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Investigating the pre-lethal cytotoxic effects of Bis(2,4-di-tert-butylphenyl)Phosphate on Chinese hamster ovary cells using high content analysis.

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Abstract

Bis(2,4-di-tert-butylphenyl)Phosphate (bDtBPP) leaches out of polyethylene films used by the biopharmaceutical industry in single-use systems (SUS) for the culturing of drug producing cell lines. Previous studies found bDtBPP (0.025 – 0.110 mg/L) negatively affects Chinese hamster ovary (CHO) cell growth and productivity. Less information is known about the potential early stages of subtle pre-lethal cytotoxic effects of bDtBPP. This study aimed to investigate the pre-lethal cytotoxic effects in CHO-K1 cells of bDtBPP (0.005 - 0.25 µg/ml) at process relevant concentrations following 2, 24 and 48 h exposure, using high content analysis to investigate multiple pre-lethal cytotoxicity markers.

After 48 h exposure, bDtBPP (0.005 - 0.25 µg/ml; $P \leq 0.001$) increased nuclear intensity. A dose- and time-dependent reduction in mitochondrial mass was seen after exposure to bDtBPP. Reactive oxygen species increased after 2 h exposure to 0.25 µg/ml bDtBPP, 24 and 48 h exposure to 0.05 - 0.25 µg/ml bDtBPP ($P \leq 0.01$ and $P \leq 0.001$). bDtBPP induced subtle pre-lethal cytotoxic effects on CHO-K1 cellular health.

This study highlights the cellular health benefits of the biopharmaceutical industry switching to alternative SUS plastics which do not leach bDtBPP, which may enhance CHO-K1 cell productivity.

Keywords: bDtBPP; Chinese hamster ovary cells; High content analysis; cellular health; pre-lethal cytotoxicity.

Abbreviations: bDtbPP, bis(2,4-di-tert-butylphenyl)Phosphate; CHO-K1, Chinese hamster ovary parental cells; CN, cell number; NA, nuclear area; NI, nuclear intensity; MMP, mitochondrial membrane potential; MM, mitochondrial mass; ROS, reactive oxygen species, SUS, single-use systems.

36 1. Introduction

37 The biopharmaceutical industry has implemented the investment of single-use
38 systems (SUS) instead of traditional fixed stainless steel bioreactors throughout the
39 manufacturing process. Flexible plastic polymeric bags utilized in SUS offer many
40 advantages over stainless steel bioreactors such as eliminating energy and time consuming
41 sterilization processes therefore reducing operational costs, allows more control over culture
42 parameters such as pH and temperature and they have proved very efficient in the cultivation
43 of cells (Butler and Meneses-Acosta, 2012; Kelly et al., 2016). However, SUS introduce
44 possible concerns in relation to the suitability of the material and their potential to impact the
45 bioprocess regarding performance, quality and patient safety (Liu et al., 2014).

46 The compound Bis(2,4-di-tert-butylphenyl)Phosphate (bDtBPP), a degradant of the
47 antioxidant stabilizer Tris(2,4-di-tert-butylphenyl)phosphite (TBPP) commonly added to
48 resin polymers and polyethylene-film used in single-use bioprocess bags. Single-use
49 bioprocessing bag films containing bDtBPP are used in the biopharmaceutical production
50 which involves using Chinese hamster Ovary (CHO) cells that are genetically engineered to
51 produce a therapeutic protein used for drug manufacturing (Hammond et al., 2013). BDtBPP
52 has been reported to be a compound that leaches out of single-use bioprocess films into the
53 cell culture media upon gamma-irradiation or sterilization by the process of autoclaving
54 where TBPP becomes oxidised and breaks down forming the degradant bDtBPP (Liu et al.,
55 2014). Numerous studies have investigated bDtBPP cytotoxic effects on CHO cell
56 proliferation, sensitivity, growth and productivity (Hammond et al., 2013; Hammond et al.,
57 2014; Kelly et al., 2016; Kelly et al., 2019). Hammond et al., (2014) found the disposable
58 material bDtBPP to be cytotoxic to CHO cell lines and leaches out of certain film bags into
59 cell culture media in concentrations (above ≈ 0.04 to 0.05 mg/L) that are deleterious to cell

growth. In another study Kelly et al., (2016) demonstrated the leachate bDtBPP to reduce growth and productivity of CHO cell lines (parental nonproducing CHO-K1 and IgG antibody producing CHO-DP12) at concentrations as low as 0.035 – 0.1 mg/L with a reduction in cell viability at 0.25 mg/L after 4 days exposure using the Guava ViaCount assay. However, bDtBPP under normal incubation conditions was found to leach out of SUS bags and into cell culture media at 0.025 – 0.110 mg/L concentrations therefore, bDtBPP 0.25 mg/L is higher than process relevant concentration levels (Hammond et al., 2014).

This study aims to investigate the potential of bDtBPP to induce pre-lethal cytotoxic effects on several cellular responses and metabolic activity using CHO-K1 cells. In the current study, high content analysis (HCA) was used to study pre-lethal cytotoxic effects on cellular health in conjunction with the conventional gross metabolic cytotoxicity thiazolyl blue tetrazolium bromide (MTT) assay. The CHO-K1 cell line was used in this study due to its predominant choice in the pharmaceutical industry as a host for stable transfection and highly efficient production of recombinant therapeutic proteins for the last three decades (Butler and Meneses-Acosta, 2012). In the current study, bDtBPP concentrations tested (0.005 - 0.25 µg/ml) were based on previous studies which found bDtBPP to inhibit cell growth at concentrations ranging from 0.12 – 0.73 mg/L and exhibit sensitivity to bDtBPP exposure at concentrations 0.035 – 0.1 mg/L (Hammond et al., 2013; Kelly et al., 2016).

HCA is a highly sensitive, high-throughput and quantitative fluorescence technique that can examine chemical induced toxicity at sub-cellular microscopic resolution (Clarke et al., 2015; Wilson et al., 2016). It combines fluorescence microscopy with automated cell analysis software allowing the simultaneous assessment of multiple morphological and functional cell parameters (Abraham et al., 2004; Giuliano et al., 2004). HCA can be more beneficial than conventional cytotoxic assays such as the MTT assay due to its ability to identify subtle pre-lethal changes in cellular health and morphology at multiple endpoints

providing substantially more information (Clarke et al., 2015; O'Brien and Haskins, 2007; Rinaldi et al., 2017). HCA measures very subtle effects on the health of cells including multiple pre-lethal and sub-cellular cytotoxicity parameters through the use of fluorescent chemical dyes. Hoechst 33342 dye can be used to measure nuclear parameters reflecting cellular health and nuclear morphology including cell number (CN), nuclear area (NA) and nuclear intensity (NI) (Wilson et al., 2016). Hoechst 33342 binds to and stains the adenine-thymine-rich regions of the minor groove in DNA which renders it specific for nuclear chromatin in live cells. Its fluorescent intensity depends on the DNA content, chromatin structure and the position of the cell within the cell cycle, on binding to DNA, the fluorescence greatly increases. Hoechst 33342 can stain the condensed nuclei of apoptotic cells to allow the identification of chromatin condensation and fragmentation (Chazotte, 2011; Purschke et al., 2010).

MitoTracker Orange CMTMRos dye is positively charged and is rapidly taken up into the negatively charged mitochondria, suggesting that the uptake of the dye would be dependent on the mitochondrial membrane potential (MMP) and therefore can be used to measure mitochondrial function parameters such as MMP and mitochondrial mass (MM) (Buckman et al., 2001; Wilson et al., 2016). MitoTracker Orange CMTMRos dye accumulates in mitochondria allowing the MM to be measured whilst the MMP measures the intensity of the stained mitochondria (Cottet-Rousselle et al., 2011). Mitochondria are essential for cellular energy production, normal cellular function and dysfunction including calcium homeostasis, cell growth and differentiation, cell cycle control, apoptotic activation and cell death. Disruption in mitochondrial function can be related to cellular damage leading to endocrine related disease such as cancer and diabetes mellitus. Mitochondria morphology is important as it is associated with mitochondrial health and function (Kashatus, 2018). Mitochondrial morphology is determined by mitochondrial bioenergetics, a balance between

the processes of fission and fusion, along with biogenesis and autophagy, ensuring an equilibrium state of organelle population (Duchen, 2000; Osellame et al., 2012; Silva et al., 2000; Susin et al., 1999). Changes in mitochondrial morphology are largely effected by disruption to fission and fusion homeostasis resulting in a heterogeneous population of organelles with non-uniform mitochondrial DNA (mtDNA) distribution, varied ability to produce adenosine triphosphate (ATP), and increased capacity to generate reactive oxygen species (ROS) and therefore increase the cells susceptibility to undergo apoptosis (Parone et al., 2008). Apoptosis is a form of programmed cell death which occurs through consequent morphological changes including cell shrinkage, plasma membrane blebbing, chromatin condensation, DNA fragmentation and formation of apoptotic bodies (Arends and Wyllie, 1991). Whereas, necrosis is a form of accidental cell death caused by a loss of membrane integrity, intracellular organelle swelling and ATP depletion leading to an increase in calcium levels (Camilleri-Broët et al., 1998; Li et al., 2018; Osellame et al., 2012).

CellROX Deep Red reagent is a fluorogenic probe designed to detect oxidative stress parameters measuring ROS intensity and ROS area. Upon oxidation, the CellROX Deep Red reagent exhibits a strong fluorogenic signal localised in the cytoplasm (Wilson et al., 2016). Excessive intracellular ROS can induce oxidative damage in cells and impair cellular functions (Harman, 1956).

In this study, a multi-parameter pre-lethal cytotoxicity analysis model for the CHO-K1 cell line was optimised on the HCA platform, focusing on assay efficiency and sensitivity, optimal seeding density, fluorescent dye selection, cytotoxic control and concentration. Previous studies have found that the leachate bDtBPP leaches out of SUS bags at concentrations (0.025 – 0.110 mg/L) that affect CHO cell growth and productivity within biopharmaceutical production (Hammond et al., 2014). However, bDtBPP exposure can negatively affect cell growth and productivity at leaching concentrations before becoming

cytotoxic and inducing cell death at concentrations above process relevant leaching concentrations (0.25 mg/L) (Kelly et al., 2016). Therefore, the overall aim of the study was to use the optimised model to assess early stage subtle pre-lethal cytotoxic effects in CHO-K1 cells following exposure to bDtBPP (0.005 - 0.25 µg/ml) at process relevant concentrations found to leach out of SUS bags into culture media. In addition, three exposure time-points were chosen to cover early cellular events/effects and the potential impact of bDtBPP on nuclear and mitochondrial health and morphology after 2, 24 and 48 h exposure.

2. Methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO), menadione and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Poole, Dorset, UK). The cell culture reagents including phosphate-buffered saline (PBS), trypsin, F-12 Nutrient Mixture (Ham) media and general foetal bovine serum were supplied by Life Technologies (Paisley, UK). TrypLE™ Express, trypan blue and CellROX Deep Red reagent was obtained from Life Technologies (Paisley, UK). Mitochondrial membrane potential dye (MMPD) MitoTracker Orange CMTMRos and Hoechst 33342 nuclear stain were purchased from ThermoFisher Scientific, UK. Triton™ X-100 solution was purchased from Analab, UK. Bis(2,4-di-tert-butylphenyl) Phosphate (bDtBPP) was supplied by Dublin City University (Dublin, Ireland).

2.2. Cell Culture

The Chinese Hamster Ovary parental (CHO-K1) cell line was obtained from SpectraGenetics, (USA) (ECACC catalogue number 85051005). The cell line was routinely grown in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5% CO₂ and 95% humidity. The CHO-K1 cell line was routinely cultured in Ham's F-12 nutrient mixture (1:1) with 2mM Glutamine supplemented with 10% Foetal bovine serum (50/500ml medium) and 1% penicillin-streptomycin.

2.3. Analysis of multiple cellular parameters by High Content Analysis (HCA)

The Cellomics® High Content Screening reagent series multi-parameter cytotoxicity assay was performed according to the manufacturer's instructions. Multi-parameter cytotoxicity dyes, Hoechst 33342, MitoTracker Orange CMTMRos and CellROX Deep Red, were used following manufacturer's instructions. CHO-K1 cells were seeded at a density of 4×10^4 cells/ml in 96-well black plates with clear, flat bottoms. The cells were allowed to attach for 24 hours before being exposed to 100 μ l of bDtBPP (0.005 - 0.25 μ g/ml) along with a cytotoxic control (menadione 50 μ M for 2 hour exposure and 10 μ M for 2, 24 and 48 hour exposures) at a final DMSO concentration of 0.2%. A solvent control of 0.2% (v:v) DMSO in media was also included. The treated plates were incubated for periods of 2, 24 and 48 hours. The incubation periods were chosen to cover early cellular events/effects and the potential consequences after 2, 24 and 48 hour exposures. The MMPD MitoTracker Orange CMTMRos was prepared by adding 117 μ l of DMSO to 50 μ g of dye to make 1 mM stock solution. The live cell staining solution was prepared by adding 5 μ l of the MMPD MitoTracker Orange CMTMRos in 5 ml of F-12 Nutrient Mixture (Ham) media and 20 μ l of CellROX Deep Red reagent in 5 ml of F-12 Nutrient Mixture (Ham) media for each assay plate. Following incubation, the plate was protected from light and 50 μ l of live cell stain added to each well for 30 mins at 37°C. Cells were fixed with 10% formalin solution for 20 minutes at room temperature and washed with 200 μ l PBS twice. Hoechst 33342 nuclear stain was prepared by adding 1 μ l of Hoechst 33342 dye stock solution (20 mM) to 10 ml PBS and 0.5 ml Triton™ X-100 solution. 100 μ l of Hoechst 33342 nuclear stain at a final concentration of 1.9 μ M was added to each well and incubated for 15 mins at room temperature. Following incubation the wells were washed with 200 μ l PBS, then filled with 100 μ l PBS and sealed with a black plate sealer.

Plates were read using CellInsight NXT High Content Analysis platform (ThermoFisher Scientific, UK). Hoechst 33342 stained nuclei were read as primary targets in the first channel (Ex/Em 350/461 nm). Fluorescent nuclei were segmented from neighbouring nuclei and evaluated based on nuclear morphology. Hoechst 33342 stain was used to measure nuclear parameters including cell number (CN), nuclear area (NA), and nuclear intensity (NI) for each well. MitoTracker Orange CMTMRos stained mitochondria as target in the second channel (Ex/Em 554/576 nm). MitoTracker Orange CMTMRos dye was used to measure mitochondrial function parameters such as mitochondrial membrane potential (MMP) and mitochondrial mass (MM). CellROX Deep Red reagent is a fluorogenic probe designed to measure reactive oxygen species (ROS) in live cells in the third channel (Ex/Em 640-665 nm). Signals from CellROX Deep Red reagent are localised in the cytoplasm and are used to detect oxidative stress parameters such as ROS intensity and ROS Area. Data was captured for each plate at x20 objective magnification. For each well, sixteen field view images were obtained to examine each parameter.

2.4. *Thiazolyl blue tetrazolium bromide (MTT) assay*

The Thiazolyl blue tetrazolium bromide (MTT) assay was used in parallel to HCA to measure cellular metabolic activity in CHO-K1 cells. CHO-K1 cells were seeded at 4×10^4 cells/ml in clear, flat-bottomed 96-well plates and incubated for 24 hours. The test compound bDtBPP (0.005 - 0.25 $\mu\text{g/ml}$) at a final DMSO concentration of 0.2% and solvent control (0.2% v:v DMSO in media) were added to the cells and incubated for 48 hours. The supernatant was discarded and cells washed once with 200 μl PBS. Then, 50 μl of thiazolyl blue tetrazolium bromide (MTT) solution (2 mg/ml stock in PBS, diluted 1:6 in media) was added to each well and incubated for 3 hours at 37 °C. In this assay, viable cells convert the soluble yellow MTT into insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. The supernatant was removed and 200 μl of dimethyl sulfoxide (DMSO) was

added to each well to dissolve the formazan crystals. The plate was incubated at 37 °C for 10 minutes with agitation. Optical density (OD) was measured using a Sunrise spectrophotometer (TECAN, Switzerland) at 570 nm with a reference filter at 630 nm. All samples were assessed in triplicate wells and in three independent exposures. Viability was calculated as the percentage absorbance of the sample when compared with the absorbance of the solvent control.

2.5. Statistical Analysis

HCA pre-lethal cytotoxicity and MTT assays were carried out in triplicate and in three independent exposures (n=3). The coefficient of variation (CV) was calculated for the three exposures; all parameters were below 15% except for the ROS intensity which was below 25%. Data collected from the HCA platform was processed with Excel and Graphpad PRISM software version 6.0 (San Diego, CA). All values shown are expressed as mean $\pm$ standard error of the mean (SEM) of three independent exposures for the compound tested. Data is expressed as a percentage of the relevant solvent control for each parameter and analysed by one-way ANOVA followed by Dunnett's procedure for multiple comparisons; the mean concentrations were tested for significant difference at the 95% confidence level. Significant effects are represented by $P \leq 0.05$ (*), $P \leq 0.01$ (**) and $P \leq 0.001$ (***).

3. Results

Multiple nuclear and mitochondrial pre-lethal cytotoxicity markers were assessed in this study in CHO-K1 cells following 2, 24 and 48 h exposure to bDtBPP (0.005 - 0.25 µg/ml). Hoechst 33342 nuclear stain was used to obtain information on cellular health; measuring cell number (CN), nuclear area (NA) and nuclear intensity (NI). MitoTracker Orange CMTMRos was used to provide information on mitochondrial health; measuring mitochondrial membrane potential (MMP) and mitochondrial mass (MM). CellROX Deep Red reagent was used to detect oxidative stress parameters such as reactive oxygen species (ROS) intensity and ROS area. Parallel to the HCA, cytotoxicity and metabolic activity were also measured using the MTT assay.

3.1. Optimisation of the pre-lethal cytotoxicity assay and HCA reading

Valinomycin was initially used as a cytotoxic control as it was previously identified to cause cell death by inducing apoptosis in CHO cells via several apoptotic events (Abdalah et al., 2006). However, after the first assay run, the method was optimized; the cytotoxic control, valinomycin (2 µM), should have increased the formation of ROS and oxidative stress when compared to the solvent control (DMSO 0.2%) however, in this study a reduction in ROS was observed when compared to the solvent control (Fig 1a, b and e). In the presence of valinomycin, a reduction in the formation of ROS may be due to the depolarizing effect of voltage-dependent potassium influx (Akopova et al., 2013). The cytotoxic control was changed from valinomycin to menadione 50 µM, as menadione had previously been found to induce ROS production when compared to the solvent control (Wilson et al., 2016). After 24 h exposure to 50 µM menadione, no cells were observed, likely due to cytotoxicity at this concentration over 24 h. The cytotoxic control for 24 and 48 h was altered to 10 µM menadione; whilst the 2 h exposure contained both 10 and 50 µM menadione (Fig 1a, b and

252 c). The cell seeding density was reduced from 6×10^4 cells/ml to 4×10^4 cells/ml to reduce
253 cell confluency in the wells. The CHO-K1 cell population after 2, 24 and 48 h exposure to the
254 solvent control is presented in Fig. 2 a, b and c.

255

Fig. 1. High content analysis composite channel images of CHO-K1 cells after exposure to the (a) solvent control (DMSO 0.2%), cytotoxic controls (b) valinomycin 2 μ M and (c) menadione 10 μ M after 2, 24 and 48 h and (d) menadione 50 μ M after 2 h exposures. Typical HCA micrographs are shown with nuclei (stained by Hoechst 33342, shown in blue), mitochondrial structure (stained by MitoTracker Orange CMTMRos, shown in green) and ROS (stained by CellROX Deep Red, shown in red) x 20 objective magnification, scale bars = 200 μ m. (e) Changes in ROS intensity in CHO-K1 cells following exposure to solvent control (DMSO 0.2%) and cytotoxic controls (valinomycin 2 μ M,

265 menadione 10 and 50 μ M) after 2, 24 and 48 hours measured by HCA. Data is expressed as a
266 percentage of solvent control; mean $\pm$ standard error of the mean (SEM), n=3. $P \leq 0.05$ (*) $P \leq 0.001$
267 (***)).

Fig. 2. High content analysis images of CHO-K1 cells after exposure to the solvent control (DMSO 0.2%) after (a) 2, (b) 24 and (c) 48 h exposures showing the CHO-K1 cell population over time. Typical HCA micrographs are shown with nuclei (stained by Hoechst 33342, shown in blue) and mitochondrial structure (stained by MitoTracker Orange CMTMRos, shown in green) x 20 objective magnification, scale bars = 200 μ m. Dyes are stated in the image, with composite images containing channels 1 and 2.

In the first assay run, the CHO-K1 cells were stained with all three dyes, Hoechst 33342, MitoTracker Orange CMTMRos and CellROX Deep Red and run together on the HCA (Fig. 3a). However, running the three dyes together induced high variations in ROS. Consequently, the dyes were applied using two separate plates in further runs, where in one plate the CHO-K1 cells were stained with Hoechst 33342 and MitoTracker Orange CMTMRos and the second plate the CHO-K1 cells were stained with Hoechst 33342 and CellROX Deep Red as shown in table 1. CellROX Deep Red uptake was improved as presented in Fig. 3b and ROS variations were reduced. The paper Wilson et al, (2016) also found high variations in ROS production using MitoTracker Orange CMTMRos and CellROX Deep Red as the CV (%) calculated for three exposures was above the ideal target (15%) but below 25%. Fig. 3 shows HCA images of CHO-K1 cells after 2 h exposure to bDtBPP (0.25 µg/ml) showing differences in fluorescent signal upon oxidation (ROS production) stained with (a) Hoechst 33342, MitoTracker Orange CMTMRos and CellROX Deep Red dyes and (b) Hoechst 33342 and CellROX Deep Red dyes.

Table 1 Optimization of the fluorescent chemical dyes used in the HCA assay runs. ✓ shows the use of the dye while ✕ shows omitting the dye from the run.

	Hoechst 33342 nuclear stain	MitoTracker Orange CMTMRos dye	CellROX Deep Red reagent
First assay run	✓	✓	✓
Second assay run plate 1	✓	✓	✕
Second assay run plate 2	✓	✕	✓

296

297

298 **Fig. 3.** High content analysis images of CHO-K1 cells after 2 h exposure to bDtBPP (0.25 $\mu\text{g/ml}$)
 299 showing differences in fluorescent signal upon oxidation (ROS production) stained with (a) Hoechst
 300 33342, MitoTracker Orange CMTMRos and CellROX Deep Red dyes and (b) Hoechst 33342 and
 301 CellROX Deep Red dyes. Dyes are stated in the image, with composite images containing channels
 302 (a) 1, 2 and 3 and (b) 1 and 2. Typical HCA micrographs are shown with nuclei (stained by Hoechst
 303 33342, shown in blue), mitochondrial structure (stained by MitoTracker Orange CMTMRos, shown in
 304 green) and ROS (stained by CellROX Deep Red, shown in red) x 20 objective magnification, scale
 305 bars = 200 μm .

306

3.2. Metabolic activity as measured by MTT assay

Gross cellular cytotoxicity was investigated in the CHO-K1 cells by quantifying metabolic activity using MTT conversion. No significant effects on metabolic activity of CHO-K1 cells were induced by bDtBPP (0.005 - 0.25 µg/ml) compared to the solvent control following 48 h exposure, as determined by the MTT assay (Fig. 4). However, not significantly, there was a 5.1% reduction in metabolic activity at the highest concentration of bDtBPP (0.25 µg/ml) after 48 h. The results suggest that there may be cytotoxic effects starting to occur therefore HCA was employed to detect subtle pre-lethal health markers of the exposed cells.

Fig. 4. MTT metabolic activity of CHO-K1 cells after exposure to bDtBPP (0.005 - 0.25 µg/ml) and solvent control (DMSO 0.2%). Data is expressed as percentage of solvent control (DMSO 0.2% v:v); mean ± standard error of the mean (SEM), n=3.

322
323 *3.3. Pre-lethal cytotoxicity as measured by cell number (CN), nuclear area (NA), and nuclear*
324 *intensity (NI)*

325 In agreement with the MTT cell viability results, CN did not significantly reduce.
326 However, not significantly, there was a 7.6% reduction in cell number at the highest
327 concentration of bDtBPP after 48 h (data not shown). After 24 and 48 h the cytotoxic control
328 menadione 50 µM was cytotoxic to the CHO-K1 cells and reduced the cell number to 0. No
329 significant decrease in NA was observed following 2, 24 or 48 h at all tested concentrations
330 (data not shown). However, not significantly, there was a 4.43% and 6.18% reduction in NA
331 after exposure to the highest concentration of bDtBPP after 24 and 48 h, respectively (data
332 not shown).

333 NI significantly increased after 48 h exposure at all tested concentrations of bDtBPP
334 (0.005 - 0.25 µg/ml), 122.20%, 119.54%, 123.06%, 125.78%, 126.32% and 127.15%
335 respectively ($P \leq 0.001$) when compared to the solvent control, Fig. 5. No significant increase
336 in NI was observed following 2 or 24 h exposure. It can be observed in Fig. 6 an increase in
337 brightness and blue intensity of the nuclei after 48 h exposure to bDtBPP 0.25 µg/ml
338 compared to 2 h.

Fig. 5. Nuclear intensity changes in CHO-K1 cells following exposure to bDtBPP (0.005 - 0.25 µg/ml), solvent control (DMSO 0.2%) and cytotoxic controls (menadione 10 and 50 µM) after 2, 24 and 48 hours measured by HCA. Data is expressed as a percentage of solvent control (DMSO 0.2% v:v); mean ± standard error of the mean (SEM), n=3. $P \leq 0.001$ (***) represent significant pre-lethal cytotoxic effects.

Fig. 6. High content analysis images of nuclear intensity changes in CHO-K1 cells after (a) 2 and (b) 48 h exposure of bDtBPP 0.25 µg/ml. Typical HCA micrographs are shown with nuclei (stained by Hoechst 33342, shown in blue) and mitochondrial structure (stained by MitoTracker Orange CMTMRos, shown in green) x 20 objective magnification, scale bars = 200 µm. Dyes are stated in the image, with composite images containing channels 1 and 2.

352 3.4. Pre-lethal cytotoxicity as measured by mitochondrial membrane potential (MMP) and
353 mitochondrial mass (MM)

354 No significant change in MMP was observed at any bDtBPP tested concentrations
355 following exposure for 2, 24 and 48 h (data not shown). After 24 h exposure, a significant
356 decrease in MM was observed for bDtBPP 0.035, 0.05, 0.1 and 0.25 µg/ml, to 90.56, 89.38,
357 88.47 and 87.27%, respectively ($P \leq 0.05$ and $P \leq 0.01$). After 48 h, all concentrations of
358 bDtBPP (0.005 - 0.25 µg/ml) were found to significantly decrease MM, to 89.49, 86.31,
359 85.42, 81.67, 79.72 and 76.18% ($P \leq 0.01$ and $P \leq 0.001$) (Fig. 7). After 24 and 48 h the
360 cytotoxic control menadione 50µM was cytotoxic to the CHO-K1 cells, reducing the cell
361 number to 0 therefore the MM result showed a decrease of 100% (Fig. 7). Changes in
362 mitochondrial mass can be observed in the HCA images of CHO-K1 cells in Fig. 8, as a
363 decrease in green staining of the mitochondria can be seen when comparing the solvent
364 control (DMSO 0.2%) image to 0.25 µg/ml bDtBPP. The changes in mitochondrial
365 morphology provide information regarding the mitochondrial health of the cells.

367

368 **Fig. 7.** Mitochondrial mass changes in CHO-K1 cells following exposure to bDtbPP (0.005 - 0.25
369 µg/ml) solvent control (DMSO 0.2%) and cytotoxic controls (menadione 10 and 50 µM) after 2, 24
370 and 48 hours measured by HCA. Data is expressed as a percentage of untreated control; mean ±
371 standard error of the mean (SEM), n=3. $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) represent
372 significant pre-lethal cytotoxic effects.

Fig. 8. High content analysis images of mitochondrial mass changes in CHO-K1 cells after 2 h exposure of (a) solvent control (DMSO 0.2%), (b) bDtBPP 0.25 µg/ml and after 48 h exposure of (c) solvent control (DMSO 0.2%) and (d) bDtBPP 0.25 µg/ml. Typical HCA micrographs are shown with nuclei (stained by Hoechst 33342, shown in blue) and mitochondrial structure (stained by MitoTracker Orange CMTMRos, shown in green) x20 objective magnification, scale bars= 200 µm. Dyes are stated in the image, with composite images containing channels 1 and 2.

4.3.5 Cellular health as measured by oxidative stress parameters ROS intensity and ROS

Area

When compared to the solvent control (DMSO 0.2%) exposure, a significant increase in ROS intensity was observed after 2 h exposure to 0.25 µg/ml bDtBPP, 24 and 48 h exposure to 0.05, 0.1 and 0.25 µg/ml bDtBPP (Fig. 9). A significant dose and time-dependent increase in ROS intensity was observed after 24 and 48 h ($P \leq 0.01$ and $P \leq 0.001$) exposure to bDtBPP. The increase in red fluorescence after 48 h exposure of the cytotoxic control (menadione 10µM) and the highest concentration of bDtBPP (0.25 µg/ml) compared to the solvent control can be seen in Fig. 10. However, no significant change in ROS area was observed at any bDtBPP tested concentrations following exposure for 2, 24 and 48 h (data not shown).

Fig. 9. Changes in ROS intensity in CHO-K1 cells following exposure to bDtBPP (0.005 - 0.25 µg/ml), solvent control (DMSO 0.2%) and cytotoxic controls (menadione 10 and 50 µM) after 2, 24 and 48 hours measured by HCA. Data is expressed as a percentage of solvent control; mean ± standard error of the mean (SEM), n=3. $P \leq 0.01$ (**) $P \leq 0.001$ (***) represent significant pre-lethal cytotoxic effects.

398

399 **Fig. 10.** High content analysis images of changes in ROS intensity of CHO-K1 cells after 48 h
 400 exposure (a) solvent control (DMSO 0.2%), (b) cytotoxic control (menadione 10μM) and (c) bDtBPP
 401 0.25 μg/ml. Typical HCA micrographs are shown with nuclei (stained by Hoechst 33342, shown in
 402 blue) and ROS (stained by CellROX Deep Red, shown in red) x20 objective magnification, scale
 403 bars= 200 μm. Dyes are stated in the image, with composite images containing channels 1 and 2.

4. Discussion

In this study, HCA was used to measure subtle pre-lethal changes in cellular health of CHO-K1 cells following exposure to the leachate bDtBPP after 2, 24 and 48 h. Multiple nuclear and mitochondrial pre-lethal cytotoxicity parameters were assessed. Parallel to the HCA, gross cellular cytotoxicity and metabolic activity were also measured using the MTT assay. Viable cells in the MTT assay convert the soluble yellow MTT to insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. Results from the HCA parameter CN and the MTT assay (Fig. 4) show no significant decrease in CN or cell viability at any concentration of bDtBPP following 2, 24 and 48 h exposure. However, numerous studies have found bDtBPP to be a leachate which negatively affects CHO cell growth. The study Hammond et al., (2014) investigated the effects of the leachate obtained on CHO cell growth and viability after 3 to 4 days incubation at 37°C and 48 h at 50°C. Multiple varieties of biopharmaceutical manufacturing single-use bags were found to leach bDtBPP and cause suboptimal cell growth and reduced cell viability due to leaching of bDtBPP at concentrations above 0.04 mg/L (Hammond et al., 2014). A reduction in cell viability was induced in both CHO-K1 and the antibody producing CHO-DP12 cells after exposure to bDtBPP at the highest concentration of 0.25 mg/L (Kelly et al., 2016). The productivity in the CHO-DP12 cells was reduced by ~50% under IC₅₀ conditions (0.1 mg/L) reflecting the reduced cell density. After incubating CHO cells for 4 days at 0.25 mg/L bDtBPP, the CHO-K1 and CHO-DP12 cells observed a 40% and 30% reduction, respectively, in viable non-apoptotic cells in early and late apoptotic stages (Kelly et al., 2016). Overall the study by Kelly et al., (2016) indicated bDtBPP (0.035-0.1 mg/L) appeared to slow down cell growth and productivity. Under normal incubation conditions bDtBPP was found to leach out of SUS bags and into cell culture media at process relevant concentrations,

0.025 – 0.110 mg/L (Hammond et al., 2014). Therefore, the concentration range of bDtBPP (0.005 – 0.25 µg/ml) tested in this study was based upon process relevant leaching concentrations of bDtBPP which induced changes in cell growth performance.

Changes in NA and NI can be used as characteristic changes involved in apoptosis or necrosis after exposure to toxic chemicals. In Fig. 5, a significant increase in NI at all tested concentrations of bDtBPP after 48 h exposure was observed when compared to the solvent control. The increase in NI can also be observed in the HCA image of the Hoechst 33342 stained nuclei in Fig. 6. The intensity of the nuclei normally correlates to the nuclear size or area as a reduction in nuclear area or size results in an increase in NI (Lucke and Mumtsidu, 2009) however, in this study no significant reduction in NA was observed following 2, 24 or 48 h exposure to bDtBPP at any of the test concentrations. However, not significantly, there was a slight reduction in NA after exposure to bDtBPP after 24 and 48 h when compared to the solvent control (data not shown). The increase in NI could be due to the onset of apoptosis which results in chromatin condensation. Chromatin condensation is characterised by an increase in DNA staining in the nuclei without a decrease in nuclei size (Cummings and Schnellmann, 2004). The increase in NI without a decrease in CN and NA indicates that bDtBPP can induce changes in nuclear morphology.

As the MTT assay determines mitochondrial activity, it is important to include the HCA mitochondrial parameters as a comparison. The leachate bDtBPP induced a significant dose and time-dependent decrease in MM ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$) (Fig. 7). After 2 h exposure, no significant changes in MM were observed however, after 24 h exposure, a significant decrease in MM was observed for 0.035, 0.05, 0.1 and 0.25 µg/ml bDtBPP, to 90.56, 89.38, 88.47 and 87.27%, respectively ($P \leq 0.05$ and $P \leq 0.01$). After 48 h, all concentrations of bDtBPP (0.005 - 0.25 µg/ml) were found to significantly decrease MM, to 89.49, 86.31, 85.42, 81.67, 79.72 and 76.18% ($P \leq 0.01$ and $P \leq 0.001$) (Fig. 7). After 24 and

48 h the cytotoxic control menadione 50 μ M was too toxic to the CHO-K1 cells, reducing the cell number to 0 therefore the MM result showed a decrease of 100% (Fig. 7). Changes in MM can be observed in the HCA images of CHO-K1 cells in Fig. 8, as a decrease in area of green staining of the intracellular content of the mitochondria can be seen when comparing the solvent control (DMSO 0.2%) image to 0.25 μ g/ml bDtBPP (Fig. 8). However, no significant changes in MMP were observed at any of the tested bDtBPP concentrations following exposure for 2, 24 and 48 h. The changes in mitochondrial morphology provide information regarding the mitochondrial health of the cells. The decrease in MM after 24 and 48 h exposure to bDtBPP could suggest that early stages of apoptosis could be occurring due to shrinking of the cytoplasm without losing the mitochondrial membrane integrity. Mitochondrial morphology is controlled by the regulated mitochondrial dynamic rates of fusion and fission. Mitochondrial fusion and fission in addition to mitophagy are key determinants of mitochondrial quality control (Arnoult et al., 2005; Twig and Shirihai, 2011). The study Arnoult et al., (2005) found that overexpression of pro-fission proteins in the mitochondria caused a decrease in mitochondrial mass by 70% in HeLa cells and thus stimulation of fission facilitates mitochondrial mitophagy under certain circumstances. The study Benischke et al., (2017) found that induction of mitophagy resulted in reduction of mitochondrial mass. Mitophagy and fission events play a role in the degradation and segregation of dysfunctional mitochondria. Mitophagy is of importance as dysfunctional mitochondria that are not degraded can produce higher amounts of ROS, be more susceptible to the release of cytochrome *c* and apoptosis-inducing factor, and thereby, amplify ROS damage (Twig and Shirihai, 2011).

There was no observed significant change in MMP this result would indicate that there was no loss of the mitochondrial membrane integrity by the formation of large conductance pore openings in the mitochondrial membrane, therefore calcium homeostasis

would be maintained, no ATP depletion or rapid progression to cell death occurring (Hunter and Hapworth, 1979; Osellame et al., 2012). The results from the MTT cell viability and the HCA CN nuclear marker in addition to the MMP marker indicates the stability of the mitochondrial membrane activity within the CHO-K1 cells after exposure to bDtBPP after 48 h. Therefore, it is possible that bDtBPP induced early apoptosis without the loss of mitochondrial membrane potential. The potential of bDtBPP to depolarize the MMP was investigated by Kelly et al., (2016) however mitochondrial membrane depolarization at 0.25mg/L was found to be a symptom of cell death and not a cause thus suggesting bDtBPP does not act exclusively at the mitochondria. The study Hammond et al., (2013), observed cellular responses to bDtBPP with a significant decrease in mitochondrial membrane potential.

Optimal functioning of the nucleus and the mitochondria in the female reproductive system and the ovary is vital for maintenance of ovarian reserve and preservation of oocyte quality. Dysfunction in the mitochondria and mitochondrial biogenesis could result in inefficient cellular energy production which could negatively affect oocyte viability and consequently embryonic development (Kasapoğlu and Seli, 2020). Mitochondrial dysfunction could also negatively affect ovarian function and reproduction leading to infertility, spontaneous abortions and birth defects (Kasapoğlu and Seli, 2020).

In the HCA assay, when compared to the solvent control (DMSO 0.2%) exposure, a significant increase in ROS intensity was observed after 2 h exposure to 0.25 µg/ml bDtBPP, 24 h to 0.05, 0.1 and 0.25 µg/ml bDtBPP and 48 h exposure to 0.05, 0.1 and 0.25 µg/ml bDtBPP (Fig. 9). A significant dose and time-dependent increase in ROS intensity after exposure to bDtBPP was observed after 2, 24 and 48 h ($P \leq 0.01$ and $P \leq 0.001$). The time and dose-dependent increase in pre-lethal cytotoxic effects of bDtBPP in this study correlates to previous studies Hammond et al., (2013) and Hammond et al., (2014) which observed a

time and temperature-dependent increase in bDtBPP leaching and a dose and time-dependent increase in cytotoxicity and cell death. The significant increase in ROS intensity after 48 h exposure to bDtBPP (0.25 µg/ml) can be seen in Fig. 10 as the red fluorescence increased from the CellROX Deep Red when compared to the solvent control and the cytotoxic control (menadione 10µM). ROS production is a natural process which occurs during the generation of energy in the mitochondria (Kasapoğlu and Seli, 2020). The generation of ROS including oxygen radicals and hydrogen peroxide are also produced during the mitochondrial oxidation of nutrients with the majority of endogenous ROS through the leakage of electrons along the electron transport chain (Kasapoğlu and Seli, 2020; Quinlan et al., 2013; Rigoulet et al., 2011). In addition, ROS generation occurs in the early events of apoptosis (Camilleri-Broët et al., 1998). The mitochondrial free radical theory of aging proposed, the detrimental effects of excessive intracellular ROS on the cell leading to oxidative damage as the determinant of cellular life span. The mitochondrial free radical theory of aging also proposed that ROS are toxic as they result in mitochondrial DNA (mtDNA) mutations that cause a bioenergetic deficit, which, in turn drives aging (Harman, 1956). A deficit in bioenergetic could result in initial damage to mtDNA leading to further mtDNA damage by increasing ROS generation, therefore leading to apoptosis (Richter, 1995). Excessive levels of ROS may be associated with mitochondrial and cellular dysfunction which is implicated in metabolic and age related disorders and neurodegenerative diseases (Osellame et al., 2012; Kasapoğlu and Seli, 2020).

One of the major pathways bDtBPP may induce oxidative stress in the CHO-K1 cells is via the activation of inducible nitric oxide synthase (iNOS). The enzyme iNOS is not present under normal conditions in most cells therefore its expression is inducible and is associated with inflammation and malignant diseases such as ovarian cancer. The activation of iNOS expression, enzyme activation and subsequent free radical gas nitric oxide (NO) production is a complex process that undergoes multilevel control and is regulated by

transcriptional from mRNA induction to post-transcriptional mechanisms including the modulation of full enzymatic activity (Kielbik, Szulc-Kielbik and Klink, 2019). Activation of the inflammatory oxidative enzyme iNOS has been linked to the generation of highly reactive ROS and NO which in turn affects various cellular components and implications in the processes of ovulation and atresia-related apoptosis (Zackrisson et al., 1996; Koppula et al., 2012). Response to cellular stress such as the production of NO can induce apoptosis through the activation of the p53 pathway and downregulating the anti-apoptotic proteins (Burke et al., 2013; Burns and El-Deiry, 1999; Singh and Gupta, 2011). Oxidative stress was also observed in the study Kelly et al., (2016) with up regulation of the gene heme oxygenase 1 (HMOX1) thus suggesting bDtBPP elicits a physiological stress and potentially elicit oxidative damage to host cell proteins in the CHO cells. Inhibited or reduced cell growth along with oxidative cellular stress could suggest activation of the p53 pathway therefore leading to apoptosis.

The high production of ROS in this study was interesting as the test compound bDtBPP is a breakdown product of the antioxidant stabiliser TBPP; antioxidants are known for their role in cellular response to prevent oxidative stress by scavenging free radicals, catalysing the oxidation of molecules and sequestering transition metal ions and preventing Fenton reactions (Chapple, 1997). The increased ROS production observed by the CellROX Deep Red dye in CHO-K1 cells after exposure to bDtBPP may be due to ROS production in the mitochondrial electron-transport chain which is not as well regulated by enzymes and therefore increase oxidative stress as antioxidant defences are inadequate to inactivate ROS production.

The study Kelly et al., (2019) identified that conditioned media generated from an older single-use bioreactor film (F-1) over time inhibits CHO cell growth and antibody productivity when compared to a more modern film (F-2) with lower levels of Irgafos 168.

Reduced growth of CHO cells in F-1 conditioned media exhibited a weakened capacity for ATP synthesis through diminished electron transport through the mitochondrial electron transport system and an increased response to oxidative stress (Kelly et al., 2019). Gene ontology analysis of the differentially expressed proteins in CHO cells cultured in F-1 conditioned media revealed the enrichment for biological processes associated with oxidative stress and mitochondrial respiratory chain complexes. Mitochondrial activity was weakened due to the downregulation of a subset of proteins involved in NADH dehydrogenase activity (Kelly et al., 2019). These potentially metabolically compromised cells exhibited a reduction in oxidative phosphorylation activity in addition to lower glycolytic metabolism, which are characteristic of poor growing cells (Kelly et al., 2019). The study Kelly et al., (2019) observed cytotoxic effects of the films after 3 (F-2) and 7 (F-1) days, whilst pre-lethal cytotoxic effects of bDtBPP on the CHO-K1 cells in the current study were observed after 24 and 48 h. Another study reported that bDtBPP accumulates to levels ranging from 0.025–0.11 mg/L over 3–4 days incubation under bioprocess-relevant conditions (Hammond et al., 2014). Therefore, it would be reasonable to suggest reducing the biopharmaceutical production period in order to prevent toxic accumulation of the leachate bDtBPP into cell culture media.

Multiple varieties of biopharmaceutical manufacturing single-use bags were found to leach bDtBPP (Hammond et al., 2014). Leachates from plastic materials such as bisphenol-A and phthalates can negatively impact human health (Moreira et al., 2015; Muncke et al., 2017; Yang et al., 2011). The leachate bDtBPP could potentially pose a threat to the patient if bDtBPP is present in the active biopharmaceutical ingredient after persisting through purification processes, the potential toxicity to the bioprocess itself, from the final container closure system or packaging components such as prefilled syringes and/or processing equipment (Kelly et al., 2019; Markovic, 2007). The study Kelly et al., (2019) reported that bDtBPP (0.1 – 0.25 µg/ml) possessed the potential to act as an endocrine disruptor,

antagonising the androgen receptor transcriptional activity in the androgen responsive TARM-Luc cell line. Androgen receptor antagonism could disrupt normal androgen signalling in humans and could lead to implications in the reproductive health of males and females potentially disrupting sexual differentiation and/or fertility (Chang et al., 2013; Luccio-Camelo and Prins, 2011). However, after the purification processes the leachate bDtBPP may potentially be removed in the later stages of the bioprocessing pipeline and therefore may not pose a threat to the patient (Owczarek et al., 2019).

Overall, exposure to bDtBPP has been found to lead to ROS accumulation and cellular oxidative stress. The mitochondrial mass can shrink and nuclear intensity increase without inducing a reduction in cell number. The level of ROS produced by the CHO-K1 cells after exposure to bDtBPP may have been sufficient to cause mtDNA mutation or damage and mitochondrial shrinkage but not enough to induce cell death. This further illustrates the ability of the HCA assay to identify early signs and/or reversible signs of cellular stress and pre-lethal cytotoxicity.

5. Conclusion

This study confirms that the leachate bDtBPP, at process relevant concentrations, can induce subtle pre-lethal cytotoxic effects on CHO-K1 cellular health. The leachate bDtBPP induced an increase in NI after 48 h exposure. A dose- and time- dependent reduction in MM, in addition a dose- and time- dependent increase in ROS intensity was observed. Exposure to bDtBPP induced the intensity of ROS and likely induced the onset of cell apoptosis due to the increase in NI and reduction in MM. These toxicological findings identify that bDtBPP can induce oxidative stress and impact nuclear and mitochondrial health and morphology without inducing a reduction in cell viability. Subtle pre-lethal changes in cellular health and

603 morphology following exposure to bDtBPP may cause CHO-K1 cells to become unhealthy
604 without reducing the cell number or viability. This study reinforces the benefits for the
605 biopharmaceutical industry and improved CHO-K1 cellular health by switching to alternative
606 SUS plastics which do not leach bDtBPP.

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