

In Vitro Cytotoxicity of Dry Powder Inhaler Medical Devices

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Abstract: Dry powder inhalers (DPIs) are widely accepted inhaled delivery dosage forms that are currently used by a large number of patients for the delivery of medications to treat asthma and chronic obstructive pulmonary disease (COPD). The cytotoxicity test of DPIs is necessary since the mouthpiece of the device is in contact with the user on a daily basis for a long period of time. In the current study, we evaluated the cytotoxicity of the mouthpiece of 4 marketed DPIs: Aerolizer[®] (Novartis), Diskus[®] (GlaxoSmithKline), Elpenhaler[®] (Elpen Pharma) and Turbuhaler[®] (AstraZeneca). The experimental procedure was designed according to the guidelines of the International Organization for Standardization (ISO), the Food and Drug Administration (FDA) and the United States Pharmacopoeia (USP). Tetrazolium salt (MTT) reduction assay and cell morphology observation are recommended by the three organizations and the results were supplemented and compared with the lactate dehydrogenase (LDH) test, an alternative cytotoxicity assay. The experiments were performed in L-929 cells using elution test. None of the tested DPI mouthpieces showed any cytotoxicity effect using the current assays, 48 and 72h after extracts application in cells, compared to negative control. Surprisingly, an increase in cytotoxic response of the negative control was observed at 72h using MTT and LDH tests. All DPI mouthpieces are equivalently safe for long term use. The methods presented in the current study offer an easy and sensitive way for the test of cytotoxicity of a biomaterial and can be applied to other medical devices.

Keywords: Biocompatibility, Cell morphology, In vitro test, MTT assay, Dry Powder Inhalers

1. INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are chronic inflammatory lung diseases. In both cases, inflammation is associated with structural alterations at large and small airway levels, which may relate to phenotypic overlaps occurring in these diseases¹⁻⁶. According to the latest World Health Organization (WHO) estimates, currently 235 million people suffer from asthma and 64 million people have COPD worldwide. During the years 2010-2012 more than 380,000 people died of asthma annually, and even though COPD is less frequent than asthma, it is estimated that, for the same period of time, more than 3 million people died of COPD. WHO predicts that COPD will become the third leading cause of death worldwide by 2030⁴⁻⁸. The treatment of asthma and COPD includes the inhalation of medication to the site of the disease process. Inhalers are the principle vehicles for the effective administration of medication. They allow high lung deposition of the drug and minimize systemic bioavailability, thus reducing possible systemic adverse drug reactions^{5,9-14}. Dry powder inhalers (DPIs), pressurized metered dose inhalers (pMDIs) and nebulizers are the devices used for respiratory drug delivery^{10,11,15,16}.

Inhalers are often being used both by adult and paediatric patients for long term therapy of asthma or COPD, hence the biological safety of the inhaler medical device is of paramount importance to the user. It must be ensured that the mouthpiece in particular, causes no toxicity whatsoever following the usual direct, frequent and daily exposure to long treatment periods. In the USA, a number of tests have been proposed by the Technical Committee of International Organization for Standardization (ISO)^{10,11,17}, by the Food and Drug Administration (FDA)¹⁸, and by the United States Pharmacopoeia (USP)¹⁹⁻²², for screening biocompatibility of materials intended for use in medical devices. The choice of a biomaterial is based on the outcome of *in vitro* and *in vivo* cytotoxicity tests. ISO describes a set of standards, designed as ISO 10993 series, of which Part 5 focuses on the *in vitro*

cytotoxicity tests and Part 6 on the *in vivo* tests for local toxicity. FDA does not recommend specific guidelines, but accepts the methodology described in ISO 10993 as also in the USP¹⁸. In relation to the latter, USP developed very specific guidelines for *in vitro* and *in vivo* cytotoxicity tests as ISO 10993-5 and 10993-6 and it is very precise regarding the use of reference standards^{23,24}. In Europe, the Technical Committee of European Committee for Standardization (CEN) adopts the guidelines of ISO 10993, providing protocols designated as EN/ISO 10993^{24,25}. The European Pharmacopoeia (EP) complies with ISO 10993^{26,27}. The European Medicines Agency (EMA) gives information related to the description of the device's material without specifying cytotoxicity tests^{28,29}. In Japan, although ISO 10993 is often accepted, the "Japanese Guidelines for Basic Biological Tests of Medical Materials and Devices" favor certain methodologies for the evaluation of biocompatibility³⁰.

The ISO, FDA and USP recommend 3 *in vitro* cytotoxicity tests for medical devices: agar diffusion test, elution test and direct contact test. The type and the shape of the material as well as the experimental conditions determine the selection of the suitable test^{18,19,21-23}.

At present, there are many studies regarding the delivery of drug, the mechanical and aerodynamic properties of the marketed dry powder inhalers (DPIs)³¹⁻³³ as well as the patients' preference for a particular inhaler³⁴, however no published data exist for the safety of the material of DPIs. Therefore, the current study has been designed so as to evaluate the *in vitro* cytotoxicity of the mouthpiece of four EU DPIs, commercially available in the European market, namely Aerolizer[®] (Novartis), Diskus[®] (GlaxoSmithKline), Elpenhaler[®] (Elpen Pharma) and Turbuhaler[®] (AstraZeneca). The elution test has been selected, since this procedure is appropriate for high density materials and allows time-, dose- and temperature- response experiments²³. In accordance to the ISO/USP elution test, medium extracts from the mouthpieces were incubated with reference cell line. The cytotoxicity was estimated using microscope and tetrazolium salt (MTT) reduction assay. The results were supplemented and compared with an alternative cytotoxicity assay that determines the levels of lactate dehydrogenase enzyme (LDH).

2. MATERIALS AND METHODS

2.1. Cell Line

ISO, FDA and USP recommend L-929 cell line for *in vitro* biological reactivity tests^{18,19,21-23}. L-929 mouse fibroblast cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured in Eagle's Minimum Essential medium with Earle's BSS and 2mM L-glutamine (EMEM) and supplemented with 1.0mM sodium pyruvate, 0.1mM nonessential amino acids, 1.5g/L sodium bicarbonate and 10% horse serum (HS), as recommended by ATCC. Cells were cultured at 37°C, 5% CO₂ and 100% humidity. When cells reached 80% confluence, they were subcultured into another flask by trypsinization with a trypsin (0.25%)–EDTA (0.03%) solution. Cell growth and confluence were examined under a phase contrast microscope.

2.2. Devices

The mouthpieces of the EU-marketed DPIs: Aerolizer[®] (Novartis), Diskus[®] (GlaxoSmithKline), Elpenhaler[®] (Elpen Pharma) and Turbuhaler[®] (AstraZeneca) were tested in this study prior to their expiration date.

2.3. Controls

USP high density polyethylene R.S. (NC) and USP positive bioreaction R.S. (PC) were used as negative and positive control, respectively^{19,21,22}. Culture medium alone was used as blank control.

2.4. Elution Test

Pieces from the mouthpiece of the devices and the controls were cut at 0.2g/ml of culture medium, and prepared as described by the ISO, FDA and USP guidelines^{18,19,23}. Briefly, the materials were sterilized by exposure to UV radiation for 15min, and were then incubated in culture medium with or without HS for 24h at 37°C and 5% CO₂. L-929 cells were incubated with extracts for up to 72h.

2.5. Tetrazolium Salt (MTT) Reduction Assay

In accordance with the ISO/FDA guidelines^{18,23}, and in order to determine whether the extracts from the devices affect the proliferation of L-929 cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide (MTT) assay was used. Briefly, cells were seeded at a density of 1.5 x

10^4 cells/well in 24-well tissue culture plates. Twenty four hours later, the medium was replaced with 0.5ml of extract medium in each well. The number of cells was estimated 48 and 72h after. MTT stock solution (5mg/ml in PBS) at a volume equal to 1/10 of the medium was added and plates were incubated at 37°C for 2h. The medium was removed, the cells were washed with PBS pH 7.4 and 100 μ l acidified isopropanol (0.33ml HCl in 100ml isopropanol) was added to each well and agitated thoroughly in order to solubilise the dark blue formazan crystals. The samples were transferred to 96-well plates and the optical density was immediately determined by a microplate photometric reader (Tecan, Sunrise, Magellan 2) at a wavelength of 540nm.

2.6. LDH Detection Assay

The amount of LDH released by the cells was determined using an LDH activity kit assay (LDH kit, Roche, Germany) according to the manufacturer's instruction. Briefly, cells were seeded at density of 8×10^3 cells/well in 48-well tissue culture plates. Twenty four hours later, the medium was replaced with 0.25ml of extract medium/well. Forty eight and 72h after incubation, the supernatants were collected and centrifuged at 250 x g for 10min. The supernatants (100 μ l/well) were transferred into 96-well plates and another 100 μ l/well of freshly prepared reaction mixture was added. Following 30min incubation, the optical density of the samples was measured by a microplate photometric reader (Tecan, Sunrise, Magellan 2) at a wavelength of 492nm with reference wavelength 620nm.

2.7. Cell Morphology

Morphological examination of cells is recommended by ISO, FDA and USP^{18,19,21-23}. L-929 cells were incubated with the mouthpiece extracts at 37°C and 5% CO₂ for 48 and 72h. Following incubation, cells were observed under an inverted microscope (Zeiss, Germany). The morphology of cells was examined by two independently reviewers. Pictures of cells were captured at a magnitude of 20X.

2.8. Statistical Analysis

Differences between groups and controls were tested by unpaired *t*-test. Each experiment included at least triplicate measurements for each condition tested. All results are expressed as mean \pm SEM from at least three independent experiments.

3. RESULTS

3.1. Mouthpiece Toxicity Measured by MTT Assay

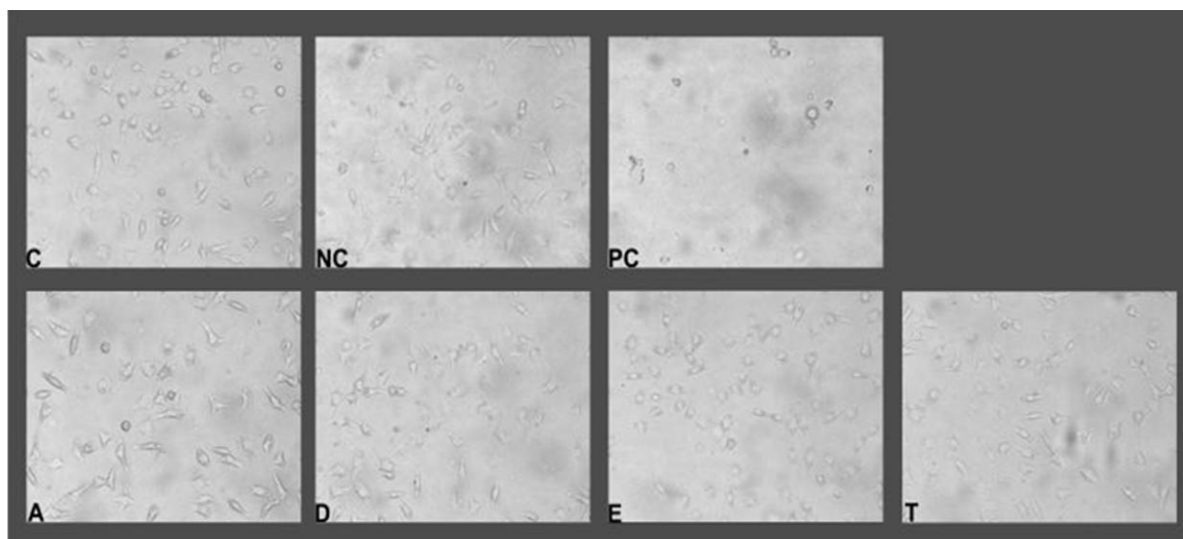


Figure1. The effect of extracts from the tested mouthpieces after **A)** 48h and **B)** 72h incubation on L-929 cells cultured with 10% HS as measured by MTT assay. Also, the effect of extracts **C)** from the tested mouthpiece eafter 48h incubation of L-929 cells in serum-free culture medium was studied. **Neg. Control:** Negative Control, **Pos. Control:** Positive Control. Results are expressed as mean \pm SEM of the number of cells from at least three independent experiments performed in triplicates. Asterisk denotes a statistically significant difference (unpaired *t*-test) compared to negative control. * $p < 0.05$ and *** $p < 0.0001$.

The L-929 cells have been incubated for 48h and 72h with the extracts of the 4 mouthpieces, the positive control, the negative control as well as the blank control. Cell proliferation was estimated by MTT assay. As shown in figure 1 A and B, all 4 mouthpieces did not affect L-929 cell proliferation as compared to the negative control. At both 48h and 72h incubation, the expected degree of cytotoxicity was observed with the positive control material as compared to negative control that is, 62% and 80%, respectively (Fig.1A and Fig.1B, $p < 0.05$ and $p < 0.001$). Although at 48h the negative control and the blank showed equal level of no effect on cell proliferation, at 72h the negative control appear to affect the cells showing a difference of 27% as compared to the blank (Fig. 1B, $p < 0.05$).

The experiment was repeated with extracts using serum-free culture medium. At 48h all mouthpieces tested did not show any cytotoxic effect as compared to the negative and blank control (Fig. 1C), whereas the positive control material was totally toxic (Fig. 1C). L-929 cells could not survive the 72h culture in serum-free medium extract, thus the results could not be evaluated.

3.2. Mouthpiece Toxicity Measured by LDH Release

L-929 cells were incubated with the 4 mouthpiece extracts, the positive control, the negative control and the blank control for 48h and 72h. The cytotoxicity was determined by measuring the LDH levels in the supernatants. As shown in Table 1, at 48h all 4 mouthpieces did not affect L-929 cell proliferation as compared to the negative control. At 72h, all tested mouthpieces appeared to be less toxic than the negative control material used, however the difference was not statistically significant (Table I). At both 48h and 72h incubation, the expected degree of cytotoxicity was observed with the positive control material showing 100% toxicity (Table I). Although at 48h the negative control and the blank showed equal level of no effect on cell proliferation, at 72h the negative control appeared to affect the cells showing an increase to 25% as compared to the blank ($p < 0.05$).

3.3. Effect of Mouthpieces on L-929 Cellular Morphology

The L-929 cells were incubated with the mouthpiece extracts, the positive control, negative control and blank control. The cell morphology was observed microscopically after 48h and 72h. Neither morphological changes nor differences in cell density were found after treatment of cells with the tested mouthpieces compared to negative and blank control (Fig. 2 and 3). No reactivity was observed with the negative control compared to blank at any time point as shown in figures 2 and 3. In contrast, a severe reactivity equal to nearly complete destruction of the cell layer was observed in positive control compared to negative and blank control at 48h and 72h (Fig. 4 and 5).

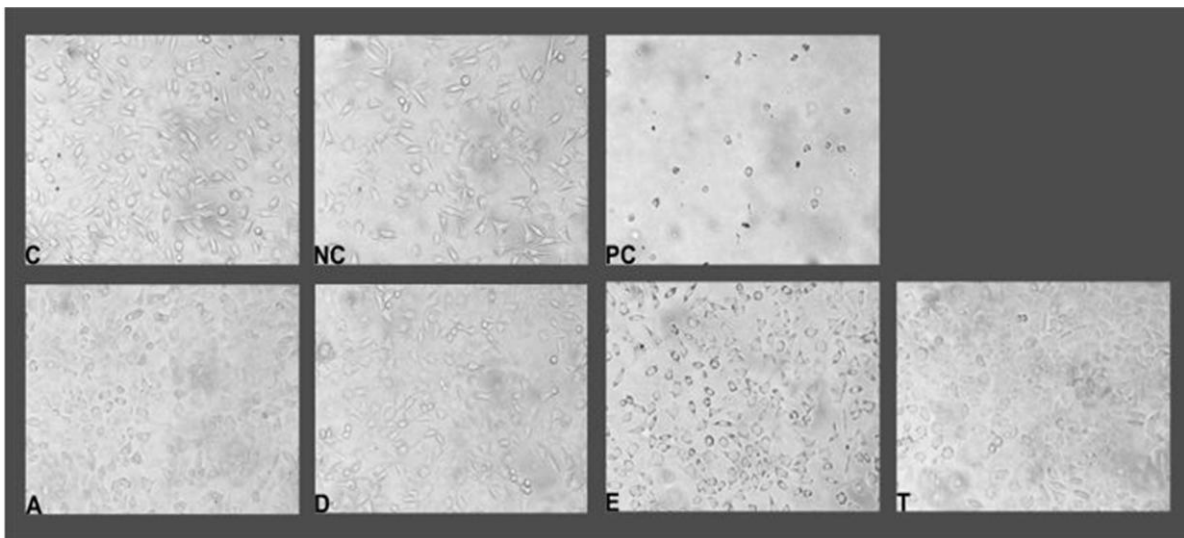


Figure2. The morphology of L-929 cells after 48h treatment with the extracts from the tested DPIs. C: blank control, NC: negative control, PC: positive control, A: Aerolizer[®] (Novartis), D: Diskus[®] (GlaxoSmithKline), E: Ellpenhaler[®] (Elpen Pharma) and T: Turbohaler[®] (AstraZeneca). The pictures are representative of three independent experiments.

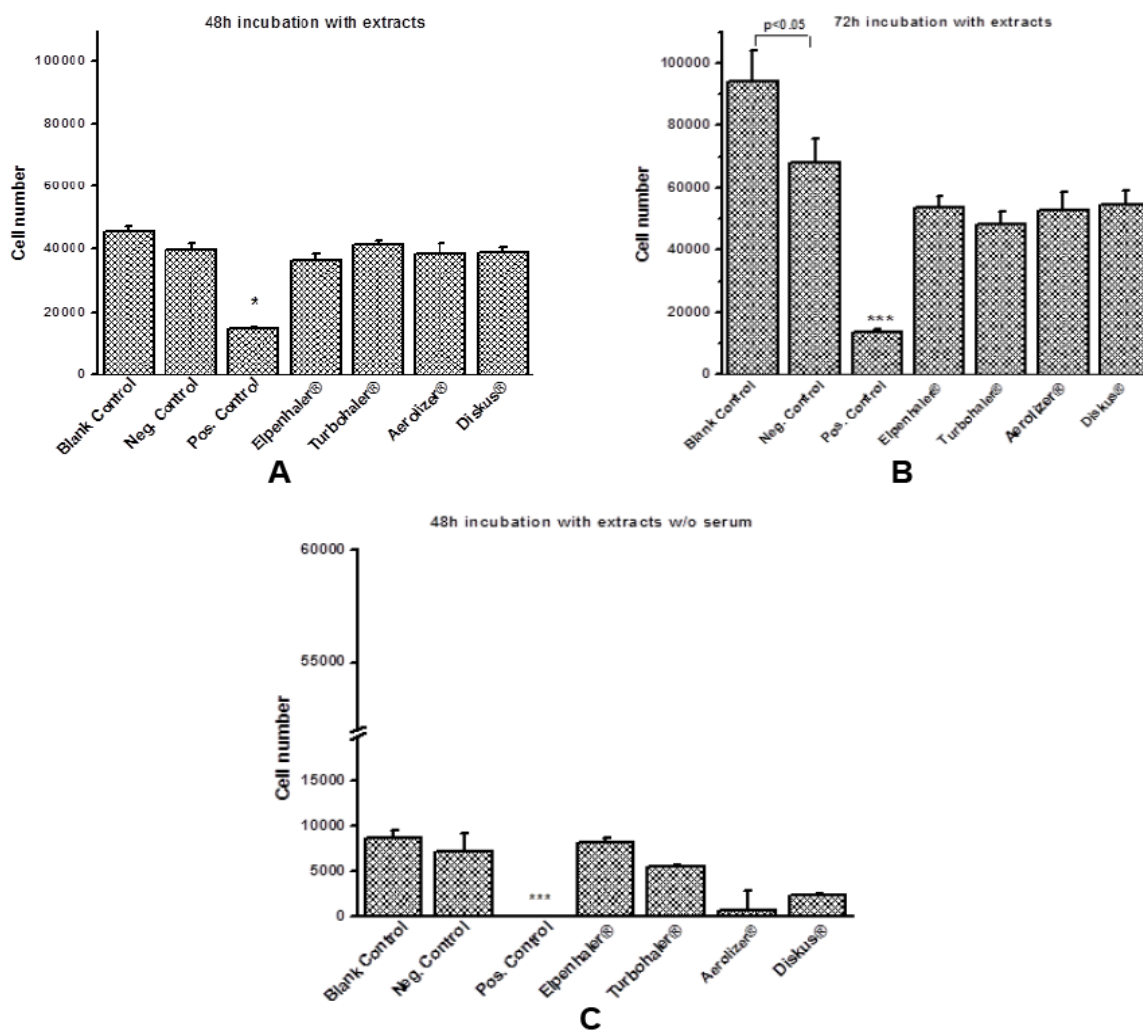


Figure 3. The morphology of L-929 cells after 72h treatment with the extracts from the tested DPIs. **C:** blank control, **NC:** negative control, **PC:** positive control, **A:** Aerolizer® (Novartis), **D:** Diskus® (GlaxoSmithKline), **E:** Ellpenhaler® (Elpen Pharma) and **T:** Turbuhaler® (AstraZeneca). The pictures are representative of three independent experiments.

4. DISCUSSION

Asthma and COPD represent the most common chronic lung diseases worldwide^{1,35-37}. In most cases asthma and COPD are controlled with a long term therapy. The treatment includes inhalation of the medication resulting in a very frequent and long term use of an inhaler device, whether DPI, pMDI or nebulizer, by patients of all age groups including children and the elderly³⁸. Although extensive literature supports the choice of the suitable combination of medical device-medication and provides detailed instructions of use^{15,38-41}, there is no reference regarding the safety of the inhaler device's mouthpiece. In the USA, the FDA does request that the inhaler devices should be tested for *in vitro* cytotoxicity in accordance with the methodology described by the ISO 10993 series and the USP^{19,21,22}. In contrast, in Europe the EMA does not as yet require the submission of *in vitro* cytotoxicity results²⁹. In any case, to date, there is no published data in relation to the effect in human, following long term exposure of the inhalers' mouthpiece.

The current study was designed to evaluate the cytotoxicity of the mouthpiece of four DPIs, commercially available in the European market, namely Aerolizer® (Novartis), Diskus® (GlaxoSmithKline), Elpenhaler® (Elpen Pharma) and Turbuhaler® (AstraZeneca). The *in vitro* cytotoxicity tests chosen, followed the guidelines of the ISO 10993 and the USP, which recommend the use of the MTT assay and cell morphological observation utilizing L-929 mouse fibroblast cells. In addition, in our experiments we employed the LDH cytotoxicity method.

It was found that all 4 mouthpieces showed no cytotoxicity as compared to the negative control, following 48h and 72h incubation with elutes, as shown by the MTT, LDH methods as well as the

morphological examination (Fig. 1, 2, 3 and Table I). No statistically significant difference was observed amongst the 4 mouthpieces tested. When the materials were eluted with serum-free medium, the same no toxic effect was observed (Fig. 1C).

Interestingly, following 72h incubation of the elutions, the negative control appeared to have a statistically significant effect on cells as compared to the blank (Fig. 1B and Table I). Although 48h rather than 72h incubation is proposed by the USP, negative control materials had shown a degree of toxicity in the past⁴². This may explain the choice of blank instead of negative material elution, in similar *in vitro* biocompatibility experiments performed in other studies⁴³. Despite the fact that the negative control material used in our experiments is specified in the USP, we believe that the utilization of a more appropriate negative control needs further investigation.

The MTT assay measures the metabolic activity of cells [28], whereas LDH detects changes in the cell membrane^{21,22,44,45}. Although only the MTT is recommended by the ISO/FDA, in our experiments the LDH assay appeared equivalent. The USP requests only cell morphological examination, but the authors believe that an additional *in vitro* cytotoxicity biochemical test, such as MTT or LDH, should be also employed.

Our *in vitro* experiments were performed on L-929 mouse fibroblast cells, as specified in the ISO, FDA and USP guidelines. These cells appear to be fairly sensitive in cytotoxicity experiments compared to McCoy and HaCaT cells as shown by others⁴². It is advisable that more cell lines should be tested in the future, which may be more representative of the human mucosa.

To our knowledge, this is the first publication dealing with the issue of cytotoxicity of the mouthpiece of commercially available and widely used DPIs. The authors feel that regulatory authorities such as the FDA and the EMA should provide clearer, more precise and additional guidelines in relation to the safety tests required for the mouthpiece in particular, since this is the part that is in constant and direct contact with man. Moreover, further research is required in the area of the choice of the negative controls and the cell line. The *in vitro* methodology may need to be adjusted in order to more accurately simulate the situation in man, avoiding the use of *in vivo* experimentation. Nevertheless, our results lead to the firm conclusion that the DPI mouthpieces tested are equivalently safe for long term use in human.

Conflict of Interest

Dr Stavroula Rozou, Dr George Ismailos and Mr Konstantinos Theofanopoulos are affiliated to ELPEN Pharmaceutical.

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