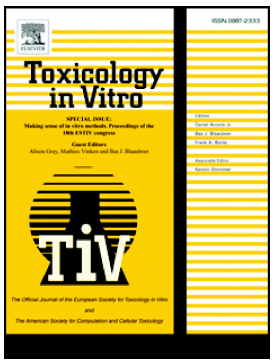

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The mechanism-based toxicity screening of particles with use in the food and nutrition sector via the ToxTracker reporter system

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Abstract

The rapid expansion of the incorporation of nano-sized materials in consumer products overlaps with the necessity for high-throughput reliable screening tools for the identification of the potential hazardous properties of the nanomaterials. The ToxTracker assay (mechanism-based reporter assay based on embryonic stem cells that uses GFP-tagged biomarkers for detection of DNA damage, oxidative stress and general cellular stress) is one such tool, which could prove useful in the field of particle toxicology allowing for high throughput screening. Here, ToxTracker was utilised to evaluate the potential hazardous properties of two particulates currently used in the food industry (vegetable carbon (E153) and food-grade TiO_2 (E171)). Due to the fact that ToxTracker is based on a stem cell format, it is crucial that the data generated is assessed for its suitability and comparability to more conventionally used relevant source of cells - in this case cells from the gastrointestinal tract and the liver. Therefore, the cell reporter findings were compared to data from traditional assays (cytotoxicity, anti-oxidant depletion and DNA damage) and tissue relevant cell types. The data showed E171 to be the most cytotoxic, decreased intracellular glutathione and the most significant with regards to genotoxic effects. The ToxTracker data showed comparability to conventional toxicity and oxidative stress assays; however, some discrepancies were evident between the findings from ToxTracker and the comet assay.

Key words: ToxTracker, comet assay, DNA damage, oxidative stress, E153, E171

1. Introduction

Humans are constantly exposed to particulates through the gastrointestinal tract (GIT). The ingestion of particles can occur directly from food (pigments or as additives), water or orally administered medicines as well the mucociliary clearance of the airways (Braakhuis *et al.* 2015). Although there is an increase in the use of (nano)particles (NPs) in the food industry, there is limited knowledge about the possible detrimental effects of oral exposure. The properties of the particles and the interactions with tissues in the GIT depends heavily on size, morphology, hydrophilic-hydrophobic balance and surface functionalization of the particle. Following ingestion, translocation of particles is a very real possibility (Panyala *et al.* 2017; Powell *et al.* 2010; Richard 2017). Once in sub-mucosal tissue, particles can enter the lymphatics and the blood capillaries and potentially be translocated to other secondary organs including the liver (Kermanizadeh *et al.* 2015a).

The exponential increase in the total number of engineered NPs for research and commercialization requires tools for rapid and efficient toxicity screening. Ideally, this screening should allow for mechanistic profiling to better inform on hazard identification and to improve risk assessment strategies. Oxidative stress has been proposed as the most predominant mechanism of NP-induced toxicity (Attia *et al.* 2018; Dasgupta *et al.* 2018; Sruthi *et al.* 2018). The increased levels of reactive oxygen species can potentially initiate a varied range of responses including but not limited to changes in the redox balance, inflammation and cell death. Another main concern following exposure to particles is their genotoxic potential. To date, the exact mechanisms of NP-induced genotoxicity is not fully understood but oxidative stress or the direct interactions of the particles and/or the produced oxidants with DNA are potentially important (Kermanizadeh *et al.* 2015b). Currently, the most commonly used method for assessing genotoxicity of NPs is the comet assay (Møller *et al.* 2015). However, this assay is technically challenging and very time-consuming while only offering limited information on the mechanism behind the observed toxicity. The ToxTracker assay is a mechanism-based reporter assay that uses green fluorescent protein (GFP)-tagged biomarkers for detection of DNA damage, oxidative stress, protein damage and general cellular stress upon exposure (Hendriks *et al.* 2016). This reporter assay can provide valuable information on the cellular signalling pathways that are activated upon exposure, thus providing an insight into the mechanisms of toxicity.

In the ToxTracker assay, GFP-tagged reporter cells for four distinct cellular signalling pathways are utilised. The DNA damage-associated Bscl2-GFP reporter depends on the ataxia telangiectasia mutated and Rad3-related-associated DNA damage pathway and is activated after exposure to genotoxic agents and the subsequent interference with DNA replication. The Rtkn-GFP reporter gene is associated with DNA damage and specifically informative on the involvement of the NF-kβ pathway. Oxidative stress is detected by the Srxn1-GFP and Blvrb-GFP reporters involved in the Nrf2

or Hmox1 anti-oxidant response pathways respectively. The Ddit3-GFP reporter is activated by the cellular unfolded protein response and the Btg2-GFP reporter is controlled by p53 and is activated by various types of cellular stress. The combination of different fluorescent reporter cell lines in a single toxicity assay allows for rapid identification of toxic properties of particles as well as enabling for mechanistic understanding of different modes of toxicity.

In this study, we investigated whether the ToxTracker assay is a suitable tool as a rapid mechanism-based tool for assessing toxic effects of particulates currently used in the food industry. The two particles were vegetable carbon-based material (E153) and food-grade TiO_2 (E171). These food-grade particles were selected due to the fact that humans are exposed to the materials on daily basis and there are no accepted limits for daily intake (ESFA 2012; 2016). Additionally, the quartz DQ12 was included as a low solubility benchmark particle (all three particles were utilised in a concertation range up to 100 µg/ml). DQ12 is a particle is often been used as a positive reactive particle due to large biologically available surface area. It should be stated, that E153 and E171 particles are not necessarily all in the nano range (< 100 nm), although a fraction are indeed NPs (size of pristine materials of less importance when considering the oral route of exposure as there will be a degree of aggregation for all particulates passing through the digestive system). In order to assess the appropriateness of the use from the ToxTracker reporter assay for this application; the generated data was compared to conventional and widely used viability (WST-1), oxidative stress (glutathione depletion assay) and genotoxicity (FPG (Formamidopyrimidine DNA glycosylase)-modified comet assay) assays. Furthermore, due to the fact that ToxTracker is based on a rodent stem cell (stem cells are generally accepted as sensitive to cellular damage) format it is crucial that the data generated is evaluated for its suitability and comparability to tissue specific cells (in this case intestinal and liver sourced cells). These tissues in particular are vital target organs for potential particulate-induced toxicity and accumulation following oral exposure. It is important to emphatically state that in this study; the traditional assays were not carried out to be a like to like comparison or supplement the ToxTracker findings but rather to establish whether the same general conclusions were possible when evaluating the datasets independently of each other (ToxTracker vs. traditional assays).

2. Materials and methods

2.1. Particles

The particle E153 (vegetable carbon powder) (Norit N.V., Amersfoort, The Netherlands) was purchased over the counter in a pharmacy (Copenhagen, Denmark). Norit is marketed as a treatment for diarrhoea (described as "activated charcoal" or "activated carbon") (Jensen *et al.* 2018a). The supplement is packaged and supplied in gelatine capsules. For use in these experiments, the capsules were emptied and the powder content stored at room temperature until use. The food-grade TiO_2 (E171) (99.8% anatase and 0.2% rutile) was purchased online (www.bolsjehuset.dk) (Jensen *et al.* 2018b). The DQ12 (crystalline silica) was sourced from Institute of Occupational Medicine (UK).

2.2. Characterisation of the panel of particles

The investigated particles were characterised by a combination of analytical techniques in order to infer primary physical and chemical properties useful to understand their toxicological behaviour. These measured physical and chemical properties have been described in detail previously (Jensen *et al.* 2018a). Furthermore, the hydrodynamic size distributions of the particles in the different biological media were determined at a concentration of (50 µg/ml) at 0, 4 and 24 hr by Dynamic Light Scattering (DLS) using a Malvern Metasizer nano series - Nano ZS (USA) (Table 1).

2.3. Preparation of particle dispersions and experimental treatments

The particles were dispersed in cell culture grade water supplemented with 2% fetal calf serum (FCS) (Gibco, UK) (stock solution of 1 mg/ml). The stock solution was sonicated for a period of 10 min without pause. This sonicated stock suspension was diluted in the appropriate complete cell culture medium for all subsequent experiments. The particles were tested at a concentration range up to 31.25 µg/cm^2 (100 µg/ml).

2.4. Cell culture

The ToxTracker reporter cells (Hendriks *et al.* 2012) were maintained by culturing them in gelatine-coated dishes in the presence of irradiated primary mouse embryonic fibroblasts in mES cell culture medium (mES KnockOut™ DMEM containing 10% FCS, 2 mM glutamax, 1 mM sodium pyruvate, 100 µM β-mercaptoethanol and leukaemia inhibitory factor). During particle exposure and reporter analysis the cells were cultured in the absence of fibroblasts in mES cell culture medium. The human

hepatoblastoma HepG2 cell line was obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in Minimum Essential Medium Eagle (MEM) (Gibco, UK) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, streptomycin, 1 mM sodium pyruvate and 1% non-essential amino acids, at 37ºC and 5% CO_2. The human Caco-2 cell line (ATCC) originated from a human colon adenocarcinoma were maintained in MEM supplemented with 10% FCS, 100 U/ml Penicillin/Streptomycin, 100 IU/ml non-essential amino acids and 2 mM L-glutamine.

2.5. The ToxTracker assay

The 6 different mES reporter cell lines were seeded in gelatine-coated 96 well cell culture plates in 200 µl of mES cell culture medium ($5x10^4$ cells per well) for 24 hr. The cells were treated with the particles as described above for a period of 24 hr. The induction of the GFP reporters was determined using a Guava easyCyte 8HT flow cytometer (Millipore). Only GFP expression in intact single cells was determined. The mean GFP fluorescence was measured and used to calculate GFP reporter induction compared to a vehicle control treatment. The cytotoxicity was estimated by cell counts using a flow cytometer and was expressed as percentage of intact cells in the exposure vs. negative controls. For cytotoxicity assessment in the ToxTracker assay, the relative cell survival for the six different reporter cell lines was averaged. The positive reference treatments with cisplatin (DNA damage) (Leiden University Medical Center pharmacy, The Netherlands), diethyl maleate (oxidative stress) (Sigma, UK) and tunicamycin (unfolded protein response - endoplasmic reticulum stress) (Sigma, UK) were included in all experiments. The mean fluorescence caused by the negative control compounds was subtracted from the results of the test particles.

The ToxTracker assay was considered to have a positive response when a compound induced at least a 2-fold increase in GFP expression in any of the reporters. Activation of the Bscl2-GFP or Rtkn-GFP reporters indicated the induction of DNA damage, Srxn1-GFP and Blvrb-GFP indicated cellular oxidative stress and Ddit3-GFP activation was associated with the unfolded protein response. Only GFP inductions at compound concentrations that showed >25% cell survival were used for the ToxTracker analysis. The data from measurements <25% cell survival was not considered as meaningful and therefore discarded. In order to allow comparison of induction levels of the ToxTracker reporter cell lines the Toxplot data analysis software package was developed. Toxplot imports raw GFP reporter data from the flow cytometer, calculates GFP induction levels and cytotoxicity, performs statistical analysis and hierarchical clustering of the tested compounds and visualises the data in a heat map.

To compare the induction of the six GFP reporters, each with different biological reactivity, dose-response relationships and kinetics, Toxplot calculates the level of GFP induction for every individual reporter at a specified level of cytotoxicity (typically 10%, 25% and 50%) for each test particle. The GFP induction levels were calculated by linear regression between two data points around the specified cytotoxicity level. In case the specified level of cytotoxicity was not reached at the highest tested concentration, Toxplot displays the GFP induction level at this top concentration.

2.6. WST-1 cell cytotoxicity assay

The HepG2 and Caco-2 cells were seeded in 96 well plates (10^4 cells per well in 100 µl of the cell culture medium) and incubated for 24 hr at 37ºC and 5% CO_2. The following day the cells were exposed to the particles or appropriate controls for 24 hr. Subsequent to particle treatment, the plates were washed twice with phosphate buffered saline (PBS), followed by the addition of 10 µl of the WST-1 cell proliferation reagent (Roche, USA) and 90 µl of fresh medium. The plates were then incubated for 2 hr at 37ºC. The supernatants was transferred to a fresh plate and the absorbance measured by dual wavelength spectrophotometry at 450 nm and 630 nm using a micro-plate reader.

2.7. Measurement of total glutathione

Briefly, a 2 ml cell suspension of Caco-2 or HepG2 cells ($1x10^6$ cells per well) were added to 6 well plates and incubated overnight at 37°C and 5% CO_2. The cells were exposed to the particles or controls in complete cell culture medium for 24 hr before being trypsinised into ice cold phosphate buffered saline and centrifuged (700 g for 2 min). The cell pellet was re-suspended in ice-cold lysis buffer (Senft *et al*. 2000), mixed and incubated on ice for 10 min before centrifugation at 15000 g for 5 min to generate lysates. Glutathione was quantified in the lysate by reaction of sulfhydryl groups with the fluorescent substrate *o*-phthaladehyde using a fluorimeter with an excitation wavelength of 350 nm and emission wavelength of 420 nm.

The protocol was slightly modified to include measurements of total glutathione by reducing oxidise glutathione dimers (GSSG) by addition of 7 µl of 10 mM sodium dithionite to all samples and incubating at room temperature for 1 hr. The results are expressed as the concentration (µM) per million plated cells.

2.8. Standard and FPG modified comet assay

The induction of oxidatively damaged DNA after particle exposure was assessed by enzymatic cleavage of oxidised bases (primarily 8-oxo-7,8-dihydroguanine) by FPG. The comet assay experiments were carried out in two different laboratories with slightly varying protocols. In order to make a fair and impartial comparison between the findings from laboratory 1 (stem cells) and 2 (HepG2 and Caco-2 cells), the data has been standardized according to the state-of-the-art procedures first developed by the European Standards Committee on Oxidative DNA damage and further developed by the European Comet Assay Validation Group. A detailed description of these procedures is described elsewhere (Møller *et al.* 2012).

In these experiments, mES cells ($5x10^5$) were exposed to the particles for 4 hr as described above (similar concentrations as tested in the ToxTracker assay). After the exposure period, the cells were trypsinised and re-suspended in 0.8% low melting point agarose before being seeded on microscopy slides (15 min 0.01 and 0.1 mM hydrogen peroxide peroxide or 2 hr of 2.5 or 5 mM $KBrO_3$ treatments were used as positive controls). After lysis (1 hr exposure with comet assay lysis solution (Trevigen)) the slides were incubated with the FPG enzyme (New England Biolabs, USA) for 30 min at 37ºC. The particle-exposed samples, hydrogen peroxide and $KBrO_3$ controls with and without FPG treatment were incubated in 0.3M NaOH, 1 mM EDTA for 30 min and subsequently subjected to electrophoresis at 1 volt/cm for a further 20 min. After washing and dehydration of the slides in 70% ethanol, DNA was stained with SYBR Gold (Invitrogen). The comets were analysed using a fluorescence microscope. The image analysis and quantification of the tail DNA damage was conducted using Opencomet software for ImageJ with 50 random cells per slide scored for each treatment. The investigations were carried out in two experimental periods (each period contained three independent replications). The first period included only E171, E153 and hydrogen peroxide treatments, whereas the second period included E171, E153, DQ12 and $KBrO_3$ treatments. $KBrO_3$ was include as a more reliable assay control for the FPG-modified comet assay in the second experiment because it predominantly generates FPG-sensitive sites and little concurrent generation of DNA strand breaks (Møller et al., 2018) The level of DNA strand breaks in unexposed cells differed from the first to the second experiment (5.9 ± 0.7 and 12.4 ± 1.5 %DNA in tail, respectively (mean ± standard error of the mean). Thus, the results have been standardized using the formula:

$$X_i = (X_{i,e}/X_{control,e}) * X_{all\ controls}$$

X_i is the standardized value in the $X_{i,e}$ experiment after standardization with the mean value of the controls (i.e. unexposed cells) from the specific day of experiment, multiplied with all controls. The

value was subsequently transformed to lesions/10^6 base pairs by use of a calibration where 1% DNA in tail is equal to 0.0273 lesions/10^6 base pairs, using the calibration curve from the European Comet assay Validation Group (Forchhammer *et al.* 2010).

In the second laboratory, the levels of DNA strand breaks and FPG sensitive sites were measured by the single cell gel electrophoresis (comet) assay as previously described (Vesterdal *et al.* 2014). Briefly, 4×10^5 HepG2 and Caco-2 were seeded in 24-well cell culture plates and incubated overnight in complete cell culture medium. The cells were exposed to the particles for a period of 4 hr, before being trypsinized and embedded into 0.75% agarose on gel bonds and lysed overnight in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, pH 10.0). The gel bonds were washed 3 times for 5 min in buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM Na_2EDTA, 200 µg/ml BSA, pH 8.0). The FPG enzyme (kind gift from Andrew Collins, University of Oslo, Norway) was added to the appropriate gels and incubated for 45 min at 37°C. The gels were then transferred to alkaline solution for 40 min (1 mM Na_2EDTA, 300mM NaOH, pH 13) and subsequently subjected to electrophoresis for 25 min in the same buffer at 300 mA and 20 V (1.1 V/cm from anode to cathode). After electrophoresis, the gels were washed 3 times for 5 min in neutralization buffer (0.4 M Trizma base, pH 7.5) and kept in 96% ethanol overnight. The samples were air-dried, stained with YOYO-1 (Molecular probes, USA) and visually scored (five-class scoring system (arbitrary score range: 0-400)). Potassium bromate-exposed THP-1 cells were included as assay control due to high levels of oxidatively damaged DNA (Møller *et al.* 2018). The levels of DNA strand breaks and FPG sensitive sites were obtained by scoring 100 nuclei/gel in 2 gels for HepG2 cells and 50 nuclei/gel in two gels for Caco-2 cells. The level of FPG sensitive sites was calculated as the difference in DNA damage between slides treated with the FPG enzyme and buffer. The experiments were carried out on three different days in duplicate. The results were standardized using the formula:

$$X_i = (X_{i,e}/X_{control,e}) * X_{all\ controls}$$

X_i is the standardized value in the $X_{i,e}$ experiment after standardization with the mean value of the controls (i.e. unexposed cells) from the specific day of experiment, multiplied with all controls. The value was subsequently transformed to lesions/10^6 base pairs by use of an investigator-specific calibration where 1 is an arbitrary unit in 0-400 comet score scale corresponding to 0.0051 lesions/10^6 base pairs. The original calibration curve was obtained as part of an inter-laboratory comet assay validation trial (Forchhammer *et al.* 2008). The genotoxicity data was analysed by the use of cubic-root transformation, which previously has been shown to be appropriate for the conversion of comet assay descriptors to achieve variance of homogeneity between groups in the comet assay (Vinzents *et al*. 2005).

2.9. Statistical analysis

For statistical analysis, the experimental results were compared to their corresponding control values using an ANOVA with Tukey's multiple comparison. The comet assay results were assessed firstly using mixed effects models with one continuous (i.e. concentration) and one categorical (i.e. type of particle) variable. Subsequently, univariate linear regression analyses was used on datasets for particles that showed a statistically significant effect in the mixed effects model. The comet assay data has also been analysed by ANOVA (including pooled results from all exposure groups for the same particle type) in order to ameliorate the chance of statistical type 2 error. The statistical analysis was carried out utilizing Minitab 18. A p value of <0.05 was considered as significant. All experiments were repeated on three separate occasions (unless otherwise stated).

3. Results

3.1. Characteristics of particles in exposure media

The investigated particles were characterised by a combination of analytical techniques in order to infer primary physical and chemical properties useful to understand their toxicological behaviour (Jensen *et al.* 2018a). In order to better understand the characteristics of the particles in the three different exposure media, the hydrodynamic size distributions and zeta potential was measured after dispersion at 0, 4 and 24 hr (Table 1).

TABLE 1

3.2. ToxTracker reporter assay

The exposure of the stem cells to E171 for 24 hr resulted in significant cytotoxicity (>50%) at concentrations above 7.8 μg/cm^2 while no cytotoxicity was observed for E153 at the investigated concentrations (Figure 1). However, the exposure of the ToxTracker cell lines to the E153 or E171 did not result in any reporter activation at any of the concentrations utilised in this study (Figure 1) (GFP reporters for DNA damage, oxidative stress and cellular stress or cell viability assessed using flow cytometry). The carcinogenic quartz DQ12 (known to induce oxidative stress and inflammation) was used as a benchmark positive insoluble particle control in the ToxTracker assay. This particle has previously been shown to induce a positive response in the oxidative stress reporter cell line at sub-lethal doses (Karlsson *et al.* 2014). The DQ12 exposure did not result in the activation of Bscl2 (DNA damage) or Btg2 (cellular stress) but did induce low level activation of Srxn1 (oxidative damage). The other three markers (Rtkn, Blvrb or Ddit3) were not activated upon DQ12 treatment at the utilised concentrations. Finally, very low levels of cytotoxicity were detected upon treatment with DQ12 at the highest exposures.

The validity of the ToxTracker assay was further confirmed using reference compounds specific for the different pathways being evaluated. The genotoxic compound cisplatin showed induction of the DNA damage response (Bscl2, Rtkn) and p53-mediated cellular stress (Btg2). Diethyl maleate induced primarily the oxidative stress related reporters (Srxn1 and Blvrb) and tunicamycin induced the unfolded/misfolded protein stress response (Ddit3). All controls showed GFP induction levels fully compliant with historical data and demonstrated the functionality of the mES reporter cell lines. Finally, $KBrO_3$ was included as an additional positive control in the ToxTracker experiments as it was a control in the comet assay (Figure 2).

FIG 1

FIG 2

3.3. Cytotoxicity of particles on HepG2 and Caco-2 cells

From the WST-1 data, it was evident that there was very little cytotoxicity to either HepG2 or Caco-2 cells exposed to E171. The exposure of the cells to the other two materials for 24 hr only resulted in cytotoxicity at the higher concentrations (Table 2).

TABLE 2

This data set was a good match with the findings in the ToxTracker assay and the observed toxicity in the stem cells.

3.4. Impact of the particles on depletion of intracellular GSH in the hepatocytes and Caco-2 cells

Analysis of the total glutathione contents in the both cell types revealed a concentration dependant decrease compared to the control cells at 24 hr following exposure to the E171 and DQ12 materials (these observations were most notable in the hepatocytes and the DQ12) (Figure 3). The assay did not show a reduction in the anti-oxidant content following exposure to E153 at any of the investigated concentration or time-point. Once again, the data interrelated very well with the reporter assay where DQ12 showed a positive response in the oxidative stress reporter cell line at a similar sub-lethal concentration.

FIG 3

3.5. DNA damage in three cell types - FPG modified comet assay

In order to investigate the DNA damage caused by the panel of particles and to compare and confirm the ToxTracker response, the three different cell types were exposed to the particles for 4 hr and genotoxicity assessed using the FPG modified comet assay (conventional and widely utilised assay for measurement of particle-induced genotoxicity). The alkaline comet assay detects DNA strand breaks and alkali labile sites whereas the FPG predominantly detects oxidized purines. Therefore, the addition of the FPG enzyme allows for detection of oxidative damage to DNA.

The findings on DNA damage in stem cells are shown in table 3. The exposure to E171 increased the level of DNA stand breaks in stem cells, assessed as both linear regression ($r=0.84$, $p<0.001$) and ANOVA on pooled exposure groups ($p<0.01$). The FPG-modified comet assay did not indicate statistically significant effects in particles exposed cells, whereas there was a statistically significant increase in the positive control (0.31 ± 0.14 and 0.06 ± 0.07 lesions/10^6 bp in the H_2O_2 and negative control samples, respectively ($p<0.001$)). Likewise, the $KBrO_3$ assay controls were concentration-dependently increased (0.05 ± 0.08, 0.36 ± 0.01 and 0.89 ± 0.29 lesions/10^6 bp in the negative control, 2.5 and 5 mM $KBrO_3$ treated cells, respectively, $P<0.01$, linear regression). The data on DNA damage in HepG2 cells is presented in table 4. In these cells, E171 induced both DNA strand breaks and FPG-sensitive sites as demonstrated by statistically significant effects in linear regression analysis and ANOVA on pooled exposure groups. There was less consistent genotoxicity for E153 as only linear regression analysis (DNA strand breaks: $r=0.56$, $p<0.05$) or ANOVA on pooled exposure groups (FPG-sensitive sites: $p<0.01$) were statistically significant. DQ12 did not generate DNA strand breaks, whereas the induction of FPG-sensitive sites was robust in both linear regression analysis ($r=0.54$, $p<0.05$) and ANOVA on pooled exposure groups ($p<0.01$). Finally, the results on Caco-2 cells are depicted in table 5. There were only minor effects on levels of DNA strand breaks after exposure to E171 ($r=0.78$, $p<0.001$) and E153 ($r=0.48$, $p<0.05$), whereas the ANOVA did not indicate statistical significance.

TABLE 3

TABLE 4

TABLE 5

4. Discussion

A large body of toxicology data is now being generated for NPs, using a wide variety of models, protocols and end-points. It is clear that not all particles are equally toxic and these disparities are largely based upon their physical and chemical properties and experimental set-ups and variations. Despite this, there has been significant progress in the field of nanotoxicology, which coincides with increasing advancements in the use of NPs in various consumer products including the food industry (packing, additives and colourants) (Antunes *et al.* 2017; Argueta-Figueroa *et al.* 2017; Lim *et al.* 2017). Therefore, it is of utmost importance to clarify whether particle exposure could result in adverse health effects in exposed individuals. Hence, the urgent need for rapid and reliable toxicity testing (Kermanizadeh *et al.* 2016; Oberdorster *et al.* 2005). In this study, we applied a fluorescence-based reporter assay as a means of rapid mechanism-based tool for assessing toxic effects of two particles currently used in the food industry (vegetable carbon-based material and food-grade TiO_2). In order to assess the suitability of the ToxTracker reporter assay for particle toxicology the generated data was compared to conventional viability (WST-1), oxidative stress (glutathione depletion assay) and genotoxicity (comet assay) assays. Furthermore, due to the fact that ToxTracker is constructed on a stem cell format the data was compared to toxicity findings from tissue specific intestinal and hepatic sourced cells.

The exposure of the stem cells to E171 for 24 hr resulted in significant cytotoxicity at concentrations above 7.8 $\mu g/cm^2$ while no cytotoxicity was observed for E153 at the investigated concentrations. Next, very low levels of cytotoxicity were detected upon treatment with DQ12 at the highest exposures (Figure 1). The conventional toxicity assay very little cytotoxicity to either HepG2 or Caco-2 cells exposed to E171. The exposure of the cells to the other two materials for 24 hr only resulted in cell death at the higher concentrations (Table 2).

Next, the exposure of the ToxTracker cell lines to the E153 or E171 did not result in p53-mediated cellular stress (Btg2) or oxidative stress related reporters (Srxn1 and Blvrb) activation at any of the concentrations utilised in this study. However, DQ12 exposure did induce low level of activation of Srxn1 (oxidative damage) (Figure 1). The analysis of the total glutathione contents in HepG2 and Caco-2 cells revealed a concentration dependant decrease compared to the control cells at 24 hr following exposure to the E171 and DQ12 materials (these observations were most notable in the hepatocytes and the DQ12) (Figure 2). The assay did not show a reduction in the anti-oxidant content following exposure to E153 at any of the investigated concentration or time-point. Overall, the data correlated very well with the reporter assay where DQ12 showed a positive response in the oxidative stress reporter cell line at a similar sub-lethal concentration.

Finally, the exposure of the ToxTracker cells to the particles did not result in Bscl2 and Rtkn reporter activation at any of the concentrations utilised in this study (no induction of the DNA damage response). The absence of Bscl2-GFP activation indicates that the tested particles do not directly interact with DNA and therefore do not interfere with DNA replication. The lack of Rtkn-GFP induction indicates that the NPs do not cause DNA double strand breaks. The absence of Srxn1/Blvrb oxidative stress reporter activation as well as the Rtkn-GFP genotoxicity reporter indicates that E153 and E171 might induce low levels of ROS but these are not sufficient to induce an anti-oxidant response and do not cause detectable levels of chromosomal damage.

The traditional genotoxicity data indicated that E171 had the strongest effect as it produced DNA strand breaks in all cell types and increased levels of FPG-sensitive sites in HepG2 cells. The E153 produced genotoxicity in HepG2 and Caco-2 cells. While, DQ12 only produced FPG-sensitive sites. In a previous study, only a marginal increase in FPG-sensitive sites was observed in lung epithelial cells after exposure to quartz, whereas no effect in terms of DNA strand breaks was noted (Jacobsen *et al.* 2007). The difference in response to oxidatively damaged DNA (i.e. FPG-sensitive sites) between the stem cells and HepG2/Caco-2 cells may be due to cell-type specific susceptibility or variances in comet assay protocols between the laboratories. A potential explanation for the variances between the two assays could be due to specific particle physicochemical characteristics. As an example, TiO_2 NPs are associated with photocatalytic activity resulting in DNA strand breaks in the comet assay, which might not be detectable in the ToxTracker assay (Di Bucchianico *et al.* 2017). In summary of the particle-induced genotoxicity, there was discrepancies between the ToxTracker data and the comet assay data which was most evident in the HepG2 cells. The summary of the particle-induced toxicity end-points in the three different cell types is presented in Table 6.

TABLE 6

It is estimated that the average human consumption of food grade TiO_2 (E171) is between 0.2-2 mg/kg body weight per day (Weir *et al.* 2012) which is below the lowest adverse effect level set at 5 mg/kg (European Commission's Scientific Committee on Consumer Safety) (Warheit *et al.* 2012). Despite this, a number of studies have demonstrated toxic effects which are attributed to the particle. In one such investigation, mono-culture of differentiated Caco-2 cell and co-cultures of the intestinal epithelial cells and goblet cells were exposed to E171 for 6 or 48 hr or repeatedly for up to three weeks. The data showed E171-induced moderate toxicity with the mechanism of cell death being oxidative stress (ROS generation and downregulation of anti-oxidants) (Dorier *et al.* 2017). Similarly, E171 was shown to have the capability to induce ROS formation in a cell-free environment and oxidative stress and genotoxicity in Caco-2 cells. Additionally, the authors showed chromosomal damage in HCT116 cells via the micronucleus assay (Proquin *et al.* 2017). However, elsewhere the

E171 did not increase the ROS production in Caco-2 cells, whereas E153 produced a 3-fold increase in ROS levels (Jensen *et al.* 2019). In another study, the exposure of Caco-2 cells to E171 was shown to be cytotoxic at similar concentrations utilised in our study (Tassinari *et al.* 2015). Finally, the oral exposure of Sprague-Dawley rats to E171 for once a week for period of ten weeks results in a modest changes in the vasocontraction and relaxation in the exposed animals as compared to the controls (Jensen *et al*. 2018a). Interestingly, the same rat study showed no effect on DNA strand breaks and FPG-sensitive sites in the liver, spleen and lungs (Jensen *et al.* 2019).

There have been relatively few studies on the safety of vegetable carbon (E153). However, in one such study the sub-chronic toxicity and genotoxicity of the particle was evaluated in Sprague-Dawley rats following a 90-day oral exposure. The authors found no toxicity in the exposed animals (clinical signs, food consumption, body and organ weights, haematological and biochemical parameters). Additionally, there was genotoxicity assessed with *Salmonella typhimurium* mutagenicity assay (Ames test), comet assay and mammalian erythrocyte micronucleus assays (Zhenchao *et al.* 2015). As a side note, the quartz DQ12 has previously been shown to cause oxidative stress and inflammation in a number of test systems (Clouter *et al*. 2001; Duffin *et al*. 2001; Schins *et al*. 2002).

To the best of our knowledge, this is one of the very few studies to have utilised the ToxTracker reporter cell assay for high throughput screening of particles. In the aforementioned investigation, CuO and NiO NPs were shown to induce a significant oxidative stress reporter cell response in the ToxTracker assay (Akerlund *et al.* 2018; Cappellini *et al.* 2018; Karlsson *et al.* 2014). The authors concluded that that the reporter system could be useful for assessing the genotoxicity of metal oxide NPs. However, for the first time the ToxTracker data is compared to tissue relevant cell types in the hepatic and digestive system to further evaluate the suitability of the assay for mechanistic high throughput screening of particles. Future utilisation of ToxTracker and subsequent data analysis of false negative and false positive rates, based on a much larger database, will further validate the assay reliably in the detection of particulate induced biological effects.

5. Conclusions

Here, we utilised the ToxTracker assay (reporter genes that are induced by DNA damage, oxidative stress or protein damage) for mechanistic toxicity screening for a panel of particles used in the food industry. The assay could be extremely useful in the field of particle toxicology as it utilises a 96-well plate format allowing for high throughput screening. The food grade TiO_2 was demonstrated to be the most cytotoxic, decreased intracellular glutathione and the most significant for genotoxic effects. The cell reporter findings were compared to data from traditional assays and tissue sourced cell types. Overall, the ToxTracker data showed comparability to conventional toxicity and oxidative stress

assays for all cell types; however, some discrepancies were noted for the genotoxicity end-point between ToxTracker and the conventional comet assay.

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Disclosures

The Authors declare no competing interest.

Author contributions

AK and GH conceived and designed the experiments. AK wrote the manuscript. DMB, PHD, RD, NM and AK performed the experiments. AK and PM analysed the data. All contributing authors have read and approved the final version of the manuscript.

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Table 1. The hydrodynamic size of E153, E171 and DQ12 (50 μg/ml) in the different cell culture media at 0, 4 and 24 hr following sonication.

	mES complete medium		**Caco-2 complete medium**		**HepG2 complete medium**	
	Size (nm)	**Zeta potential (mV)**	**Size (nm)**	**Zeta potential (mV)**	**Size (nm)**	**Zeta potential (mV)**
E153 - 0 hr	281	-7.35	187	-7.44	192	-7.33
E153 - 4 hr	284	-9.26	324	-8.04	433.5	-7.63
E153 - 24 hr	231	-7.31	208	-8.7	325	-9.13
E171 - 0 hr	392	-5.89	408	-9.07	395	-6.44
E171 - 4 hr	426	-8.97	468.5	-8.74	472.5	-9.06
E171 - 24 hr	503	-8.99	488.5	-9.88	509	-9.34
DQ12 - 0 hr	195.4	-9.41	205.5	-9.62	243.1	-8.01
DQ12 - 4 hr	365.1	-8.32	295.6	-8.71	274.9	-9.74
DQ12 - 24 hr	326.8	-9.45	310.1	-8.43	226.3	-9.16

Table 2. WST-1 cytotoxicity following 24 h exposure of hepatocytes and Caco-2 cells to E153, E171 and DQ12 (lethal concentration 20 - LC_{20} values). The LC_{20} refers to the concentration of particles which caused the death of 20% of the cells.

	HepG2 (LC_{20})	**Caco-2 (LC_{20})**
E153	Not reached	Not reached
E171	15.6 µg/cm^2	31.25 µg/cm^2
DQ12	15.6 µg/cm^2	31.25 µg/cm^2

Table 3. Levels of DNA strand breaks and FPG-sensitive sites in stem cells after exposure to E171, E153 and DQ12 for 4 hr.

	Concentration (µg/cm^2)	**DNA strand breaks (lesions/10^6 bp)**	**FPG-sensitive sites (lesions/10^6 bp)**
Negative control	0	0.28	0.05
Positive control	100 µM	1.65 ± 0.28	0.25 ± 0.09
Positive control	2.5 mM	0.11 ± 0.03	0.30 ± 0.08
	5.0 mM	0.17 ± 0.03	0.83 ± 0.21
E171	0.98	0.45 ± 0.06	0.05 ± 0.06
	1.95	0.39 ± 0.02	0.04 ± 0.07
	3.9	0.51 ± 0.06	No damage
	7.8	0.53 ± 0.04	0.03 ± 0.09
	15.6	0.89 ± 0.06$^{###}$	No damage
	All exposure groups	0.55 ± 0.04***	Not calculated
	Linear regression (r-value)	*0.84****	*Not calculated*
E153	0.98	0.26 ± 0.03	0.06 ± 0.08
	1.95	0.27 ± 0.03	No damage
	3.9	0.25 ± 0.02	No damage
	7.8	0.24 ± 0.04	No damage
	15.6	0.24 ± 0.04	No damage
	All exposure groups	0.25 ± 0.01	Not calculated
	Linear regression (r-value)	*-0.17*	*Not calculated*
DQ12	0.98	0.30 ± 0.08	0.01 ± 0.11
	1.95	0.24 ± 0.06	No damage
	3.9	0.24 ±0.02	0.07 ± 0.06
	7.8	0.26 ± 0.04	No damage
	15.6	0.23 ± 0.05	0.07 ± 0.05
	All exposure groups	0.26 ± 0.02	Not calculated
	Linear regression (r-value)	*-0.19*	*Not calculated*

The data is presented as mean ± standard error of the mean of three independent experiments. The results from the assay controls are reported as net differences compared to their respective controls (not shown), whereas the negative control are standardized results with inter-assay variation. The values are lesions/10^6 base pairs after calibration of the primary comet assay descriptor (% DNA in tail) by use of the ECVAG calibration where 1% DNA in tail corresponds to 0.0273 lesions/10^6 base pairs. *** $p<0.001$ (linear regression), $^{###}P<0.001$ (ANOVA, compared to negative control).

Table 4. Levels of DNA strand breaks and FPG-sensitive sites in HepG2 cells after exposure to E171, E153 and DQ12 for 4 hr.

	Concentration (µg/cm^2)	**DNA strand breaks (lesions/10^6 bp)**	**FPG-sensitive sites (lesions/10^6 bp)**
Negative control	0	0.23 ± 0.09	0.23 ± 0.21
Positive control	5 mM	0.09 ± 0.03	1.44 ± 0.03
E171	3.9	0.40 ± 0.09	1.25 ± 0.97
	7.8	0.48 ± 0.22	1.00 ± 0.68
	15.6	0.48 ± 0.33	1.28 ± 0.90
	All exposure groups	0.45 ± 0.21$^{\#}$	1.18 ± 0.76$^{\#\#}$
	Linear regression (r-value)	*0.52**	*0.56**
E153	3.9	0.19 ± 0.03	1.01 ± 0.13
	7.8	0.24 ± 0.02	0.87 ± 0.37
	15.6	0.47 ± 0.25	0.85 ± 0.01
	All exposure groups	0.30 ± 0.18	0.86 ± 0.46$^{\#\#}$
	Linear regression (r-value)	*0.56**	*0.40*
DQ12	3.9	0.23 ± 0.04	0.86 ± 0.46
	7.8	0.37 ± 0.16	0.67 ± 0.41
	15.6	0.34 ± 0.12	0.89 ± 0.50
	All exposure groups	0.31 ± 0.12	0.81 ± 0.41$^{\#\#}$
	Linear regression (r-value)	*0.43*	*0.54**

The data is presented as mean ± standard error of the mean of three independent experiments, except the controls (n=9). The values are lesions/10^6 base pairs after calibration of the primary comet assay descriptor (% DNA in tail) by use of an investigator-specific calibration where 1 arbitrary unit in the 5-class visual scoring system (0-400 scale) corresponds to 0.0051 lesions/10^6 base pairs.* $p<0.05$ linear regression), $^{\#}$ $p<0.05$ or $^{\#\#}$ $p<0.001$ (ANOVA, compared to control).

Table 5. Levels of DNA strand breaks and FPG-sensitive sites in Caco-2 cells after exposure to E171, E153 and DQ12 for 4 hr.

Variable	Concentration (μg/cm^2)	DNA strand breaks (lesions/10^6 bp)	FPG-sensitive sites (lesions/10^6 bp)
Negative control	0	0.23 ± 0.02	0.23 ± 0.06
Positive control	5 mM	0.14 ± 0.04	1.39 ± 0.04
E171	3.9	0.24 ± 0.01	0.23 ± 0.21
	7.8	0.25 ± 0.01	0.32 ± 0.34
	15.6	0.33 ± 0.04	0.40 ± 0.51
	All exposure groups	0.27 ± 0.05	0.31 ± 0.33
	Linear regression (r-value)	*0.78****	*0.05*
E153	3.9	0.22 ± 0.02	0.24 ± 0.21
	7.8	0.28 ± 0.07	0.10 ± 0.08
	15.6	0.29 ± 0.07	0.22 ± 0.04
	All exposure groups	0.27 ± 0.06	0.19 ± 0.13
	Linear regression (r value)	*0.48**	*-0.17*
DQ12	3.9	0.24 ± 0.04	0.15 ± 0.20
	7.8	0.27 ± 0.05	0.16 ± 0.09
	15.6	0.26 ± 0.04	0.20 ± 0.14
	All exposure groups	0.25 ± 0.04	0.17 ± 0.13
	Linear regression (r-value)	*0.30*	*-0.14*

The data is presented as mean ± standard error of the mean of three independent experiments, with the exception of the controls (n=9). The values are lesions/10^6 base pairs after calibration of the primary comet assay descriptor (% DNA in tail) by use of an investigator-specific calibration where 1 arbitrary unit in the 5-class visual scoring system (0-400 scale) corresponds to 0.0051 lesions/10^6 base pairs.* $p<0.05$ or *** $p<0.001$ (linear regression).

Table 6. Summary of the investigated toxicological end-points/assay in cell types following 4/24 hr exposure to E171, E153 and DQ12.

Biomarker	E171	E153	DQ12
ToxTracker (mES) - DNA damage	-	-	-
ToxTracker (mES) - oxidative stress	-	-	+
ToxTracker (mES) - p53 response	-	-	-
ToxTracker (mES) - cell survival	+	-	-
WST-1 assay (HepG2)	+	-	+
WST-1 assay (Caco-2)	+	-	+
Comet assay - SB (mES)	++	-	+
Comet assay - FPG (mES)	-	-	-
Comet assay - SB (HepG2)	++	+	-
Comet assay - FPG (HepG2)	++	+	+
Comet assay - SB (Caco-2)	+	+	-
Comet assay - FPG (Caco-2)	-	-	-
GSH (HepG2)	+	-	+
GSH (Caco-2)	-	-	+

(++) robust statistical significance in linear regression and ANOVA on pooled exposure groups
(+) only statistical significance in one test
(-) no statistical significance in test

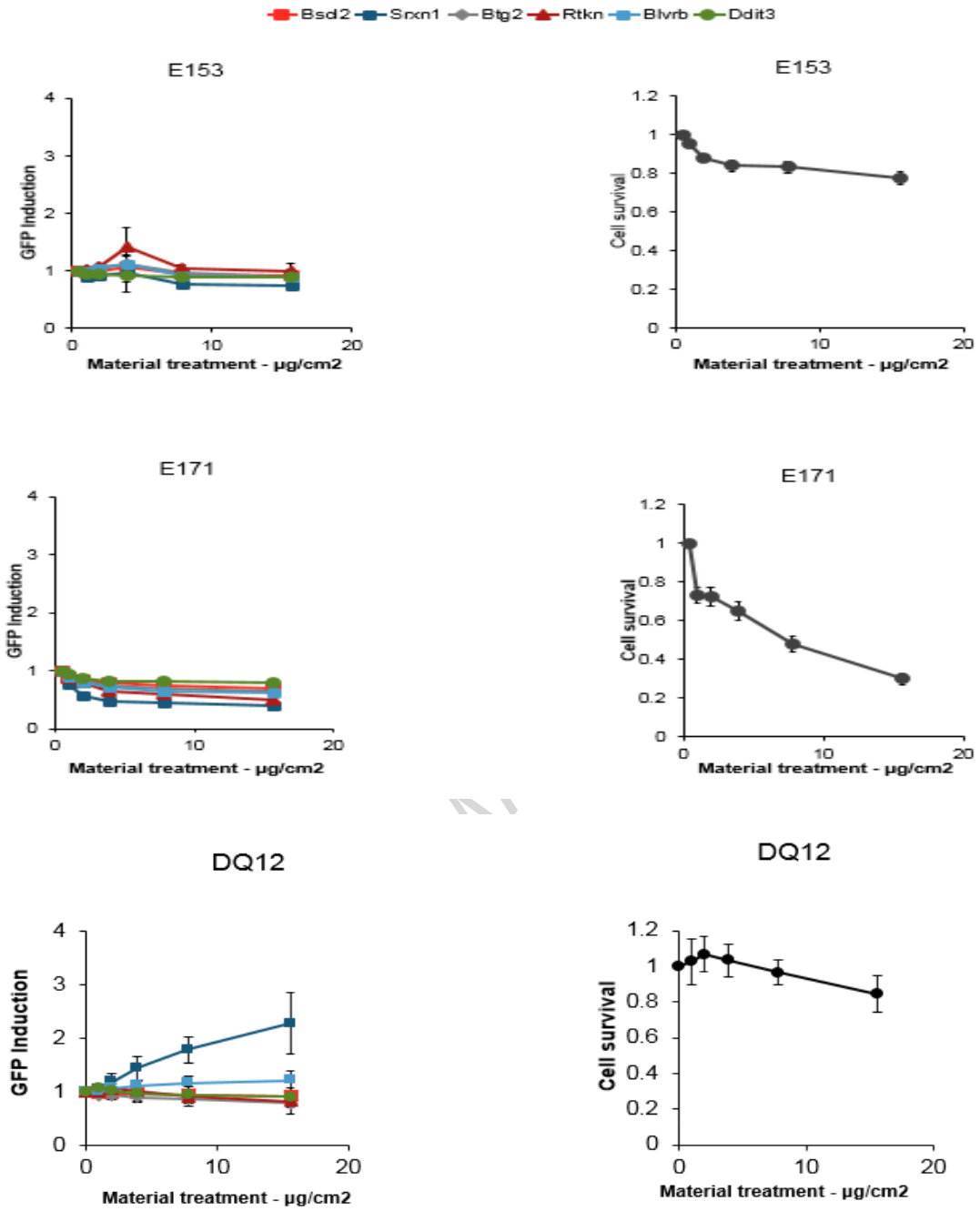
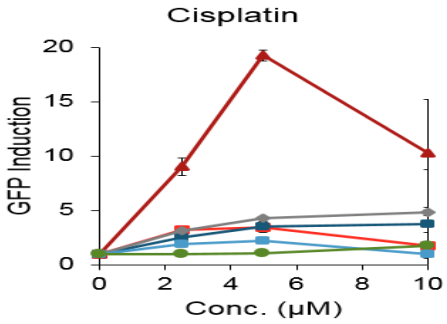
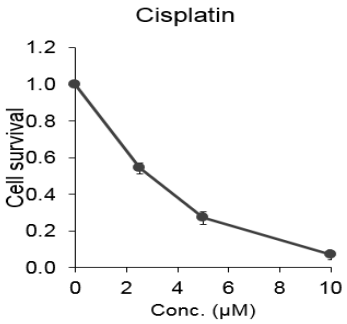
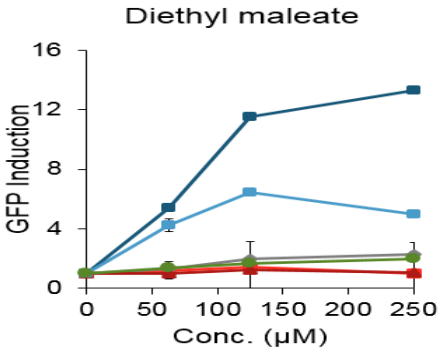
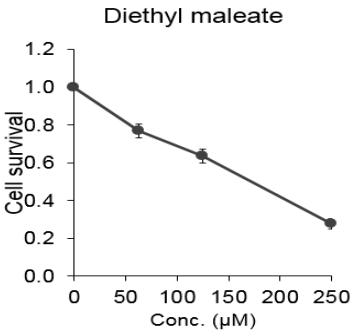


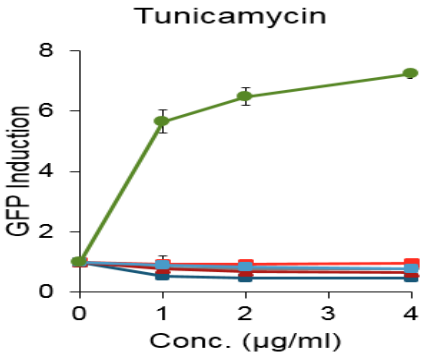
Figure 1. The ToxTracker reporter cell lines Bscl2-GFP and Rtkn-GFP (DNA replication stress), Btg2-GFP (p53-associated cellular stress), Srxn1-GFP and Blvrb-GFP (oxidative stress) and Ddit-GFP (protein damage) observed by flow cytometry after 24 hr exposure to the E153, E171 or the DQ12. The cell survival detected via flow cytometry measuring the fraction of intact cells. The ToxTracker reporter experiments were discarded if the survival rate was less than 25% (i.e. cut-off). The values depict mean ± SEM (n=3).

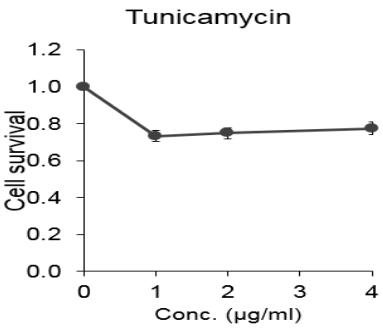


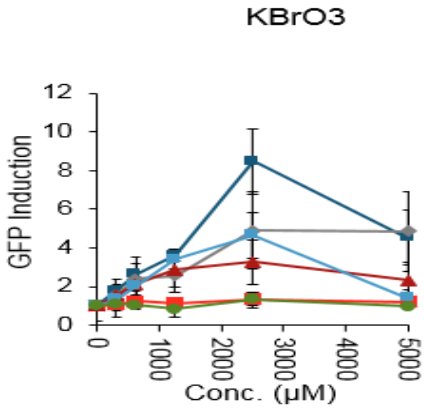












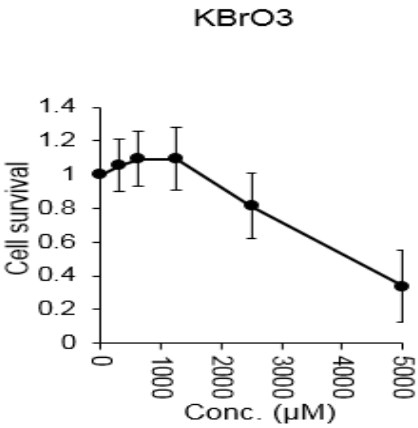


Figure 2. The validity of ToxTracker was confirmed by the inclusion of positive controls - cisplatin - induction of the DNA damage response (Bscl2, Rtkn) and p53-mediated cellular stress (Btg2); Diethyl maleate induction of oxidative stress related reporters (Srxn1 and Blvrb); tunicamycin induction of protein stress response (Ddit3) and $KBrO_3$ oxidative DNA damage.

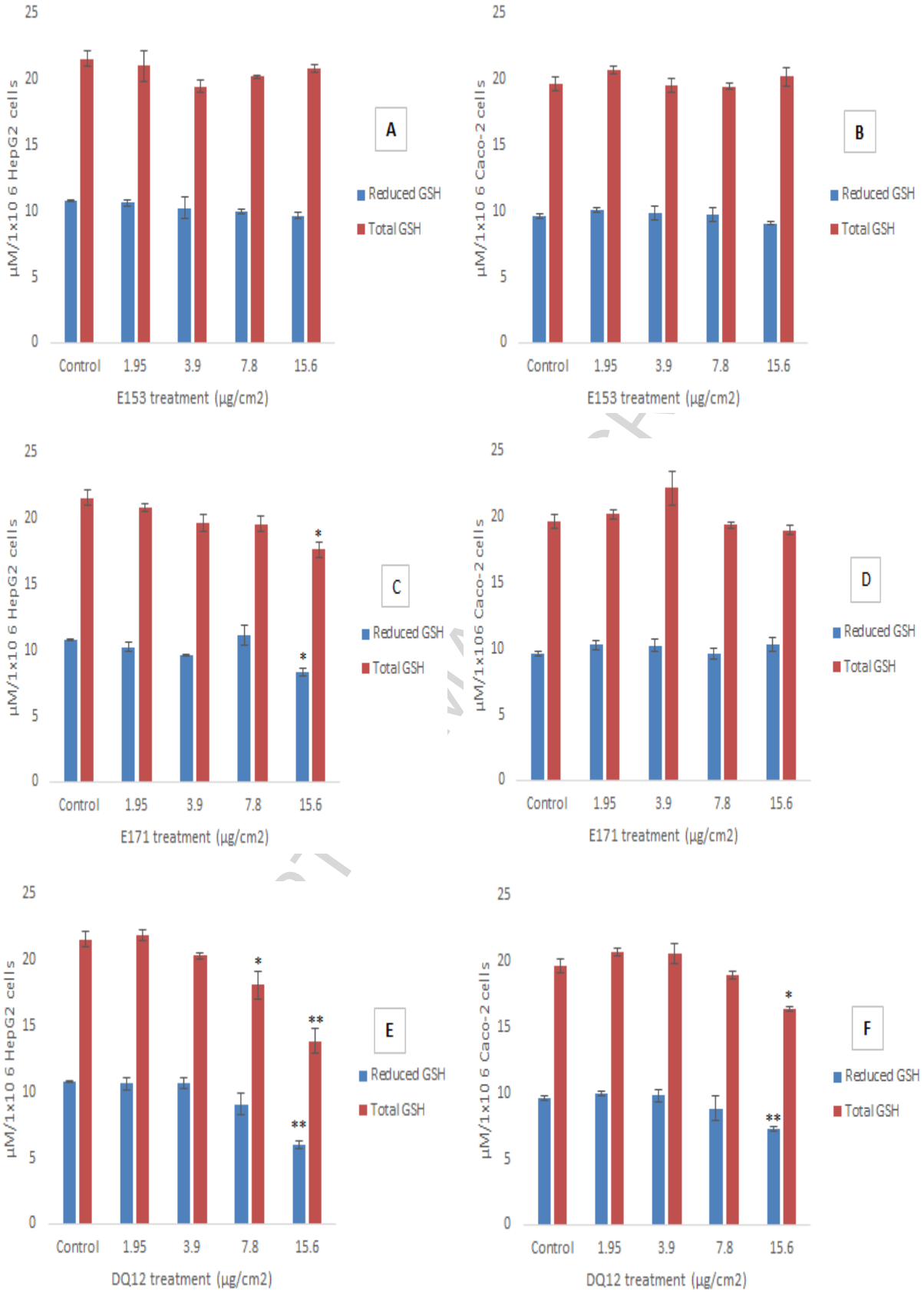


Figure 3. The effect of particle exposure on reduced and total glutathione levels in HepG2 and Caco-2 cells. The cells were exposed to medium (Control) and increasing concentrations of the selected particles for 24 hr. The values represent mean ± SEM (n=3), significance indicated by * = $p<0.05$ and ** = $p<0.005$ compared to the control. **A)** E153 - HepG2 **B)** E153 - Caco-2 **C)** E171 - HepG2 **D)** E171 - Caco-2 **E)** DQ12 - HepG2 and **F)** DQ12 - Caco2 cells.

Highlights

- ToxTracker system very useful tool in the field of particle toxicology as it utilises a 96-well plate format allowing for high throughput screening
- food grade TiO_2 was demonstrated to be the most cytotoxic, decreased intracellular glutathione and the most significant for genotoxic effects to stem cells, Caco-2 and HepG2 cells
- ToxTracker data showed very good comparability to conventional toxicity and oxidative stress assays
- Discrepancies were noted for the genotoxicity end-point between ToxTracker and the conventional comet assay