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Detection of Nanocarrier Potentiation on Drug Induced Phospholipidosis in Cultured Cells and Primary Hepatocyte Spheroids by High Content Imaging and Analysis

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Abstract

Considerable effort has been made to develop nanocarriers for controlled drug delivery over the last decade, while it remains unclear how the strength of adverse drug effect will be altered when a drug is loaded on the nanocarrier. Drug-induced phospholipidosis (DIP) is characterized with excessive accumulation of phospholipids in cells and is common for cationic amphiphilic drugs (CAD). Previously, we have reported that PEGylated graphene oxide (PEG-GO) loaded with several CAD can potentiate DIP. In current study, we extended our study on newly identified phospholipidosis (PLD) inducers that had been identified from the Library of Pharmacologically Active Compounds (LOPAC), to investigate if PEO-GO loaded with these CAD can alter DIP. Twenty-two CAD were respectively loaded on PEG-GO and incubated with RAW264.7, a macrophage cell line. The results showed that when a CAD was loaded on PEG-GO, its strength of PLD induction can be enhanced, unchanged or attenuated. PEG-GO loaded with Ifenprodil exhibited the highest PEG-GO potentiation effect compared to Ifenprodil treatment alone in RAW264.7 cells, and this effect was confirmed in human hepatocellular carcinoma HepG2, another cell line model for PLD induction. Primary hepatocyte culture and spheroids mimicking in vivo conditions were used to further validate nanocarrier potentiation on DIP by Ifenprodil. Stronger phospholipid accumulation was found in PEG-GO/Ifenprodil treated hepatocytes or

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spheroids than Ifenprodil treatment alone. Therefore, evidences were provided by us that nanocarriers may increase the adverse drug effects and guidance by regulatory agencies need to be drafted for the safe use of nanotechnology in drug delivery.

Keywords

phospholipidosis; nanocarrier; primary hepatocyte; spheroid; high content screening

1. Introduction

In the past several decades, a numerous of small molecule drugs have been developed by pharmaceutical companies and approved by the U.S. Food and Drug Administration (U.S. FDA) and other regulatory agencies around the world. However, many drug candidates have failed in the clinical trials, during which hepatotoxicity was the common reason for their discontinuance in drug development. There are many types of drug-induced hepatotoxicity including necrosis, cirrhosis, and cholestasis, while debate remains open regarding to the relationship between phospholipidosis (PLD) and hepatotoxicity. PLD is a condition of excessive phospholipid accumulation in the lysosomes of cells, shown as single or multi-center lamellar bodies observed under the transmission electron microscope (Anderson and Borlak, 2006). Lamellar bodies as the hallmark of PLD were mainly found in the alveolar macrophages or hepatocytes treated with cationic amphiphilic drugs (CAD) (Pirovino et al., 1988; Reasor et al., 1988). Particularly, cationic amphiphilic drugs (CAD) containing hydrophilic side chains and hydrophobic fatty ring structures allow them to penetrate easily into the cell membrane, being protonated and retained instead of metabolized in the lysosomes (Halliwell, 1997). At least fifty CAD are commercially available, including many antifungal drugs (ketoconazole), antidepressants (amitriptyline), antihistamines (chlorpromazine), anticancer drugs (tamoxifen) and antiarrhythmia agent (amiodarone) (Whitehouse et al., 1994; Pospischil and Walther, 2010). Researchers have proposed several mechanisms that these CAD may either block phospholipase activity directly, or form complex with phospholipid preventing its access to the digestive enzyme (Shayman, 2009; Shayman and Abe, 2013). Although the relationship between drug induced PLD (DIP) and drug toxicity is not clear, the occurrence of PLD suggests the simultaneous accumulation of CAD in the lysosomes (Funk and Krise, 2012). Therefore, DIP has become a concern during drug research and development that is currently regulated by the U.S. FDA.

Many efforts have been made to develop liposomes, micelles, nanoemulsions, polymeric nanoparticles and even carbon/metal based nanomaterials for controlled and targeted drug delivery (Webster et al., 2013; Al et al., 2017). Nanocarriers have many advantages due to their unique physicochemical properties such as small sizes for efficient cellular uptake and large areas for surface modification and high capacity of drug loading (Kingsley et al., 2006; Suri et al., 2007). However, research in drug safety and occupational health strongly suggested that some nanocarriers were mainly distributed and accumulated in the liver after exposure, indicating their potential adverse effects when used for a long period of time (Kermanizadeh et al., 2014; Sonmez et al., 2016; Jia et al., 2017). With the advances made in nanotechnology, inherent toxicity of nanocarriers can be minimized, and nanocarriers for

drug delivery will be the trend of the future in drug development. However, very few researchers have focused on the possibility of nanocarrier potentiation on the adverse effects of small molecule drugs.

Currently, the U.S. FDA has approved total 361 small molecule drugs during the fifteen-year period of 2000-2015 (Lin et al., 2017). Some of these drugs can be loaded into nanocarriers or assembled into nanohybrids, aiming to reduce their doses and presumably the drug toxicity (Vibe et al., 2015). There are 190 active compounds available in the FDA database, indicating the important role of DIP in the regulatory point of view (Kruhlak et al., 2008). The U.S. FDA has also set up a PLD working group and the critical path initiative funding to draft the guidance for DIP testing and provide a tiered approach to risk assessment of DIP since 2004 (Chatman et al., 2009). In addition, Shahane *et al.* identified twenty-two novel CAD capable of PLD induction by screening 1280 compounds from Library of Pharmacologically Active Compounds (LOPAC) using a cell-based assay in high-throughput and high-content screening format (Shahane et al., 2014). Our previous results have demonstrated for the first time that PEGylated graphene oxide (PEG-GO), a model nanocarrier can potentiate DIP induced by amiodarone, tamoxifen, ketoconazole and other CAD (Yang et al., 2016). Here in this study, we extended our study to above-mentioned CAD, aiming to systemically analyze the potential effects of PEG-GO on DIP.

To screen the potentiation effects of nanocarrier PEG-GO on DIP, RAW264.7 cell line (a mouse macrophage cell line) was used because RAW264.7 cells possess strong phagocytic ability and literatures have shown PLD occurrence in foamy macrophages from amiodarone treated animals (Mccloud et al., 1995; Bedrossian et al., 1997). Since liver is the principle site for drug metabolism and toxicity, several investigators selected human HepG2 (a human hepatocellular carcinoma cell line) cells to carry out PLD testing assay *in vitro* (Atienzar et al., 2007; Loa et al., 2016). Although HepG2 is widely used for DIP prediction during drug screening, its metabolism profile is significantly different from *in vivo* scenario. In addition, freshly isolated primary hepatocytes fully expressing functional drug metabolizing enzymes can be a more suitable cell based drug toxicity prediction model than cell lines (Fagerland, 2001; Pradip et al., 2016). However, within the past decades, researchers gradually found that traditional monolayer culture of primary hepatocytes can only maintain their physiological function with phase I and phase II drug metabolic enzyme activities up to one week with morphologically and physiological changes afterwards (Walker et al., 2000; Sakai et al., 2010). In order to retain hepatocyte function for a longer period of time, three dimensional spheroids have been proposed to be more physiologically relevant to *in vivo* condition than the cultured primary hepatocytes (Landry et al., 1985). The characteristics of spheroid include enhanced cell morphology, abundant cytoplasmic organelles, functional tight junctions and gap junctions, and sufficient cell-cell contact, all of which are helpful to maintain hepatocyte differentiation status characterized by albumin and transferrin secretion (Brophy et al., 2009). Importantly, rat primary hepatocyte spheroids have been used to predict the PLD and the results suggested that loratadine metabolites instead of the parent drug caused PLD (Takagi et al., 2016).

To screen the potentiation effects of nanocarrier PEG-GO on DIP, RAW264.7 cell line was used due to its high phagocytic ability and we further confirmed DIP potentiation effects by

PEG-GO in HepG2 cell line. In addition, mouse hepatocytes were freshly isolated to provide a CYP450 based metabolic environment to investigate PEG-GO enhancement on DIP. Finally, three-dimensional hepatocyte-derived spheroids were made as a microtissue model to predict PEG-GO effects on DIP. By using alternative strategies for PLD evaluation, we aim to determine whether nanocarriers loaded with different CAD can further enhance DIP.

2. Material and method

2.1 Reagents

Paraformaldehyde, dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), Amiodarone and compounds listed in Table 1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), penicillin/streptomycin solution (100X) were purchased from HyClone Laboratories (Logan, UT, USA). Hoechst33342, fetal bovine serum (FBS), MEM non-essential amino acids (100X), L-glutamine and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium Salt (NBD-PE) were purchased from Thermo Fisher Scientific (St. Wyman, MA, USA). Insulin, transferrin and dexamethasone were purchased from Solarbio (Beijing, China). Epidermal growth factor was purchased from Pepro Tech (Rocky Hill, NJ, USA). Cell Counting Kit-8 was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Glutaraldehyde was purchased from SPI Supplies Division of Structure Probe (West Chester, PA, USA).

2.2 Synthesis of PEGylated graphene oxide and silica oxide nanoparticles

Graphene oxide (GO) was synthesized by a modified Hummers method (Sun et al., 2008). Briefly, expandable graphite flakes (Graftech, Parma, Ohio, USA) were ground with sodium chloride crystals for two days, and dried in sulfuric acid for 8h. Potassium permanganate was slowly added into the mixture and stirred, after which water was added and maintained above 100°C for 15min. Finally, distilled water and H₂O₂ solution were added to terminate the reaction. For GO functionalization, mPEG-NH₂ was dissolved in deionized water and GO solution was added with N-(3-dimethylaminopropyl-N-ethylcarbodiimide) hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as the catalyst. PEG-GO were purified by filtration through 100kDa molecular weight cut-off Amicon filters to remove free PEG (Yang et al., 2013).

For silica oxide nanoparticle (SiO₂NP) synthesis, sodium hydroxide and cetyltrimethylammonium bromide were completely dissolved in deionized water under vigorous stirring at 80°C. When the solution became clear, tetraethoxysilane was added dropwise. The vigorous stirring was continued for 20h, and milk-white product was collected by centrifugation. In order to remove the surfactant, the products were refluxed in a mixed solution of ethanol and hydrochloric acid at 78°C for 12h, then centrifuged and washed with ethanol. The final products were dried for 12h at 120°C in vacuum (He et al., 2010).

2.3 Microscopic characterization of PEG-GO and SiO₂NP

PEG-GO or SiO₂NP suspension was diluted to 50µg/mL, dropped on a copper grid and dried overnight. PEG-GO morphology was imaged on transmission electron microscope (Tecnai G2 Spirit BioTWIN, FEI, USA). PEG-GO was also deposited on a mica slice and characterized by the atomic force microscope (AFM, Dimension Icon, Bruker, USA). In addition, SiO₂NP were also observed and imaged by dark field microscopy (FENYE, Shanghai, China).

2.4 Cell Culture

RAW264.7 and HepG2 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells were cultured in DMEM supplemented with 10% FBS, 100U/mL penicillin and 100µg/mL streptomycin. Cells were maintained at 37°C under a humidified atmosphere and 5% CO₂.

For primary hepatocyte culture, cell culture plates were coated with rat tail collagen I (Sigma-Aldrich, St. Louis, MO, USA) for 1h and washed with PBS before cell seeding. Cell number and viability of the isolated cells were determined. The cell pellets were re-suspended by gentle pipetting and seeded gently at 2.5×10^5 cells/mL and incubated in a tissue culture incubator with 5% CO₂ at 37°C. Two hours later, non-adherent cells were removed and attached cells were incubated overnight with maintenance medium.

2.5 Hepatocyte isolation

Five-week-old BALB/c male mice were obtained from Cavens laboratory animal company (Changzhou, China), and retained in a specific pathogen-free condition at room temperature with a humidity of 40-60% and were approved by the Animal Ethics Committee of Soochow University. A classic two-step collagenase perfusion technique was used to isolate primary hepatocytes as per other group's instructions (Gonçalves et al., 2007). A peristaltic pump with a constant flow rate at 6mL/min was used for perfusion and the water bath temperature was maintained at 42-44°C so that the perfusion fluid can reach 37°C at the portal vein. Mice were received a peritoneal injection of 2% pentobarbital sodium (0.15mL/each). Peritoneal cavity was open and the venous indwelling needle (24G) was inserted into the hepatic portal vein and perfused with HBSS for blood removal, followed by digestion with collagenase IV (Sigma-Aldrich, St. Louis, MO, USA). Digested liver was further dissected to release hepatocytes and cell viability was determined by trypan blue exclusion assay, and 80% of cell viability of mouse hepatocytes was acceptable. Percoll solution at 1.08mg/mL Percoll density solution (GE Healthcare Life Sciences, Pittsburgh, PA) was used for dead cell removal when cell viability was lower than 80%. Isolated mouse hepatocytes were used for cell culture or for 3D spheroid formation.

2.6 Hepatocyte spheroid formation

Isolated hepatocytes were inoculated at 500 cells/well in each agarose-coated well of 96-well plates. Within three days, cells began to aggregate each other, forming a single spheroid. Spheroids were treated with PLD inducers or PEG-GO/inducers in the presence of fluorescent NBD-PE for 48h to evaluate phospholipid accumulation using high content screening system (see section 2.8) or an Olympus FV1200 confocal laser scanning

microscopy (CLSM) with FV10-ASW 4.2 software (Olympus, Tokyo, Japan). Viability of mouse primary hepatocyte spheroids was determined using the cell counting kit-8 (CCK-8) assay by measuring intracellular formazan content. In details, CCK-8 solution was added into each well and assay plates were incubated in the dark at 37°C for 1-4h. The absorbance of each well was measured at 450nm via using microplate reader (Synergy NEO, Biotek, Winooski, VT, USA).

2.7 Resin embedding of cell spheroid and observation in TEM

Mouse primary hepatocytes were cultured, forming cell spheroids in argarosesolidified 96-well plates, treated with 8μM Ifenprodil or Ifenprodil loaded PEG-GO (25μg/mL) for 48h. Spheroids were harvested and fixed in Trump's fixative (4% formaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.3) for 3h at 4°C, rinsed with 0.1M phosphate buffer and then post-fixed with 1% osmium tetroxide in 0.1M phosphate buffer for 1h. Spheroids were rinsed in distilled water and re-suspended in 3% low melting point agarose at 70°C. Spheroids were quickly centrifuged in 1.5mL Eppendorf tube at 8000rpm for 1min. Agarose containing spheroids were transferred into 5mL tube, dehydrated with 50%, 70%, 95%, 100% ethanol and acetone for 10min each. Spheroids were infiltrated with 50% spur (1:1, spur: acetone) once and 100% spur (Polysciences, Inc., Warrington, PA, USA) three times. Finally, the samples were embedded in fresh spur for polymerization in 70°C oven for 24h. Plastic sections were cut at a 1μm and stained with azure blue and toluidine blue for pre-view. Thin sections of 80nm were cut using an ultramicrotome (Leica EM UC7, Wetzlar, Germany), stained with lead citrate and uranyl acetate, placed on 200 mesh copper grids and imaged by TEM operated at an accelerating voltage of 80kV.

2.8 Phospholipidosis imaging and analysis using high content screening system

Fluorescent-labeled phospholipid probe NBD-PE was used to quantify the phospholipid accumulation (phospholipidosis, PLD) in 96-well plates through RAW264.7, HepG2 and mouse primary hepatocytes using high content screening system. In brief, RAW264.7 at 8,000 cells/well, HepG2 at 20,000 cells/well and mouse primary hepatocytes at 25,000 cells/well were incubated at 37°C and 5% CO₂ in 96-well plates overnight. Individual CAD were mixed with 25μg/mL PEG-GO and vortexed overnight at room temperature for drug loading on the nanocarrier. PEG-GO/CAD were added into the assay plates in the presence of NBD-PE at 37°C for 48h. Assay plates were fixed with 4% paraformaldehyde solution, stained with Hoechst33342 (Thermo Fisher Scientific, Waltham, MA, USA) and rinsed with PBS. The fluorescence images (ex463nm/em536nm for NBD-PE; ex352nm/em461nm for Hoechst33342) were taken by ImageXpress Micro Widefield High Content Screening System (Molecular Devices, Sunnyvale, CA, USA) with a 20X PlanFluor objective, and analyzed using MetaXpress software (Molecular Devices, Sunnyvale, CA, USA).

For PLD quantification, cells were first acquired with four images per well and total five wells per sample were recorded. For each image, total green fluorescence indicating intracellular NBD-PE accumulation were quantified and divided by the total number of cell nuclei stained with Hoechst dye (W2 Stained Integrated Intensity/total cells), which was defined as the mean fluorescence intensity (MFI) for one image. Secondly, four sites of images per well were taken and the average MFI (aMFI) were calculated to represent the

MFI of each cell in one well. Finally, aMFI from five wells were calculated and averaged to obtain the aMFI $\pm$ SE for each treated sample, which was further normalized to non-treated control samples in the histograms indicative the fold increase of PLD, and plotted by simulation curves (Figure S1) in this work.

The quality control for nanocarrier enhanced DIP was based on Amiodarone treatment group. Specifically, 8 μ g/mL Amiodarone or PEG-GO/Amiodarone were incubated with cells for 48h. When the ratio of the fold increase between samples for PEG-GO/Amiodarone and Amiodarone alone was more than 1.5, we considered a positive response of the entire plate that can pass the quality control. In addition, 0.5% DMSO was used as the negative control because all the compounds were all solubilized in DMSO.

2.9 Statistical analysis

Acquired images were analyzed using MetaXpress software in high content imaging system. All data were expressed as mean $\pm$ SE. The differences with statistical significance between mean values were analyzed by one-way ANOVA in combination with Student's t-test (SPSS V16.0, Chicago, Illinois, USA), and a probability value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Physicochemical characterization of PEG-GO

GO was synthesized by a modified Hummers method and functionalized with amine-terminated polyethylene glycol (PEG, molecular weight of 5kDa) according to our previous studies (Yang et al., 2013). PEG modification endowed GO with high water solubility and stability in physiological solutions such as PBS and cell culture medium. The structure formula of PEG-GO was illustrated in Figure 1A. PEG-GO morphology was shown in transmission electron microscope (TEM) and atomic force microscope (AFM), both of which indicated the small sizes of PEG-GO ranging from 10 to 50nm with the mean size of 30nm (Figure 1B).

Phospholipidosis (PLD) is characterized with intracellular phospholipid accumulation when cationic amphiphilic drugs (CAD) are applied, and drug induced PLD (DIP) is a type of adverse drug effect that may be associated with drug toxicity (Laurent et al., 1990; Halliwell, 1997). In our previous work, PEG-GO have been used as the model nanocarrier for drug loading and demonstrated to enhance PLD induced by amiodarone, tamoxifen, ketoconazole and other CAD (Yang et al., 2016). Here we extended our studies on twenty-two PLD inducers previously identified from LOPAC (Shahane et al., 2014), as seen below.

3.2 Detection of PEG-GO potentiation on DIP by high content imaging

Previously, twenty-two PLD inducers with cationic amphiphilic structure were successfully identified from LOPAC, a library containing 1280 pharmacologically active compounds, and these CAD were all listed in Table 1 (Shahane et al., 2014). Based on their work, we aimed to investigate if PEG-GO as the model drug nanocarrier can further enhance PLD induced by these CAD. Individual compound was mixed and loaded on PEG-GO (PEG-GO/CAD).

NBD-PE as the fluorescent phospholipid is the indicator for intracellular phospholipid accumulation, which can be imaged and analyzed by high content screening system (HCS). CAD alone or PEG-GO/CAD at four concentrations were incubated with RAW264.7, a commonly used mouse macrophage cell line used for DIP studies (Lecureux et al., 2011). Fluorescence images indicative of phospholipid accumulation were taken by HCS. As per section 2.8, the mean fluorescence intensity (MFI) per cell from five wells in each treated group was calculated, normalized to non-treated group, and shown as fold increase.

In this work, we have set up a standard method to evaluate the potentiation strength of nanocarrier on drug induced PLD (DIP), taken amiodarone as an example. HCS imaging showed a dose dependent increase of NBD-PE accumulation (green color) in cells treated with amiodarone compared to non-treated control (Figure 2A). Amiodarone loaded on PEG-GO at the same concentration significantly potentiated DIP, compared to amiodarone treatment alone (bottom images vs top images of Figure 2A). To quantitate the potentiation strength of PEG-GO on DIP, simulation curves (concentration of CAD vs fold increase of PLD) were plotted and area under the curve (AUC) for CAD alone or PEG-GO/CAD was calculated respectively, as shown in Figure 2B. Ratio between the AUC for PEG-GO/CAD and AUC for CAD alone was defined as Ratio[AUC], which can represent the potentiation strength of PEG-GO on CAD induced PLD. Ratio[AUC] in Figure 2B was calculated to be 2.008, indicating that amiodarone induced PLD was strongly potentiated when amiodarone was loaded on PEG-GO.

Accordingly, twenty-two novel CAD as PLD inducers identified by Shahane *et al.* were performed in RAW264.7 cells to investigate if their loading on PEG-GO can further potentiate DIP (Shahane et al., 2014). All the results including simulation curves (CAD alone or PEG-GO/CAD) were illustrated in Figure S1, while compound concentrations used for PLD induction, EC₅₀ for PLD by the free drug, Ratio[AUC] and category for each compound were listed in Table 1.

Based on Ratio[AUC] value, namely, index for the potentiation strength of PEG-GO on DIP, these CAD were arranged in order and classified into categories of -3, -2, -1, 0, +1, +2, +3 in Table 1. Among these CAD, Ifenprodil obtained the highest Ratio[AUC] value at 2.129 and was scored in +3 category, indicating a strong PLD induction of PEG-GO/Ifenprodil compared to Ifenprodil treatment alone. HCS images presented in Figure 3A showed a dose-dependent increase in NBD-PE accumulation indicative PLD in RAW264.7 cells by Ifenprodil treatment alone from 2-16μM, while Ifenprodil loaded on PEG-GO further increased PLD (bottom vs top images in Figure 3A, and left panel of Figure 3C for PLD quantification).

Other than Ifenprodil, PLD induction by a series of CAD can be further potentiated when they were loaded on PEG-GO, such as Spiperone, NAN-190, and other eight compounds falling categories between +1 and +3. Four compounds (Mibefradil, Methiothepin, Metergoline and Flupenthixol) can induce PLD as free drugs, but their loading on PEG-GO did not alter their PLD induction strength, and they fell into the category of 0. On the contrary, PEG-GO were capable of weakening PLD when some other CAD were loaded on PEG-GO, for example, Ellipticine, 5-(N, N-hexamethylene) amiloride, GR 127935 and so

on with categories between -1 to -3. In conclusion, results from Table 1 and Figure S1 gave us new insights that CAD loaded on the nanocarrier does not always further increase CAD induced PLD, but PLD effects may be also weakened when the compound is loaded on nanocarrier, which highly depend upon drug individuals.

3.3 PLD potentiation by PEG-GO/Ifenprodil in HepG2 cells

Previous studies indicated that DIP not only occurred in alveolar macrophages, but also in hepatocytes from the liver (Guigui et al., 1988). Since PEG-GO was demonstrated to significantly increase PLD induced by Ifenprodil initially screened in RAW264.7 cell line (Figure 3A), further validation in HepG2, a human hepatocellular carcinoma cell line commonly used for PLD research, was performed (Miyamoto et al., 2009; Water et al., 2011). HepG2 cells were treated with Ifenprodil alone, or PEG-GO/Ifenprodil in the presence of NBD-PE for 48h followed by HCS imaging and analysis. Overall, Ifenprodil loaded on PEG-GO significantly potentiated Ifenprodil induced PLD (bottom vs top images, Figure 3B). Quantitatively, a 4-fold increase in NBD-PE accumulation by 8 μ M Ifenprodil in HepG2 was evident compared to non-treated control. In comparison, PEG-GO/Ifenprodil (8 μ M) group exhibited a 14-fold increase in PLD compared to non-treated control, or an extra 3.5-fold increase of PLD compared to Ifenprodil treatment alone (right panel of Figure 3C). When simulation curves were generated and AUC analyzed, we found a very nice potentiation value of Ratio[AUC] at 2.85, again demonstrating the strong potentiation effect of PEG-GO on Ifenprodil induced PLD in HepG2 cells.

3.4 Confirmation of PEG-GO potentiation on Ifenprodil induced PLD in mouse primary hepatocytes

In order to improve the accuracy of PLD prediction, attention has been paid to the use of primary cells for the detection of PLD during early drug development (Godoy et al., 2013). Freshly isolated hepatocytes can provide a CYP450 based metabolic environment to investigate PEG-GO enhancement on DIP. Therefore, here we focused on the validation work on PEG-GO/Ifenprodil potentiated PLD in mouse primary hepatocytes (MPH).

As per section 2.5, mouse primary hepatocytes were successfully isolated. To verify the PLD response of MPH, the positive control amiodarone at four different concentrations with or without PEG-GO were applied. As seen from Figure S2A, amiodarone treated cells displayed DIP in a dose-dependent manner, indicating the ability of amiodarone to induce PLD in MPH. Quantitative column chart also demonstrated that PEG-GO/Amiodarone further enhanced DIP compared to free drug (Figure S2B). Therefore, MPH can respond to PLD positive control with PEG-GO potentiation effect, indicating that primary hepatocyte model is suitable to predict nanocarrier potentiation on DIP.

For follow up studies, MPH were incubated with Ifenprodil alone or PEG-GO/Ifenprodil to study if Ifenprodil loaded on PEG-GO can further increase PLD induction ability of Ifenprodil in MPH. As observed in Figure 4B, NBD-PE accumulation became evident with the increase of Ifenprodil concentrations. Intracellular fluorescence intensity of the cells significantly increased in PEG-GO/Ifenprodil treated sample compared to Ifenprodil treatment alone (bottom vs top images in Figure 4B). Quantatively, 16 μ M Ifenprodil

introduced a 3-fold increase than non-treated control group, while a further double increase in PLD was found in PEG-GO/Ifenprodil (16 μ M), as shown in Figure 4C. The above data strongly suggested that if Ifenprodil is loaded on the nanocarrier, severe phospholipid accumulation may occur and can be at risk of human health.

3.5 PEG-GO/Ifenprodil potentiated DIP in hepatocyte spheroids

Although previous studies showed that primary hepatocytes may be suitable *in vitro* model for drug toxicity testing or PLD screening (Hewitt et al., 2007; Ferslew and Brouwer, 2014), monolayer MPH were found functional for up to one week and dedifferentiation may occur thereafter (Koide et al., 1989; Ang et al., 2013). In comparison, three-dimensional cell culture system is superior to monolayer cell culture in the gene expression, matrix secretion and functional activities, and is structurally and functionally similar to tissue or organs *in vivo* (Juillerat et al., 1997; Hamilton et al., 2001). In this study, 500 mouse hepatocytes were seeded on the agarose concave in 96-well plates and three-dimensional spheroids were formed after three days, followed by treatment with Ifenprodil or PEG-GO/Ifenprodil for additional 48h in the presence of NBD-PE. Spheroids were transferred into confocal dishes, Z-stack imaged and re-constructed by confocal laser scanning microscopy (CLSM), or processed for TEM observation. As shown in Figure 5A, MPH spheroids showed an increase of PLD in a dose-dependent manner when treated with Ifenprodil alone (top images in Figure 5A), and intracellular accumulation of green fluorescent NBD-PE in each individual cell was evident. In comparison, PEG-GO/Ifenprodil further enhanced PLD compared to Ifenprodil treatment alone (bottom images in Figure 5A).

The hallmark of PLD induced by drug candidates is the finding of lamellar bodies observed by TEM. As a standard detection method, TEM was utilized to observe the intracellular content in MPH spheroids and confirm if PEG-GO as the nanocarrier was able to potentiate DIP when Ifenprodil was loaded on nanocarrier. TEM image of non-treated control spheroids showed a few intact intracellular vacuoles sporadically distributed in the cytoplasm (left images of Figure 5B). Unicentric and multicentric lamellar bodies (black arrows) were found in the cells of spheroids treated with 8 μ M Ifenprodil for 48h (middle images of Figure 5B). In comparison, MPH spheroids treated with PEG-GO/Ifenprodil revealed a significant increase in the amount of lamellar bodies (black arrows) compared to Ifenprodil treatment alone. As the result, both CLSM and TEM demonstrated that PEG-GO significantly potentiated PLD induction ability of Ifenprodil in MPH spheroids.

3.6 Other nanocarriers may potentiate DIP

Since PEG-GO as the nanocarrier can further increase DIP when drugs are loaded, we wonder if other nanocarriers such as silica dioxide nanoparticles (SiO₂NP) can enhance DIP. From TEM image, it was showed that SiO₂NP had the average particle size at 48nm (left image, Figure 6A) with bright dots available in dark field microscopy (right image, Figure 6A). SiO₂NP exhibited high solubility and homogeneity with PDI value of 0.28. Amiodarone at 8 μ M increased NBD-PE accumulation in HepG2 cells, as shown in the top-right image of Figure 6B. In comparison, a significant PLD potentiation by SiO₂NP/Amiodarone was found compared to amiodarone treatment alone (bottom-right image of

Figure 6B). This experiment strongly indicated that other than PEG-GO, alternative nanocarriers such as SiO₂NP may also potentiate DIP.

4. Discussion

Previously, we have established a high content screening (HCS) assay for phospholipidosis (PLD) detection in 96-well plate format and reported that nanocarrier such as PEG-GO may further increase drug induced PLD (DIP) when drugs were loaded on nanocarriers, indicating the concern of nanocarriers for drug delivery that nanocarrier may enhance adverse effects of drugs (Yang et al., 2016). However, nanocarrier potentiated DIP may highly depend on the inherent physicochemical properties of nanocarriers, the hydrophobicity and structure of small molecule drugs, phagocytic activity of cells, and microenvironment around the tissues. Our current concerns focus on the potentiation effect of nanocarrier on PLD induced by the potential druggable compounds in the Library of Pharmacologically Active Compounds.

In this study, we first screened twenty-two compounds identified as CAD by Shahane *et al.* in the presence of PEG-GO, to investigate if nanocarrier potentiated DIP is universally applicable to a wide range of CAD (Shahane et al., 2014). RAW264.7 cell line was used for primary screening, due to its high sensitivity in PLD assay *in vitro* (Lecureux et al., 2011). We showed that amiodarone caused PLD in a dose-dependent manner in RAW264.7 cells, while PEG-GO/Amiodarone significantly exacerbated DIP compared to Amiodarone treatment alone (Figure 2A). To quantitate the potentiation strength of PEG-GO on DIP, area under curve (AUC) of Amiodarone and PEG-GO/Amiodarone were both simulated and ratio between two AUC were calculated. Our results demonstrated the strong potentiation effects of PEG-GO on amiodarone induced PLD with Ratio[AUC] of 2.008 (Figure 2B). Based on the above definition, the effects of PEG-GO on PLD induced by twenty-two novel PLD inducers were tested in RAW264.7 cells, quantified by HCS, and plotted for Ratio[AUC] calculation, as summarized in Table 1 and Figure S1. Strikingly, we found a strong PLD potentiation ability of PEG-GO/Ifenprodil compared to Ifenprodil treatment alone in RAW264.7 cells, and two compounds such as Spiperone and NAN-190 also had moderate DIP enhancement effects. There are several reasons that PEG-GO can synergistically potentiate DIP: (1) PEG-GO possess high drug loading capacity on CAD due to its high surface area and therefore a significant amount of CAD was enriched on the nanocarrier for cellular uptake (Yang et al., 2011; Yang et al., 2016); (2) Nanocarrier loaded with CAD can be readily taken up by cells owing to its two dimensional structure and small sizes, leading to severe DIP (Balandin et al., 2008; Shen et al., 2012); (3) Nanocarrier may have difficulty to escape from lysosomes and CAD may be retained together with nanocarrier and continue to produce its DIP effects (Bussy et al., 2013; Wan et al., 2013). However, not all drugs loaded on PEG-GO can significantly enhance DIP, such as Ellipticine, 5-(N, N-hexamethylene) amiloride, GR 127935, etc. with categories between -1 to -3, likely due to the possibly low loading capacity of PEG-GO on CAD, or drug trapping by PEG-GO without release at low pH in lysosomes (Tietz et al., 1990). In addition, drug absorbed by PEG-GO may prevent the access of drug into the active site of phospholipase or phospholipid, leading to the diminishment of PLD induction effect by CAD (Ikeda et al., 2008). Although our results suggested that DIP can be significantly influenced by

nanocarrier loading, the mechanisms remained unclear. Since PEG-GO exhibited a strong potentiation effect on Ifenprodil induced PLD, we were attempting to confirm this phenomenon in other physiologically relevant *in vitro* models.

Liver is a principal site for drug metabolism and PLD was often reported in liver with foamy vacuoles and lipid droplets found in hepatocytes (Goldman et al., 1985; Asaoka et al., 2013). PEG-GO enhancement on Ifenprodil induced PLD was confirmed in a series of hepatocyte related models, including HepG2 cell line, primary hepatocytes and hepatocyte spheroids. Using HepG2 model, we confirmed that PEG-GO also potentiated Ifenprodil induced PLD (Figure 3). However, unavoidable disadvantages of HepG2 cell lines are evident. For example, the protein profile for drug-metabolizing enzymes, especially the phase I enzymes in HepG2 cell line were significantly different from that in primary hepatocytes, which reduced the PLD prediction accuracy (Wilkening et al., 2003). Therefore, freshly prepared primary hepatocytes may be an alternative *in vitro* model due to their similarity to the *in vivo* condition. If isolated and handled appropriately, primary hepatocytes can retain sustainable metabolic enzyme activities including cytochrome P450 and transporters (Chang and Hughesfulford, 2014). In our study, we successfully isolated and purified mouse primary hepatocytes (MPH) and verified MPH response for DIP using PLD positive control amiodarone. Amiodarone treated cells displayed DIP in a dose-dependent manner and PEG-GO/Amiodarone further enhanced DIP compared to free Amiodarone, indicating that MPH were capable of responding to Amiodarone and PEG-GO/Amiodarone (Figure S2). MPH were further applied with PEG-GO/Ifenprodil or Ifenprodil alone. Our results again indicated that DIP strength in PEG-GO/Ifenprodil treated MPH were significantly stronger than Ifenprodil treatment alone (Figure 4B, 4C).

Because three-dimensional culture has some unique advantages superior to two-dimensional cell culture, we produced hepatocyte spheroids in agarose concave surface in 96-well plates. Cells aggregated together during the first day and formed tight spheroids three days later. CCK-8 assay was used to quantify spheroid viability and we found cell viability did not differ much at different seeding densities (Figure S3). Spheroids were more condense with tight cell-cell contact at the seeding density of 500 cells per spheroid. Spheroids were treated with Ifenprodil or PEG-GO/Ifenprodil and observed by CLSM and TEM. Consistent with previous HepG2 cells result, PEG-GO/Ifenprodil further enhanced intracellular accumulation of green fluorescence (PLD) in a dose-dependent manner compared to Ifenprodil treatment alone. In addition, PEG-GO/Ifenprodil treated spheroids revealed a significant increase in the amount of lamellar bodies. As the result, both CLSM and TEM demonstrated that Ifenprodil loaded on PEG-GO significantly potentiated PLD induction ability of Ifenprodil (Figure 5). Thus, we proposed some novel CAD such as Ifenprodil may not be suitable to be loaded on nanocarriers, which may introduce severe phospholipid accumulation and may be at a risk to human health.

Finally, we examined if other types of nanocarriers can enhance drug induced PLD. Silica dioxide nanoparticle (SiO₂NP) was widely used for drug delivery, owing to its high surface area, minimized toxicity and easy uptake by cells (Chu et al., 2013; Chen et al., 2014). SiO₂NP were synthesized according to our previous work and amiodarone was selected as the positive control to study if amiodarone loaded on SiO₂NP can also potentiate DIP (He et

al., 2010). Amiodarone at 30 μ M was mixed with SiO₂NP at 114 μ g/mL in water for 12h and free drug was removed by ultrafiltration centrifugation and quantified by UV-Vis spectroscopy (UV-3600, Shimadzu). Our results revealed 73.3% loading efficiency of amiodarone on SiO₂NP. HepG2 cells were treated with Amiodarone and SiO₂NP/Amiodarone for 48h and then analyzed by HCS. Our results showed that SiO₂NP/Amiodarone increased DIP compared to Amiodarone alone, indicating that other nanocarriers such as SiO₂NP can also potentiate DIP (Figure 6B).

Overall, based on the above findings for nanocarrier potentiation effects on DIP, we highly suggested that adverse drug effects may be further amplified when drugs are loaded on the nanocarriers. Long-term treatment of drug loaded nanocarriers may be accompanied with the simultaneous accumulation of nanocarriers and drugs, leading to unexpected risks.

5. Conclusion

In summary, we have screened from the twenty-two CAD in RAW264.7 cell *in vitro* model and found that PLD induction ability may be potentiated when some CAD were loaded on PEG-GO, especially for Ifenprodil. We also confirmed that PEG-GO can further enhance Ifenprodil induced PLD in other *in vitro* models such as HepG2 cell line, primary hepatocytes and hepatocyte spheroids. As the result, novel PLD inducers such as Ifenprodil may not be suitable to be loaded on nanocarrier, which may trigger severe phospholipid accumulation and impact human health. While more experiments need to be conducted to further validate our results, nanocarrier potentiation on drug induced phospholipidosis (DIP) may highly depend on the types of the nanocarriers, the physicochemical properties of the drugs, and biological characteristics of cell types. More importantly, our studies have provided evidence regarding to the cautious use of nanocarriers for drug delivery that nanocarriers may significantly increase the adverse effects of clinical drugs or active compounds. Advices or guidance may need to be drafted by the regulatory agencies on investigational new drug applications concerning the use of nanocarriers for drug delivery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AFM	Atomic force microscope
CAD	Cationic amphiphilic drug
CLSM	Confocal laser scanning microscope

DIP	Drug induced phospholipidosis
GO	Graphene oxide
IND	Investigational new drug
LOPAC	Library of Pharmacologically Active Compounds
NBD-PE	N-(7-Nitrobenz-2-Oxa-1, 3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, triethylammonium Salt
NP	Nanoparticle
PEG-GO	PEGylated graphene oxide
PEG-GO/CAD	PEG-GO loaded with CAD
PLD	Phospholipidosis
SiO₂NP	Silica dioxide nanoparticles
TEM	Transmission electron microscope

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Figure 1. Physicochemical characterization of PEG-GO. (A) Structural formula of PEG-GO. (B) TEM (left) and AFM (right) images of PEG-GO.

Figure 2.

High content screening (HCS) imaging and calculation for the potentiation strength of PEG-GO on amiodarone induced phospholipidosis (PLD). RAW264.7 cells were treated with Amiodarone at 0, 2, 4, 8, and 16 μ M or PEG-GO/Amiodarone for 48h in the presence of NBD-PE. (A) Intracellular phospholipid accumulation imaged by HCS. Green color represents NBD-PE accumulation and blue color indicates nuclei staining. (B) Simulation curves for the fold increase of PLD vs amiodarone concentration with or without loading on PEG-GO. Simulated curves were created by Origin software and area under the curve (AUC) was calculated. Potentiation strength of PEG-GO as the nanocarrier on Amiodarone induced PLD was presented as Ratio[AUC], calculated as $AUC_{PEG-GO/Amiodarone}/AUC_{Amiodarone}$.

Figure 3.

Potential of PEG-GO on Ifenprodil induced PLD in RAW264.7 and HepG2 cell lines. (A) Representative images of PLD induction in Raw 264.7 cells by Ifenprodil or PEG-GO/Ifenprodil. RAW264.7 cells were treated with Ifenprodil or PEG-GO/Ifenprodil for 48h in the presence of NBD-PE. Cells were fixed/nuclei stained and images for PLD were recorded by HCS. (B) Representative images of PLD induction in HepG2 cells by Ifenprodil or PEG-GO/Ifenprodil. HepG2 cells were treated same as RAW264.7 cells, and PLD images were recorded by HCS. (C) Quantification of PLD induction for Figure 3A (in RAW264.7) and 3B (in HepG2). PLD induction difference between Ifenprodil and PEG-GO/Ifenprodil was presented as P value (* $0.01 < P < 0.05$, ** $0.01 < P < 0.001$, and *** $P < 0.001$).

Figure 4.

Potentiation of PEG-GO on Ifenprodil induced PLD in mouse primary hepatocytes (MPH).

(A) Representative images for cell viability and culture for isolated hepatocytes. Isolated MPH were obtained and cell viability was evaluated by trypan blue assay. Fresh isolated MPH were seeded and cultured in DMEM containing FBS and other growth factors, showing typical binuclear morphology. (B) Representative images of PLD in MPH by Ifenprodil or PEG-GO/Ifenprodil. MPH were treated with Ifenprodil or PEG-GO/Ifenprodil for 48h in the presence of NBD-PE, and cells were fixed/nuclei stained and images for PLD were recorded by HCS. (B) Quantification of PLD induction for Figure 4B. PLD induction difference between Ifenprodil and PEG-GO/Ifenprodil was presented as P value (* $0.01 < P < 0.05$, ** $0.01 < P < 0.001$, and *** $P < 0.001$).

Figure 5. Potentiation of PEG-GO on Ifenprodil induced PLD in primary hepatocyte spheroids. (A) Representative images of PLD in mouse hepatocyte spheroids by Ifenprodil or PEG-GO/Ifenprodil. Hepatocyte spheroids were treated with Ifenprodil at 0, 2, 4, 8, and 16 μ M or PEG-GO/Ifenprodil for 48h in the presence of NBD-PE, and spheroids were fixed, imaged and re-constructed by confocal laser scanning microscopy (CLSM). (B) PLD in non-treated, Ifenprodil (8 μ M) and PEG-GO/Ifenprodil treated spheroids by TEM. The area from the rectangular marks from images on the top are enlarged and shown on the bottom. Lamellar bodies were indicated with arrows. Strong PLD induction is evident in PEG-GO/Ifenprodil treated spheroids.

Figure 6. Physicochemical characterization of SiO₂ nanoparticles (SiO₂NP) and the potentiation on amiodarone induced PLD. (A) TEM and dark field image of SiO₂; (B) SiO₂NP can potentiate drug induced PLD. HepG2 were treated with 8 μ M Amiodarone or SiO₂NP/Amiodarone for 48h in the presence of NBD-PE, and cells were fixed/nuclei stained and images for PLD were recorded by HCS.

Table 1.

CAD loaded on PEG-GO can alter the strength of DIP.

Name	Concentrations Applied (μ M)	Free Drug EC ₅₀ (μ M)	Ratio [AUC]	Category
Ellipticine	0, 0.5, 1, 2, 4	3.0	0.038	-3
5-(N,N-hexamethylene) amiloride	0, 2, 4, 8, 16	13.6	0.226	-3
GR 127935	0, 0.5, 1, 2, 4	3.2	0.479	-2
BIX 01294	0, 0.25, 0.5, 1, 2	1.6	0.639	-1
NNC 55-0396	0, 1, 2, 4, 8	7.2	0.745	-1
CGS-12066A	0, 2, 4, 8, 16	12.0	0.834	-1
Octoclotheptin	0, 2, 4, 8, 16	14.6	0.839	-1
Mibefradil	0, 1, 2, 4, 8	6.2	0.905	0
Methiothepin	0, 1, 2, 4, 8	7.3	0.971	0
Metergoline	0, 0.5, 1, 2, 4	3.4	0.995	0
cis-(Z)-Flupenthixol	0, 1, 2, 4, 8	7.3	1.087	0
L-765,314	0, 0.5, 1, 2, 4	1.3	1.114	+1
GW405833	0, 2, 4, 8, 16	13.9	1.141	+1
Ebastine	0, 0.25, 0.5, 1, 2	1.6	1.160	+1
BW 723C86	0, 2, 4, 8, 16	13.8	1.162	+1
Propranolol	0, 4, 8, 16, 32	25.8	1.166	+1
Ouabain	0, 0.5, 1, 2, 4	2.1	1.262	+1
(+)-Butaclamol	0, 2, 4, 8, 16	3.1	1.289	+1
Eliprodil	0, 2, 4, 8, 16	1.3	1.311	+1
NAN-190	0, 0.5, 1, 2, 4	2.7	1.406	+1
Spiperone	0, 0.5, 1, 2, 4	2.9	1.564	+2
Ifenprodil	0, 2, 4, 8, 16	12.3	2.129	+3

Twenty-two novel PLD inducers with cationic amphiphilic structure identified from LOPAC (Library of Pharmacologically Active Compounds containing 1280 chemicals) (Shahane et al., 2014) were tested at multiple concentrations in RAW264.7 cells. All these compounds are capable of inducing PLD with EC₅₀ value shown in the table and were comparable with our previous studies (Shahane et al., 2014). RAW264.7 cells were incubated with each CAD or CAD loaded on PEG-GO (PEG-GO/CAD) for 48h in the presence of NBD-PE, fixed and stained with Hoechst33342. Cells in 96-well plates were imaged and average Mean Fluorescence Intensity (aMFI) for each group was quantified by high content imaging and analysis system. aMFI was further compared to control cells (without drug treatment) for fold increase calculation. Fold increases at different concentrations were plotted, simulated and area under the curve (AUC) was calculated. AUC ratio between PEG-GO/CAD and free CAD (Ratio[AUC]) indicated the potentiation strength of PEG-GO on DIP (see Figure S1 for simulation curves and AUC). All twenty-two CAD were classified into the following categories based on Ratio[AUC] values: -3 (0-0.3); -2 (0.3-0.6); -1 (0.6-0.9); 0 (0.9-1.1); +1 (1.1-1.5); +2 (1.5-2); +3 (2-2.5).