In vitro toxicological assessment of an organosulfur compound from *Allium* extract: cytotoxicity, mutagenicity and genotoxicity studies

Pilar Mellado-García[1], Sara Maisanaba[1], María Puerto[1], Ana Isabel Prieto[1], Ricard Marcos[2], Silvia Pichardo[1], Ana María Cameán[1]*

[1]Area of Toxicology, Faculty of Pharmacy, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

[2]Group of Mutagenesis, Department of Genetics and Microbiology, Universidad Autónoma of Barcelona, 08193 Cerdanyola del Valles, Barcelona, Spain.

*Corresponding author:

Ana M. Cameán

Area of Toxicology, Faculty of Pharmacy, Universidad de Sevilla, Profesor García González n°2, 41012 Seville. Spain.

E-mail address: camean@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

Garlic (Allium sativum) and onion (Allium cepa) are being used in the food industry as flavoring but also for their antimicrobial activities.These activities are mainly derived from the organosulfur compounds (OSCs). Propyl propane thiosulfinate (PTS) is an OSC with potential use in the active packaging, but its safety should be guaranteed before being commercialized. The aim of this work was to investigate for the first time the cytotoxicity of PTS as well as its in vitro mutagenic/genotoxic potential using the following battery of genotoxicity tests:(1)the bacterial reverse-mutation assay in S. typhimurium (Ames test, OECD 471, 1997);(2) the micronucleus test (MN, OECD 487, 2014);(3) the mouse lymphoma thymidine-kinase assay (MLA, OECD 476, 2015), and (4)the comet assay (standard and modified with restriction enzymes). The results revealed that PTS was not mutagenic neither in the Ames test nor in MLA. However, genotoxic effects were recorded in the MN test on mammalian cells (L5178Y Tk+/-cells) after PTS exposure at the highest concentration tested (17.25 µM) without S9, and also its metabolites (+S9, from 20 µM). Moreover, in the comet assay, PTS induced DNA breaks damage in Caco-2 cells at the highest concentration tested (280 µM) but it did not induce oxidative DNA damage.

Keywords: propyl propane thiosulfinate (PTS), Ames test, mouse lymphoma TK assay; micronucleus test; comet assay.

1. Introduction

Garlic, *Allium sativum* L., is considered one of the twenty most important vegetables with various uses throughout the world, not only for culinary purposes but also as an ingredient of traditional and modern medicine (Martins et al., 2016). In addition, onion*, Allium cepa*, has similar applications (Corzo-Martínez et al., 2007). They both exhibit a peculiar odour, which is associated with the presence of organosulfur compounds (OSCs), which are also responsible of their beneficial properties (Lekshmi et al., 2015). Essential oils (EOs) from garlic and onion have been studied for their use in the food industry (Mnayer et al., 2014). In fact, onion EO has been pointed out as a potential source of natural antimicrobial and antioxidant agents to be applied in food systems, due to its interesting properties (Benkeblia et al., 2004; Ye et al., 2013; Prakash et al., 2015). In this regard, the toxic assessment of extracts from garlic and onion, as well as their components, need to be addressed. The toxic assessment of OSCs is of great interest because their content can vary substantially depending on the different conditions (Benkeblia and Lanzotti, 2007). Previous works from our research group have studied the cytotoxicity and mutagenicity/genotoxicity of several OSCs such propyl propane thiosulfonate (PTSO) (Llana-Ruiz-Cabello et al. 2015a; Mellado-Garcia et al. 2015), dipropyl sulfide (DPS) and dipropyl disulfide (DPDS) (Llana-Ruiz-Cabello et al. 2015b). The obtained results evidenced a lack of toxic effects at the concentration ranges intended to be used for food packaging. Nevertheless other authors have reported both genotoxic (Musk et al., 1997) and antigenotoxic effects (Guyonnet et al., 2000, 2001; Belloir et al., 2006; Arranz et al., 2007; Chiu et al., 2016) for some OSCs. The contradictory results reported for these OSCs (LLana-Ruiz-Cabello et al., 2015c) made necessary to evaluate the toxicological profile of them before their use in food industry, particularly in food contact materials (FCM).

Despite the beneficial effects attributed to *Allium* plants, growing awareness exist on the hazards associated with the use of plants and their extracts as antibiotic and chemical feed additives (Wallace, 2004), as well as actives in food packaging (LLana-Ruiz-Cabello et al., 2015c;

Maisanaba et al., 2016). Among the OSCs, propyl propane thiosulfinate (PTS) is an organosulfur compound obtained by decomposition of initial components present in *Allium* plants, that has been stabilized and characterized by DOMCA Research Center (DMC, Granada, Spain). This compound has previously shown to have beneficial effects on goats as methanogenesis inhibitor in the rumen (Martínez-Fernández et al., 2013, 2015). PTS is considered as a secondary metabolite of garlic, derived of the formation of the sulfenic acids, which nowadays has been studied for evaluate its antimicrobial activity against the faecal microbiota of pigs (Ruiz et al., 2015), or as dose-dependently killer of invasive sporozoites and stimulating of higher spleen cell proliferation in chickens (Kim et al., 2013). Nevertheless, little is known about its safety for human consumption.

According to the Guidelines of the Scientific Committee on Food for Safety Assessment of Substances Used in FCM, information on the genotoxic potential is a key component in the risk assessment of substances used in active packaging (EFSA, 2011; 2016). In the new European Food Safety Authority (EFSA) Scientific Committee recommendations (EFSA, 2016) on genotoxicity testing strategies, a basic battery of two *in vitro t*ests, a bacterial reverse mutation assay (OECD 471, 1997) and an *in vitro* mammalian cell micronucleus test (MN) (OECD 487, 2014) are recommended. The bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test) is a short-term mutation study specifically designed to detect a wide range of chemical substances that can produce genetic damage leading to gene mutation. It is rapid and relatively easy to perform (Mortelmans and Zeiger, 2000). Concerning the MN test using mammalian cells, it is one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage, together with chromosome non-disjunction to be measured reliably (Fenech, 2000; EFSA, 2016). In the case of inconclusive, contradictory or positive results from these two *in vitro* tests, it may be appropriate to conduct further *in vitro* tests to optimize any subsequent *in vivo* testing, or to provide additional useful mechanistic information (EFSA, 2011, 2016). In this sense, the *in vitro*

mammalian cell gene mutation assay (MLA) (OECD 476, 2015) is an assay commonly used, to evaluate the mutagenicity of chemical and physical agents This can detect a wide range of genetic alterations, including both point and chromosomal, gene, base pair substitutions and frame-shift mutations (Wang et al., 2009). Finally, the effect on DNA damage can be studied through the comet assay which is a reproducible and sensitive test for the detection of DNA damage (mainly DNA breaks) in eukaryotic cells. Incorporation of oxidative DNA damage repair enzymes (for example, formamidopyrimidine DNA glycosylase (FPG) and endonuclease III (EndoIII)) in the standard alkaline comet assay procedure allows the detection and measurement of oxidatively DNA damaged (Collins, 2004; Pu et al., 2016).

In the present work, a specifically toxicological approach has been carried out on PTS for the first time. The cytotoxicity of PTS was studied in the Caco-2 cell line by means of three endpoints: total protein content (TP), neutral red uptake (NRU) and MTS tetrazolium salt reduction. Afterwards, an *in vitro* mutagenicity and genotoxicity assessment of PTS has been performed using a prokaryotic system for the Ames test, and two different mammalian cell lines, L5178Y $Tk^{+/-}$ (for MLA and MN tests) and Caco-2 cells (for the standard and enzyme-modified comet assay).

2. Materials and Methods

2.1. Supplies and chemicals

Culture medium, cell culture reagents and foetal bovine serum (FBS) were obtained from BioWhittaker (Spain). PTS (95.5% purity) was kindly supplied by DOMCA S.L. (Granada, Spain). Cyclophosphamide (CP, CAS No. 6055-19-2), mitomycin C (MMC, CAS No. 50-07-7), methyl methanesulfonate (MMS, 99% purity; CAS No. 66-27-3), hypoxanthine (99% purity; CAS No. 68- 94-0), thiazolyl blue tetrazolium bromide (MTT, 99.7% purity; CAS No. 298-93-1), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt), and tetrazolium compound (CAS No. 351330-42-2) were purchased from (Promega

Biotech Ibérica, Madrid, Spain); Coomassie Brilliant Blue G-250 (CAS No. 6104-59-2) was purchased from (BioRad, Madrid, Spain), trifluorothymidine (TFT, ≥ 99% purity; CAS No. 70-00-8), thymidine (CAS No. 4449-43-8), THMG medium (thymidine 9 µg/mL, methotrexate 0.3 µg/mL, hypoxantine 15 µg/mL, glycine 22.5 µg/mL) (CAS No. 59-05-2), glycine (≥99% purity; CAS No. 56-40-6), cythochalasin B (Cyt-B, 98%, CAS No. 14,930-96-2), Giemsa stain (CAS No. 51,811-82-6), dimethyl sulfoxide (DMSO) (CAS No. 67-68-5), 2-nitrofluoerene (2-NF) (CAS No. 607-57-8), sodium azide (NaN_3) (CAS No. 26628-22-8), 2-aminofluorene (2-AF) (CAS No. 153-78-6), Neutral Red (CAS No. 553-24-2), and trypan blue solution 0.4% (CAS No. 72-57-1) were purchased from Sigma–Aldrich (Madrid, Spain). RPMI 1640 medium, horse serum, L-glutamine solution (CAS No. 56-85-9), sodium pyruvate solution (CAS No. 113-24-6), penicillin/streptomycin solution and amphotericin B solution (CAS No. 1397-89-3) were from Gibco (Biomol, Sevilla, Spain). S9 fraction was purchased from Moltox (Trinova, Biochem, Germany). Endo III (EC 3.1.21.5) was purchased from C-viral S.L. (Sevilla, Spain), and FPG (EC 3.2.2.23) from Sigma-Aldrich (Madrid, Spain).

2.2. Cells and culture conditions

Caco-2 cells, used for the cytotoxicity and for the standard and enzyme-modified comet assays, are derived from a human colon carcinoma (ATCC© HTB-37). They were maintained at 37 °C in a humidified incubator gassed with 5% CO_2 in air at 95% relative humidity (CO_2 incubator, NuAire, Spain), in Eagle's medium supplemented with 1% non-essential amino acids (NEAA), 2 mM l-glutamine, 1.25 µg/mL fungizone, 1 mM pyruvate, 50 µg/mL gentamicin and 10% FBS. Cell viability and cell number were determined with the trypan blue exclusion test.

Five *S. typhimurium* histidine-auxotrophic strains were used for the Ames test, according to Organisation for Economic Cooperation and Development recommendations (OECD 471, 1997).

For the MN and MLA tests, L5178Y $Tk^{+/-}$ mouse lymphoma cells were used. This cell line was originally provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France). L5178Y $Tk^{+/-}$ cells were cultured according to Mellado-Garcia et al. (2015). Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. To perform the assays, 2×10^5 cells/mL for the MN test and 1×10^7 cells/mL for the MLA were seeded.

2.3 Test solutions

The concentration ranges of PTS were selected according the results obtained in the cytotoxicity assays carried out in Caco-2 and L5178Y $Tk^{+/-}$ cells. Stock solution of PTS (400 mM) was prepared in DMSO, and the different exposure concentration solutions were made by dilution in MilliQ sterile water (Ames test), RPMI 1640 medium (MN and MLA assays) or MEM medium (cytotoxicity, standard and modified comet assays). To avoid the toxic effects for the cells, the final percentages of DMSO were always less than 0.1% in all the assays.

2.4. Cytotoxicity assays.

PTS cytotoxicity was measured in Caco-2 and L5178Y $Tk^{+/-}$ cells, exposed to different concentrations of PTS (range 0–500 µM) at two different exposure times (24 and 48 h). Caco-2 cells were plated at density of 7.5×10^5 cells/mL in 96 well tissue-culture plates. The basal cytotoxicity endpoints were the total protein content (TP), neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) inner salt (Puerto et al., 2009).

L5178Y $Tk^{+/-}$ cells were seeded in 24-well tissue culture treated plates at density of 2.0×10^5 cells/mL and exposed to PTS in absence of the S9 metabolic activation system during 24 h, and in presence of S9 during 4 h, according to OECD 487 (2014) procedures. The cytotoxicity was assessed using the trypan blue exclusion test (Strober, 2001).

The concentrations to be tested in the standard and enzyme-modified comet assay in the Caco-2 cells, and in the MN test in L5178Y $Tk^{+/-}$cells were selected according to the half

maximal effective concentration (EC_{50}) calculated from the results obtained in the above cytotoxicity tests mentioned.

2.5. Ames test

The mutagenicity test was performed following the principles of OECD guideline 471 (1997) and Mellado-Garcia et al. (2015). Briefly, cultures of five *Salmonella typhimurium* histidine-auxotrophic strains (TA97A, TA98, TA100, TA102 and TA104) were prepared from their main strain plates and used in their late exponential growth phase. PTS was assessed in three independent experiments, which included one positive control for each strain, one negative control (distilled sterile water), one solvent control (DMSO) and five increasing concentrations of the test compound (0-280 µM). Each experiment was conducted in the presence and in the absence of the S9 metabolic activation system from rat livers, using triplicate plates for all test substance concentrations in each case.

2.6. Micronucleus test

This assay was performed according to the OECD guideline 487 (2014). L5178Y $Tk^{+/-}$ cells were seeded at a concentration of 2.0×10^5 cell/mL and treated with five different concentrations of PTS (0-17.25 µM in the absence of S9 during 24 h, and 0-25 µM in the presence of S9 during 4 h). As a negative control RPMI medium was used, and CP 8 µg/mL (in the presence of S9) and MMC 0.0625 µg/mL (in the absence of S9) were used as positive controls. The exposure of PTS (4 or 24h, in presence and absence of S9 mix, respectively), exposure of Cyt-B (20h) to induced binucleated cells, hypotonic treatment, fixation, dying and quantification of the cells were carried out according to Mellado-Garcia et al. (2015).

2.7. Mouse lymphoma thymidine-kinase assay (MLA)

The MLA assay was carried out based on Soriano et al. (2007) and Mellado-García et al. (2015). As first step, the cells were purified to purge excess possible $Tk^{-/-}$ mutants and they were transferred to THG medium (THMG without methotrexate) for 2 days. Preliminary

experiments were performed to determine the cytotoxicity of PTS by the relative total growth (RTG) after a treatment of 4 and 24 h with the test substance (0-500 µM) in absence of S9 fraction. The highest concentration selected in the experiment showed a RTG at least of 20%, according to ICH Expert Working Group (2008). Each main experiment comprised a negative control (RPMI 1640 medium), a positive control (MMS, 10 µg/mL), and five increasing concentrations for PTS. RTG values were used to decide on the acceptability of the toxicity at each dose level. Thereby, the selected ranges for PTS taking into account the RTG values were 0.9-15.65 µM for both exposure times. Then, the cells were plated at a density of 10^4 cells/mL in 96-well plates (two replicates/experimental group) to evaluate the viability of cells after an incubation time of 12 days at 37 °C in a humidified incubator with 5% CO_2 in air. After this period, viable colonies were counted. Moreover, other two replicates/experimental group were plated at the same density and exposed to 4 µg/mL TFT for the mutation analysis. Plates were also incubated the same period (12 d, 37 °C, 5% CO_2). To support the scoring of TFT mutation colonies, MTT 2.5 mg/mL was added to each well and the plates were incubated for other 4 h. After this time, the mutant colonies of each plate were counted. Colony size was estimated taking into account the recommendations described by Honma et al. (1999). The MLA assay was carried twice by time assayed.

2.8. Standard and enzyme-modified comet assay

Caco-2 cells were seeded at 3.5×10^5 cells/mL into 24-well tissue culture treated plates (Corning Costar Corporation, New York, USA) and in each well were treated with increasing concentrations of PTS (70, 140 and 280 µM) for 24 h and 48 h, according to the value obtained in the most sensitive cytotoxicity endpoint assayed.

The standard and enzyme-modified comet assays were performed, as previously described by Collins et al. (1997), with slight modifications (Corcuera et al., 2011; Llana-Ruiz-Cabello et al., 2014). To investigate oxidative DNA damage two different enzymes, Endo III and

FPG were applied (Llana-Ruiz-Cabello et al., 2014; Mellado-García et al., 2015). Cells were treated with H_20_2 (100 µM) for 5 min as a positive control for the standard comet assay (DNA strand-breaks) and Endo III. About 2 µM of Ro 19-8022 photosensitiser (Hoffman-La Roche, Switzerland) together with light irradiation (1.5 min) was used as a positive control for FPG.

A fluorescence microscope (Olympus BX61) with comet assay IV software (Perceptive Instruments, UK) was used to score at least 100 cells per sample, and the results were expressed as mean % DNA in tail. The percentage of Buffer F and Buffer F + Enzyme was presented as % of DNA in tail. For each concentration tested only was determined the difference between Buffer F + Enzyme *VS* Buffer F.

2.9. Calculations and statistical analysis

The mutagenicity experiment (Ames test) was performed three times in at least triplicate per concentration. The data are presented as the arithmetic mean percentage ± standard deviation (SD) compared to the control group, which is the unexposed bacterial strains. Statistical analysis was performed using the analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests using GraphPad InStat software (GraphPad Software Inc., La Jolla, USA). The normality of the distribution and the homogeneity of variances were confirmed using Kolmogorov and Smirnov's test, and Bartletťs test, respectively.

In the MN test, the statistical approach included the one-way ANOVA followed by Chi-square test. Significant differences about nuclear division index (NDI) were calculated by Kruskal–Wallis test (nonparametric ANOVA) between the control and treated cultures.

In the MLA, the induced mutant frequency (IMF) was determined according to the formula IMF = MF − SMF, where MF is the test culture mutant frequency and SMF is the spontaneous mutant frequency. Positive responses are determined as those that for any treatment meet or exceed the global evaluation factor (GEF, 126 for the microwell assay) and also when a positive

trend test is obtained. The statistical approach was the one-way ANOVA followed by Dunnett's test, which was used to evaluate the significance of the difference in MF between the control and treated cultures.

For the comet assays, the data are presented as the means ± SD of three different independent experiments. The data (% DNA in tail) are presented as the means ± SD. The total scores of the different groups were compared using Tukey-Karmer test (parametric) and Kruskal–Wallis test (non-parametric).

For all the assays significant differences were considered at $P < 0.05$.

3. Results

3.1 Cytotoxicity assays

After 24 h of exposure to PTS (Fig. 1a) the protein content of Caco-2 cells was not dramatically altered, and only significant ($P<0.001$) reductions were recorded for 300 μM. Similarly, no significant changes were observed in the NRU assay at the lowest concentrations used; nevertheless at higher concentrations the viability of cells decreased considerably, especially between 300 and 500 μM PTS. Finally, MTS metabolization declined in a concentration-dependent pattern being significant from 200 μM of PTS.

More severe effects could be observed after 48 h of exposure to PTS (Fig. 1b). At this exposure time all endpoints analysed decreased in a concentration-dependent manner, with reductions from 200 μM PTS (93% and 83 % in NRU and MTS metabolization, respectively). Regarding to the TP assay the highest decrease was observed at 500 μM PTS (80% reduction in comparison to the control). The most sensitive endpoint was NRU, independently of the exposure time(Table 1), therefore the mean effective concentration (EC_{50}) value for NRU after 24 h (280 μM) was chosen as the highest exposure concentration for the genotoxicity studies in Caco-2 cells, along with the fractions $EC_{50}/2$ and $EC_{50}/4$ (140 and 70 μM PTS, respectively).

L5178Y $Tk^{+/-}$ cells exposed to different concentrations of PTS exhibited an EC_{50} value (in absence of S9 fraction) of 17.25 μM (24 h) while in the presence of S9 (4 h) this value reached 25 μM (Table 1).

3.2 Ames test

After exposure of bacterial strains to PTS (8.75-280 μM), no increase in the number of revertants colonies was observed for three of the strains assayed (TA97, TA98 and TA100) at any of the tested concentrations, either in the presence or absence of S9 (Table 2). However, a significant decrease of the TA102 colonies was obtained in the absence of S9 metabolic activation system from 8.75-140 μM PTS, and in the presence of S9 from 8.75-35 μM of PTS. Furthermore, a significant increase in the number of bacterial colonies of TA104 respect to the control was showed at the two lowest concentrations tested in the presence of S9. Moreover, for all the strains the mutagenic index (IM) was below to 2; consequently, the results revealed that PTS was not mutagenic under the conditions tested. Positive controls produced statistically significant increases in the number of revertant colonies ($P \leq 0.01$), as well as the IM was ≥2, confirming the sensitivity of the test system and the activity of the S9 mix.

3.3 Micronucleus test

The results of the frequency of binucleated cells with micronuclei (BNMN %) in L5178Y $Tk^{+/-}$ and the NDI in absence and presence of S9 metabolic activation system are shown in Table 3. PTS induced significant ($P<0.001$) increase in the frequency of MN in binucleated cells at the highest concentration tested (17.25 μM) in absence of S9 mix; furthermore, significant ($P<0.01$; $P<0.001$) enhancements were also observed after exposure to 20 and 25 μM of PTS, in presence of S9. Regarding to the NDI, no significant variations were observed in absence and presence of S9 at any concentration tested in comparison to the control group. As expected,

treatment with MMC and CP (positive control) induced significant (P<0.001) increases in the frequency of MN.

3.4 Mouse lymphoma assay (MLA)

The results obtained in the MLA after treatment of L5178Y $Tk^{+/-}$cells with PTS for 4 and 24 h are shown in Tables 4 and 5, respectively. After 4 h of exposure to PTS no significant differences were found under the conditions tested (Table 4). In the same manner, PTS did not induce mutagenic response at any concentration tested after 24h treatment. A similar concentration-response pattern in the decreased of RTG was found at both periods of exposure times (Table 5). Positive controls with MMS (10 μg/mL) were run in parallel, appearing significantly increased mutant frequencies. A good concordance was observed between the two conducted experiments for each exposure time.

3.5 Comet assay

In the standard comet assay, PTS did not induce DNA breaks in Caco-2 cells at 70 and 140 μM after 24 and 48 h of exposure (Fig. 2a). However, significant increases (P <0.05; P <0.001) after both times of exposure were observed at 280 μM PTS compared to the control group. The percentage of DNA in the tail at 280 μM PTS after 48 h was approximately 2-fold higher compared to the same concentration of PTS after 24 h (P < 0.05).

According to the results obtained after application of the enzyme digestion of the nuclei, no increase in DNA damage was observed when Endo III (Fig. 2b) or FPG (Fig.2c) were used in the cells exposed to PTS after 24 and 48 h.

In all the experiments, Caco-2 cells treated with the positive controls, significant increases of % DNA in tail (P <0.001) were observed under the conditions tested, as expected.

4. Discussion

An exhaustive toxicological assessment is needed to elucidate the potential risks associated with the use of EOs in food industry and in order to limit their incorporation into food packaging. The study of single compounds from the EOs facilitates establishing a relationship between the damage observed and the toxic mechanism of EOs (Maisanaba et al., 2016); moreover, the complex and variable composition of EOs complicates the interpretation of the results when studying the toxicity directly on them. Only few works have dealt on the toxicological assessment of different components of *Allium* EOs, and, specifically, the studies concerning the mutagenicity/genotoxicity of the main components of garlic and onion EOs are practically non-existent. In the case of OSCs, the scarce mutagenic/genotoxic studies carried out revealed the absence of mutagenicity, such is the case of DPS and DPDS in the range of 0-200 µM (Llana-Ruiz-Cabello et al., 2015b), whereas diallyl sulfide (DAS) and diallyl disulfide (DADS) induced significant chromosomal aberrations (CA) and sister chromatid exchanges in CHO cells (Musk et al., 1997). In this sense, mutagenic and genotoxic assays are needed to establish the safety of these compounds, which are required before their approval use in active packaging (Llana-Ruiz-Cabello et al., 2015c). In the case of PTS, no data are available so far about its cytotoxicity, mutagenic or genotoxic potential.

In the present work, the results obtained in the cytotoxicity assays revealed that PTS reduced cell viability from 150 µM in Caco-2 cells and the EC_{50} values obtained showed that the most sensitive endpoint after 24 h of exposure was MTS (EC_{50}: 280 µM). Previous studies have demonstrated that PTSO, another volatile OSC, induced damage from 350 µM in two mammalian cell lines, Caco-2 and Hep-G2 cells (the latter derived from human hepatocellular carcinoma), being the most sensitive endpoint NRU (EC_{50}: 368 µM) (Llana-Ruiz-Cabello et al., 2015a). In the case of DPDS, DPS and their mixture, neither of the endpoints assayed (TP, NRU, MTS) have shown remarkable changes respect to the control at the concentrations assayed (0-

200 µM) (Llana-Ruiz-Cabello et al., 2015b). Therefore, according to these results, PTS resulted to be more cytotoxic than the OSCs above mentioned.

Concerning the mutagenicity/genotoxicity, according to the Scientific Opinion of EFSA (2016) the starting point should be a combination of the bacteria reverse mutation assay and the *in vitro* mammalian cell MN test (EFSA, 2016). With regards to the Ames test, the negative results obtained for PTS with and without S9 fraction, correlate well with the negative response showed for PTSO in any of the strains assayed (0-20 µM) (Mellado-Garcia et al., 2015). Similarly, DPS, DPDS and their mixture 1:1 did not show mutagenic activity under the same conditions tested up to 200 µM (Llana-Ruiz-Cabello et al, 2015b). In the same manner, allyl propyl sulphide (APDS) and DAS exhibited no mutagenic potential in the Ames test (TA98, TA100, TA1535, TA1537 and TA1538) (Eder et al., 1982). Furthermore, fresh garlic juice and an extract of garlic demonstrated no evidence of mutagenicity using TA98, TA100, and TA1537 *S. Typhimurium* strains (Yoshida et al., 1984). And even other authors have evidenced antimutagenic activity of the digested onion extracts as well as several OSCs (DAS, DADS, DPS and DPDS) tested by the Ames test (Guyonnet et al., 2000, 2001; Shon et al., 2004). This fact evidences the dual genotoxic/antigenotoxic effects of the EOS and their main components (Belloir et al., 2006; Horvathova et al., 2006; Slamenova et al., 2013).

Concerning the genotoxicity/mutagenicity approach, data from the gene mutation test in bacteria and in the *in vitro* MN test allow the detection of all the relevant *in vivo* carcinogens (Kirkland et al., 2011).The *in vitro* MN assay is being increasingly used in the evaluation of EOs (Llana-Ruiz-Cabello et al., 2015c). In the last years, MN test has been recommended as an alternative to the *in vitro* CA test (Corvi et al., 2008) due to its advantages compared with this assay; in fact, the MN test has been incorporated in the core set of genotoxicity studies recommended by EFSA (EFSA, 2011, 2016). The results showed in the present work indicated the significant increase in the frequency of MN in binucleated cells in absence (at 17.25 µM) and presence (20-25µM) of S9 mix, so the potential genotoxic damage could be due to the

parent compound (PTS) and/or its metabolites. Previous studies demonstrated that PTSO metabolites increased BNMN frequency but not the original OSC (Mellado-García et al., 2015). These findings highlight the importance of incorporating the cofactor-supplemented S9 fraction in the experiments to mimic human liver metabolism, being microsomal S9 fraction the most commonly used system (EFSA, 2011). In this regard, Guyonnet et al. (2000) observed that OSCs have capacity to induce several isoforms of Cyt P450 (CYP) belonging to CYP1A and 2B subfamilies that involved in the activation of different carcinogens.

The lack of agreement between the results obtained in Ames and MN test in the presence of S9, could be explained by differences in the experimental models employed, prokaryotes or eukaryotes cells, which have different uptake, metabolism, chromosome structure and DNA repair processes (EFSA, 2011). In order to clarify the contradictory results obtained by both assays, and following indications published by EFSA (2011), *in vitro* MLA assay was carried out to add information on the potential effects at the mammalian gene mutation level. Although the MLA mainly detects gene mutation, different types of chromosomal damage are also detects (Clements, 2000). The negative results obtained in the present work after 4 and 24 h of PTS treatment (15.65 μM) are in agreement with the data observed in the bacterial assays, showing an absence of mutagenic potential of PTS in prokaryotic and eukaryotic systems. By contrast, PTSO induced significant mutation frequencies from 2.5 to 20 μM after 24 h of exposure (Mellado-Garcia et al., 2015), whereas negative results were obtained after short time treatment (4h).

Finally, the standard comet assay was performed on Caco-2 cells in order to complete the assessment of the potential genotoxicity of PTS. The comet assay finds extensive use in the area of testing new chemicals for genotoxicity, monitoring environmental contamination with genotoxins, human biomonitoring and molecular epidemiology, diagnosis of genetic disorders and fundamental research in DNA damage and repair (Ganapathy et al., 2016). In this work, PTS did not induce DNA strand breaks after 24 and 48 h of exposure to 70 and 140 μM,

although the highest concentrations assayed (280 μM) induced damage after both exposure times. Our finding would agree with those observed with PTSO which did not induce increase in the % DNA in tail at any of the assayed concentrations (0-50 μM) in Caco-2 cells (Mellado-García et al., 2015). Similarly, when HepG2 cells were incubated with allicin, DAS, DADS, S-allyl cysteine (SAC) and allyl mercaptan (AM) (5–100 μM) for 20 h, no DNA (single and double) strand breaks were observed (Belloir et al., 2006). In another study, none of the OSCs tested (DAS, DPS at 1-50 μM, and DADS and DPDS at 1-5 μM) caused DNA damage *per se* in HepG2 cells after 24 h using comet assay; nevertheless, positive results were obtained at higher concentrations (>5 μM) for DADS and DPDS (Arranz et al., 2007).

The results obtained in the standard comet assay were completed by the use of restriction enzymes in order to evidence the possible oxidative damage in DNA caused by PTS, due to the dual prooxidant/antioxidant potential of EOs and specifically in the case of OSCs. In general, fewer modified comet assay have been reported on EOs in comparison to other toxicity studies, and most of them did not show oxidative DNA damage at low concentrations tested (Ündeğer et al., 2009; Llana-Ruiz-Cabello et al., 2015c). Different studies have demonstrated that OSCs possess antioxidant capacities (Fei et al., 2015); hence, consuming organosulfide rich foods might be beneficial because of their protection of biomolecules from oxidative damage (Wang and Huang, 2015). Nevertheless, antioxidants at higher concentrations could react as prooxidants which can oxidize DNA, proteins and lipids (Bakkali et al., 2008). In the present work, PTS did not induce oxidation of pyrimidines (by Endo III) and purines bases (by FPG) at any concentration tested in Caco2 cells, and these results agree with previous data reported after exposure to PTSO (Mellado-Garcia et al., 2015). Arranz et al. (2007) showed that DAD and DPS (1-50 μM) and DADS and DPDS (1-5 μM) in presence of FPG enzyme did not cause DNA damage in human Hep-G2 cells.

In summary, the results have shown that PTS did not exhibit mutagenic activity neither in bacterial *S. typhimurium* nor in mammalian L5178Y $Tk^{+/-}$ cells through the Ames and MLA

tests, respectively. However, in this eukaryotic system L5178Y $Tk^{+/-}$, PTS showed a genotoxic activity in the MN test in both presence and absence of S9. Moreover, a positive response in Caco-2 cells at the highest concentration assayed has been also detected in the standard comet assay, although PTS did not induce DNA oxidative damage. Taking into account all the obtained results, PTS has demonstrated a clastogenic effect after application of battery of genotoxicity tests. Following the recommendations of the EFSA Scientific Committee a single rodent *in vivo* study combining MN analysis (in bone marrow or blood) and a comet assay in the liver to know the involvement of liver-specific clastogenic metabolites, should be considered for further studies (EFSA, 2011). Moreover, these *in vivo* studies have been useful to clarify the absence of genotoxicity effects in the case of substances showing contradictory *in vitro* results, such as PTSO (Mellado-Garcia et al., 2016).

5. Conclusion

PTS was not mutagenic neither in the Ames test (in the presence/absence of S9) nor in the MLA (after 4 and 24 h of exposure) at any of the tested concentrations. However, genotoxic effects were recorded for the MN assay inL5178Y $Tk^{+/-}$ cells in both absence and presence of S9. Similarly, positive results were also found in the comet assay at the highest concentration tested (280 µM) in Caco-2 cells after 24 and 48 h of exposure. However, any DNA-oxidative damage (by using the Endo III and FPG-modified comet assay) was observed. A substance is only considered negative for genotoxicity if all tests performed are assessed negative. According to the obtained results further *in vivo* genotoxic studies are recommended in order to confirm the genotoxic profile of PTS.

Acknowledgements: the authors would like to thank the Spanish Ministry of Science and Innovation (AGL2012-38357-C02-01) co-financed by FEDER funds and Junta de Andalucía (AGR-

7252) for the financial support for this study, the Microscopy Service of CITIUS and the animal housing from the Faculty of Pharmacy in the Universidad de Sevilla. We thank F. Hoffmann-La Roche for the gift of Ro 19-8022.

7. References

Arranz, N., Haza, A.I., García, A., Möller, L., Rafter, J., Morales, P., 2007. Protective effects of organosulfur compounds towards N-nitrosamine-induced DNA damage in the single-cell gel electrophoresis (SCGE)/HepG2 assay. Food Chem. Toxicol. 45, 1662-1669.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils—a review. Food Chem. Toxicol. 46, 446–475.

Belloir, C., Singh, V., Daurat, C., Siess, M.H., Le Bon, A.M., 2006. Protective effects of garlic sulphur compounds against DNA damage induced by direct-and indirect-acting genotoxic agents in HepG2 cells. Food Chem. Toxicol. 44, 827-834.

Benkeblia, N., Onodera, S., Yoshihira, T., Kosaka, S., Shiomi, N., 2004. Effect of temperature on soluble invertase activity, and glucose, fructose and sucrose status of onion bulbs (*Allium cepa*) in store. Int. J. Food Sci. Nutr. 55, 325-31.

Benkeblia, V., Lanzotti., 2007. *Allium* thiosulfinates: chemistry, biological properties and their potential utilization in food preservation. Food 1, 193–201.

Chiu, C.K., Chen, T.Y., Lin, J.H., Wang, C.Y., Wang, B.S., 2016. Protective effects of five *allium* derived organosulfur compounds against mutation and oxidation. Food Chem. 197, 829-835..

Clements, J., 2000. The Mouse Lymphoma assay. Mutat. Res. 455, 97–110.

Collins, A.R., Mitchell, D.L., Zunino, A., de Wit, J., Busch, D., 1997. UV-sensitive rodent mutant cell lines of complementation groups 6 and 8 differ phenotypically from their human counterparts. Environ. Mol. Mutagen. 29, 152–160.

Collins, A.R., 2004. The comet assay for DNA damage and repair principles, applications, and limitations. Mol. Biotechnol. 26, 249–261.

Corcuera, L.A., Arbillaga, L., Vettorazzi, A., Azqueta, A., López de Cerain, A., 2011. Ochratoxin A reduces aflatoxin B1 induced DNA damage detected by the comet assay in Hep G2 cells. Food Chem. Toxicol. 49, 2883–2889.

Corvi, R., Albertini, S., Hartung, T., Hoffmann, S., Maurici, D., Pfuhler, S., van Benthem, J., Vanparys, P., 2008. ECVAM retrospective validation of the in vitro micronucleus test (MNT). Mutagenesis 271, 271-283.

Corzo-Martínez, M., Corzo, N., Villamiel, M., 2007. Biological properties of onions and garlic. Trends Food Sci. Technol. 18, 609–625.

Eder, E., Neudecker, T., Lutz, D., Henschler, D., 1982. Correlation of alkylating and mutagenic activities of allyl and allylic compounds: standard alkylation test vs. kinetic investigation. Chem-Biol. Interact. 38, 303–315.

European Food Safety Authority, 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA J. 9 (9), 2379, 69. http://www.efsa.europa.eu/en/efsajournal/pub/2379 (accessed 09/11/16).

European Food Safety Authority, 2016. Recent developments in the risk assessment of chemicals in food and their potential impact on the safety assessment of substances used in food contact materials. EFSA J. 14, 4357, 28. https://www.efsa.europa.eu/en/efsajournal/pub/4357 (accessed 09/11/2016).

Fei, M., Tong, LI., Wei, LI., Long, De Yang., 2015. Changes in antioxidant capacity, levels of soluble sugar, total polyphenol, organosulfur compound and constituents in garlic clove during storage. Ind. Crop. Prod. 69, 137–142.

Fenech, M., 2000. The in vitro micronucleus technique. Mutat. Res. 455, 81–95.

Ganapathy, S., Muraleedharan, A., Sathidevi, P.S., Chand, P., Rajkumar, R.P., 2016. CometQ: An automated tool for the detection and quantification of DNA damage using comet assay image analysis. Comput. Method. Program. Biomed. 133, 143-54.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2000. Liver subcellular fractions from rats treated by organosulfur compounds from *Allium* modulate mutagen activation. Mutat. Res. 466, 17-26.

Guyonnet, D., Belloir, C., Suschetet, M., Siess, M.H., Le Bon, A.M., 2001. Antimutagenic activity of organosulfur compounds from *Allium* is associated with phase II enzyme induction. Mutat. Res. 495, 135–145.

Honma, M., Hayashi, M., Shimada, H., Tanaka, N., Wakuri, S., Awogi, T., Yamamoto, K.I. Kodani, N., Nishi, Y., Nakadate, M., Sofuni, T., 1999. Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test. Mutagenesis 14, 5-22.

Horváthová, E., Sramkova, M., Labaj, J., Slamenova, D., 2006. Study of cytotoxic, genotoxic and DNA-protective effects of selected plant essential oils on human cells cultured in vitro. Neuro Endocrinol. Lett. 27, 44–47.

ICH Expert Working Group, 2008. International Conferences on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, in ICH Harmonised Tripartite Guideline Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use. Draft Version 5.3.

Kim, D.K., H. S., Lillehoj, Lee, S. H., Lillehoj, E. P., Bravo, D., 2013. Improved resistance to *Eimeria acervulina* infection in chickens due to dietary supplementation with garlic metabolites. Brit. J. Nutr. 109, 76-88.

Kirkland, D., Reeve, L., Gatehouse, D., Vanparys, P., 2011. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutat. Res. 721, 27-73.

Lekshmi, C.J., Packia, S., Viveka, S., Viswanathan, M.B., Shobi, Mini., T., 2015. GC-MS characterization of organic sulphurcompounds and other volatile odorous compounds from *Allium sativum*. Asian J. Microbiol. Biotech. Env. Sc. Vol. 17, 73-78.

Llana-Ruiz-Cabello, M., Maisanaba, S., Puerto, M., Prieto, A.I., Pichardo, S., Jos, A., Cameán, A.M., 2014. Evaluation of the mutagenicity and genotoxic potential of carvacrol and thymol

using the Ames Salmonella test and alkaline, Endo III- and FPG-modified comet assays with the human cell line Caco-2. Food Chem. Toxicol. 72, 122–128.

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Moreno, F.J., Baños, A., Núñez, C., Guillamón, E., Cameán, A.M. 2015a. Acute toxicological studies of the main organosulfur compound derived from *Allium* sp. intended to be used in active food packaging. Food Chem. Toxicol. 82, 1- 11.

Llana-Ruiz-Cabello, M., Maisanaba, S., Gutiérrez-Praena, D., Prieto, A.I., Pichardo, S., Jos, A., Moreno, F.J., Cameán, A.M. 2015b. Cytotoxic and mutagenic in vitro assessment of two organosulfur compounds derived from onion to be used in the food industry. Food Chem. 166, 423- 431.

Llana-Ruiz-Cabello, M., Pichardo, S., Maisanaba, S., Puerto, M., Prieto, A.I., Gutiérrez-Praena, D., Jos, A., Cameán, A.M., 2015c. In vitro toxicological evaluation of essential oils and their main compounds used in active food packaging: a review. Food Chem. Toxicol. 81, 9–27.

Maisanaba, S., Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Pichardo, S., Puerto, M., A.I. Prieto, A.I., Jos, A., Cameán, A.M., 2016. New advances in active packaging incorporated with essential oils or their main components for food preservation. Food Rev. Int. 1-69.

Martínez-Fernández, G., Abecia, L., Martín-García, AI., Ramos-Morales, E., Hervás, G., Molina-Alcaide, E., Yáñez-Ruiz, DR., 2013. *In vitro-in vivo* study on the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats. Animal 7, 1925-34.

Martínez-Fernández, G., Abecia, L., Martín-García, AI., Ramos-Morales, E., Denman, SE., Newbold, CJ., Molina-Alcaide, E., Yáñez-Ruiz, DR., 2015. Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. FEMS Microbiol. Ecol. 91, 79.

Martins, N., Petropoulos, S., Ferreira, I.C., 2016. Chemical composition and bioactive compounds of garlic (*Allium sativum* L.) as affected by pre-and post-harvest conditions: A review. Food Chem. 211, 41-50.

Mellado-García, P., Maisanaba, S., Puerto, M., Llana-Ruiz-Cabello, M., Prieto, AI., Marcos, R., Pichardo, S., Cameán, AM., 2015. Genotoxicity assessment of propyl thiosulfinate oxide, an organosulfur compound from *Allium* extract, intended to food active packaging. Food Chem. Toxicol. 86, 365-73.

Mellado-García, P., Puerto, M., Prieto, A.I., Pichardo, S., Martin-Cameán, A.M., Moyano, R., Blanco A., Cameán A., 2016. Genotoxicity of a thiosulfinate compound derived from *Allium* sp. Intended to be used in active food packaging: *In vivo* comet assay and micronucleus test. Mutat. Res-Gen. Tox. En. 800, 1-11.

Mnayer, D., Fabiano-Tixier, A.S., Petitcolas, E., Hamieh, T., Nehme, N., Ferrant, C., Fernandez, X., Chemat, F., 2014. Chemical Composition, Antibacterial and Antioxidant Activities of Six Essentials Oils from the *Alliaceae* Family. Molecules 19, 20034-20053.

Mortelmans, K., Zeiger, E., 2000. The Ames Salmonella/microsome mutagenicity assay. Mutat. Res. 445, 29–60.

Musk, S.R.R., Clapham, P., Johnson, I.T., 1997. Cytotoxicity and genotoxicity of diallyl sulfide and diallyl disulfide towards Chinese hamster ovary cells. Food Chem. Toxicol. 35, 379–385.

OECD (Organisation for Economic Cooperation and Development), 1997. Guideline for Testing of Chemicals: Mutation Test, pp. 1–11 Guideline 471.

OECD (Organisation for Economic Cooperation and Development), 2014. Guideline for Testing of Chemicals: in vitro Mammalian Cells Micronucleus Test, pp. 1–26 Guideline 487.

OECD (Organisation for Economic Cooperation and Development), 2015. Guideline for Testing of Chemicals: In vitro Mammalian Cell Gene Mutation Test Using the Hprt and Xprt Genes, pp. 1–18 Guideline 476.

Prakash, B., Kedia, A., Mishra, P.K., Dubey, N.K., 2015. Plant essential oils as food preservatives to control moulds, mycotoxin contamination and oxidative deterioration of agri-food commodities, potentials and challenges. Food Control 47, 381-391.

Pu, X.,Wang, Z.,Klaunig, James E., 2016. Cryopreservation of human blood for alkaline and Fpg-modified comet assay. Toxicol. Mechan. Method. 26,196-201.

Puerto, M., Pichardo, S., Jos, A., Cameán, AM., 2009. Comparison of the toxicity induced by microcystin-RR and microcystin-YR in differentiated and undifferentiated Caco-2 cells. Toxicon 54, 161-9.

Ruiz, R., Peinado, M. J., Aranda-Olmedo, I., Abecia, L., Suárez-Pereira, E., Ortiz Mellet, C., García Fernández, J.M., Rubio, L. A., 2015. Effects of feed additives on ileal mucosa–associated microbiota composition of broiler chickens. J. Anim. Sci. 93, 3410-3420.

Shon, S., Kahng, G., Namb, S., Sunga, N., 2004. Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. Food Chem. Toxicol. 42, 659–666

Slamenova, D., Kozics, K., Hunakova, L., Melusova, M., Navarova, J., Horvathova, E., 2013.Comparison of biological processes induced in HepG2 cells by tert-butyl hydroperoxide (t-BHP) and hydroperoxide (H2O2): the influence of carvacrol. Mutat. Res. 757, 15–22.

Soriano, C., Creus, A., Marcos, R., 2007. Gene-mutation induction by arsenic compounds in the mouse lymphoma assay. Mutat. Res. 634, 40–50.

Strober, W., 2001. Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. 21 3B:A.3B.1–A.3B.2, Appendix 3 : Appendix 3B.

Ündeğer, A., Başaranb, G.H, Degenc., Başarana, N., 2009. Antioxidant activities of major thyme ingredients and lack of (oxidative) DNA damage in V79 Chinese hamster lung fibroblast cells at low levels of carvacrol and thymol. Food Chem. Toxicol. 47, 2037–2043.

Wallace, R.J. 2004. Antimicrobial properties of plant secondary metabolites. P. Nutr. Soc. 63, 621-629.

Wang, J,. Sawyer, J.R., Chen, L., Chen, T., Honma, M., Mei, N., Moore, M., 2009. The Mouse Lymphoma Assay detects recombination, deletion, and aneuploidy. Toxicol. Sci. 109, 96–105.

Wang H., Huang, D., 2015. Dietary organosulfur compounds from garlic and cruciferous vegetables as potent hypochlorite scavengers. J. Funct. Foods 18, 986–993

Ye, C.H., Dai, D.H., Hu, W.L., 2013. Antimicrobial and antioxidant activities of the essential oil from onion (*Allium cepa* L.). Food Control 30, 48–53.

Yoshida, S., Hirao, Y., Nakagawa, S., 1984. Mutagenicity and cytotoxicity tests of garlic.

J. Toxicol. Sci. 9, 77–86.

Table captions

Table 1. Cytotoxicity assays carried out with PTS. EC_{50} values were determined for Caco-2 and L5178Y *Tk*$^{+/-}$ cells using different approaches. For Caco-2 cell the endpoints were total protein content (TP); neutral red uptake (NRU) ; MTS tetrazolium compound (MTS). For L5178Y *Tk*$^{+/-}$ was trypan blue exclusion test.

Table 2. Ames test results for PTS. In three independent experiments. Milli Q water was used as negative control and DMSO (10 µl) as solvent for positive controls. Data are given as mean ± SD revertants/plate for three replicates for each concentration in each experiment. Positive controls without S9 for TA 97A/TA98/TA102/TA104: 2-NF (0.1µg/plate) and for TA100: NaN_3 (1 µg/plate). Positive control for all strains with S9: 2-AF (20 µg/plate). * *P*<0.05 significant differences from controls, ** *P*<0.01 very significant differences from controls.

Table 3. Percentage of binucleated cells with micronuclei (BNMN%) and the nuclear division index (NDI) in cultured mouse lymphoma cells (L5178Y *Tk*$^{+/-}$) treated with PTS in the absence or presence of S9. Values are expressed as mean ± SD. ** *P*<0.0005, *** *P*<0.0001 significantly different from negative control.

Table 4. Results obtained for PTS in the MLA (L5178Y *Tk*$^{+/-}$ cells) in two different experiments. Exposure to PTS last for 4 h. *** *P*≤0.001 significantly different from negative control. [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

Table 5. Results obtained for PTS in the MLA (L5178Y *Tk*$^{+/-}$ cells) in two different experiments. Exposure to PTS last for 24 h. ***$P \leq 0.001$ significantly different from negative control. [a] Total mutant frequency divided into small/large (S/L) colony mutant frequencies.

Figure captions

Figure 1. a) Cell viability of PTS. Total protein content (TP), neutral red uptake (NRU) and reduction of tetrazolium salt (MTS) of Caco-2 cells exposed for 24 h (a) and 48h (b). All values are expressed as mean ±SD. * Significant differences at $P < 0.001$.

Figure 2. DNA damage measured in Caco-2 cells after 24 and 48 h of exposure to 0, 70, 140, and 280 µM PTS expressed as the formation of strand breaks (a) and oxidative DNA damage as Endo III-sensitive sites (b) and FPG-sensitive sites (c). The levels of DNA strand-breaks (SBs), oxidized pyrimidines and oxidized purines are expressed as % DNA in tail. All values are expressed as mean ± SD. ***$P < 0.001$, *$P < 0.05$ significantly different from negative control; # $P<0.05$ significantly different from groups exposed for 48 h in comparison to 24 h;& $P<0.05$ significant differences between FPG enzyme digested and non digested group.

Table 1

Caco-2 cells			**L5178Y *Tk*$^{+/-}$ cells**		
Endpoints	EC_{50} values (µM)		Endpoint	EC_{50} values (µM)	
	24 h	48 h		4 h	24 h
TP	-	228	Trypan blue	25	17.25
NRU	280	187			
MTS	340	193			

Table 2

	[µM]	TA97A				TA98				TA100				TA102				TA104			
		-S9	IM	+S9	IM	-S9	IM	+S9	IM	-S9	IM	+S9	IM	-S9	IM	+S9	IM	-S9	IM	+S9	IM
PTS	Negative controls	234±37	-	207±8	-	27±3	-	36±4	-	120±31	-	128±7	-	291±2	-	243±9	-	255±8	-	323±31	-
	8.75	303±16	1.3	221±33	1.1	34±2	1.2	64±6	1.8	153±10	1.3	143±3	1.1	169±10**	0.6	170±10**	0.7	209±27	0.8	485±69**	1.5
	17.5	246±11	1.1	234±31	1.1	32±1	1.2	69±12	1.9	136±5	1.1	81±8	0.6	223±3*	0.8	190±19**	0.8	312±17	1.2	527±8**	1.6
	35	246±19	1.1	213±9	1	23±1	0.9	40±5	1.1	108±7	0.9	98±2	0.8	225±14*	0.8	168±21**	0.7	304±14	1.2	392±14	1.2
	70	212±11	0.9	221±18	1.1	31±2	1.2	30±1	0.8	91±2	0.8	91±18	0.7	204±7**	0.7	228±28	0.9	285±4	1.1	381±30	1.2
	140	244±33	1.1	251±26	1.2	33±1	1.2	34±3	1.1	100±5	0.8	86±11	0.7	218±11*	0.8	204±7	0.8	321±11	1.3	380±22	1.2
	280	199±26	0.8	261±58	1.3	33±3	1.2	32±4	0.9	96±6	0.8	92±17	0.7	265±21	0.9	209±12	0.9	290±7	1.1	237±21	0.8
	Positive controls	837±274**	3.6	831±233**	4	742±50**	27.5	469±50**	13	1035±48**	8.6	1474±106**	11.5	748±68**	2.6	729±20**	3	837±105**	3.3	730±61**	2.3
	DMSO	229±12	1	210±18	1	24±3	0.9	28±2	0.8	111±8	0.9	115±12	0.9	273±16	0.9	261±12	1.1	249±13	1	298±16	0.9

Table 3

Test substance	Absence of S9				Presence of S9			
	Treatment time (h)	**Concentrations**	**BNMN (%) ± SD**	**NDI ± SD**	**Treatment time (h)**	**Concentrations**	**BNMN (%) ± SD**	**NDI ± SD**
Negative control	24	-	1.5±0.7	1.6±0.0	3-6	-	1.7±0.7	1.6±0.1
Positive control	24	Mitomycin C 0.0625 µg/mL	6.8±2.1***	1.4±0.2	3-6	Cyclophosfamide 8 µg/mL	6.0±2.0***	1.6±0.0
PTS	24	2.1 µM	1.1±0.7	1.5±0.1	3-6	5 µM	1.6±0.8	1.7±0.0
	24	4.3 µM	1.2±0.7	1.5±0.1	3-6	10 µM	1.9±1.2	1.7±0.1
	24	8.6 µM	1.1±0.6	1.6±0.1	3-6	15 µM	2.0±0.8	1.7±0.1
	24	12.2 µM	1.8±0.6	1.6±0.1	3-6	20 µM	3.5±1.8**	1.7±0.1
	24	17.25 µM	3.7±1.6***	1.8±0.1	3-6	25 µM	5.0±1.8***	1.6±0.0

Table 4

Concentration (µM)	Percent plating efficiency	Mutant frequency (x 10^{-6})	Relative total growth	MF (S/L)[a]	IMF (MF-SMF)
Experiment 1					
0	93	163	100	81/82	-
0.9	95	182	92	69/113	19
1.9	82	179	93	69/110	16
3.9	90	226	70	85/141	63
7.8	90	244	61	92/152	81
15.65	60	276	28	152/124	113
MMS (10 µg/mL)	34	2820***	42	1944/876	2657
Experiment 2					
0	130	149	100	36/113	-
0.9	76	217	86	72/145	68
1.9	87	178	81	62/116	29
3.9	76	194	81	64/130	45
7.8	76	222	71	131/91	73
15.65	80	212	35	81/131	63
MMS (10 µg/mL)	48	2510***	38	1479/1031	2361

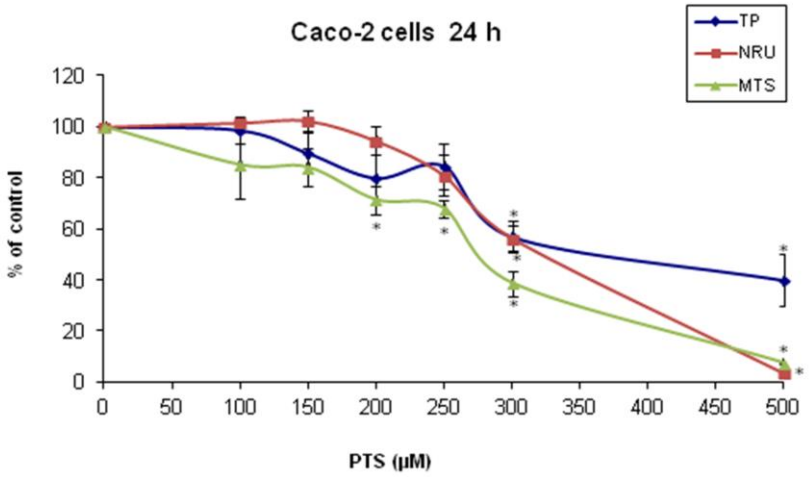
Table 5

Concentration (µM)	Percent plating efficiency	Mutant frequency (x 10^{-6})	Relative total growth	MF (S/L)[a]	IMF (MF-SMF)
Experiment 1					
0	124	142	100	67/75	-
0.9	106	99	98	24/76	-43
1.9	111	101	74	46/55	-41
3.9	102	102	70	54/48	-40
7.8	73	111	60	35/76	-31
15.65	85	122	21	42/80	-20
MMS (10 µg/mL)	34	1730***	30	1013/717	1588
Experiment 2					
0	111	153	100	77/77	-
0.9	111	80	89	37/43	-73
1.9	93	100	82	56/44	-53
3.9	90	85	85	35/50	-68
7.8	95	80	42	35/45	-73
15.65	102	118	23	42/76	-35
MMS (10 µg/mL)	40	2400***	26	1273/1127	2247

Figure 1

Figure 1.

a)



b)

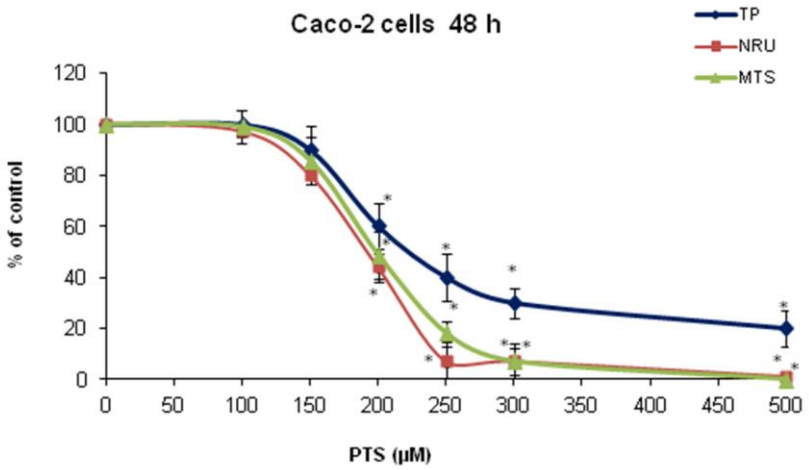


Figure 2

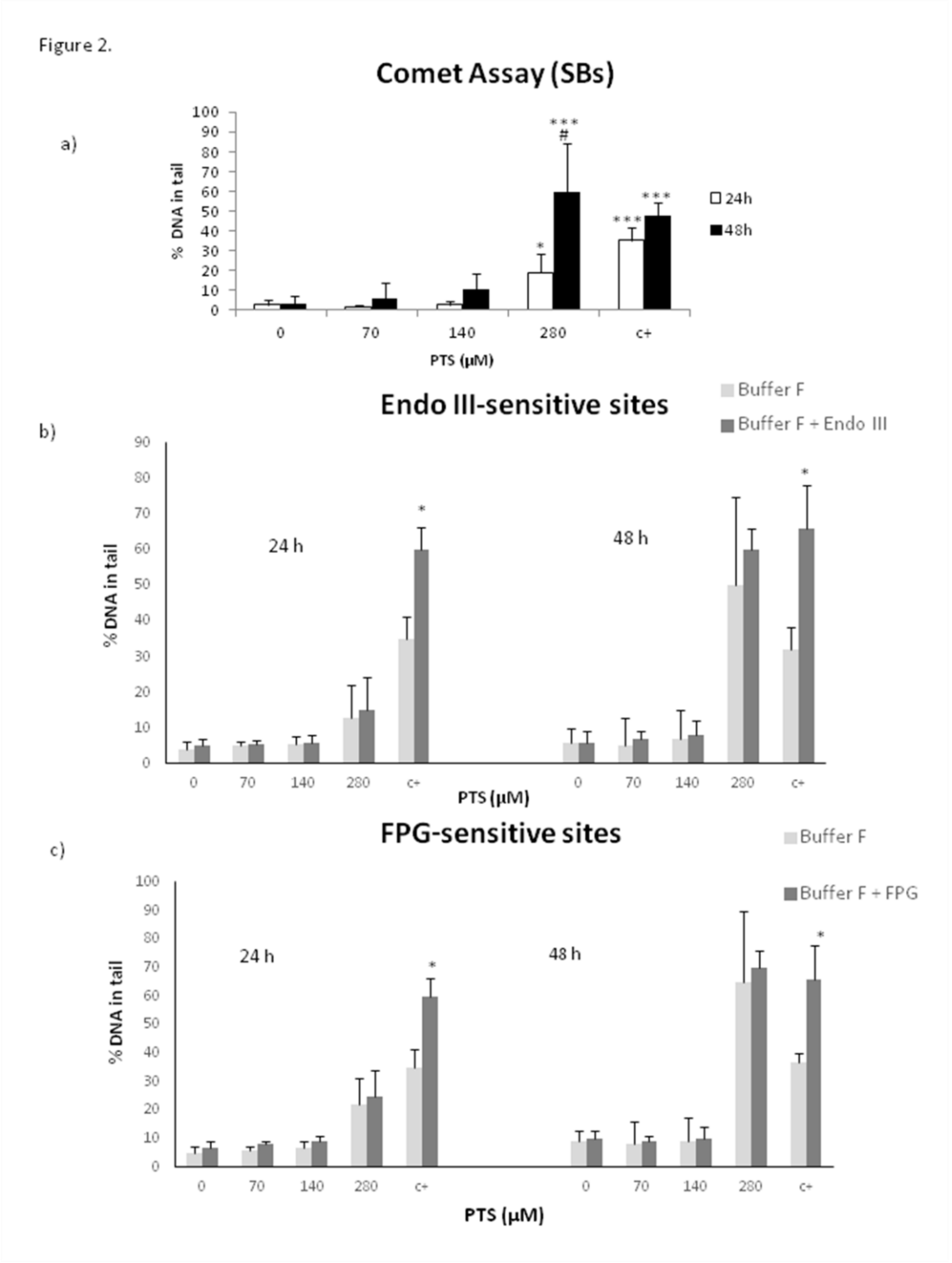


Figure 2.
Comet Assay (SBs)
a)
% DNA in tail
PTS (µM)
0
70
140
280
c+
24h
48h
b)
Endo III-sensitive sites
Buffer F
Buffer F + Endo III
24 h
48 h
% DNA in tail
PTS (µM)
c)
FPG-sensitive sites
Buffer F
Buffer F + FPG
24 h
48 h
% DNA in tail
PTS (µM)