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Insights into the effect of polyethylene terephthalate (PET) microplastics on HER2 signaling pathways

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ABSTRACT

Plastic pollution poses a significant threat to both ecosystems and human health, as fragments of microscale size are daily inhaled and ingested. Such tiny specks are defined as microplastics (MPs), and although their presence as environmental contaminants is ubiquitous in the world, their possible effects at biological and physiological levels are still not clear. To explore the potential impacts of MP exposure, we produced and characterized polyethylene terephthalate (PET) micro-fragments, then administered them to living cells. PET is widely employed in the production of plastic bottles, and thus represents a potential source of environmental MPs. However, its potential effects on public health are hardly investigated, as the current bio-medical research on MPs mainly utilizes different models, such as polystyrene particles. This study employed cell viability assays and Western blot analysis to demonstrate cell-dependent and dose-dependent cytotoxic effects of PET MPs, as well as a significant impact on HER-2-driven signaling pathways. Our findings provide insight into the biological effects of MP exposure, particularly for a widely used but poorly investigated material such as PET.

1. Introduction

Plastic debris is a major and growing environmental concern all over the world (da Silva Brito et al., 2022) as almost 400 million tonnes of plastics are produced each year (Lim, 2021), and its environmental contamination is now ubiquitous (Horton et al., 2017). Globally, plastic particles are far-reaching and represent a multifaceted problem (da Silva Brito et al., 2022), especially for their fragmentation into microplastics (MPs). MPs are defined as solid polymer particles with a size <5 mm (Thompson et al., 2004; Toussaint et al., 2019), and represent a greater risk to organism health due to their increased likelihood of uptake, increased surface area and a greater number of particles per unit of bulk mass (Horton et al., 2017; Jeong et al., 2016a; Lee et al., 2013). It is known that the possible adverse effects of MPs are mainly due to the intrinsic properties of the fragments (e.g., size, hydrophobicity, and chemical composition), and the possible presence of co-contaminant, i.e., (Campanale et al., 2020). Such physical and chemical effects determine the biological response of the exposed organisms, including humans, thus posing manifold risks. For these reasons, great efforts are being done to assess human exposure to MPs and evaluate the potential consequences on public health. As an instance, the primary pathways of human exposure to MPs have been identified as gastrointestinal ingestion, pulmonary inhalation, and dermal infiltration (Yuan et al., 2022). Ingested MPs mainly accumulate in the gastrointestinal tract and, if smaller than 2 μm, may cross the gastrointestinal epithelium, thus leading to systemic exposure (Campanale et al., 2020). Then, translocation to the liver, spleen, and other secondary target organs may occur, and cause an inflammatory response in the surrounding tissues (Merkley et al., 2022; Weber et al., 2022; Yuan et al., 2022). In vitro and in vivo experiments revealed also that MPs affect oxidative stress, energy and lipid metabolism, and cause neurotoxicity (Barboza et al., 2018; Deng et al., 2017). The chemical composition of the fragments is one of the main factors determining the possible adverse effects of MPs. In this regard, polyethylene terephthalate (PET) is among the most employed polymers involved in plastic production. PET is primarily used as a packaging material, especially for drinking water and beverage. Indeed, it has low production costs, light weight, and is considered safe. However, albeit PET shows resistance to weathering, fragmentation

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processes occur upon photooxidation and hydrolysis, especially due to pH variation that may lead to chemical leach from their surface (Issac and Kandasubramanian, 2021). In this work, we collected and characterized PET fragments from commercially available plastic bottles and evaluated their potential toxicity effects in vitro. We studied the impact of MPs exposure on human embryonic kidney-293 (HEK-293) cells and human immortalized keratinocytes (HaCaT), intended as model systems of healthy tissues, and on two breast cancer (BC) cell lines (BT-474 and SK-BR-3). Indeed, despite BC is the most diagnosed cancer in females and the leading cause of cancer death, the relationship between exposure to MPs and BC has never been considered so far. Moreover, in very recent reports, scientists pointed out that MPs can induce resistance to therapeutics in human epidermal growth factor receptor 2 (HER2)-positive cancers (Kim et al., 2022). HER2 is a tyrosine kinase receptor that, once activated by dimerization, enhances cell survival and proliferation mainly through activation of the MAPK/ERK and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathways.

Our results suggest that cell viability is affected by exposure to PET MPs, mostly in a dose-response way, and dependently on the exposure time and cell type. Furthermore, we found that high concentrations of PET MPs interfered with the HER2-signaling pathways in both BT-474 and SK-BR-3 BC cells, resulting in a downregulation of the phosphorylated HER2, AKT, and ERK effector molecules. These findings may help elucidate the potential consequences of MP accumulation on pathophysiological molecular pathways.

2. Materials and methods

2.1. Preparation and characterization of PET samples

Polyethylene terephthalate (PET) was purchased as commercially bottled water and scraped into powder form to generate microplastic fragments using a hand file tool. Grinding powder was stored in a glass bottle. These PET microparticles were washed once with 70% ethanol and twice with phosphate-buffered saline (PBS) by centrifugation, then suspended in PBS at a concentration of 2000 μg/mL. To obtain smaller particles for cell treatment, PET samples were subsequently sonicated three times for 15 min (Fig. 1a). Before cell treatment, the sizes and shapes of PET particles were estimated by the bright field microscope (Motic, AE2000) and analyzed through Matlab software (MathWorks, Natick, MA). The hydrodynamic diameter and the zeta potential of PET particles were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern, UK) at room temperature.

2.2. Cell culture

Human embryonic kidney-293 (HEK-293), human immortalized keratinocytes (HaCaT), BT-474 (estrogen receptor (ER)+/progesterone receptor (PR)+/HER2+) and SK-BR-3 (ER-/HER2+) breast cancer cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Essential Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Life Technologies, USA). Cells were maintained at 37 °C with 5% CO_2 under a humidified atmosphere.

2.3. Cell viability

Cells were seeded (8000 cells/well) in 96 well plates and stabilized for 24 h in an incubator. All the cell lines were treated with various concentrations of PET samples (from 25 to 400 μg/mL by serially diluting stock sample in growth media) for 72 h. Then the cell viability of HEK-293 and HaCaT cells was measured by XTT [sodium 3′-[1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy6-nitro) benzene sulfonic acid hydrate)] assay. Briefly, 50 μL XTT solution, prepared as indicated in the kit protocol, was added to each well, and cells were incubated at 37 °C for 3 h. After 3 h, the absorbance was measured with a Glomax Discover System (Promega, Madison, WI, USA) at 560 nm. As for BT-474 and SK-BR-3 cell viability, they were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, according to the specific operational details quoted in (Cui et al., 2022).

2.4. Western blot

BT-474 and SK-BR-3 breast cancer cell lines were treated with PET for 72 h. Cells' proteins were then harvested using RIPA buffer (0.1% SDS, 1% NP40, 0.5% CHAPS) supplemented with protease inhibitors aprotinin, sodium orthovanadate, and phenylmethylsulfonyl fluoride

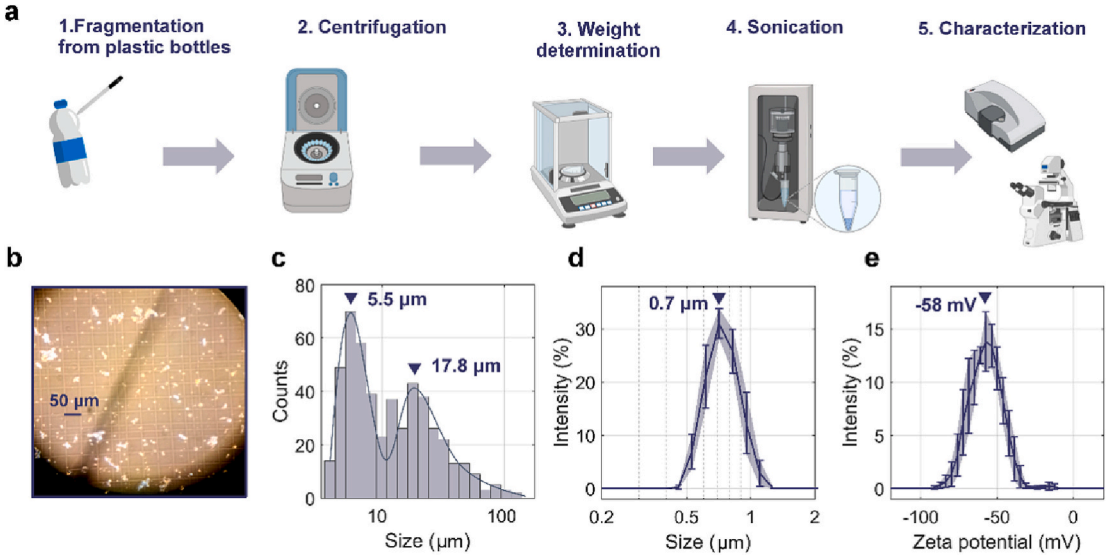

Fig. 1. Production and characterization of PET microplastics (MPs). (a) Schematic workflow to produce PET MPs: fragments from plastic bottles were scraped into powder, centrifuged, and weighted, then sonicated. Finally, the obtained MPs were characterized by optical microscopy and dynamic and electrophoretic light scattering. (b) Representative optical image of PET particles dispersed in PBS on the Hemocytometer (width 50 μm) and (c) corresponding size distribution. (d) Submicron size of PET samples and (e) corresponding zeta potential, measured using dynamic light scattering and electrophoretic light scattering. Error bars represent mean ± S.D. ($n = 3$).

(Sigma-Aldrich, St. Louis, MO). Proteins were quantified by Bradford assay (Bio-Rad) and were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilion P, Millipore) using Criterion™ Blotter (Bio-Rad) and then incubated with primary antibodies (anti-pHER2, anti-pERK, anti-pAKT, anti-β-actin antibodies were from Cell Signaling Technology; 1:1000) at 4 °C overnight. After washing three times, membranes were incubated with secondary antibodies (Sigma-Aldrich, 1: 20000) at room temperature for 1 h. Target protein bands were detected with enhanced chemiluminescence solution LiteAblot PLUS (Euroclone) and images were taken using Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA). Original Western blot images can be found in Fig. S1 in the Supplementary Material. Densitometric analysis was performed through ImageJ software.

2.5. Statistical analysis

Statistical analysis was carried out with GraphPad Prism 9 Software, using one-way ANOVA test. P value <0.05 was considered statistically significant. All data are represented as mean ± standard deviation or standard error of the mean.

3. Results and discussion

3.1. Characterization of PET particles

PET was obtained from commercial products as plastic bottles, and it was mechanically ground into powder, as described in *Materials and methods* section and summarized in Fig. 1a. As a first step of our analysis, we assessed the size and zeta potential of the obtained fragments. Optical microscopy revealed that PET samples were within the micro-range, heterogeneous, with irregular shapes (Fig. 1b). In detail, by analyzing the images, we detected a bimodal distribution, containing small objects (average size 5.5 μm) and larger fragments (average size 17.8 μm) (Fig. 1c). Furthermore, we were able to detect even smaller fragments in PET samples. Indeed, DLS experiments revealed a sub-micron population of PET particles, with an average size of about 744.1 ± 40.74 nm, and a polydispersity index (PdI) equal to 0.593 (Fig. 1d). Accordingly, the zeta-potential measurements revealed that PET MPs were anionic, as they exhibited a negative zeta potential of −57.6 ± 3.5 mV (Fig. 1e).

It is to be noted that plastics are polymers and mostly prepared through the polymerization process, which often requires high temperature, high pressure, and catalyst. Some catalysts may contain negatively charged terminal groups, such as the catalyst with terminal sulfate group used in styrene polymerization to produce polystyrene (PS). These terminal sulfate groups populate the surface of particles, resulting in negative surface charges (Banerjee et al., 2022). Globally, the characterization of PET fragments obtained by plastic bottles revealed the presence of anionic particles with a broadly distributed size distribution that ranged within the sub-micro- and micro-scale. Furthermore, fragments were heterogeneous in shape, as spherical and non-spherical objects were detected by optical microscopy. Due to these features, samples can be regarded as models of environmental systems, in which degradation of macroscopic debris into MPs may lead to highly polydisperse suspensions of smaller fragments with irregular shapes.

3.2. PET MPs impair cell viability

A very recent report points out that almost all we know about the effects of MPs derives from studies that use commercial polystyrene (PS) particles (Ekvall et al., 2022), but little is known about the effects of other polymers, especially PET. In fact, environmental systems are much more heterogeneous than commercially available beads and deserve to be carefully evaluated. To advance understanding of this gap, we explored the cytotoxic effects of mechanically produced PET MPs. For this purpose, four different human cell lines, including HEK-293, HaCaT, BT-474 and SK-BR-3 cells, were treated for 72 h with PET MPs concentrations ranging from 25 μg/mL to 400 μg/mL. SK-BR-3 cells and BT-474 cells represent HER2-overexpressing and luminal B subtypes of breast cancer (BC), respectively. As shown in Fig. 2, PET MPs reduced the viability of HEK-293 and HaCaT cells in a dose-dependent way, although a statistically significant inhibition of about 20–25% was reached only at the highest PET MPs concentrations (200 μg/mL and 400 μg/mL). Of note, BC cell lines seem to be more sensitive to PET MPs exposure than normal cells. A dose-dependent reduction of viability was observed in SK-BR-3 cells, reaching a 40% inhibition at the minimum MP concentration (25 μg/mL). Different sensitivity to MPs exposure of cancer cells with respect to normal cells might be ascribed to their altered mechano-responsiveness (Tijore et al., 2021). Indeed, MPs induce mechanical stress on the cell membrane (Fleury and Baulin, 2021) and tumor cells are much more sensitive than normal cells to mechanical stretching, which can cause apoptosis (mechanoptosis) (Tijore et al., 2022). Nevertheless, the effect of MPs exposure on different BC cells might be influenced by their expression of hormone receptors (Fiocchetti et al., 2021). It is known that MPs, i.e., phthalates, release estrogenic chemicals that can activate the ER signaling pathways and modulate the proliferation of BC cells (Fiocchetti et al., 2021). These estrogen mimetics can potentially counterbalance, at least in part, the cytotoxic effect of PET MPs in ER-positive BT-474 cells and might explain their lower sensitivity to MPs exposure with respect to ER-negative SK-BR-3 cells. However, both two studied BC cell lines are HER2-positive. Thus, we proceeded by studying the effects of PET MPs on the HER2-driven signaling pathways.

3.3. The effects of PET MPs on HER2-driven signaling pathways

To better understand the molecular mechanisms behind the inhibitory effect of PET MPs on cell viability, we performed a Western blot analysis of HER2 signaling pathway in SK-BR-3 and BT-474 cells, treated or not with 200 μg/mL and 400 μg/mL PET MPs for 72 h. We analyzed phosphorylated HER2 (pHER2), corresponding to the activated form of HER2, and phosphorylated ERK (pERK) and AKT (pAKT), as HER2 downstream effector molecules. As shown in Fig. 3, results clearly indicate that PET MPs were responsible for a downregulation of HER2 signaling pathways by reducing phosphorylation of HER2, AKT, and ERK (Fig. 3). These outcomes are in agreement with the current literature, as the downregulation of phosphorylated ERK was also reported for a different type of polymers, i.e. for PS MPs in HUVEC cells (H.-S. Lee et al., 2021).

Finally, considering the possible long-term exposure effects, a 7-day exposure period to PET MPs was established for Western blot analysis on BT-474 cells. SK-BR-3 cells were not included in this long-term analysis, due to the remarkable cytotoxic effects of MPs observed already for 72 h-treatments (Fig. 2). After 7-day exposure, the relative levels of pHER2, pAKT, and pERK were significantly lower in PET MPs treated BT-474 cells compared to the untreated group (Fig. 4b) and were further decreased with respect to the 72 h results (Fig. 3b and d).

4. Conclusions

In conclusion, this work explores some of the biological impacts of a widely employed but often overlooked plastic material, i.e., PET. The studied PET MPs were fragments of irregular shape, with anionic surface charge and size ranging from the sub-micron to the micro-scale. Such morphological heterogeneity resembles the complexity of environmental systems, which usually contain particles with broad size distributions and non-spherical structures. The cytotoxicity of PET MPs varied in a cell-dependent way. In detail, we detected dose-response trends of cell viability, which at the highest MP concentration decreased by about 20% in three out of four cell lines (i.e., HEK-293, HaCaT, and BT-474) but was remarkably reduced (i.e., of 60%) in SK-BR-3 cells. We

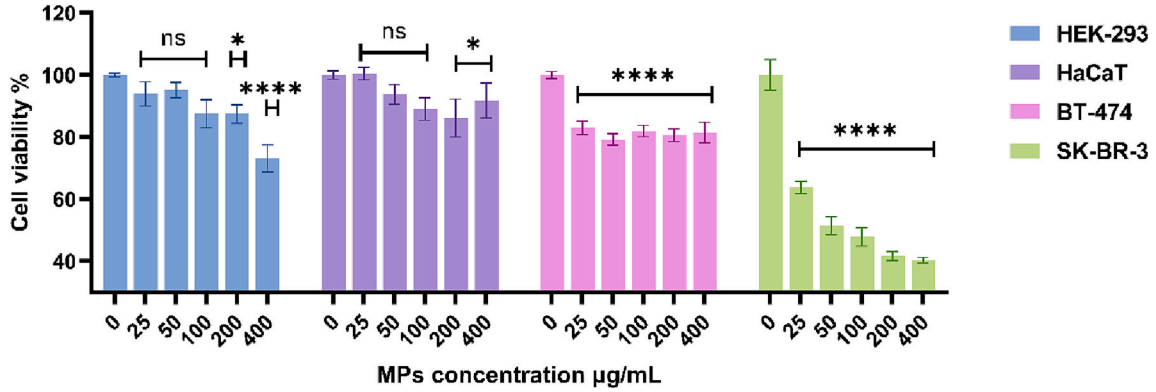

Fig. 2. The effect of PET microplastics (MPs) on cell viability. Cell viability of HEK-293, HaCaT, BT-474 and SK-BR-3 cells upon incubation with PET MPs for 72 h. Results are expressed as the percentage of living cells with respect to untreated cells. Data are reported as mean ± SEM of three separate experiments. * $p < 0.05$; **** $p < 0.0001$; One-way ANOVA followed by Dunnett's test.

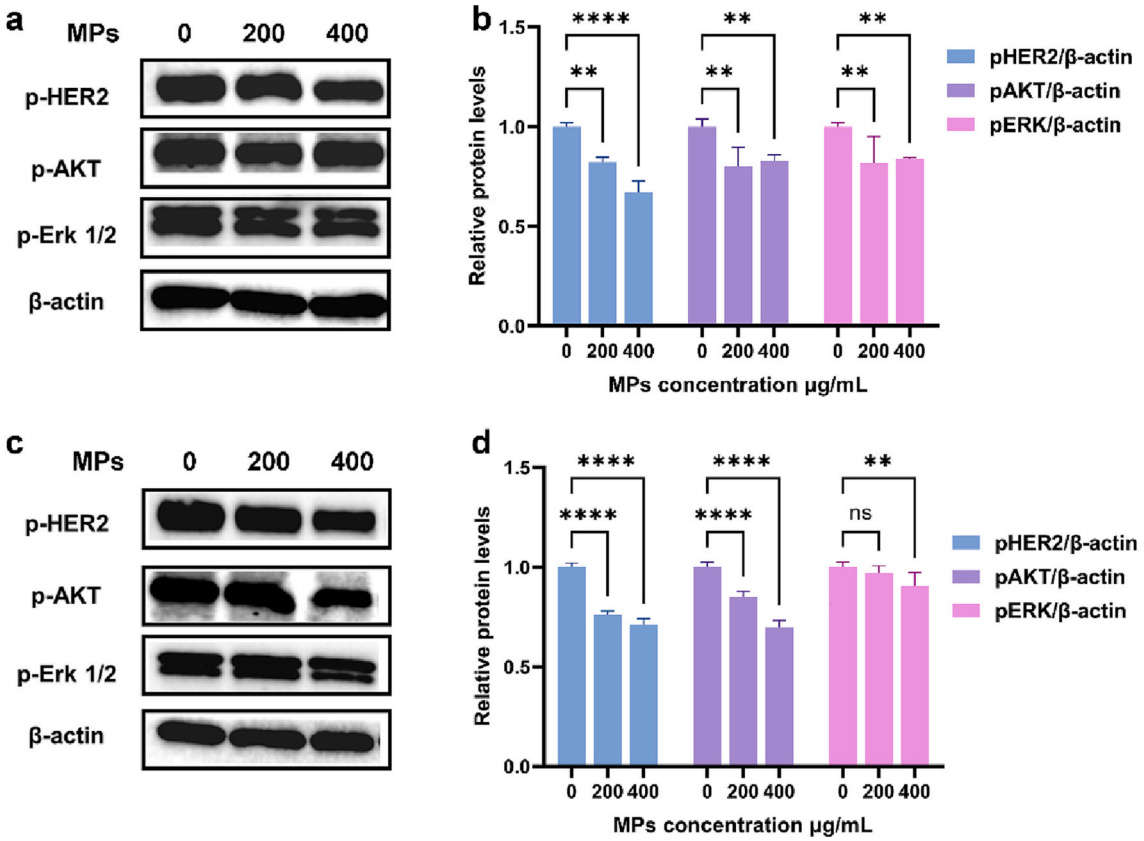

Fig. 3. PET MPs impact on HER2-driven signaling pathway. Representative Western blot analysis of pHER2, pAKT, pERK and beta-actin (loading control) in SK-BR-3 cells (a) and BT-474 cells (c) incubated for 72 h with indicated concentrations of PET MPs. (b, d) Densitometric analysis of pHER-2, pAKT, and pERK in SK-BR-3 cells and BT-474 cells, respectively. Data are represented as mean ± S.D. ($n = 3$). Statistical results of two-way ANOVA test followed by Dunnett's test (** $p \leq 0.01$; **** $p \leq 0.0001$).

hypothesize that a different sensitivity to MP exposure might be determined by a different mechano-responsiveness as well as a differential expression of hormone receptors and HER2 protein in the cell lines considered as experimental models. In this study, we focused on the effect of PET MPs on HER2-driven signaling pathways. Globally, our results suggest that PET MPs can interfere with the HER2-driven signaling pathways that are required for proliferation and survival in BT-474 and SK-BR-3 cells. Although this study gives insight into molecular mechanisms of MPs toxicity, further investigations are required to fully elucidate the difference in response to PET MPs by different cells. Studies focused on mechanoptosis and on the molecular pathways implicated in this calcium-dependent apoptosis may shed light on the effects of PET MPs in both normal and cancer cell lines. Moreover, since it is known that mechanoptosis is triggered by calcium influx and that increased intracellular calcium inhibits HER2 signaling and stimulates HER2 internalization (Jeong et al., 2016b), it may be interesting to correlate MPs exposure with these molecular events in SK-BR-3 cells. Future studies will be aimed at exploring also the role of the bio-nano interactions between MPs and biological fluids (e.g., human plasma, saliva, urine, etc.) on their biological response.

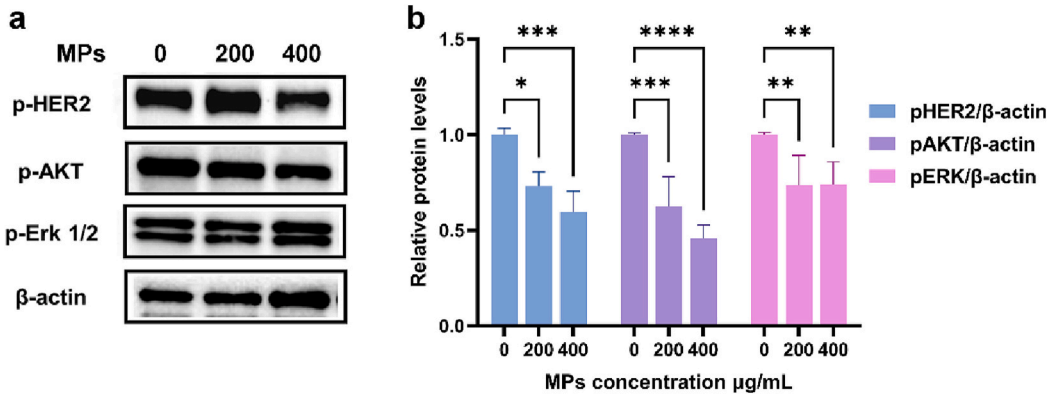

Fig. 4. Long-term effect of PET MPs on HER2-driven signaling pathway. Representative Western blot analysis of pHER2, pAKT, pERK and beta-actin (loading control) in BT-474 cells incubated for 7 days with indicated concentrations of PET MPs (a). Densitometric analysis of pHER-2, pAKT, pERK in BT-474 cells (b). Data are represented as mean ± S.D. (n = 3). Statistical results of two-way ANOVA test followed by Dunnett test (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tiv.2023.105632.

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