Effects of Titanium Dioxide Nanoparticles on the Liver and Hepatocytes in vitro

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Abstract

Background:

Nanoscale titanium dioxide (TiO$_2$) is used in a variety of consumer products, including toothpastes, food colouring and sunscreens. There is evidence of potential translocation of nanoparticles (NPs) into the bloodstream following inhalation and ingestion, which is associated with subsequent accumulation in the liver. Since dose is often related to toxicity it is essential to investigate the liver for potential adverse effects.

Results:

This study examined toxicity, inflammation and oxidative stress in the liver following direct application of TiO$_2$ NPs, either to C3A cell cultures *in vitro*, or by intravenous (i.v.) injection *in vivo* in female Wistar rats (50 µg).

TiO$_2$ NPs showed low cytotoxicity *in vitro*, and no effects on hepatocyte homeostasis as measured by albumin release. However, exposure to high doses of 250 µg/cm$^2$ of nano-TiO$_2$ *in vitro* resulted in depletion of reduced glutathione, indicating oxidative stress. This effect was also observed *in vivo* in rat liver following i.v. injection of TiO$_2$.

TiO$_2$ NPs were readily taken up by hepatocytes both *in vivo* and *in vitro* as assessed by TEM, and could be detected in large agglomerates in the cytoplasm (C3A cells), or in both cytoplasm and the nucleus (*in vivo*). In addition to the glutathione depletion, we found dys-regulation of gene transcription, with increased IL-8/MIP-2 and TNF$_\alpha$ expression in both models 24 h after particle application.

Conclusions:
These results show that while nano-TiO$_2$ may not have direct cytotoxic effects in the liver, inflammogenicity and oxidative stress can occur at relatively low doses. The similarities between the \textit{in vitro} and \textit{in vivo} observations are promising indicators for the suitability of \textit{in vitro} hepatotoxicity models in nanoparticle risk assessment for injected particles.

\textbf{Keywords:}

Nanotoxicology, hepatotoxicity, titanium dioxide, nanoparticles, oxidative stress
**Background**

Titanium dioxide nanoparticles (TiO$_2$ NPs or nano-TiO$_2$) are amongst the most commonly used NPs in consumer products. They are included as pigments in paints and as reflecting agents in sunscreens [1]. TiO$_2$ particles have long been used for whiteners in products designed for direct exposure of humans, such as toothpastes or foods such as confectionery, sauces or creamers [1, 2].

In the 1990s the TiO$_2$ intake in the average daily diet was estimated to be 5.4 mg per person per day [3], which corresponds to approximately $10^{12}$ particles per person per day, of which some part is expected to be in the nano-range of 100 nm and smaller [4]. These figures were recently updated by Weir *et al.* [1], listing average exposures of 1-2 mg/kg bodyweight of TiO$_2$ for children and of 0.2-0.7 mg/kg bodyweight for adults, mainly depending on dietary habits. The mean diameter of TiO$_2$ in food colourants such as E171 was measured at 200 nm [3] and 110 nm [1], respectively, and Weir *et al.* report that 36% of particles were in the range of 100 nm and below [1]. Therefore, dietary exposure to nano-TiO$_2$ is a realistic and everyday scenario.

Exposure to nano-TiO$_2$ by inhalation can also happen, for example during the production of paints containing TiO$_2$, one of the most commonly used pigments, or during sanding and polishing of surfaces to which these paints have been applied [5]. Finally, there has been some concern about absorption of NPs such as ZnO through the skin after application of sunscreens and cosmetics [6]. While NP uptake through intact skin has been shown to either not take place, or be very low, there is still some concern about potential uptake of NPs or dissolved ions through damaged skin [7], and since TiO$_2$ NPs are contained in many different cosmetics, they are a main target for investigation.

A number of *in vivo* studies have shown that NPs can enter the bloodstream following exposure via inhalation and diet [8, 9]. Inhaled nano-TiO$_2$ was found to have distal effects on antioxidant levels in the
liver (Gosens et al., manuscript in preparation). Indeed, the liver frequently acts as a sink for NPs after their translocation into the bloodstream [10]. Therefore, even though direct application of TiO$_2$ NPs into the bloodstream is an unlikely scenario, it is important to test effects of these particles on the liver, since translocation may occur. In the present study, female Wistar rats were exposed to 50 µg (approx. 250 µg/kg bodyweight) of ST-01 TiO$_2$ NPs in a single-dose experiment via injection into the tail vein. Particles were anatase with a primary particle size of 7 nm and a Z-average diameter in suspension prior to application of 72.2 nm, close to the average diameter found in the diet and described by Weir et al [1].

In parallel studies, the human hepatoblastoma cell line C3A was exposed to NM-101 TiO$_2$ NPs (anatase, primary particle size 8 nm), to assist in establishing and improving in vitro models of NP hepatotoxicity in comparison with the in vivo study. Finding and improving suitable in vitro models for the accurate prediction of in vivo toxicity of NPs is a current priority in NP research, due to the high number of NPs already in use and currently being developed [11]. The present study was undertaken as part of the European FP7 project InLiveTox (www.inlivetox.eu) which aims to develop advanced in vitro models for ingested NPs and to assess NP toxicity across a number of different organs and cell culture models. The project was conducted in collaboration with a second European FP7 project ENPRA (www.enpra.eu).
Results and Discussion

Nanoparticle Characterisation

NM101 NPs were previously characterised as a powder and in C3A cell culture medium by Kermanizadeh et al [12]. NM101 particles were anatase with less than 1% rutile. The primary particle size was 4-8 nm (TEM), but agglomerates of 50 nm and larger were present. Dynamic light scattering (DLS) in C3A culture medium (10% serum) showed peaks in the particle-size distribution at 185 and 742 nm, indicating agglomeration in the medium.

ST-01 NPs were prepared as described in the Methods section. Suspensions were characterised in water prior to injection into the tail vein. Dynamic Light Scattering (DLS) analysis gave a z-average of 75.66 nm.

NM101 and ST-01 TiO$_2$ NPs were very similar in their physicochemical properties, with almost identical primary particle sizes (4-8 and 7 nm, respectively) and identical surface areas as measured by BET (320 m$^2$/g). Both particles tended to form agglomerates larger than 50 nm in suspension, either cell culture medium (NM101) or water for injections (ST-01).

Cytotoxicity of NM101 in C3A cells and effect on hepatocyte homeostasis

No cytotoxicity was evident in C3A hepatocytes after TiO$_2$ exposures of up to 625 μg/cm$^2$, using both the AlamarBlue (Fig. 1A) and the lactate dehydrogenase (Fig. 1B) assays. A small decrease in viability at extremely high concentrations, which was observed in the LDH assay was not significant. A low, medium and high exposure (4, 32 and 250 μg/cm$^2$) were chosen to investigate hepatocyte functionality by albumin ELISA in culture medium. No significant changes in albumin release were detected at any concentration (Fig. 1C).

Using two different methods of analysing cell viability, the LDH assay measuring membrane permeability and the AlamarBlue assay for mitochondrial activity, NM101 TiO$_2$ NPs were found to be essentially non-
cytotoxic up to very high doses of 625 µg/cm². In addition, cellular function as measured by albumin release was not negatively affected at a low (4 µg/cm²), a medium (32 µg/cm²) and a high dose (250 µg/cm²). A small increase was not found to be significant.

These data confirm findings by Kermanizadeh et al. [12], who exclusively used mitochondrial activity assays for cytotoxicity assessment. In general, TiO₂ NPs are considered a low-toxicity, low-solubility dust, following experiments in vivo and in vitro. Adverse in vivo effects, excepting for example the overload phenomenon observed in rodents after inhalation [13], are usually attributed to inflammation and oxidative stress rather than cytotoxicity. Even at extremely high and unphysiological exposures, for example a 5 mg/kg intravenous injection in Wistar rats [14], no gross lesions were observed. Rather than cytotoxicity, our work focused on uptake and sub-lethal effects of TiO₂ NPs on hepatocytes, and we analysed production and gene transcription of inflammatory mediators and markers of oxidative stress and apoptosis.

**Morphology and NP uptake into C3A cells and liver tissue**

Confocal microscopy of C3A cells exposed to cell culture medium (Fig. 2A) and NM101 (Fig. 2B) indicated uptake of TiO₂, but no gross changes to the morphology of the cells. Further examination of cells by transmission electron microscopy (TEM) after 4 h of exposure confirmed the presence of large agglomerates of NM101 particles in C3A cells, with cytoplasmic and perinuclear localisation (Fig. 2D-E). Particles were confirmed as TiO₂ by energy-dispersive x-ray spectroscopy, and showed the characteristic bands at 4.5 and 4.9 keV (EDX; Fig. 2F).

Liver tissue of rats exposed to 50 µg of TiO₂ (ST-01) via the tail vein showed no gross morphological, inflammatory or other pathological changes when compared to control tissue (Fig. 3A,B).
TEM of rat livers exposed to TiO$_2$ NPs via the tail vein revealed uptake into hepatocytes, both in the cytoplasm, particularly the perinuclear area (Fig. 3D), and the nucleus (Fig. 3E,F). Particles were identified as containing titanium by EDX (Fig. 3G) by characteristic bands at 0.5, 4.5 and 4.9 keV. No membranes surrounding the particles were identifiable. Particle location was confirmed as being within the section by Fresnel fringes, the blurring of image borders when defocusing which indicates whether or not an object is in the plane of the section.

NM101 at a low dose of 5 µg/cm$^2$ was taken up into C3A cells and appeared within the cell in large clusters (Fig. 2 D,E), which were concentrated in membrane-bound vesicles. In contrast, ST-01 particles injected into the tail veins of rats were present as small agglomerates, in the cytoplasm and nucleus, and were not contained in vesicles (Fig. 3 D-F). It is possible that the inclusion of larger agglomerates in vesicles inhibits oxidative effects of nano-TiO$_2$, or contains them within the vesicles, whereas a comparatively small number of particles present in the cytoplasm and nucleus can interact directly with the surrounding environment.

There are two main reasons for the presence of smaller agglomerates in livers compared to cell culture: Firstly, the particle number per cell is much smaller, and secondly, particles in static cell culture tend to settle on the cell surface, where they can agglomerate before uptake. With injection into the bloodstream and exposure to shear stress as well as distribution in blood vessels and capillaries, delivery to hepatocytes would likely occur in smaller agglomerates or even as single particles.

**Glutathione depletion in vitro and in vivo**

Reduced glutathione (GSH) was quantified in C3A cells after 2, 6 and 24 h exposure to 0, 4, 32 and 250 µg/cm$^2$ of NM101 and 0.5 mM CuSO$_4$ as a positive control for oxidative stress (Fig. 4A). NM101 NPs at low (4 µg/cm$^2$) and medium (32 µg/cm$^2$) doses did not cause a decrease in reduced glutathione (GSH)
content of the hepatocytes. However, exposure to a very high dose (250 µg/cm²) resulted in an initial decrease to 78.6% (+/- 13.9%) of the untreated control after 2 h, and GSH levels did not return to control levels after 6 h (67.6% +/- 10.1%) or 24 h (65.9% +/- 10.6%; n=3, means +/- standard error of the mean).

An in vivo study using ST-01 TiO₂ NPs (50 µg) applied via intravenous injection showed a similar decrease in GSH levels in the liver 24 h after injection (71.3% of control +/- 5.9%; Fig. 4B).

Few studies examining the effects of TiO₂ NPs on oxidative stress in hepatocytes are available.

Kermanizadeh at al. [15] found no changes in GSH levels in C3A cells following 24 h exposure to up to 80 µg/cm² of NM101, however, intracellular levels of reactive oxygen species as measured by the DCFH assay were increased after 6 h of exposure at 10 µg/cm² and higher. These increases were inhibited by pre-incubation of C3A cells with the antioxidant Trolox. This indicates that NM101 can potentially cause oxidative stress not affecting GSH levels at lower doses, or that the increase in reactive oxygen species is initially buffered by the cellular defences. Our results show that high doses of 250 µg/cm² can cause GSH depletion in C3A hepatocytes, however, these doses are unlikely to be reached in a physiological situation.

The effects of NPs, including TiO₂, on GSH depletion was first established in the lungs, and is related to the high surface area of the NPs [16]. Gosens et al. (manuscript in preparation) found no decrease in GSH in livers of c57/bl6 mice exposed to up to 128 µg of NM101 particles via intratracheal instillation, however, following instillation of a positively charged rutile nano-TiO₂, a distal effect of GSH reduction in the liver was observed.

Our results suggest that nano-TiO₂-induced oxidative stress in the liver can happen at very low exposures. In comparison, oxidative stress in hepatocytes is observed at relatively higher doses. A possible reason for this observation can be derived from uptake studies of nano-TiO₂ into hepatocytes discussed above, and the location and compartmentalisation of these particles in C3A cells vs. rat liver.
**mRNA expression following nano-TiO\textsubscript{2} exposure of C3A cells and rat livers**

mRNA expression of the inflammatory mediators IL-1\textbeta, IL-8, IL-10, TNF\textalpha and MCP-1, and the IL-1RI receptor, as well as for the apoptotic mediator Fas Ligand and glutathione peroxidase, was assessed by RT-PCR following 4 and 24 h of incubation to 4, 16 and 64 \( \mu \text{g/cm}^2 \) of NM101. Levels of IL-1\textbeta, IL-10 and MCP-1 mRNA were below the detection limit after 40 cycles for all samples (not shown). IL-8 levels were decreased at all exposure concentrations after 4 h, and increased at 4 and 64 \( \mu \text{g/cm}^2 \) after 24 h (Fig. 5A). IL-1RI expression was unchanged at all concentrations and time points (Fig. 5B). TNF\textalpha expression was changed similar to that of IL-8, with a strong decrease at all concentrations 4 h after exposure, and a dose-dependent increase after 24 h (Fig. 5C).

Expression of IL-8 and TNF\textalpha mRNA in C3A cells followed an interesting pattern at all concentrations tested: There was a significant initial reduction in gene expression after 4 h of exposure, which was followed by moderate but significantly increased expression around 2-fold of the untreated control at 24 h. These results are difficult to interpret. The reduction at 4 h could be a lag phase following an initial increase in gene expression, since gene expression changes for cytokines often occur within less than an hour (for example [17]).

Twenty-four hours after intravenous injection of 50 \( \mu \text{g} \) of ST-01 and water (control), there was an increase in mRNA expression of MIP-2 and TNF\textalpha in liver tissue (Fig. 6). MIP-2 is one of the functional homologues of IL-8 in rats [18]. No significant changes were detected in the expression of IL-1RI, IL-1b, IL-10, MCP-1, as well as FasL and GSHpox.

The results above provide further evidence to support the conclusion that, in terms of inflammatory gene expression, C3A cells are a useful model for hepatotoxicity of NPs. Kermanizadeh et al., (manuscript submitted) have also demonstrated that the response of C3A cells to 10 different nanomaterials were
highly comparable to the results of primary human hepatocytes with respect to cytotoxicity, inflammation and uptake. However, the results of the present study also point to the importance of choosing appropriate concentrations and time points, since the in vitro results differ greatly between 4 and 24 h, and while the 24 h in vitro and in vivo results show similar changes in gene expression. An additional time course study would be beneficial to monitor early changes and their similarity between the two models.

**Quantification of inflammatory and apoptotic mediators released from C3A cells**

We analysed release of IL-8, TNFα, Fas Ligand (FasL) and soluble ICAM-1 (sICAM-1) from C3A cells 4 and 24 h after exposure to NM101 NPs. TNFα and FasL release were below the detection limit across the range of TiO₂ concentrations used at both time points.

A small but significant increase in IL-8 cytokine release was detected at low TiO₂ concentrations after 4 h of exposure (Fig. 7A), and supports the interpretation that the decreased gene expression observed after 4 h of exposure as discussed above could be in response to an initial up-regulation. However, the increase in IL-8 gene expression observed after 24 h was not translated to the protein level, and the increase at 4 h was only observed at very low concentrations of particles (2 µg/cm²).

Soluble ICAM-1 was slightly increased at high concentrations of TiO₂ (62.5 µg/cm² after 4 h, and 125 and 500 µg/cm² after 24 h; Fig. 7B). The release of sICAM-1 is a sign of inflammation and signalling for leukocyte activation [19], and sICAM-1 was increased following subcutaneous injection of nano-TiO₂ in a mouse model [20]. The presence of large numbers of particles on the cell surface is likely to induce signalling for recruitment of leukocytes. Unfortunately, analysing the release of cytokines in vivo was beyond the scope of the present study, so that we were not able to confirm how these in vitro results relate to the animal model.
The fact that both TNFα and FasL secretion was below detection levels at all concentrations was surprising, particularly in the case of TNFα, for which an increase in gene expression had been found. We hypothesize that this may be linked to disabled pathways of apoptosis in C3A cells, a cancer cell line, since we were unable to identify publications describing elevated levels of either molecule on the protein level, despite evidence of up-regulation at the mRNA level. Publications suggest a link between TNFα and apoptosis in the liver [21] and the release of TNFα from primary hepatocytes [22]; this is a probable explanation for the lack of TNFα protein in the in vitro studies.
Conclusions

In conclusion, non-functionalised anatase TiO$_2$ NPs exhibit little toxicity, and low effects on cytokine expression and release, cellular functionality, and oxidative stress. However, it is obvious from the present study that there is the potential for uptake of TiO$_2$ not only into the cell body of hepatocytes but also into nuclei, and that this is accompanied with an oxidative stress which is observed at concentrations which could be reached by uptake from the intestine in subjects with a diet high in TiO$_2$. 
Methods

Animals

All experiments followed German federal guidelines for the use and care of laboratory animals. Studies were approved by the Institutional Animal Care and Use Committee of the Helmholtz-Zentrum Munich, Germany and the Government of District of Upper Bavaria (Approval No. 55.2-1-54-2531-26-10).

Eight to ten weeks old Female Wistar rats (WKY/Kyo@Rj; Janvier, Le Genest Saint Isle, France) of 190-210 g body weight were housed in ventilated cages controlled for humidity and temperature on a 12 h day/night cycle in pairs, and granted ad libitum access to a rodent diet and water. Three animals were used per treatment group, and all animals were free of clinical symptoms throughout the 24 h exposure.

Particles and particle characterisation

NM101 is part of the OECD Working Party on Manufactured Nanomaterials Sponsorship Programme of Representative Manufactured Nanomaterials and was purchased from Mercator GmbH. ST-01 was purchased from Ishihara Corporation.

NM101 and ST-01 both have anatase crystalline structure with a primary particle size of 8 nm (NM101) and 7 nm (ST-01). Both have a specific surface area of 320 m²/g (BET). Since another part of the in vivo studies for the project involved analysis of biokinetics of irradiated particles (manuscript in preparation), and NM101 was unsuitable for this purpose, ST-01 was chosen instead. Both particles were extensively characterised in the relevant media before use [12, 13].

Cells and Cell Culture

C3A cells (ATCC, Manassas, USA) were cultured in M2279 medium (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/0.1 mg/l streptomycin, 1 mM sodium pyruvate and 1% non-essential amino acids (all Sigma) at 37°C and 5% CO₂ as described in Gaiser et al [14]. For all experiments, cell culture surfaces were pre-treated with 0.1 mg/ml of type I collagen from rat tail (Sigma Aldrich).
Nanoparticle preparation and exposures

For use in *in vitro* experiments, NM101 particles were prepared in stock suspension by sonication in water containing 2% FCS in a waterbath for 16 minutes, and diluted in cell culture medium for exposures.

For the preparation of ST-01 TiO$_2$ NPs, a protocol was used which was previously described by Ramirez et al [13]. Briefly, 10 mg powdery anatase ST-01 TiO$_2$ were suspended in 1 ml of saturated pyrophosphate solution and diluted with 3 ml distilled water. For effective dispersion, this suspension was bath-sonicated for 1 minute and heat-treated at 70°C for 3 days in an incubator, and afterwards centrifuged for 1 minute at 4000 g. After removing the supernatant, the pellet was re-suspended with 2 ml of 0.1 mM pyrophosphate solution. Another heat treatment was made for 2 h, followed by 30 minutes of bath sonication and 30 minutes of heating. After 30 minutes of settling, the deposit was removed. The supernatant was centrifugation-washed by a 3kDa centrifugation filter (Amicon) for 50 minutes at 4000 g to reduce the pyrophosphate concentration and filled up again with distilled water to 2 ml. In the final size-selective step, this suspension was centrifuged for 6 minutes at 4000 g, and the supernatant was used for the subsequent *in vivo* experiments. Size distribution measurements by DLS (Nano Zetasizer ZS, Herrenberg, Germany) was determined immediately after preparation and additionally just prior to *in vivo* suspension administration. TiO$_2$ mass concentration was determined by separate preparations which were completely dried and weighed.

*In vivo* application of ST-01 was performed at HMGU in Munich by injection of 50 µg per animal (approximately 250 µg/kg bodyweight) of aqueous ST-01 suspension (1 µg/µl) into the catheterized tail vein of female Wistar rats under isoflurane anaesthesia. After 24 h, rats were exsanguinated under isoflurane anaesthesia and the liver was prepared for experiments as described below.

AlamarBlue and Lactate Dehydrogenase (LDH) Assays

C3A cells (300,000/cm$^2$) were exposed to 0-625 µg/cm$^2$ of NM101 NPs or 0.1% Triton X-100 for 24 h. Culture supernatants were removed, centrifuged (10 min, 10,000 g) and used for LDH analysis as described by Brown et al [15]. Cells were rinsed with PBS before fresh medium containing 10%
AlamarBlue reagent (Invitrogen) was added. After 90 minutes of incubation (37°C, 5% CO₂), fluorescence was quantified on a fluorescent plate reader (Fluostar Optima, BMG Labtech; 544/590 nm).

**Albumin Quantification**

Albumin was quantified in cell culture supernatants after 24 h of exposure to medium containing 0 and 20% FCS (inhibiting and enhancing of albumin release), and 4, 32 and 250 µg/cm² of NM101. The Human Albumin ELISA Quantitation Set (Bethyl Laboratories) was used according to the manufacturer’s instructions, with a sample dilution of 1:200, and a detection antibody dilution of 1:16667. The assay was validated by using high and low FCS controls and Triton X-100 as controls for conditions in which albumin release increases and decreases [16].

**Confocal Microscopy**

Cells seeded at low densities (approx. 40,000 cells/cm²) on collagen-coated glass coverslips and left to adhere overnight were exposed to 1-200 µg/cm² NM101. After 2 h of exposure, medium was removed, and cells were washed in PBS. Following fixation in 4% p-formaldehyde in PBS and permeabilisation with 0.2% Triton X-100 in PBS, actin was stained with TRITC-conjugated phalloidin (Sigma). Coverslips were washed and mounted on glass slides using Vectashield (Vector Laboratories, Peterborough, UK). Cells were examined under a Zeiss LSM510 Meta confocal microscope with LSM510 Meta v3.2 SP2 software. NM101 NPs were visualised by reflexion of light at 550 nm.

**Histology**

The right lateral liver lobe of rats exposed to TiO₂ NPs was rinsed in 4% formaldehyde in PBS, and transferred to a new vial containing 10% formaldehyde in neutral buffer solution overnight, before being rinsed in 70% ethanol and transferred into tubes containing 70% ethanol for storage. The fixed samples were shipped to Histologix Ltd (Nottingham, UK) to be embedded, sectioned and stained with Haematoxylin/Eosin according to standard protocols.
Transmission Electron Microscopy (TEM)

TEM was performed using a Tecnai TF20 field emission gun transmission electron microscope. Chemical composition of particles detected by microscopy was confirmed using energy-dispersive x-ray spectroscopy (EDX).

Confluent C3A cells were exposed to NM101 TiO$_2$ NPs at a concentration of 5 µg/cm$^2$ for 4 h. Cells were washed with medium, trypsinised, transferred to BEEM capsules and centrifuged (1000 g, 5 min), before fixation (2.5% glutaraldehyde in PBS, 2 h).

Tissue from the left lateral liver lobe was sampled and fixed by cutting cubes of 2 mm side length from the left lateral lobe and immersing them in 2.5% glutaraldehyde in 100 mM phosphate buffer for 2-3 h, transferring them into fresh phosphate buffer. Samples were processed according to standard protocols for transmission electron spectroscopy preparation at the University of Edinburgh’s Biology department.

Glutathione Quantification

The method described by Senft et al. [17] was used for quantification of reduced glutathione (GSH) in cell cultures and liver tissue. Briefly, C3A cells seeded at 300,000/cm$^2$ were grown to confluence overnight, exposed to NM101 NPs for 24 h, and subsequently scraped into PBS, centrifuged and lysed in a redox-quenching buffer. GSH was measured following conjugation with o-phthalaldehyde (5 mg/ml in methanol, Sigma-Aldrich) in a fluorescent plate reader (Spectramax M5, Molecular Devices) at an excitation wavelength of 350 nm, and an emission wavelength of 420 nm. GSH content was normalised to cell culture protein content as determined by the Bradford assay [18].

For in vivo samples, approximately 0.5 g of left lateral liver lobe tissue was shock-frozen in liquid nitrogen. Samples were stored at -80°C and homogenised (PowerGen 125, Fisher Scientific) in redox-quenching buffer containing 5% trichloroacetic acid on ice. After 15 min incubation on ice, homogenates
were further processed according to Senft et al. [17]. Liver lysates were diluted 1:20 before o-
phthalaldehyde conjugation, and GSH levels were normalised to tissue mass.

**RNA isolation, RT reaction and PCRs**

Confluent C3A cells were exposed to 4, 16 and 64 µg/cm² of NM101 TiO₂ NPs for 4 and 24 h. Cells were
lysed and processed according to the protocol for the MagMAX™-96 Total RNA Isolation Kit (Ambion).

Between 0.2 and 0.5g of the left lateral liver lobe of animals exposed to 50 µg of ST-01 via injection were
minced and immersed in a tube containing RNAlater reagent (Ambion, UK). Samples were stored at -20°C
until RNA isolation took place, and homogenised with mortar and pestle in liquid nitrogen. Homogenised
tissues were stored for up to 3 weeks at -80°C until RNA was extracted using the MagMAX™ kit
(Ambion).

The Nanodrop 2000c system (Thermo Scientific) was used to determine RNA concentration and purity.
RNA was transcribed into cDNA using the High Capacity cDNA RT kit (Applied Biosystems, UK) according
to the protocol. Equal quantities of RNA from 3-4 animals per treatment group (300 ng total), or 3 cell
culture experiments (100 ng total), were pooled for RT reactions.

PCRs were conducted in triplicate on a 7900 RT fast PCR system, in 384 well plates and using SDS 2.3
software (all Applied Biosystems). The following TaqMan kits with FAM dye (Applied Biosystems) were
used under standard TaqMan conditions for 50 cycles.

*In vivo* (rat) kits: Rn00562055_m1 (tumor necrosis factor, TNFα), Rn00580432_m1 (interleukin 1 beta,
IL1β), Rn00565482_m1 (interleukin 1 receptor, IL1R1), Rn00586403_m1 (chemokine cxcl2, MIP-2),
Rn00569886_m1 (complement regulatory protein, MCP-1), Rn00563409_m1 (interleukin 10, IL10),
Rn00577994_g1 (glutathione peroxidase, GSHpox), Rn00563754_m1 (FAS ligand, FASL), Rn00564227_m1
(ICAM 1), Rn01775763_g1 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH).
In vitro (human) kits: Hs02758991_g1 (GAPDH), Hs01555410_m1 (IL1β), Hs00991010_m1 (IL1RI), Hs00961622_m1 (IL10), Hs00174128_m1 (TNFα), Hs00174103_m1 (interleukin 8, IL8), Hs00234140_m1 (ccl2, MCP-1).

FACSAarray analysis of soluble mediators

Culture medium of C3A cells exposed to 4, 16 and 32 µg/cm² of TiO₂ NPs was collected 4 and 24 h after exposure and stored at -80°C. Samples were analysed on the BD FACSAarray, using Flex Sets for human TNFα (558273), IL8 (558277), FAS Ligand (558330) and ICAM-1 (560269) according to the instrument and Flex Set manuals.

Statistics and software

Data were controlled for normal distribution and processed using Minitab 15 software with analysis of variance (ANOVA) followed by t-test (significance: 95%). PCR results were analysed with SDS 2.3, RQ Manager 1.2.1 and DataAssist 2.0 (all Applied Biosystems). An 1.8-fold change in gene expression was used as a cut-off value.
Competing Interests

The authors declare that they have no competing interests.

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References:


**Figure legends:**

**Figure 1:**
Cytotoxicity and albumin release: C3A cells were exposed to NM101 TiO$_2$ NPs for 24 h. Cytotoxicity was determined by AlamarBlue (A) and LDH (B) assays, and albumin in cell culture was measured by ELISA (C). Data represent means ± standard error of the mean, n=3-5.

**Figure 2:**
Imaging of C3A cells after exposure to NM101.

A: Confocal image of control cells. B: Confocal image of cells exposed to 12.5 μg/cm$^2$ of NM101 for 2 h. A,B show actin (red), nuclei (blue) and particles (yellow; arrows); bar represents 10 μm.

C: TEM of control cell (bar: 2μm). D: TEM of cell exposed to 5 μg/cm$^2$ of NM101 (arrows) for 4 h (bar: 2 μm). E: Close-up of TiO$_2$ NPs in C3A cell, TEM (bar: 200 nm). F: EDX of NM101 cluster in the cytoplasm of C3A cells.

**Figure 3:**
Imaging of liver tissue after exposure to ST-01 TiO$_2$ NPs:

Light microscopy of H&E-stained control livers (A) and livers of rats exposed to 50 μg of ST-01 for 24 h (B). Bars represent 100 μm.

TEM images of control livers (C, bar: 2 μm) and livers of rats exposed to 50 μg of ST-01 for 24 h (D,E: bars: 1μm and F: bar: 50 nm). Arrows indicate TiO$_2$ clusters in the perinuclear area (D) and the nucleus (E, F).

G: EDX spectrum of the TiO$_2$ cluster in the nucleus shown in Fig. 3F.
Figure 4:
Quantitation of reduced glutathione (GSH) in C3A cells following exposure to 4, 32 and 250 µg/cm$^2$ of nano-TiO$_2$ (NM101) for 2, 6 and 24 h (A) and in rat liver 24 h after injection of 50 µg of nano-TiO$_2$ (ST-01) into the tail vein. Values are normalised by mg protein and standardised to the control. Bars represent means + stder. n=3-6; * p<0.05.

Figure 5:
Messenger RNA expression of inflammatory cytokines and receptors in C3A cells. Cells were exposed to sub-toxic concentrations of TiO$_2$ NPs for 4 and 24 h, and mRNA expression was analysed by RT-PCR with GAPDH as an endogenous control. IL-8 (A), IL-1RI (B) and TNF$\alpha$ (C) mRNA were analysed. Bars represent expression relative to the untreated control, error bars represent minimum and maximum expression. * describes expression changes 1.8-fold or higher.

Figure 6:
Fig. 6: mRNA expression of inflammatory cytokines and receptors, FasL and GSHpox in rat liver. Female Wistar rats were exposed to 50 µg of ST-01 TiO$_2$ NPs for 24 h by injection via the tail vein, and mRNA expression was analysed by RT-PCR with GAPDH as an endogenous control. Bars represent expression relative to the control exposure (water). Error bars represent minimum and maximum expression. * describes expression changes 1.8-fold or higher.

Figure 7:
Fig. 7: IL-8 (A) and ICAM-1 (B) release from C3A cells exposed to 0-500 µg/cm$^2$ of NM101 for 4 and 24 h. Bars represent means (n=3) of cytokine concentrations in cell culture medium + standard error of the mean. *p<0.05.
Figure 1
Figure 4

A: GSH/Protein (Standardised to Control) vs Concentration (μg TiO₂/cm²)

- 2h
- 6h
- 24h

B: GSH/mg tissue (Standardised to Ctrl)

- Control
- TiO₂
Figure 5

A

IL-8 fold change

B

IL-1RI fold change

C

TNF fold change

4h ■ 24h

µg/cm²
Figure 7

A

IL-8 release (pg/ml)

4 h □ 24 h

B

ICAM-1 release (pg/ml)

TiO₂ (µg/cm²)

0 2 4 8 16 31 62.5 125 500

*