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Highlights

- 1 TMPTA induces γH2AX and Caspase-3 in HepG2 cells
- 2 PEG-400 does not prevent *in vitro* genotoxicity effect of TMPTA
- 3 The genotoxic effect of TMPTA is more pronounced when it is diluted in PEG400 than in DMSO

ABSTRACT

Small hydrophobic chemical compounds require solvents to produce suitable solutions for toxicological studies. However, some solvents can modify the biological properties of substances and therefore their toxicity. This specific issue has been raised for PEG-400 as an anti-inflammatory and anti-oxidative compound. Recently, in the context of the REACH Regulation, PEG-400 was used to test the *in vivo* genotoxicity of trimethylolpropane triacrylate (TMPTA) in the comet assay. TMPTA failed to increase DNA damage whereas it induces genotoxicity *in vitro* in DMSO. Therefore, we questioned whether PEG-400 could modify the genotoxicity of TMPTA. The aim of this study was to determine the potential impact of PEG-400 on the *in vitro* genotoxicity of TMPTA, compared to DMSO. TMPTA was dissolved in either PEG-400 or DMSO, and the induction of γH2AX and Caspase-3 was analyzed in HepG2 cells. TMPTA induced γH2AX and Caspase-3 with both PEG-400 and DMSO. However, TMPTA induced effects at 4-fold lower concentrations when PEG-400 is used as the solvent compared to DMSO. While genotoxic effects are observed at much lower concentrations with PEG-400, it does not modify the *in vitro* genotoxicity of TMPTA. However, further *in vitro* studies with small hydrophobic compounds should be done to clarify the effect of PEG-400. Moreover, *in vivo* studies should be performed to confirm that PEG-400 remains suitable for *in vivo* genotoxicity tests.

Keywords

PEG-400, DMSO, TMPTA, genotoxicity, HepG2, γH2AX

1. Introduction

Many chemicals, pesticides, drugs, environmental contaminants are insoluble in water and require the use of a solvent for *in vitro* and *in vivo* toxicology studies. Among them, dimethylsulfoxyde, DMSO, is the solvent the most commonly used, which solubilizes small hydrophobic molecules at high concentrations. Other hydrophobic solvents such as ethanol or acetone are also frequently used depending on the nature of chemicals and their condition of administration (Buggins et al., 2007). Polyethylene glycols (PEGs) are widely used in pharmaceutical and cosmetic formulations because they are not toxic, very well tolerated and easy to use (Fruijtier-Pölloth, 2005; Gullapalli and Mazzitelli, 2015). Although not commonly used as a solvent to assess the toxicity of chemicals, the use of PEG-400 as a solvent has been recently reported in several toxicity studies according to the registration dossier of TMPTA (ECHA, 2018).

However, it is important to ensure that the solvent chosen, in particular for *in vitro* and *in vivo* toxicity studies, does not interfere with the biological activity of the substance studied and also does not protect from its intrinsic toxicity. For example, the cytotoxicity of platinum salts differs in function of solvent used. Indeed, the *in vitro* cytotoxicity of cis-platinum is drastically reduced when it is dissolved in DMSO compared to other solvents such as PEG-400 in different human cell lines (Hall et al., 2014; Marzano et al., 2009). Indeed, DMSO reacted with the platinum complex, and ligand displacement and changes in the structure of the complex inhibited its cytotoxicity and its ability to initiate cell death (Hall et al., 2014).

Similarly, in the context of *in vivo* studies, it is possible that the solvent not only modifies the chemical substance itself (e.g; ionization, structure, etc.) but also modifies the toxicokinetics of the substance and therefore its toxicity. The solvent can also have protective antioxidant and anti-inflammatory properties and interfere with the biological response induced by the substance. For example, polyethylene glycol 400 (PEG-400) is generally listed as an inactive

ingredient in drug formulation but it can influence the toxicokinetics of the substances studied by affecting its gastrointestinal absorption (Ma et al., 2017) or the renal elimination of drugs (Hodoshima et al., 2004). In addition, PEG of low molecular weight has been associated with antioxidant and anti-inflammatory potential (Ackland et al., 2010; Ferrero-Andrés et al., 2020; Juarez-Moreno et al., 2015).

The solvents must not interfere with the biological activity of the compounds in toxicology studies, which the aim is to adequately identify and characterize the effects of chemical substances for human health. Recently, in the context of the REACH Regulation (CEC, 2006), trimethylolpropane triacrylate (TMPTA, CAS 15625-89-5) was tested in different solvents, including two studies performed with PEG-400. Whereas maternal mortality was observed in a prenatal toxicity study at a dose of 500 mg/kg/day of TMPTA diluted in corn oil, no mortality was reported in pregnant rats exposed up to 1000 mg/kg/d TMPTA in PEG-400 for 14 days. TMPTA was also prepared in PEG-400 before being injected intravenously in mice, in the *in vivo* Comet genotoxicity assay performed in liver and bone marrow. No statistical effect was noted in the liver. The statistically significant increase of DNA migration observed in the bone marrow was not dose-related. Moreover, in the negative (solvent) controls, the mean tail intensity in the bone marrow was lower with PEG 400 (0.18) than that reported with the historical controls with carboxymethylcelluloseas solvent (0.24-0.72). Based on the results, the authors considered the substance as devoid of genotoxicity potential *in vivo*, whereas *in vitro* studies indicated that TMPTA induced genotoxicity in lymphoma cells lines and primary human lymphocytes (ANSES, 2019; Kirkland and Fowler, 2018). DMSO was used as a solvent in the *in vitro* studies. However, it cannot be excluded that PEG-400 could mask the genotoxicity of TMPTA *in vivo*, in particular due to its antioxidant and anti-inflammatory potential (Ackland et al., 2010; Juarez-Moreno et al., 2015).

The aim of the present study was to investigate the impact of the solvents, DMSO or PEG-400, on the *in vitro* genotoxicity of TMPTA in human hepatocytes HepG2 cell lines by using the γH2AX test as a marker of DNA damage and the induction of Caspase-3 as a marker of apoptosis.

2. Material and methods

2.1.Chemicals and reagents

Dimethylsulfoxide (DMSO), etoposide (CAS Number 33419-42-0, purity>98%), TMPTA (CAS Number 15625-89-5, purity 81.3%), PEG-400 (CAS Number 25322-68-3, purity >99%) and insulin were purchased from Sigma (St. Quentin-Fallavier, France). Williams' E medium, Fetal Bovine Serum Fetalclone II (FBS), penicillin and streptomycin were purchased from Invitrogen Corporation (Illkirch, France). The primary and secondary antibodies were purchased from Abcam (Cambridge, UK): mouse monoclonal anti γH2AX ser139 (ab26350), rabbit polyclonal anti Caspase-3 (ab13847), goat anti-mouse IgG H&L AlexaFluor 647 (ab150115), goat anti-rabbit IgG H&L 488 (ab150077).

2.2.Cell Culture and treatment

Human hepatoblastoma HepG2 cells (ATCC N° HB-8065) were routinely grown in 75-cm2 culture flasks in αMEM medium supplemented with 10% FBS, 100 U mL-1 penicillin and 100 µg mL-1 streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO2 at 37 °C and the medium was refreshed every 2-3 days during sub-culturing.

HepG2 were seeded at a density of 60,000 cells/cm2 in 96-well plates. Twenty-four hours after seeding, HepG2 cells were treated with different concentrations of PEG-400 for 24 hours in serum-free culture medium.

To investigate the impact of solvent on the cytotoxicity of TMPTA, TMPTA was prepared either in PEG-400 with a final concentration of 0.1 and 1% (based on a preliminary cytotoxicity assay) or in DMSO, with a final concentration of 0.1% (higher concentrations of DMSO are toxic in HepG2 cells, data not shown) in exposure medium.

To distinguish the intrinsic effect of the solvent from the solvation of TMPTA, TMPTA was prepared in PEG-400 at 1% and DMSO at 0.1% was added concomitantly in the culture

medium. In the same way, TMPTA was prepared in DMSO at 0.1% and PEG-400 at 1% was added concomitantly in the culture medium.

2.3.Cellular imaging and High Content Analysis (HCA)

After 24 h treatment, cells were fixed 10 min with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Plates were then incubated in blocking solution (PBS with 1% BSA and 0.05% Tween-20) for 30 min before addition of primary antibodies. All antibodies were prepared in blocking solution. Primary antibodies (1:1000) were incubated 2 hours at room temperature. After three washing steps with PBS, secondary antibodies (1:2000) were incubated for 45 min at room temperature. Cells were then washed three times with PBS + 0.05% Tween-20. Nuclei were stained with 1 µg/mL DAPI in PBS for 5 min for automated cell identification by HCA. Plates were scanned with an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, USA) and analyzed using the Target Activation module of the BioApplication software. For each well, 10 fields (10X objective) were scanned and analyzed for immunofluorescence quantification. Cell numbers were determined by quantification of cell nuclei from DAPI staining and were expressed as percentage of cells compared to control. γH2AX and Caspase-3 were quantified in cell nuclei and cytoplasm, respectively and expressed as fold changes compared to control cells.

2.4.Statistical analysis

One-way analysis of variance (ANOVA) and Dunnet's multiple comparison test were used and P values <0.05 were considered statistically significant (Prism 6.0, GraphPad, La Jolla, CA, USA). Data are presented as mean ± S.E.M of at least 3 independent experiments.

3. Results

3.1.Cytotoxicity of PEG-400 in HepG2

In order to select non-cytotoxic concentrations of PEG-400 to be used as a solvent, its cytotoxicity on HepG2 after 24-h exposure was investigated (Figure 1). PEG-400 induced a concentration-dependent decrease in HepG2 cells numbers starting from 1.25%, and induced γH2AX and Caspase-3 at the cytotoxic concentration of 2.5%. Based on these results PEG-400 was used at final concentrations of 0.1 and 1%.

3.2.Differential cytotoxicity of TMPTA prepared in DMSO or PEG-400

Figure 2 shows the results obtained for the HepG2 cells treated for 24 hrs with increasing concentrations of TMPTA prepared in DMSO at a final concentration of 0.1%, or in PEG-400 at final concentrations of 0.1 and 1%. Whereas TMPTA is cytotoxic in HepG2 cells when it is prepared in PEG-400 (0.1 or 1%) with an 80% decrease in cell numbers at 80 µM, the reduction of cell numbers when TMPTA is prepared in DMSO is only around 50% at 80 µM (Figure 2A).

Moreover, TMPTA in PEG-400 significantly increased γH2AX and Caspase-3 levels at concentrations greater than 40 µM at 24hrs, while statistically significant increases in γH2AX and Caspase-3 were only observed at concentrations greater than 80µM when DMSO was used as a solvent (Figure 2B and 2C). The absence of effect of TMPTA in PEG-400 on Caspase-3 levels at 80 µM can be explained by the considerable cytotoxicity at this concentration. The positive clastogenic compound, etoposide (1 µM), statistically increased γH2AX in HepG2 cells, as expected.

3.3.DMSO did not protect from cytotoxicity induced by TMPTA prepared in PEG-400 in HepG2 cells

In order to determine a potential protective effect of DMSO on the cytotoxicity of TMPTA that could explain the differential cytotoxicity observed above, DMSO was added at final concentration of 0.1 % in cell culture medium concomitantly to TMPTA prepared in PEG-400 (0.1 or 1%).

When DMSO was added at 0.1% in cell medium containing increasing concentrations of TMPTA prepared in PEG-400 at final concentration of 0.1 or 1%, we did not observe a protective effect of DMSO (Figure 3). DMSO at 0.1% did not modify the cytotoxicity of TMPTA prepared in PEG-400 (Figure 3A and 3D for final concentrations of PEG-400 of 0.1 or 1%, respectively), or the induction of γH2AX (Figure 3B and E) and Caspase-3 (Figure 3C and 3F). We only observed an increase of cytotoxicity and γH2AX induction at the higher concentration of TMPTA, 80 µM, when DMSO was added at 0.1% in TMPTA prepared in PEG-400 at final concentration of 0.1% and 1% (Figure 3A, 3B, 3D and 3E).

3.4.PEG-400 did not influence the cytotoxicity induced by TMPTA prepared in DMSO in HepG2 cells

In order to determine if PEG-400 could influence the cytotoxicity of TMPTA, we added PEG-400 at a final concentration of 1% in DMSO-TMPTA treatment medium. Interestingly, we observed that the addition of PEG-400 did not modify the cytotoxicity profile (cell number, γH2AX and caspase-3 activation) of TMPTA prepared in DMSO (Figure 4A, B and C).

1. Discussion

The primary aims of the present study was to determine the impact of PEG-400 on the *in vitro* genotoxic potential of TMPTA. In both solvents, PEG-400 and DMSO, TMPTA generated a similar cytotoxic profile, with a decrease in the number of cells, associated with an induction of γH2AX and caspase-3. However, the effects were more pronounced, and occurred at lower concentrations when TMPTA was dissolved in PEG-400 compared to DMSO. While the induction of these effects was observed at concentrations as low as 40 µM TMPTA in PEG-400, these effects were observed at the highest concentration of 80 µM when DMSO was used as solvent.

The phosphorylated form of histone H2AX at serine 139, γH2AX, is formed in response to double-stranded breaks elicited by DNA damage (Kopp et al., 2019; Rogakou et al., 2000). Induction of γH2AX indicates that TMPTA is a clastogenic compound *in vitro*. These results are in agreement with the results of the clastogenicity tests already carried out on TMPTA (Anses, 2019; Kirkland and Fowler, 2018). Indeed, TMPTA induced chromosome aberrations in human lymphocytes and CHO cells and mutagenic responses likely by a clastogenic mode of action in L5178Y cells (Anses, 2019; Kirkland and Fowler, 2018).

The comparison of results obtained with PEG-400 or DMSO as solvents do not provide evidence that PEG-400 interferes with the *in vitro* genotoxicity of TMPTA. In contrast, the results obtained suggest that DMSO can decrease the cytotoxicity and genotoxicity of TMPA since TMPTA is 4 fold less toxic in DMSO than when it is dissolved in PEG-400. Similar effects have already been observed by previous studies with other compounds, such as cisplatin (Hall et al., 2014; Marzano et al., 2009). The authors suggest that DMSO interacts with cisplatin and modifies its structure and its biological reactivity (Hall et al., 2014; Marzano et al., 2009). In our case, it is difficult to determine if DMSO modifies the structure of TMPTA and thus its biological reactivity.

The differential cytotoxicity observed between PEG-400 and DMSO might be due to the higher stability of TMPTA in PEG-400 that could prevent its degradation, but in absence of further information of its behavior in solution (e.g. by spectral analysis), it is impossible to conclude.

It is also possible that the solvation of TMPTA in PEG-400 improves its bioavailability and its capacity to cross the plasma membrane and therefore to induce more toxicity at equivalent concentration in culture medium. In fact, PEG-400 is more hydrophobic than DMSO and it is expected that TMPTA in PEG-400 could more easily cross the cell membrane (Lentz, 2007; Wojcieszyn et al., 1983).

The results suggest that PEG-400 does not prevent the *in vitro* genotoxicity of TMPTA. However, it is difficult to extrapolate these results to the *in vivo* condition. Indeed, while PEG-400 does not seem to modify the biological reactivity of TMPTA, PEG-400 could modify the toxicokinetics of TMPTA by disturbing the metabolism, distribution and elimination. Indeed, PEG-400 is known to modify the activities of CYP3A4 and Pgp (Buggins et al., 2007; Ma et al., 2017; Pestel et al., 2006). Moreover, PEG-400 has antioxidant and anti-inflammatory properties that cannot be adequately investigated in an *in vitro* study. Further investigation is therefore required *in vivo*, such as genotoxicity studies comparing the use of PEG-400 and DMSO as vehicles to respond to this issue. Before PEG-400 is further used as a solvent in toxicity studies, comparison of toxicity results with solvents other than DMSO, and other test substances besides TMPTA should be conducted to better understand if and how PEG-400 may interfere with the detection of toxic effects.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest in this work.

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AUTHOR CONTRIBUTIONS

LLH, EP and SC conceived the study and designed the experiments; SH performed the experiments; LLH analyzed the data; LLH, EP and SC wrote the manuscript. All authors reviewed the manuscript and finally approved this version to be published.

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Figure legends

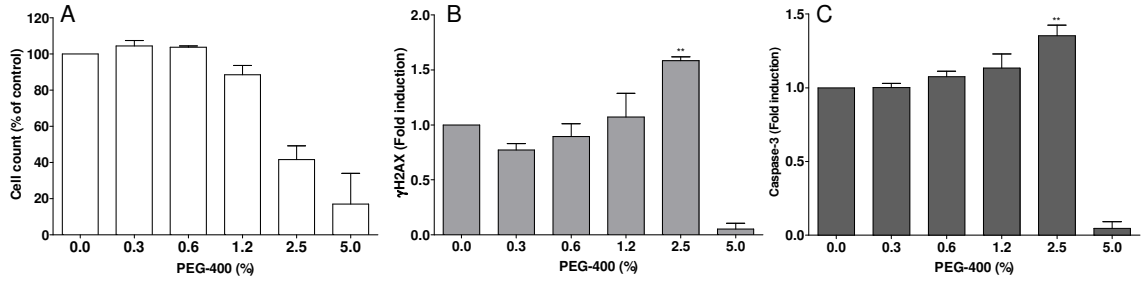
Figure 1: Cytotoxic effects of PEG-400 on human HepG2 cells. Effect of increasing concentrations of PEG-400 on cell numbers (A), induction of γH2AX (B) and induction of Caspase-3 (C) following a 24h treatment. The results shown are the mean (±SEM) of 3 independent experiments. ** $p < 0.01$ one way Anova followed by Dunnet's post-hoc test.

Figure 2: Cytotoxic effects of TMPTA dissolved in PEG-400 or DMSO on human HepG2 cells following a 24h treatment. Effect of increasing concentrations of TMPTA dissolved in PEG-400 (0.1 or 1%) or in DMSO (0.1%) on cell numbers (A), induction of γH2AX (B) and induction of Caspase-3 (C). The results shown are the mean (±SEM) of 3 independent experiments. * $p <0.05$, ** $p < 0.01$, ***$p < 0.001$ one way Anova followed by Dunnet's post-hoc test.

Figure 3: Effect of DMSO on the cytotoxic effects of TMPTA dissolved in PEG-400 on human HepG2 cells following a 24h treatment. Effect of increasing concentration of TMPTA dissolved in PEG-400 (0.1 % (A, B and C) or 1% (D, E, F)) in presence of DMSO (0.1%) on cell numbers (A and D), induction of γH2AX (B and E) and induction of Caspase-3 (C and F). The results shown are the mean (±SEM) of 3 independent experiments. * $p <0.05$, ** $p < 0.01$, ***$p < 0.001$ one way Anova followed by Dunnet's post- hoc test.

Figure 4: Effects of PEG-400 at final concentration of 1% on the cytotoxic effects of TMPTA dissolved in DMSO (at final concentration of 0.1%) on human HepG2 cells following a 24h treatment. Effect of increasing concentrations of TMPTA dissolved in DMSO 0.1% in presence of PEG-400 (1%) on cell numbers (A), on induction of γH2AX (B) and induction of Caspase-3 (C). The results shown are the mean (±SEM) of 3 independent experiments. * $p <0.05$, ** $p < 0.01$, ***$p < 0.001$ one way Anova followed by Dunnet's post-hoc test

Figure 1: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80
60
40
20
0
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0.3
0.6
1.2
2.5
5.0
PEG-400 (%)
B
γH2AX (Fold induction)
2.0
1.5
1.0
0.5
0.0
**
PEG-400 (%)
C
Caspase-3 (Fold induction)
1.5
1.0
0.5
0.0
**
PEG-400 (%)

Figure 2:

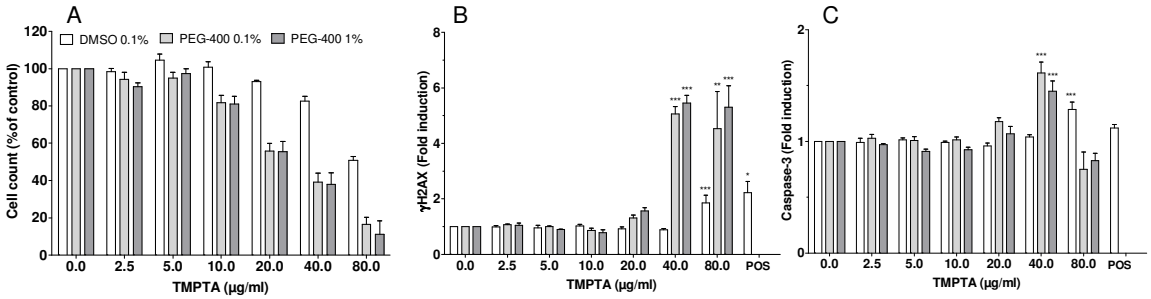

A
DMSO 0.1%
PEG-400 0.1%
PEG-400 1%
Cell count (%of control)
TMPTA (µg/ml)
B
γH2AX (Fold induction)
TMPTA (µg/ml)
C
Caspase-3 (Fold induction)
TMPTA (µg/ml)
0.0
2.5
5.0
10.0
20.0
40.0
80.0
POS

Figure 3:

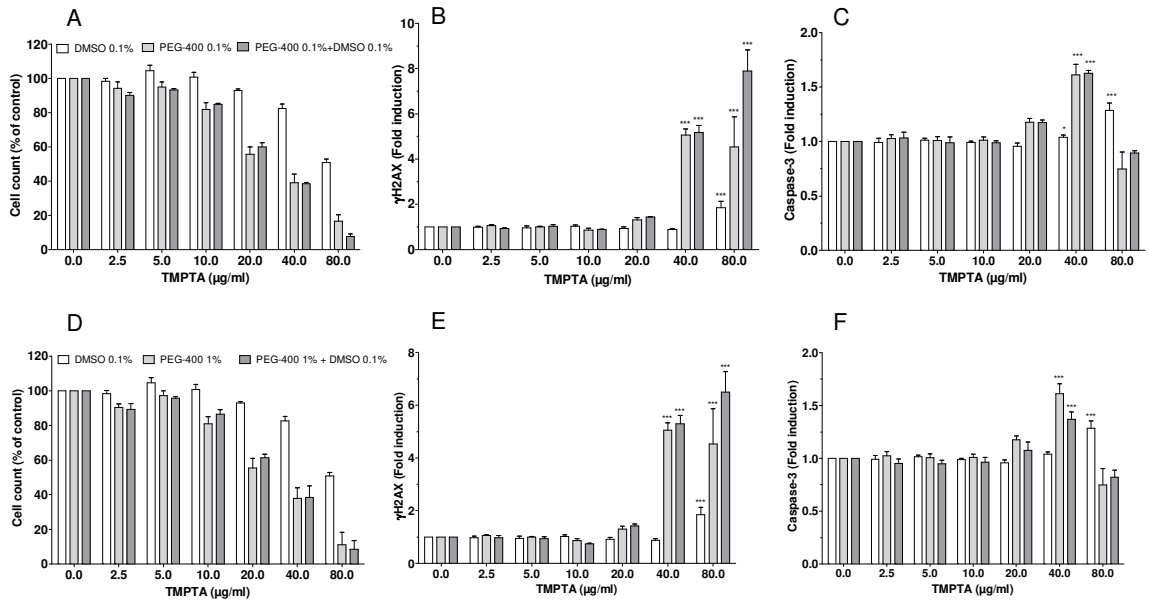
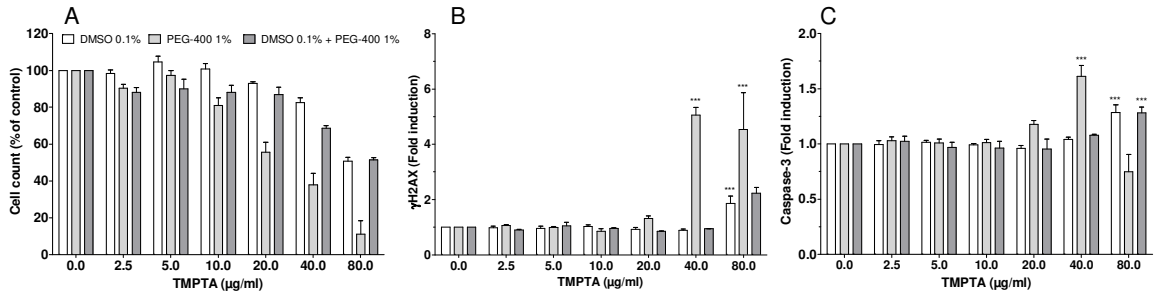

A
DMSO 0.1%
PEG-400 0.1%
PEG-400 0.1%+DMSO 0.1%
Cell count (% of control)
TMPTA (µg/ml)
B
γH2AX (Fold induction)
TMPTA (µg/ml)
C
Caspase-3 (Fold induction)
TMPTA (µg/ml)
D
DMSO 0.1%
PEG-400 1%
PEG-400 1% + DMSO 0.1%
Cell count (% of control)
TMPTA (µg/ml)
E
γH2AX (Fold induction)
TMPTA (µg/ml)
F
Caspase-3 (Fold induction)
TMPTA (µg/ml)

Figure 4: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PEG-400 1%
DMSO 0.1% + PEG-400 1%
Cell count (%of control)
TMPTA (µg/ml)
B
γH2AX (Fold induction)
TMPTA (µg/ml)
C
Caspase-3 (Fold induction)
TMPTA (µg/ml)