis, CTGF reporter gene assay or an in-house malondialdehyde ELISA.

Results: The in vitro 50% inhibitory dose (ID50) of 1,7-DMX was 0.95 mM for protein expression in PC and 1.25 mM for protein expression in HSC. Oral 1,7-DMX application (1 mM) attenuated cholestatic hepatocellular injury in vivo as determined by biochemical serum analysis and reduced intercellular collagen deposition in the cholestatic rat liver (HE- and Sirius-red staining). Western Blot analysis of whole liver lysates revealed a reduction of intrahepatic concentrations of Smad2/3 and CTGF following oral 1,7-DMX intake. However, serum CTGF concentrations were not reduced in 1,7-DMX treated BDL rats. Oral 1,7-DMX furthermore led to a reduction of intrahepatic lipid peroxidation (malondialdehyde concentrations) as markers of oxidative stress in BDL rats.

Conclusion: Our pilot study warrants further studies of 1,7-DMX as a potential new drug to fight fibrotic processes, not just of the liver.

Key words: 1,7-dimethylxanthine, caffeine, fibrogenesis, paraxanthine, Smad signaling, transforming growth factor β

INTRODUCTION

CONNECTIVE TISSUE GROWTH factor (CTGF/CCN2) is a 38 kDa cysteine-rich peptide whose synthesis and secretion is strongly induced by transforming growth factor-β (TGF-β) in liver parenchymal and non-parenchymal cells. The molecular mechanisms of CTGF in the pathogenesis of hepatic fibrosis are not yet fully understood, but one suggested role is that of an upstream amplifier of the effects of TGF-β, strengthening the binding of the latter cytokine to its cognate receptors.1,2 Despite the remaining uncertainties regarding its pathophysiological function, the overall pivotal role of CTGF in the fibrogenic process of the liver has been convincingly proven in experimental rat liver fibrosis with silenced CTGF,3,4 and by its strong overexpression in both, fibrotic and tumor tissues.5-13
Regulated SMADs (R-SMADs; for TGF-β activated type I receptor then phosphorylates receptor-the phosphorylation of the Type I receptor. The activation of type I receptor then phosphorylates receptor-regulated SMADs (R-SMADs; for TGF-β; Smad2 and 3), which can now bind the coSMAD SMAD4. R-SMAD/coSMAD complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression.

In many diseases, excessive TGF-β activity contributes to a pathological excess of extracellular matrix formation and deposition that compromises normal organ function, a topic that has been the subject of numerous reviews and for why TGF-β is now often designated as the "profibrogenic master cytokine". Furthermore, TGF-β secreted during liver carcinogenesis was demonstrated to inhibit the proliferation of normal cells while providing a selective advantage for the growth of cells that are "partially transformed" and are unresponsive to the growth factor, thus also promoting the development of hepatocellular carcinoma.

Scientists of the American National Institutes of Health reported that increased coffee consumption is associated with a slower progression of liver fibrosis in patients with chronic, particularly alcoholic, hepatopathy. However, a causal, mechanistic explanation for this phenomenon has been pending for a long time. Emerging results now indicate that the methylxanthine caffeine might, at least partially, be responsible for this phenomenon as it inhibits the synthesis of CTGF in hepatocytes (PC), primarily by inducing degradation of Smad2 (and, to a lesser extent, Smad3), thus impairing TGF-β signaling. Further investigations, initiated to define the repressive capacities of the primary demethylated caffeine metabolites on hepatocellular CTGF expression finally suggested paraxanthine or 1,7-dimethylxanthine (1,7-DMX) as the most potent caffeine-derived pharmacological inhibitor of hepatocellular CTGF expression.

Based on these observations, the present study is the first to describe the pharmacological effects of oral 1,7-DMX application on the formation of experimental liver fibrosis in the rat induced by prolonged bile-duct ligation. The findings are interesting and provide a new ray of hope in the search for drugs useful in the treatment of patients with chronic fibrogenic (liver) diseases.

**METHODS**

Isolation of primary rat hepatocytes and hepatic stellate cells

RAT HEPATIC STELLATE cells (HSC) were isolated from livers of male Sprague–Dawley rats (250–320 g body weight) as described previously. The purity (ranging from 93% to 95%) of HSC preparations was assessed by light microscopic appearance, vitamin A autofluorescence and positive immunofluorescence stainings. HSCs were harvested at time points day 1, 2, 5 and 15 (resembling myofibroblasts [MFB]; cultures were passaged at confluency [day 7 of primary culture]).

Rat hepatocytes (PC) were also isolated from livers of male Sprague–Dawley rats (180–250 g body weight) by the two-step collagenase method of Seglen modified as described before. The viability of the cell suspension was around 90% (trypan blue exclusion). Contamination with non-parenchymal cells was less than 2%.

Animal surgery and experimental protocol

Twenty Adult Sprague–Dawley rats (body weight 140–160 g) were obtained from Harlan Winkelmann (Borken, Germany). They were housed in the animal facilities of the Institute for Laboratory Animal Science and Experimental Surgery at the RWTH-University Hospital on a 12:12 h light : dark (L : D) cycle, and they were fed food and water ad libitum for 1 week before the experiments were initiated. 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.

For the in vivo experiment, animals were randomly divided into four study groups: six for sham operation alone, two for sham operation plus paraxanthine (1,7-DMX; 1 mM; 0.18 g/L) per os, four for bile duct ligation (BDL) plus 1,7-DMX (1 mM) per os, and eight for BDL alone. Chemically pure 1,7-DMX was purchased from Sigma-Aldrich (St. Louis, MI, USA; Art. No. D5385).

Bile duct ligation was performed using a standard technique. In brief, rats were anesthetized with isoflurane. After midline laparotomy, the common bile duct was exposed and twice ligated with 6-0 silk suture before dissection. Sham operation was performed by gently touching the bile duct. The abdomen was closed in layers, and the animals were allowed to recover on a heat pad under oxygen insufflation.

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In 1,7-DMX-treated animals, 1,7-DMX was added to the drinking water at a dosage of 1 mM, beginning 24 h before BDL or sham operation, and their daily intake was recorded by weighing the water bottles of the animals in their regular home cages. Rats were killed at 30 days after surgery. Livers were harvested and the inferior vena cava was punctured and 5 mL blood was collected in serum tubes (Sarstedt, Nümbrecht, Germany). Serum was separated by centrifugation (1500 g for 10 min at room temperature) for biochemical studies and analyzed within 4 h from death.

**SDS-PAGE and Western blotting**

Whole-cell and liver lysates were prepared as previously described. Equal amounts of protein were loaded and resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). The membrane was blocked and probed with the primary antibodies against CTGF (goat anti-CTGF/CCN2, L-20, sc-14939, Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:500), total Smad2 (Santa Cruz Biotechnology; dilution 1:500), total Smad3 (Rabbit anti-SMAD3, ab28379, Abcam, Cambridge, UK; dilution 1:500), α-smooth muscle actin (αSMA), and collagen α1 type 1 (Col1) overnight at 4°C, and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein signals were visualized using the Supersignal West Dura Extended chemiluminescent (Pierce Biotechnology, Rockford, IL, USA) or 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) substrate (Perbio Science, Rockford, IL, USA). β-actin (Cymbus Bioscience; Southampton, UK) served as loading control. Blot results were subject to densitometric quantification using the Lumi-Imager System (Roche Diagnostics, Mannheim, Germany) and the LUMIANALYST 3.0 software (Roche Diagnostics) or Adobe Photoshop CS5 (Adobe, Dublin, Ireland).

**CTGF luciferase reporter gene assay**

Cells cultured in black 96-well plates were infected with 1x10^8 virions/mL of Ad-hCTGF-luc reporter virus, and after specific treatment, the Luciferase activity was measured. Generation of the Ad-hCTGF-luc reporter virus and performance of the assay were described previously.

**WST1 cell vitality assay**

To quantify cell proliferation as marker of vitality, the water-soluble tetrazolium (WST)-1 assay using a sulphonated tetrazolium salt, 4-(3-[4-iodophenyl]-2-[phenyl-2H-tetrazolio-1,3- benzene disulphonate]) (Roche Diagnostics) was performed according to the manufacturer’s instructions.

**Sirius-red and HE stain**

Formalin-fixed, paraffin-embedded liver sections were cut at 3 μm in thickness using a routine procedure. Extracellular matrix accumulation in liver sections was determined by staining with either hematoxylin-eosin (VWR Chemicals, Philadelphia, PA, USA) or Sirius red-Fast green FCF (Sigma), according to manufacturer’s protocols.

**CTGF ELISA**

Concentrations of the full-length CTGF molecule in serum were determined using an in-house sandwich enzyme-linked immunosorbert assay (ELISA) as described previously. In short, 96-well plates were coated with a rabbit anti-CTGF antibody (H-55, Santa Cruz Biotechnology) previously diluted in phosphate-buffered saline (PBS)-coating buffer (pH 7.4) for 24 h at RT with gentle shaking. Unspecific binding sites were blocked with blocking solution (10 g/L bovine serum albumin, 5 g/L sodium azide in PBS pH 7.4, 50 g/L sucrose). After pipetting 50 μL of reagent diluent (10 g/L bovine serum albumin in PBS; pH 7.4) to each microwell, 50 μL of calibrators (BioVendor, Brno, Czech Republic) or serum samples were added in duplicate and the assay plate incubated overnight at 4°C with gentle shaking. After washing with PBST (four times; PBS including 0.5 mL/L Tween 20), a goat anti-CTGF (L-20, Santa Cruz Biotechnology) antibody was added and the plate incubated for another 2 h with shaking. Wells were washed and then incubated with biotinylated rabbit antigen IgG (DakoCytomation, Hamburg, Germany) for 1 h. After another series of four washings and incubation with streptavidin-HRP (DakoCytomation), all wells were thoroughly rinsed and substrate reagent TMB (R&D Systems, Minneapolis, MN, USA) added. Color development was stopped after incubation for 20 min at 37°C, and absorbance was measured at 450 nm. Calibration curves were fitted using a 4-parameter logistic function provided by the VICTOR Multilabel Counter (Wallac/Perkin Elmer, Waltham, MA, USA).

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Malondialdehyde ELISA
Malondialdehyde (MDA) levels in liver homogenate were determined by fluorometric detection, as described previously.\textsuperscript{33,34}

Additional serum analytics
The serum concentrations of total and direct bilirubin, $\gamma$-glutamyl transpeptidase (GGT), alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine and lactate dehydrogenase (LDH; in cell culture media) were determined by standard auto-analyzer methods on a Roche Modular Analytics autoanalyzer. Quantitative determination of interleukin-6 in rat serum was performed using a chemiluminescence assay on the Immulite 2000 autoanalyzer (Siemens Medical Healthcare, Erlangen Germany). Serum concentrations of 1,7-DMX were determined using a Finnigan SSQ 710 liquid chromatography/mass spectrometer (LC-MS; GenTech, Girvan, Scotland). Chemically pure 1,7-DMX (purity ~98%; D5385, Sigma-Aldrich) served as internal standard.

Statistical analysis
SPSS 16.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. To compare the responses of the different groups to the respective treatment, a matched-pair $t$-test. $P \leq 0.05$ was considered statistically significant. Results are expressed as mean ± standard error of the mean (SEM). We furthermore performed regression analysis for the association of CTGF protein expression or promoter activation, and 1,7-DMX concentrations in cell culture media.

RESULTS
Inhibitory effect of 1,7-DMX on parameters of hepatocellular TGF-β signaling and hepatic stellate cell activation in vitro
Based on our previous findings of an inhibitory effect of caffeine and 1,7-DMX on hepatocellular TGF-β signaling,\textsuperscript{22} we performed a dose-response on the reduction of CTGF expression and promoter activity by 1,7-DMX in primary rat PC (Fig. 1) and on parameters of HSC activation, including CTGF promoter transcriptional activation. (Fig. 2).

Treatment of PC with 1,7-DMX confirmed the previously observed inhibitory effect of 1,7-DMX on the activation of the CTGF promoter as classical downstream TGF-β target gene and on CTGF protein expression (Fig. 1a). The estimated ID50 was set at 0.95 mM for both, protein expression (Fig. 1A, C) and transcriptional activation in PC (Fig. 1b,d).

In order to exclude a toxic effect on cultured PC at the tested concentrations, a WST-1 assay was performed (Fig. 1e) and release of LDH to the culture media was measured (Fig. 1f). However, 1,7-DMX application had no relevant effect on hepatocellular viability (Fig. 1e), the LDH release was even reduced with increasing 1,7-DMX concentrations (Fig. 1f).

As expected, CTGF was increasingly expressed during transdifferentiation of HSC to MFB.\textsuperscript{35} We observed an enforced cleavage of the full length molecule in MFB, resulting in an additional fragment of ~18 kDa – a phenomenon, for which we have no explanation yet (Fig. 2a,b). Results showed a transdifferentiation dependent inhibitory effect of 1,7-DMX on CTGF expression in HSC (Fig. 2a,b), being particularly effective in the progressive stage of transdifferentiation. A representative hCTGF-luciferase reporter assay performed in 2-day-old HSC confirmed their responsiveness towards 1,7-DMX also on the transcriptional level (Fig. 2e). The estimated ID50 was calculated as being 1.25 mM for transcriptional activation of the CTGF promoter in HSC cultured for 48 h (Fig. 2f). The observed inhibition of CTGF expression was accompanied by a continuous 1,7-DMX dependent reduction of expression of collagen α1 type 1 but not of α-smooth muscle actin throughout the entire process of transdifferentiation (Fig. 2a,c,d).

Oral intake, and serum concentrations of 1,7-DMX in the rat
To test a possible in vivo relevance of our results obtained in the in vitro situation, we subjected 20 male Sprague–Dawley rats to either prolonged BDL or sham operation. Some of the animals received oral 1,7-DMX application in the drinking water (1 mM), and the effect of 1,7-DMX on the formation of cholestatic liver fibrosis was examined.

Table 1 gives an overview on the daily 1,7-DMX intake and on saturated serum 1,7-DMX concentrations in the four different study groups. 1,7-DMX treated rats were in excellent general condition. BDL rats showed a distinct gallbladder hydrops but displayed no liver abscess formation or other relevant complications. Renal insufficiency and/or systemic or local inflammation was excluded by the quantification of serum creatinine and interleukin-6 concentrations (data not shown).
CTGF

\[\frac{\text{Ad-hCTGF-Luc intensity}}{\text{CTGF/\(\beta\)-actin [% of control]}}\]

(a)

CTGF\[\rightarrow\]
\[\beta\text{-actin}\[\rightarrow\]

(b)

100
90
80
70
60
50
40
30
20
10
0

Ad-hCTGF-Luc intensity [% of control]

Untreated
0.03125
0.0625
0.1563
0.3125
0.625
1.25
2.5

1,7-DMX [mM]

(c)

1,7-DMX concentrations [mM]

1,7-DMX [mM]

0.5 1

Untreated

(d)

0.25
1,7-DMX [mM]

2.5

WST-1 (OD450nm/ culture)

2.5

0.5 1

LDH [U/mL]

(e)

Untreated

1,7-DMX [mM]

0.3125
0.625
1.25
2.5

2

1

(f)

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Oral 1,7-DMX attenuates cholestatic hepatocellular injury and intercellular collagen deposition in the cholestatic rat liver

Cholestasis was assessed by the biochemical quantification of GGT and AP activities, as well as of total and direct bilirubin concentrations in serum. Hepatocellular damage was assessed by the determination of AST and ALT activities in serum. As expected, BDL significantly increased serum activities of GGT (sham: 16.0 ± 2.8 U/L; BDL: 96.0 ± 14.4 U/L; average 500%) and AP (sham: 118.8 ± 19.9 U/L; BDL: 457.8 ± 55.9 U/L; average 285%) as well as total (sham: 0.1 ± 0.0 mg/dL; BDL: 10.8 ± 2.1 mg/dL; average 9.9%) and direct bilirubin (sham: 0.1 ± 0.0 mg/dL; BDL: 7.5 ± 1.5 mg/dL; average 7.4%) concentrations compared with sham-operated animals (Fig. 3).

Parameters of hepatocellular damage, i.e. AST (sham: 94.7 ± 13.2 U/L; BDL: 654.7 ± 181.1 U/L; average 592%) and ALT (sham: 62.0 ± 9.0 U/L; BDL: 211.2 ± 60.9 U/L; average 241%) were also significantly elevated. There was no pathological elevation of any of the parameters in both, untreated sham-operated rats and sham-operated rats receiving oral 1,7-DMX. However, concomitant oral application of 1,7-DMX relevantly but not significantly reduced serum concentrations of total (BDL: 10.8 ± 2.1 mg/dL; BDL + 1,7-DMX: 8.5 ± 1.8 mg/dL; average 15.5 %) and direct bilirubin (BDL: 7.5 ± 1.5 mg/dL; BDL + 1,7-DMX: 5.0 ± 2.9 mg/dL; average 32.6%), as well as activities of GGT (BDL: 96.0 ± 14.4 U/L; BDL + 1,7-DMX: 76.8 ± 21.4 U/L; average 20.1%) and AP (BDL: 457.8 ± 55.9 U/L; BDL + 1,7-DMX: 401.3 ± 39.7 U/L; average 12.4%). AST (BDL: 654.7 ± 181.1 U/L; BDL + 1,7-DMX: 468.0 ± 108.3 U/L; average 28.5%), and ALT (BDL: 211.2 ± 60.9 U/L; BDL + 1,7-DMX: 157.8 ± 24.4 U/L; average 25.3%) activates in serum were also lowered in those cholestatic animals receiving oral 1,7-DMX treatment (Fig. 3).

Hematoxylin-eosin and Sirius-Red staining showed extensive bile infarcts, as could be seen by confluent foci of hepatocyte feathery degeneration due to bile acid cytotoxicity, bile duct proliferation, and bridging fibrosis in untreated BDL rats. All lesions were attenuated in 1,7-DMX-treated BDL rats (representative results shown in Figure 4a [Sirius-Red stain] and 4B [HE-stain]). No pathological changes were observed in liver tissues from rats receiving sham operation or sham operation and oral 1,7-DMX.

Hepatic TGF-β signaling is inhibited in vivo following oral 1,7-DMX intake

Consistent with the data of the presented in vitro studies showing a repressive effect of 1,7-DMX on TGF-β and Smad signaling in both, primary rat PC and transdifferentiating rat HSC, we observed that oral treatment with 1,7-DMX significantly decreased steady state levels of CTGF (Fig. 5a) as well as total Smad2 (Fig. 5b) and Smad3 (Fig. 5c) in homogenized whole liver lysates of 1,7-DMX treated animals when compared to untreated BDL rats. However, we observed a considerable individual variability. In order to see whether the degree of cholestasis, and thus had an influence on the repressive capacities of 1,7-DMX on hepatic TGF-β signaling, we...
divided BDL rats receiving oral 1,7-DMX into two groups, one group with serum direct bilirubin concentrations between 0 and 5 mg/dL (slight to moderate cholestasis) and one group with serum direct bilirubin concentrations between 5 and 10 mg/dL (moderate to severe cholestasis). Of note, animals with moderate to severe cholestasis displayed a much stronger response towards oral 1,7-DMX treatment compared to those animals with slight to moderate cholestasis, as shown by a much stronger reduction of intrahepatic CTGF (Fig. 5d), Smad2 (Fig. 5e) and Smad3 (Fig. 5f) concentrations.

Serum CTGF concentrations are not reduced in 1,7-DMX treated bile-duct-ligated rats

As expected from previous studies,32 serum CTGF concentrations were significantly elevated (approximately 5-fold) 30 days after BDL (sham: 4.2 ± 0.1 ng/mL; BDL: 20.4 ± 2.7 ng/mL; Fig. 6). Of note, different to the results of protein expression obtained in whole liver lysates, oral application of 1,7-DMX did not alter serum CTGF concentrations in BDL animals, making this parameter unsuitable for monitoring the efficacy of 1,7-DMX treatment (Fig. 6).

Oral 1,7-DMX intake reduces hepatic MDA concentrations

Lipid peroxidation is a well-established mechanism of cellular injury due to reactive oxygen species (ROS), and is used as an indicator of oxidative stress in cells and tissues.36 To evaluate the effect of pharmacological 1,7-DMX application on oxidative stress in the cholestatic liver, we therefore measured intrahepatic MDA as suitable biomarker36 in 1,7-DMX treated and untreated rats with or without cholestatic liver injury.

In line with previous findings,37 we found a strong increase of oxidative damage to lipids in the hepatic tissue of BDL rats. MDA concentrations were 2.6-fold higher in BDL rats vs. sham operated controls (sham: 146.2 ± 20.8 mmol/g dry weight; BDL: 377.0 ± 88.1 mmol/g dry weight; Fig. 7). After oral 1,7-DMX treatment lipid peroxidation and thus MDA concentrations in the liver were reduced compared to those rats, only receiving BDL (BDL: 377.0 ± 88.1 mmol/g dry weight; BDL + 1,7-DMX: 312.7 ± 112.3 mmol/g dry weight; average 17.1%; Fig. 7).

Table 1 Oral water and 1,7-DMX intake as well as resulting saturated serum 1,7-DMX concentrations in the four study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>n</th>
<th>Water intake/24 h [mL]</th>
<th>1,7-DMX intake/24 h [mg]</th>
<th>Saturated 1,7-DMX concentration in serum [mg/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: sham</td>
<td>6</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0</td>
</tr>
<tr>
<td>2: sham + 1,7-DMX [1 mM]</td>
<td>2</td>
<td>48.4 ± 7.8</td>
<td>8.7 ± 1.4</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>3: BDL</td>
<td>8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0</td>
</tr>
<tr>
<td>4: BDL + 1,7-DMX [1 mM]</td>
<td>4</td>
<td>54.7 ± 5.0</td>
<td>9.9 ± 0.9</td>
<td>2.5 ± 0.9</td>
</tr>
</tbody>
</table>

1 mM 1,7-DMX = 0.18 g/L.
BDL, bile duct ligation; sham, sham-operation.

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**DISCUSSION**

Our data are the first to evaluate a potential therapeutic application of the caffeine derived primary metabolite paraxanthine, or 1,7-dimethyl xanthine (1,7-DMX), on the formation of experimental cholestatic liver fibrosis in the rat.

*In vivo* studies investigating methylxanthines as potentially antifibrotic agents have been missing so far, but just in recent years, more and more epidemiological studies have pointed to a beneficial effect not just of coffee, but also of caffeine (1,3,7-trimethylxanthine) consumption, on the progression of chronic fibrogenic liver diseases. For example, the study by Modi *et al.*
Figure 4  Bile duct ligation (BDL)-induced histopathological changes in livers 30 days after surgery with or without concomitant oral 1,7-DMX application compared to livers of sham operated animals. Representative photomicrographs of liver sections processed for Sirius-red – (a) or hematoxylin-eosin (HE) (b) staining: sham (upper left), sham + 1,7-DMX (upper right), BDL (lower left), and BDL + 1,7-DMX (lower right). Extensive bile infarcts, bile duct proliferation, and bridging fibrosis in untreated BDL rats are shown. All these lesions were markedly attenuated in 1,7-DMX-treated BDL rats. No pathological changes were observed in liver tissues of rats with sham operation or sham operation and oral 1,7-DMX treatment. Original magnification, 100×.
impressively demonstrated that those patients with chronic hepatitis C virus (HCV) infection who consumed more than 308 mg of caffeine daily (particularly from caffeinated coffee) had reduced fibrogenic activity, compared with patients who were no coffee consumers. For each 67 mg increase in caffeine consumption a 14% decrease in the odds of advanced fibrosis for patients with chronic HCV could be demonstrated.

Our previous in vitro findings identified 1,7-DMX as the most potent inhibitor of profibrogenic TGF-β in PC among caffeine and its three primary demethylated metabolites, by dose dependently inducing degradation of the TGF-β effector Smads 2 and 3. The intestinal resorption of 1,7-DMX in human is 100%. Following oral intake (2 and 4 mg/kg body weight), 3 and 2 h post-ingestion, maximum plasma concentrations of 2.8 or 5.6 mg/L, respectively, were achieved. Bortolotti et al. thoroughly investigated the pharmacokinetics of 1,7-DMX in the rat and found that up to 10 mg/kg body weight, 1,7-DMX followed first order kinetics. The fraction bound to plasma proteins in the concentration range of 1–100 micrograms/mL was determined as 15%. Intravenous administration of [1-Me-14C]-1,7-DMX to rats showed that it is mostly excrated.
through the urine, where 85 ± 3% of the initially administered dose could be recovered; however, it is also highly reabsorbed in the renal tubule. In addition to renal excretion, 1,7-DMX is enzymatically metabolized in the liver to 1-methylxanthine and 7-methylxanthine by CYP1A2, to 1,7-dimethyluric acid (17U) and 3,7-dimethyluric acid (37U) by the FAD/molybdopterin-dependent xanthine oxidase/dehydrogenase, as well as to 5-acetylamino-6-formylamino-3-methyluracil by the arylamine N-acetyltransferase 2. Mean half-life and elimination rate constant of 1,7-DMX in the rat were calculated as being 1 h and 0.70 h⁻¹, respectively.

Considering this, we therefore chose continuous oral ingestion through the drinking water as the best type of application to achieve continuously elevated 1,7-DMX serum concentrations in the therapy group. Arnaud and Enslen could show an equilibrium between blood and tissue concentrations with no particular organ-specific accumulation (except for the brain). Our *in vitro* experiments allowed a calculation of an ID50 for 1,7-DMX of 0.95 mM for CTGF protein expression and promoter activity in rat PC and of 1.25 mM for CTGF promoter activity in 2-day-old rat HSC. Considering an average blood volume of male Sprague-Dawley rats of 6.86 ± 0.53 mL/100 g and an average weight of the rats of 150 g, we therefore defined 0.1 mM as therapeutically justifiable concentration for long time continuous *in vivo* exposure that should not have any serious side-effects.

![Figure 6](image1.png)

**Figure 6** No difference in serum connective tissue growth factor (CTGF) concentrations of bile duct ligation (BDL) rats receiving oral 1,7-DMX administration and untreated BDL rats. Serum CTGF levels (*n* = 14) were determined by an in-house enzyme-linked immunosorbent assay. BDL led to a significant increase of serum CTGF concentrations. However, there was no significant difference between the sham and sham + 1,7-DMX group as well as BDL and BDL + 1,7-DMX groups. Data represent means ± standard error of the mean (SEM) for each group. * = *P* < 0.05.

![Figure 7](image2.png)

**Figure 7** Intrahepatic malondialdehyde (MDA) concentrations are reduced in bile duct ligation (BDL) rats receiving concomitant oral 1,7-DMX application. MDA concentrations in liver tissue homogenates (*n* = 14) were determined by fluorometric measurement as described in Methods. Intrahepatic MDA levels in rats with BDL were significantly higher than in the sham-operated group. Oral 1,7-DMX application led to a relevant but not yet significant decrease of lipid peroxidation in liver tissue. * = *P* < 0.05.
But let’s get back to the in vitro results presented in this study. They are, in part, in line with our previous findings of inhibitory capacities of both, caffeine and 1,7-DMX on PC. Our results obtained in PC should therefore not be discussed at this point any more. Not thoroughly investigated so far has been the effect of 1,7-DMX on transdifferentiating rat HSC. The observed increasingly strong repression of CTGF and also collagen α1 type 1 expression by 1,7-DMX in the course of transdifferentiation may be explained by the known selective inhibition of the Smad2/3 pathway by methylxanthines. As all three forms of TGF-β receptors, types I, II, and III (betaglycan), just as TGF-β itself, are synthesized by HSC, and TGF-β1 binding and responsiveness are greatly enhanced during their activation and transdifferentiation to MFB in vivo and in vitro, facilitating an autocrine stimulation, and as Smad signaling evolves with HSC activation, an increasing sensitivity of HSC towards the pharmacological effects of methylxanthines in the course of transdifferentiation is very likely. The mode of action of 1,7-DMX as inhibitor of TGF-β induced Smad signaling also explains the lacking effect on α-smooth muscle actin expression in HSC, as the transcriptional regulation of α-smooth muscle actin is (largely) TGF-β independent, whereas its re-organization to stress fibers and the enhancement of focal adhesion formation during transdifferentiation is not.

Now let’s take a look at the in vivo results obtained. Following oral 1,7-DMX application in rats with cholestatic liver injury, we show (i) that cholestatic hepatocellular damage and intercellular collagen deposition in the liver is attenuated; (ii) that hepatic TGF-β signaling and de novo hepatic CTGF synthesis are inhibited in vivo through interference with the Smad2/3 pathway; and (iii) that hepatic lipid peroxidation, and thus, oxidative stress in the liver is reduced.

Next to these intriguing results, our in vivo study opens a few questions; however, for example, why we observe a relatively large interindividual variability in the response to oral 1,7-DMX treatment, which makes it difficult to yield statistically significant results in the small number of cases that is presented here. As environmental factors may be largely excluded in our group of animals, such variability may very likely be the result of genetic variation, either in the CYP1A2-, xanthine oxidase/dehydrogenase-, or arylamine N-acetyltransferase 2 gene, of its regulatory partners, or in other genes involved in the disposition of this xanthine derivative (e.g. uptake and/or other transporters, metabolizing enzymes or plasma binding proteins), and thus in the pharmacokinetic properties of each animal.

Up to now, several genetic polymorphisms in the CYP1A2-, xanthine oxidase/dehydrogenase-, or arylamine N-acetyltransferase 2 genes have been identified and functionally characterized through comprehensive single nucleotide polymorphism (SNP) discovery efforts. Most intriguing were the findings by Labedzki et al., showing that the main metabolite of 1,7-DMX was 1-methylxanthine in wild-type mice liver microsomes, whereas CYP1A2 knockout mice mostly produced 7-methylxanthine. However, further studies on the effect of metabolic variants on the pharmacological effect of 1,7-DMX in the liver are certainly required at this point.

But it may also be that the effects of 1,7-DMX are dependent on the extent of exogenous TGF-β stimulation, which, of course, displays high interindividual variability. Several studies have demonstrated that cholestasis increases the expression of TGF-β mRNA in liver tissue, and that serum TGF-β concentrations are significantly increased already 3 days after BDL. It was frequently suggested that this phenomenon of an upregulation of TGF-β results in delayed liver regeneration in conditions of obstructive jaundice. In line with this, after liver resection, TGF-β1 blockage with anti-TGF-β1 monoclonal antibody improved liver regeneration both functionally and morphologically.

In our previous study, we could show that the proteasomal degradation of the TGF-β effector Smads 2 and 3 that is triggered by methylxanthines takes place through a functional enhancement of the hepatocellular activities of SMURF2, an E3 ubiquitin ligase.

Proteasomal degradation of Smad2 in response to TGF-β requires receptor-mediated phosphorylation of its C-terminal serines. This indicates a facilitation of proteasome-mediated Smad2 degradation in the presence of exogenous TGF-β. However, Smurf2 is also able to bind the activated TGF-β receptor complex, leading to TGF-β type 1 receptor degradation, which in turn results in degradation of total Smad3 protein allosterically bound to the type 1 receptor in the Smad3/Sara (Smad anchor for receptor activation)/TGF-β type 1 receptor kinase complex. Therefore, it may be suggested that animals displaying a higher activity of the cytokine TGF-β would be more sensitive towards the inhibitory actions of methylxanthines, such as 1,7-DMX. This suggestion is supported by our in vivo data showing that the stronger the degree of obstructive cholestasis (being an inducer of TGF-β activities), as determined by serum concentrations of direct bilirubin,
the stronger the depletory effect of 1,7-DMX on intrahepatic CTGF, Smad2 and Smad3 concentrations. As the degree of cholestasis is interindividually different, the response towards oral 1,7-DMX treatment is as well.

Another aspect of our study that remains unclear is that even though we find a suppression of TGF-β signaling, and, as a result, of CTGF expression in liver tissue of 1,7-DMX treated BDL rats, serum CTGF concentrations are not reduced in this group. Several explanation approaches are possible, but they all remain speculative: it may be that reduced CTGF expression results in a compensatory increased secretion from liver cells or other tissues; that 1,7-DMX reduces renal CTGF clearance, thereby leading to a retention of the full-length molecule; that CTGF is increasingly released from necrotic or apoptotic cells that do not respond to transcriptional regulation by TGF-β anymore and, thus, are not sensitive towards the inhibitory action of methylxanthines; that 1,7-DMX triggers the release of CTGF from protein binding, or, very simple; that the investigated study group is too small to reveal any relevant differences in serum CTGF concentrations. Also here, further investigations are certainly necessary.

In conclusion, our study gives a hint on a potential application of the methylxanthine derivative 1,7-DMX in the prevention, therapy support and, maybe, treatment of fibrogenic process of the rat liver, without showing significant side effects at the tested dosage. Based on these findings and on the role of TGF-β (and CTGF) in hepatic fibrogenesis (and, very likely, carcinogenesis), a suppressive effect of 1,7-DMX also on human liver fibrogenesis may be suggested. Even though the study number is rather small, and further experiments (not just on the dose-response relationship) are certainly required, this pilot study warrants further studies of 1,7-DMX as a ray of hope in the search for new drugs to fight fibrotic processes, not just of the liver.

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