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High content analysis of cytotoxic effects of pDMAEMA on human intestinal epithelial and monocyte cultures

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ABSTRACT

Poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is a cationic polymer with potential as an antimicrobial agent and as a non-viral gene delivery vector. The aim was to further elucidate the cytotoxicity of a selected pDMAEMA low molecular weight (MW) polymer against human U937 monocytes and Caco-2 intestinal epithelial cells using a novel multi-parameter high content analysis (HCA) assay and to investigate histological effects on isolated rat intestinal mucosae. Seven parameters of cytotoxicity were measured: nuclear intensity (NI), nuclear area (NA), intracellular calcium ($[Ca^{2+}]i$), mitochondrial membrane potential (MMP), plasma membrane permeability (PMP), cell number (CN) and phospholipidosis. Histological effects of pDMAEMA on excised rat ileal and colonic mucosae were investigated in Ussing chambers. Following 24–72 h exposure, 25–50 μg/ml pDMAEMA induced necrosis in U937 cells, while 100–250 μg/ml induced apoptosis in Caco-2. pDMAEMA increased NA and NI and decreased $[Ca^{2+}]i$, PMP, MMP and CN in U937 cells. In Caco-2, it increased NI and $[Ca^{2+}]i$, but decreased NA, PMP, MMP and CN. Phospholipidosis was not observed in either cell line. pDMAEMA (10 mg/ml) did not induce any histological damage on rat colonic tissue and only mild damage to ileal tissue following exposure for 60 min. In conclusion, HCA reveals that pDMAEMA induces cytotoxicity in different ways on different cell types at different concentrations. HCA has potential for high throughput toxicity screening in drug formulation programmes.

1. Introduction

Poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is a mucoadhesive polymer, that is cationic in acidified media or when quaternised with an alkylating agent [1,2]. It is being investigated as a potential antimicrobial coating on medical devices [3–5] and as a non-viral gene delivery vector [6,7]. It induces cytotoxicity in U937 human monocyte-like cells, but less cytotoxicity in human Caco-2 intestinal epithelial cells [3]. Intravenous delivery of 5.1 mg/kg pDMAEMA was fatal to rats, but 2.1 mg/kg was well tolerated [8]. In order to eventually use pDMAEMA in a clinical setting, its cytotoxic potential should therefore be further investigated with improved assays since *in vitro* cytotoxicity data from standard assays is equivocal and lacks predictive power.

High Content Analysis (HCA) is a novel technology that allows quantitative analysis of each cell at sub-cellular microscopic resolution using a selection of multi-coloured fluorescence-based non-toxic dyes [9]. It allows combinations of numerous fluorescent probes to be used concurrently to investigate different parameters of cytotoxicity following acute and chronic exposure at varying concentrations [10,11]. The information obtained and throughput is more than an order of magnitude higher than conventional cytotoxicity assays. For example, a 96-well plate is read in 30 min with a large proportion of cells in each well analysed. Multiple biochemical, functional and morphological parameters are measured on individual cells and there is a higher sensitivity of cytotoxicity detection as indicated by the lower IC_{50} values for HCA compared to the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. HCA has recently been validated against *in vivo* human toxicity data for hundreds of marketed drugs (10–13). Additional advantages of HCA over conventional cell-based cytotoxicity assays include detailed data on numerous cell physiological and morphological parameters measured kinetically up to 72 h, measurements on individual cells, and mechanistic information on elucidating pathophysiology.

HCA has also been used to investigate the influence of siRNA on cell cycling [14], to screen for activators of DNA damage [15], as well as in the study of vision-related diseases to investigate neurite outgrowth in retinal ganglion cells [16]. The assay used in this study was based on the multi-parameter cytotoxicity assay protocol developed by O'Brien et al. [11]. This assay allows concurrent analysis of 6 parameters of cell cytotoxicity (nuclear intensity (NI), nuclear area (NA), intracellular calcium ($[Ca^{2+}]i$), mitochondrial membrane potential (MMP), plasma membrane permeability (PMP) and cell

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number (CN)). HCA therefore provides high sensitivity (predictive detection of known cytotoxic agents) and high specificity (predictive correlation with human toxicity) compared to conventional cytotoxicity end-point assays [11]. By combining flow cytometry, intracellular fluorescence probes and image analysis of multiple parameters, extensive data is provided to examine sub-lethal effects, which can relate to *in vivo* toxicological outcomes.

HCA was also used here to investigate the potential of pDMAEMA to induce phospholipidosis. Drug-induced phospholipidosis is a lipid storage disorder that results in the accumulation of phospholipids in lysosomes in tissues throughout the body due to inhibition of phospholipid breakdown [17]. Evidence of drug-induced phospholipidosis in pre-clinical studies can lead to delays or removal of drugs from development [18]. Despite this, there are over 50 marketed pharmaceuticals that are known to induce phospholipidosis [18]. Many of these molecules are cationic amphiphilic drugs, which are generally characterised by a structure that is comprised of a hydrophobic region and a hydrophilic side chain with a charged amine group [19]. As pDMAEMA has a similar structure with charged amine groups, it is possible that it may also induce phospholipidosis.

Due to the variation in pDMAEMA cytotoxicity between red blood cells and human cell lines [3], it is possible that the range of different cell types in intact tissue may afford differential protection. In addition, the mucus layer present in many epithelial tissues may reduce toxicity [20]. To investigate this, freshly excised rat ileal and colonic intestinal epithelial tissue was incubated for 60 min with pDMAEMA in Ussing-type chambers. This allowed for a fast and simple assessment of the histological effects of pDMAEMA on whole tissue. Using HCA, we report that high concentrations of a relatively low molecular weight pDMAEMA polymer induced necrosis in U937 cells, while apoptosis was induced in Caco-2 cells. pDMAEMA did not induce phospholipidosis in either cell line. The cytotoxicity induced in U937 cells was seen at lower concentrations than in Caco-2 cells. pDMAEMA induced only minimal histological damage to rat intestinal tissue *in vitro*, and isolated intestinal tissue mucosae was clearly more resilient to the polymer than cell cultures.

2. Materials and methods

2.1. Materials

All cell culture and HCA reagents were obtained from Invitrogen Corporation (CA, USA), unless otherwise stated. Cell lines were obtained from the American Tissue Type Culture Collection (ATCC). Adult male Wistar rats (210–300 g) were obtained from the Biomedical Facility, UCD. All other general chemicals and reagents used were of analytical grade and were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK), unless otherwise stated.

2.2. Synthesis of pDMAEMA

pDMAEMA was synthesized by atom transfer living radical polymerisation (ATRP) in accordance with our previous descriptions [2,3]. The molecular weight and polydispersity index of pDMAEMA were 12.8 kDa and 1.16, respectively, as determined by size exclusion chromatography (SEC-HPLC) [3]. pDMAEMA had high purity and minimal levels of catalyst residue and we used the specific polymer that was described and characterised in [3].

2.3. HCA assay of pDMAEMA against U937 and Caco-2 cells

The HCA cytotoxicity assay was based on the methods of O'Brien et al. [11]. For HCA, either phenol red-free supplemented DMEM or RPMI media was used. Caco-2 epithelial cells (ATCC: HTB-37, passage numbers 65–69) were cultured in DMEM containing GlutaMAX™, supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin (Pen–Strep). U937 monocytes (ATCC: CRL-1593.2, passage numbers 12–14) were cultured using RPMI medium supplemented with FBS, NEAA, Pen–Strep and 1% L-glutamine. All cells were grown in a humidified 37 °C incubator with 5% CO_2 in air. The Caco-2 cells were seeded in 96-well cell culture plates at a density of 500 cells/well and incubated for 24 h at 37 °C with 5% CO_2. After incubation, Caco-2 cell media was replaced with 50 µl fresh media in each well. U937 cells were seeded in 96-well plates by adding 50 µl cells at a density of 5000 cells/well; these were incubated for 60 min to allow cells to settle. 100 µl pDMAEMA-containing solutions were added to all wells (at 1.5 times final concentration) and plates were further incubated for 24, 48 or 72 h. Plate lids were left on during acquisition to prevent evaporation. Media alone was added as untreated controls for the 24 to 72 h incubations. Dye cocktails were made up (Table 1). Positive control treatments were mixed with the dye cocktails (Table 2) and added to selected wells after incubations, 30 min prior to data acquisition.

After 24–72 h incubations with or without pDMAEMA, 50 µl of dye cocktail was added to wells. Plates were then incubated for 30 to 40 min and images were acquired on the In Cell® 1000 High Content Analyzer (GE Healthcare, UK), using a 10× objective. Ten random fields were viewed per well and concentrations of pDMAEMA were repeated in triplicate. Experiments were repeated on at least three separate occasions.

Fluorescence of the dyes were monitored at excitation and emission wavelengths respectively of: (1) 360 nm and 460 nm for Hoechst 33342; (2) 480 nm and 535 nm for Fluo 4-AM; (3) 535 nm and 600 nm for TMRM; and (4) 620 nm and 700 nm for TOTO®-3. Exposure times were varied between experiments to optimise image quality. However, typical exposure and hardware autofocus (HWAF) values were: (1) 100 ms and 11.1 µm for Hoechst 33342; (2) 250 ms and −1.1 µm for Fluo 4-AM; (3) 300 ms and −2.1 µm for TMRM; and (4) 200 ms and −0.6 µm for TOTO®-3. After acquisition of the images, the data was analysed using In Cell® 1000 Workstation software (GE Healthcare, UK) using multi-target analysis. Table 3 summarises the analysis settings with a glossary of In Cell® 1000 analysis terms. For $[Ca^{2+}]i$ and MMP analyses, values were calculated minus background readings.

2.4. HCA phospholipidosis assay of pDMAEMA

Evidence for phospholipidosis includes intracellular accumulation of phospholipids and lamellar bodies in cells. To accurately view lamellar bodies, electron microscopy is normally used [27]. The accumulation of phospholipids also correlates to induction of phospholipidosis as visualised by Nile red [28]. The HCA phospholipidosis assay used was based on the method of Halstead et al. [29]. U937 cells were selected to test for phospholipidosis as they have been found to be sensitive for evaluating this parameter [30]. In addition, lamellar bodies that are associated with phospholipidosis can be found in intestinal epithelial cells [31], so Caco-2 cells were also used. 100 µl of Caco-2 cells were seeded in 96-well cell culture plates at a density of 5×10^4 cells/ml. Plates were incubated at 37 °C in 5% CO_2 for 24 h. After incubation, Caco-2 cell media was replaced with 100 µl fresh media. For U937 cells, wells were seeded with 100 µl of

Table 1
Dye cocktails for HCA assay.

Dye	Volume	[Stock]
Hoechst 33342	32 µl	1 mM, in dH_20
Fluo 4-AM	40 µl	1 mM, in DMSO
TOTO®-3 iodide (642/660)	40 µl	1 mM, in DMSO
TMRM	8 µl	100 µM in dH_20, from 100 mM stock (DMSO)

TMRM: tetramethyl rhodamine methyl ester; TOTO®-3: TOTO®-3 iodide (642/660); total volume = 10 ml.