Tetracyclines cause cell stress-dependent ATF4 activation and mTOR inhibition

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Abstract

Tetracyclines have long been used as valuable broad-spectrum antibiotics. The high antibacterial activity of tetracyclines, combined with their good tolerability, has led to their widespread use in treating various infectious diseases. However, similar to other antibiotics, tetracyclines are also known for their adverse effects on different human tissues, including hepatic steatosis. We observed that tetracyclines, including doxycycline and minocycline, caused enhanced expression of the liver chalone inhibin βE in HepG2 cells, mediated by the cell stress-regulated transcription factor ATF4. ATF4 and its target genes ATF3, CHOP, and inhibin βE are involved in cell cycle control, cell survival, cell metabolism, and modulation of cytokine expression. Furthermore, we observed that long term tetracycline incubation also caused inhibition of the mTOR complex, a central regulator of cell metabolism, further contributing to the observed cell-cycle arrest and autophagy in doxycycline- and minocycline-treated cell lines. ATF4 activation and mTOR inhibition link two crucial regulators of the cellular stress response and cell metabolism to the effects of tetracyclines on eukaryotic cell metabolism, and may help to understand the antibiotic-independent influence of these drugs on human tissues. Since the observed effects of tetracyclines on human cells were also found to be dependent on the magnesium ion concentrations supplied, the data further indicate the importance of magnesium supplementation to reduce or prevent side effects of long term treatment with tetracyclines.

Introduction

Tetracyclines are broad-spectrum antibiotics active against a wide range of bacteria [1,2]. The good tolerability, oral applicability, and low costs of this drug class have led to their wide use in human medicine and in animal husbandry. However, despite their general lack of pronounced adverse effects, tetracyclines may exert negative influences on the gastrointestinal tract, skin, and central nervous system [3]. Although similar in their antibiotic spectrum, the types and frequencies of adverse effects of the most commonly prescribed tetracyclines appear to differ among patients. For example, drug-induced hepatotoxicity and steatosis, reported rarely in the context of doxycycline treatment [3–5], is more frequently associated with minocycline [3,5,6] and tetracycline [4,7].

Tetracyclines are strong metal ion-chelating agents, leading to their accumulation in calcium-rich organs such as bones and teeth [8]. Several of their adverse effects, as well as their beneficial non-antibiotic applications, are associated with their divalent metal ion-chelating properties [9]. Sequestration of zinc ions has been regarded as the main cause of matrix metalloproteinase inhibition,
resulting in reduced inflammation, angiogenesis, and cancer-cell invasion and metastasis [2,8,9]. Independent of their effects on matrix metalloproteinases, high concentrations of tetracyclines also cause direct cytostatic and cytotoxic effects in cancer cells both in vitro and in vivo [8,10,11].

Serum levels of tetracyclines after standard oral intake fall within the range of 2–5 μg/ml [1], but it is to be expected that gastrointestinal cells and liver cells encounter markedly higher concentrations of tetracyclines during drug uptake and detoxification. Hepatoma-derived cell lines have frequently been used to study the cellular effects of hepatotoxic agents or to facilitate preclinical screening of drugs for possible hepatotoxicity [12]. We recently showed that induction of inhibin βE in HepG2 cells can be used as a sensitive marker to screen for drugs that may cause endoplasmic reticulum stress [13]. Endoplasmic reticulum stress can arise as a consequence of diverse physiological and drug-induced disturbances of cellular homeostasis, and may lead to cell-cycle arrest, apoptosis, or autophagy [14–15]. Autophagy is also controlled by the mTOR kinase complex, which is a key regulator and integrator of cellular metabolism and growth-factor responses [16,17]. While screening antibiotic drugs for their ability to induce endoplasmic reticulum stress in HepG2 hepatoma cells, we noticed that drugs of the tetracycline family were able to strongly induce expression of the peptide hormone inhibin βE in HepG2 cells, and were also associated with reduced cell viability and marked morphological and physiological changes. The aim of this study was to identify the molecular mechanisms that cause these physiological changes in human cell lines, including the use of cells of hepatic and gastrointestinal origin, whose tissues are expected to be exposed to high concentrations of tetracyclines during routine oral antibiotic therapy. Since newly identified growth-inhibiting effects of established medications may also be of interest for a possible drug repurposing in cancer science, confirmed by recent studies on minocycline [10,11], experiments on HeLa cervical cancer cells were also included.

### Drugs and drug treatment

Doxycycline, minocycline, and nelfinavir were purchased from Sigma (Munich, Germany). Tetracyclines were kept at −20 °C in stock solutions of 10 mg/ml in H2O, nelfinavir was kept at −20 °C in a stock solution of 100 mg/ml in dimethyl sulfoxide.

### PCR analysis

Semi-quantitative and real-time PCR analysis for XBP1 splicing was performed as previously described [13,19]. Whole RNA was extracted from cells with the RNA extraction kit (Macherey-Nagel, Düren, Germany) and synthesis of cDNA was performed with the MMLV-reverse transcriptase kit (Promega, Mannheim, Germany). The primer sequences for semi-quantitative amplification and real-time PCR analysis of INHBE, ATF3, CHOP, XBP1, and β-actin have previously been described [13]. Calculation of gene expression was by the 2−ΔΔCt method [20], using β-actin expression as a reference value.

### MIT assay

Cells were seeded in 96-well cell culture plates (5 × 103 cells per well) in the presence or absence of the indicated drugs and incubated for 72 h under cell culture conditions. For MITT assay analysis, 20 μl of an MITT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide, Sigma, Germany) stock solution (5 mg/ml PBS) was added to 200 μl of cell culture medium and incubated for 1 h under cell culture conditions. Precipitates were dissolved in 100 μl of dimethyl sulfoxide and analyzed by an ELISA reader at 595 nm.

### Cell organelle staining

The MitoCapture Mitochondrial Apoptosis Detection Kit (Axxora, Lörrach, Germany) was used for mitochondrial membrane potential analysis, according to the manufacturer’s instructions. Autophagy was visualized with the autophagy detection marker (Cyto-ID, Enzo Life Sciences, Lörrach, Germany) and liposomes were stained with lipophilic dye Nile red (MP Biomedicals, Illkirch, France). Cells were incubated under cell culture conditions for 1 h with the organelle trackers and photographed as viable cells by means of a Zeiss Axioskop fluorescence microscope (Zeiss, Germany).

### Western blot analysis

Cells were incubated in 10 cm diameter Petri dishes and proteins were extracted with RIPA-buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% doxycolline, 0.1% SDS). 20 μg of protein (BioRad Bradford Assay, BioRad, München, Germany) were used for SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes in a BioRad Mini Protean II Cell (BioRad, Munich, Germany) at 1 mA/cm2 membrane in 10% methanol, 192 mM glycine, 25 mM Tris, pH 8.2. Membranes were blocked with 4% non-fat milk powder in PBS-0.05% Tween for 4 h. Primary antibodies were applied in blocking buffer and incubated at room temperature overnight. Antibodies against 4E-BP1, p70S6 kinase, phospho-p70S6 kinase, cyclin D3, cdk1, mc1, smac/diablo, and COX-IV were all purchased from Cell Signaling Technology.
(NEB, Frankfurt, Germany). The PathScan multiplex Western blot cocktail III was from Cell Signaling. Antibodies against Bcl2 (100), ATF4 (C-20), GAPDH (O411), and β-actin (C4) were from SantaCruz Biotech (Heidelberg, Germany). Secondary, alkaline phosphatase (AP)-coupled antibodies against the corresponding primary antibodies were from Dianova, Hamburg, Germany. Alkaline phosphatase detection was performed by the chromogenic BCIP/NBT substrate (Promega, Mannheim, Germany).

Results

Tetracyclines cause ATF4 activation and upregulation of inhibin βE in HepG2 cells

We recently showed that transcriptional induction of inhibin βE (INHBE) is a response mechanism to cell stress and can be used as a sensitive marker for drug-induced endoplasmic reticulum stress [13]. RT-PCR expression analysis of inhibin βE in HepG2 cells incubated with tetracyclines revealed a pronounced induction of inhibin βE by doxycycline and minocycline treatment (Fig. 1A). RT-PCR analysis additionally revealed upregulation of the cell-stress markers ATF3 (activating transcription factor 3) and CHOP (C/EBP homologous protein) in HepG2 cells treated with either minocycline or doxycycline (Fig. 1A). Inhibin βE, ATF3, and CHOP are common transcriptional target genes of the endoplasmic reticulum stress-inducible ATF4 transcription factor [13]. However, XBP1 splicing, an exclusive marker of endoplasmic reticulum stress, was barely detectable in doxycycline- and minocycline-treated HepG2 cells; by contrast, XBP1 splicing was strongly activated by nelfinavir, a strong inducer of endoplasmic reticulum stress used as a positive control (Fig. 1A). Real-time PCR analysis of inhibin βE expression and specific analysis of XBP1 splicing further confirmed induction of inhibin βE in the absence of pronounced endoplasmic reticulum stress in doxycycline- and minocycline-treated HepG2 cells (Fig. 1B), suggesting endoplasmic reticulum stress-independent ATF4 activation by tetracyclines. Enhanced levels of ATF4 protein expression could be confirmed by Western blot analysis in HepG2 cells treated with minocycline, doxycycline, and nelfinavir (Fig. 1C,D).

Tetracyclines reduce cell viability in human cell lines

High concentrations of tetracyclines induce cell death in human cancer cells [2,10,11], but the concentrations we used to induce inhibin βE expression in HepG2 cells were subtoxic, and phase-contrast microscopy revealed no visible signs of apoptosis (Fig. 2). However, treatment of HepG2 cells with 20 μg/ml of either doxycycline or minocycline resulted in a different phenotype that was more pronounced in TC7 cells and HeLa cervical-cancer cells (Fig. 2). In these cell lines, tetracycline treatment was associated with a marked increase in cytoplasmic granules and an overall decrease in dividing cells, but no visible signs of apoptosis or necrosis (Fig. 2). Assessment of general cell viability by the MTT assay revealed that even low concentrations (5–10 μg/ml) of tetracyclines caused a reduction in cell viability of HepG2, TC7, and HeLa cells that became pronounced after prolonged incubation (Fig. 3).

In minocycline-treated cells in particular, a massive accumulation of strong light-scattering vesicles occurred (Fig. 2). Staining with Nile red, a fluorescent lipophilic dye, confirmed that in HeLa and HepG2 cells, most of these vesicles represented lipid droplets (Fig. 4A). Although lipid droplets were poorly visible by...
phase-contrast microscopy in minocycline-treated TC7 cells (Fig. 2), Nile-red staining also confirmed massive accumulation of small lipid droplets in TC7 cells (Fig. 4A). In doxycycline-treated cell lines, lipid-droplet accumulation also occurred (Fig. 4A) but was less distinct than in minocycline-treated cells. In both minocycline- and tetracycline-treated cell lines, accumulation of granular vesicles (Fig. 2) and larger autophagosome-like vacuoles could also be observed (Fig. 2). Staining with an autophagy-marker dye confirmed a marked increase in autophagosome formation in all doxycycline- and minocycline-treated cell lines tested (Fig. 4B).

**Tetracyclines cause mTOR inhibition**

Autophagy is under direct control of the mTOR protein kinase complex, and we therefore investigated the effect of tetracyclines on mTOR activity by analyzing the phosphorylation status of mTOR substrates. Erythromycin, structurally unrelated to tetracyclines but with similar anti-bacterial activity, was tested in comparison. A marked reduction in phosphorylation of the mTOR downstream targets 4E-BP1 and p70S6 kinase was revealed by Western-blot analysis of doxycycline and minocycline-treated HeLa, TC7, and HepG2 cells, but not in erythromycin-treated cells (Fig. 5). Western-blot analysis also revealed pronounced downregulation of cell-cycle regulatory proteins cyclin D3 and Cdk1 (Fig. 5). To investigate whether mTOR inhibition by tetracyclines was an immediate or late effect, a time-course analysis was performed. Fig. 6 shows that activation of ATF4 was an early effect in minocycline-treated HepG2 and TC7 cells, having already occurred at 8 h of treatment. By contrast, mTOR inhibition by minocycline occurred after ATF4 activation and downregulation of the cell-cycle regulatory protein cyclin D3, and thus apparently represents a secondary effect (Fig. 6). Expression of mitochondrial proteins Smac/Diablo and COX-IV (cytochrome c oxidase IV) remained largely unchanged; however, in TC7 cells, a slight upregulation of Bcl-2 and a slight downregulation of Mcl-1 occurred after prolonged minocycline treatment (Fig. 6B). Because tetracyclines preferentially accumulate in mitochondria and can lead to mitochondrial dysfunction [21,22], we assessed the uptake of a mitochondrial viability marker in the presence of doxycycline and minocycline. Whereas the uptake and accumulation of the color dye in doxycycline-treated HeLa cells was similar to that of control cells, minocycline-treated HeLa cells exhibited a marked reduction in the uptake of the MitoTracker dye (Fig. 7).

**Magnesium supplementation protects from growth-inhibitory effects of tetracyclines**

Tetracyclines are strong divalent cation-binding agents, and in particular, the pronounced effect of minocycline on mitochondria was described to be due to either calcium or magnesium binding,
depending on the experimental setting [21,22]. In addition, calcium, magnesium, and zinc ions are important interaction partners of a variety of cellular organelles, signaling cascades, and transcription factors. We therefore tested the effect of supplementing media with calcium, magnesium, and zinc ions on the tetracycline-induced reduction in viability of HeLa and HepG2 cells. In both cell types, magnesium supplementation markedly inhibited the growth-inhibitory effect of both doxycycline and tetracycline, whereas calcium- and zinc-ion supplementation revealed either only marginal or no effect on the growth-inhibitory effects of tetracyclines (Fig. 8A). Since tetracyclines also interact with serum components and are transported by serum albumin, we additionally tested the effect of tetracyclines in cell culture medium supplemented with or without fetal bovine serum. Serum also contains growth factors which may influence the effects of tetracyclines on cellular metabolism (Fig. 8A). Since tetracyclines also interact with serum components and are transported by serum albumin, we additionally tested the effect of tetracyclines in cell culture medium supplemented with or without fetal bovine serum. Serum also contains growth factors which may influence the effects of tetracyclines on cellular metabolism. Fig. 8B shows that ATF4 activation by tetracyclines occurs both in the absence and presence of serum supplementation, although the induction of ATF4 was found to be more pronounced under conditions of serum supplementation.

**Discussion**

We initially analyzed inhibin βE expression in HepG2 cells as a sensitive tool to screen for ER stress-inducing agents. However, further analysis of endoplasmic reticulum stress markers revealed only weak induction of endoplasmic reticulum stress by tetracyclines but an endoplasmic reticulum stress-independent ATF4 activation and ensuing inhibin βE expression. In fact, a toxicogenic approach previously identified inhibin βE as one of the genes most significantly upregulated by drug-induced phospholipidosis [23]. Inhibin βE is a peptide-hormone subunit of the TGFβ family with poorly understood autocrine or paracrine functions. In untransformed liver cells, inhibin βE is expressed at high concentrations and is believed to exert growth-regulatory functions [24,25]. Inhibin βE expression is down-regulated in hepatoma cells (including HepG2 cells) and hepatocellular cancer [24,25], and may also have growth-inhibitory and tumor-suppressive functions in other tissues [26]. Thus, induced inhibin βE expression may contribute to the growth-restricting effects of tetracyclines. Because the inhibin βE-regulating transcription factor ATF4 controls the expression of a variety of other stress-related target genes [14–16,27] the roles of these factors in tetracycline-treated cells or tissues remain to be investigated. The ATF4 target gene ATF3 functions primarily as a transcriptional repressor, attenuating the expression of cell-cycle regulatory proteins (cyclin D) and pro-inflammatory cytokines (interleukins, interferons) via interactions with NF-κB and AP1 [28]. ATF3 activation by tetracyclines may therefore contribute to cell-cycle arrest as well as some of the anti-inflammatory effects of these drugs.

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**Fig. 3** - Tetracyclines reduce cell viability in human cell lines. HeLa, CaCo2-TC7, and HepG2 cells were seeded in 96-well cell tissue plates (5000 cells/well in duplicate) and incubated for 1–3 days with the indicated concentrations of either doxycycline or minocycline. Cell viability was analyzed by the MTT assay and expressed as the percentage of viability (optical density at 595 nm) compared to untreated cells. Representative cell survival curves from two independently performed experiments are shown.
Tetracyclines exert pleiotropic effects in humans, leading to both beneficial and detrimental side effects [1,2]. Minocycline recently attracted a great deal of interest because of its assumed neuroprotective effects, leading to its application and/or clinical testing in treatment of ischemia, multiple sclerosis, Huntington’s disease, Parkinson’s disease, and Alzheimer’s disease [29,30]. Some of these benefits are believed to be due to the anti-inflammatory effects of tetracyclines [2,30]. mTOR activity is connected to NF-κB [31] and eNOS/iNOS activity [32]; therefore, mTOR inhibition by tetracyclines may also contribute to the anti-inflammatory and cytoprotective effects of minocycline. The cytoprotective effect of minocycline was further deduced from its ability to reduce caspase expression [33], to increase mitochondrial Bcl-2 expression [34], and to inhibit mitochondrial cytochrome c release [35]. Minocycline has repeatedly been shown to lead to a “mild uncoupling” of the mitochondrial membrane potential [21,22,36]. The observations presented in this study confirmed attenuation of the mitochondrial membrane potential (Fig. 7) in minocycline-treated cancer cells and upregulation of Bcl-2 by minocycline in HeLa cells (Fig. 6).

Besides the common effects of both doxycycline and minocycline, cytosolic lipid accumulation (Fig. 2) and reduction in mitochondrial membrane potential (Fig. 7) were primarily observed in minocycline-treated cell lines, suggesting that minocycline has the more pronounced effect on mitochondrial integrity. Cyttoplasmic lipid accumulation can be caused by impaired mitochondrial β-oxidation, as described for tetracycline-induced microvesicular steatosis [6,37,38].

Fig. 4 – Tetracyclines cause lipid-droplet accumulation and autophagy in human cell lines. HeLa, CaCo2-TC7, and HepG2 cells were incubated on glass cover slips in the absence or presence of 20 μg/ml of either minocycline or tetracycline and incubated with Nile red (Sigma, Munich, Germany) to stain for lipid droplets (A) or with Cyto-ID Green Detection Reagent (Enzo Life Sciences; Lörrach, Germany) to stain for autophagic vesicles (B). (40 x lens.)

Fig. 5 – Tetracyclines inhibit phosphorylation of mTOR substrates. Lysates of HeLa, CaCo2-TC7, and HepG2 cells treated for 48 h with 20 μg/ml of doxycycline (D), minocycline (M), or erythromycin (E) were subjected to Western-blot analysis to analyze the expression and phosphorylation of mTOR substrates and cell-cycle regulatory proteins. The p70S6 kinase antibody stains for both the p70 and p83 isoforms. Representative Western blot analyses are shown.
Hepatotoxicity caused by minocycline is not a rare phenomenon [3,5], and our results on different human cell lines indicate that intracellular lipid accumulation upon minocycline treatment is not restricted to liver cells, but may also occur in a variety of other cell types.

Some of the effects of minocycline on mitochondria have been attributed either to mitochondrial calcium or magnesium depletion by minocycline [21,22]. In this study, magnesium supplementation markedly inhibited the effects of tetracyclines on HepG2 and HeLa cells. In addition to its function in mitochondrial energy metabolism, magnesium is important for the catalytic activity of most ATP-generating and -consuming processes, including phosphorylation cascades such as those occurring in the mTOR signaling pathway. Therefore, a complex interplay of various metabolic processes is expected to be influenced by tetracyclines, of which only some could yet be identified (Fig. 9).

The mTOR complex is also of crucial importance in animal cell growth and development [39]. Tetracyclines are contraindicated for pregnant women [40], and the inhibition of mTOR activity by tetracyclines therefore provides a further rationale for refraining from the use of this class of antibiotics during pregnancy.

This study has the limitation to be performed on transformed cell lines and not on primary cells directly isolated from liver or gastrointestinal tissues. However, since the observed effects in our cell culture system are known to occur as side effects in humans and in animal models, and could also differentiate between common adverse effects of tetracyclines (ATF4 activation and mTOR inhibition) and a specific steatosis-promoting effect of minocycline, we conclude that this cell-based analysis might be suited to reveal and investigate the molecular mechanisms of adverse effects of tetracyclines in human cells. In addition, further studies including the use of additional cell types from different tissue origin, different cell culture conditions with varying types of media supplemented with different divalent cation concentrations and sera components are necessary to confirm and validate the transferability of the here presented observations before they can be generalized and transferred to all tissue types and all physiological conditions. Since growth-inhibiting effects of tetracyclines are also of possible interest for cancer treatment and because Pourgholami et al. [10,11] identified growth-inhibiting effects of minocycline on ovarian cancer cells in vitro and in vivo, further identification of cancer cell types that may preferentially respond to tetracyclines may also be of interest for cancer investigators.

We conclude that tetracyclines have growth-inhibitory effects on human cells, associated with cell stress-induced ATF4 activation and mTOR inhibition. These effects are expected to primarily occur in gastrointestinal cells and liver cells because both tissues encounter high concentrations of tetracyclines applied by the standard oral route. Since both tissues have to rely on a regenerative capacity for optimal function, growth-restricting effects of tetracyclines on these tissues are of further concern. The study may also help to explain many of the known pleiotropic
effects of tetracyclines in human tissues, and this knowledge may further help to understand or prevent some of their adverse effects. For example, although a calcium-rich diet during oral tetracycline medication is not recommended due to an impaired gastrointestinal uptake of tetracyclines, the supplementation with magnesium should be considered for persons who are prone to adverse effects of tetracyclines or with a history of previous liver damage.

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**Flict of interest**

The authors declare no conflicts of interest.

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