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Cytotoxicity of folpet fungicide on human bronchial epithelial cells

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ABSTRACT

Folpet, a widely used dicarboximide fungicide, has been detected in the ambient air of several vine-growing regions of France. It is present in particle form in the environment; however, no study exploring its potential health impact on airways and the respiratory system has been published. Here, the biological effect of these particles was investigated *in vitro* on human bronchial epithelial cells (16HBE14o-). To be close to the real-life conditions of exposure, Folpan 80WG®, a commercial form of folpet, was tested.

Folpan 80WG® particles showed dose- and time-dependent cytotoxic effects on 16HBE14o- cells. This effect was compared to that produced by technical-grade folpet and both were found to induce a toxicity with similar IC_{50} values after 24 h of exposure. After 4 h and at least until 48 h of exposure, the IC_{50} values of Folpan 80WG® particles were between 2.4 and 2.8 μg/cm^2. Investigation of the cytotoxicity found that Folpan 80WG® particles at 1.85 μg/cm^2 induced an increase in ROS production from the first hour of exposure. Evidence that oxidative processes occur in folpet-exposed cells was confirmed by the presence of membrane lipid peroxidation. Furthermore, early apoptosis and late apoptosis/necrosis were both present after the first hour of exposure.

These findings indicate that exposure to Folpan 80WG® particles result in a rapid cytotoxic effect on human bronchial epithelial cells *in vitro* that could be in part explained by oxidative stress, characterised by membrane lipid peroxidation and ROS production.

1. Introduction

Folpet is a contact fungicide belonging to the dicarboximide family that has been used for the past 50 years. It is still widely employed on vines and several vegetable crops as a preventive or curative treatment against diverse fungi such as mildew but is also present in certain paints and wood stains. Folpet acts as a multisite inhibitor and is able to inhibit spore germination (Corbett et al., 1984).

In vivo studies have shown a carcinogenic activity (Quest et al., 1993) and a teratogenic effect (Verrett et al., 1969). Studies performed *in vitro* have found folpet to induce cell-cycle deregulation (Perocco et al., 1995) and to have mutagenic (O'Neill et al., 1981) and clastogenic effects on mammalian cells (Sirianni and Huang, 1978). Folpet and its degradation product, thiophosgene, interact with thiol compounds such as glutathione and it has been suggested that this is its main mode of action (Liu and Fishbein, 1967; Janik and Wolf, 1992; Siegel, 1971; Davidek and Seifert, 1975).

In spite of its extensive use, data regarding the health risks associated with prolonged exposure to folpet remains limited. Only skin lesions including hand eczema or dermatitis have been recognized among persons exposed during its manipulation (Peluso et al., 1991). However, during its application, it has been detected in the air at an average concentration of 40 μg/m^3 (Isabelle Baldi, unpublished data). It has also been found in rural and urban air in several vine-growing regions of France where it was found to be the main air-polluting pesticide (Chretien, 2004a,b). Being only slightly water-soluble, folpet is present in particle form in the environment.

In a previous study, we reported that under their typical conditions on use, particles of two commercial forms of folpet have a size that could be inhaled by humans and deposit on the tracheobronchial region and that these particles have a cytotoxic effect on cultured human bronchial epithelial cells (Canal-Raffin et al., 2007). Here, this study aimed to gain a better understanding of the cellular events leading to folpet-mediated toxicity on such cells. To be more close to the real-life conditions of exposure, the

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commercial form of folpet, Folpan 80WG® was used. Its cytotoxicity was compared to technical-grade folpet and reactive oxygen species (ROS) generation, membrane lipid peroxidation and cell death were investigated.

2. Materials and methods

2.1. Chemicals

Eagle's Minimum Essential Medium (EMEM), Roswell Park Memorial Institute (RPMI) 1640 medium without phenol red, Hank's Buffered Salt Solution (HBSS) w/o phenol red w/o Ca^{2+}/Mg^{2+}, L-glutamine, penicillin, streptomycin, fungizone and trypsine/EDTA were obtained from Cambrex (Verviers, Belgium). Foetal calf serum (FCS) was purchased from Eurobio (Courtaboeuf, France) and type I bovine collagen from Becton Dickinson (Le Pont-de-Claix, France). Folpan 80WG® (Makhteshim-Agan), a commercial formulation containing 80% (w/w) folpet as the active ingredient was purchased from Euralis (Bruges, France). Folpet (purity > 99%), dimethyl sulphoxide (DMSO), micronic titanium dioxide, neutral red, bovine serum albumine (BSA, fraction V) and 2,7-dichlorodihydroflurescein-diacetate were obtained from Sigma–Aldrich (Saint Quentin-Fallavier, France). The TBARS assay kit was purchased from Sobioda (Grenoble, France) and the FITC–anti human Annexin V/PI Kit from Beckman-Coulter (Roissy, France).

2.2. Cells

Human bronchial epithelial cells were chosen for this investigation owing to the granulometry of Folpan 80WG® particles (Canal-Raffin et al., 2007) which defines the probability of deposition on the bronchial area. The human bronchial epithelial cells line sub clone 14o- (16HBE14o-) was kindly provided by Dr. D. Gruenert. These are SV40 large T antigen-transformed human bronchial epithelial cells (Gruenert et al., 1988). These have differentiated epithelial morphology and functions such as tight junctions, directional ion transport, a morphological polarity (microvillosity) and cytokeratine production but have lost cilia (Cozens et al., 1994). These cells are routinely employed to investigate the death and/or injury mechanisms on respiratory epithelial cells induced by environmental air contaminants (Baulig et al., 2003; Jiang et al., 2001; Pulfer and Murphy, 2004).

2.3. Cell culture conditions

Cells were cultured as mono-layers in EMEM supplemented with 1% (v/v) penicillin (10^4 U/ml), 1% (v/v) streptomycin (10 mg/ml), 1% (v/v) fungizone (25 μg/ml), 1% (v/v) L-glutamine (200 mM) and 10% (v/v) heat-decomplemented FCS, at 37 °C in a humidified environment containing 5% (v/v) CO_2. To keep the morphological polarity, cells were seeded at 50 000 cells/cm^2 on 75 cm^2 plastic flasks coated with 4 μg/cm^2 collagen. Culture medium was replaced twice a week. Sub-confluent cells were released by incubation with trypsine/EDTA (500 mg/l/200 mg/l) for 10 min at 37 °C.

2.4. Measurement of cytotoxicity

2.4.1. Exposure conditions

Technical-grade folpet is characterised by large crystals difficult to manipulate and that float at the surface of aqueous solutions. For this reason, it was necessary to first dissolve these crystals in DMSO before incubation with cells. In experiments, technical-grade folpet and Folpan 80WG® were dissolved in DMSO. The final concentration of DMSO did not exceed 1% and at this concentration, DMSO alone has no effect on the morphological appearance or viability of 16HBE14o- cells. Titanium dioxide (1 μm) particles with a granulometry similar to that of Folpan 80WG® particles were used as negative control. Stock suspensions of Folpan 80WG® particles and micronic titanium dioxide particles were prepared in serum-free culture medium.

Cells were exposed to concentrations ranging of 0.185–18.5 μg/cm^2 (active ingredient) corresponding to 0.1–100 μM, prepared in serum-free culture medium. Concentrations are expressed in μg/cm^2 because particles rapidly deposed onto cells. Cells were exposed to folpet in DMSO, Folpan 80WG® in DMSO, Folpan 80WG® particles or TiO_2 particles for 24 h. Cells were also exposed to Folpan 80WG® particles for 1, 2, 4, 6, 16, 24 and 48 h.

2.4.2. Neutral red release assay

Cytotoxicity was studied using sub-confluent 16HBE14o- mono-layers cultured on collagen-coated 96 well plates using the neutral red release assay according to Borenfreund and Puerner (1985). The neutral red release assay is an *in vitro* viability test, based on the incorporation of neutral red stain into the lysosomes of viable cells. This test is often used for assessing the cytotoxicity of contaminants such as pesticides (Dierickx, 2004), particles (Kuper-Smith et al., 1994) or gas (Bakand et al., 2006).

After the exposure period, cells were washed with 200 μl/well of 0.9% (w/v) NaCl aqueous solution. Filtered neutral red stock solution (4%, w/v, 0.9% NaCl) was diluted 1:60 in serum-free EMEM and 200 μl were added to each well. After 3 h of incubation, cells were rinsed with 0.5% (v/v) formaldehyde, 1% (w/v) $CaCl_2$ aqueous solution. Cells were then lysed with 200 μl/well of 1% (v/v) acetic acid, 50% (v/v) ethanol aqueous solution. Absorbance at 540 nm (reference 630 nm) was measured using a spectrophotometric microplate reader (Titertek multiskan® plus, Labsystem, France).

Neutral red release assay gave an absorbance signal (arbitrary unit; au), proportional to the number of viable cells within the well. All results were expressed as percentage of non-viable cells as calculated using the formula: $100 - (\text{absorbance}_{540-630\,nm}\ \text{drug-treated sample} \times 100/\text{absorbance}_{540-630\,nm}\ \text{control sample})$.

2.5. Reactive oxygen species (ROS) detection

2.5.1. Toxic exposure

Sub-confluent 16HBE14o- cultured on collagen-coated 60 mm dishes were exposed to Folpan 80WG® particles corresponding to 0, 0.92, 1.85 and 3.70 μg/cm^2 folpet (0, 5, 10 and 20 μM) for 1, 2 and 4 h in RPMI-1640 medium w/o phenol red, w/o FCS, supplemented with 1% L-glutamine (200 mM), 1% penicillin (10^4 U/ml), 1% streptomycin (10 mg/ml) and 1% fungizone (25 μg/ml).

2.5.2. ROS detection

ROS generation was measured by using 2,7-dichlorodihydrofluorescein-diacetate (DCFH-DA) as a probe. Before exposure to Folpan 80WG® particles, 1.2 ml of HBSS w/o phenol red w/o Ca^{2+}/Mg^{2+}, supplemented with 2 mM $CaCl_2$, 1 mM $MgSO_4$ and containing 10 μM DCFH-DA were loaded for 15 min at 37 °C. DCFH-DA is a non-fluorescent molecule that is hydrolyzed by intracellular esterases to non-fluorescent 2,7-dichlorofluorescein (DCFH), which is rapidly oxidized in the presence of peroxides to a highly fluorescent adduct (Crow, 1997). After exposure to Folpan 80WG® particles, cells were scraped in the medium culture using a rubber policeman. Suspended cells were sonicated for 30 s. The fluorescence was measured in the supernatant using a spectrofluorimeter (SFM 25, Kontron instruments, Montigny le Bretonneux, France) with an excitation and emission wavelength of 480 and 520 nm, respectively. Results are reported as fluorescence ratio between exposed cells against unexposed cells.

2.6. Lipid peroxidation assay

2.6.1. Toxic exposure

Sub-confluent 16HBE14o- plated on collagen-coated 60 mm dishes were exposed to Folpan 80WG® particles corresponding to 0, 1.85 and 3.70 μg/cm^2 folpet (0, 10 and 20 μM) for 2, 4 and 24 h in RPMI-1640 medium w/o phenol red (Cambrex), w/o FCS, supplemented with 1% L-glutamine (200 mM), 1% penicillin (10^4 U/ml), 1% streptomycin (10 mg/ml) and 1% fungizone (25 μg/ml).

2.6.2. TBARS test

Lipid peroxidation was evaluated by measuring the fluorescence of 2-thiobarbituric acid reactive substances (TBARS) in both exposed and unexposed cells using the TBARS assay. Briefly, cells were scraped in the medium culture and lysed by sonication for 30 s. Five hundred microlitres of the cell lysate was boiled under acidic conditions (1.875 ml of Working Solution) in the presence of thiobarbituric acid (TBA), at 95 °C for 1 h in a Pyrex® tube. After cooling in an ice bath for at least 10 min, each sample was extracted with 1 ml *n*-butanol followed by vortexing for 3 min and centrifugation at 3000 × *g* for 10 min. The fluorescence intensities in the butanol fraction were measured at excitation and emission wavelengths of 532 and 553 nm, respectively, using a spectrofluorimeter. TBARS values were calculated using the linear regression obtained from the standard (tetraethoxypropane), and normalized to the cell protein content. Protein content of each sample for TBARS experiment was measured by the Bradford method (Bradford, 1976) using BSA as standard and Bio-Rad protein reagent (Bio-Rad, Marnes La coquette, France). Because TBA is reactive with other products of lipid peroxidation besides malondialdehyde (MDA), results are expressed as nmol TBA-reactive substances (TBARS) per mg of protein.

2.7. Apoptosis determination

2.7.1. Exposure conditions

16HBE14o- cells plated on 60 mm collagen-coated plastic dishes at 70–80% confluence were exposed to Folpan 80WG® particles corresponding to 0, 0.89, 1.33, 2.37 and 3.72 μg/cm^2 folpet (0, 6, 9, 16 and 25 μM) for 1 h in EMEM medium supplemented with 5% FCS, 1% L-glutamine (200 mM), 1% penicillin (10^4 U/ml), 1% streptomycin (10 mg/ml) and 1% fungizone (25 μg/ml).

2.7.2. Dual Annexin V/propidium iodide staining

A marker of apoptosis occurring in the early stages is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane (Dong et al., 2005). This alteration was evaluated by flow cytometric analysis after double staining of cells using the fluorescein isothiocyanate (FITC)–anti human Annexin V/PI Kit carried out according to the manufacturer's instructions. This method is frequently used and has been shown as a reliable assay in the quantification of