Induction of chromosomal aberrations and micronuclei by 2-hydroxy-4-methoxybenzophenone (oxybenzone) in human lymphocytes

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Title: Induction of chromosomal aberrations and micronuclei by 2-Hydroxy-4-methoxybenzophenone (Oxybenzone) in human lymphocytes.

Short Title: Effects of Oxybenzone on Human Lymphocytes

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Abstract

Oxybenzone or benzophenone-3 (2-hydroxy-4-methoxybenzophenone; BP-3) is a filter used in a variety of personal care products for protection of human skin and hair from damage by ultraviolet radiation. BP-3 is suspected to exhibit endocrine disruptive properties. Indeed, it was found to be able to interact with the endocrine system causing alteration of its homeostasis, with consequent adverse health effects. Moreover, it is ubiquitously present in the environment, mostly in aquatic ecosystems, with consequent risks to the health of aquatic organisms and humans. In the present study, we analysed the cytogenetic effects of BP-3 on human lymphocytes using *in vitro* chromosomal aberrations and micronuclei assays. Blood samples were obtained from 5 healthy Italian subjects. Lymphocyte cultures were exposed to five concentrations of BP-3 (0.20, 0.10, 0.05, 0.025 and 0.0125 µg/mL) for 24 and 48 hrs (for chromosomal aberrations and micronuclei tests, respectively). The concentration of 0.10 µg/mL represents the acceptable/tolerable daily intake reference dose established by European Union, whereas 0.20, 0.05, 0.025 and 0.0125 µg/mL represent multiple and sub-multiple of this concentration value. Our results reported cytogenetic effects of BP-3 on cultured human lymphocytes in terms of increased micronuclei and chromosomal aberrations frequencies at all tested concentrations, including concentrations lower than those established by European Union. *Vice versa*, after 48-h exposure, a significant reduction of the cytokinesis-block proliferation index value in cultures treated with BP-3 was not observed, indicating that BP-3 does not seem to produce effects on the proliferation/mitotic index when its concentration is equal or less than 0.20 µg/mL.

Keywords:

BP-3, sunscreen, UV filters, genotoxicology.

Abbreviations

Ab.C = Aberrant Cell

AF = Acentric Fragments

BP = Benzophenone

BP-1 = 2,4- dihydroxybenzophenone

BP-2 = 2,2′,4,4′-tetrahydroxybenzophenone

BP-3 = 2-hydroxy-4-methoxybenzophenone

BP-8 = 2,2′-dihydroxy-4-methoxybenzophenone

4-OH-BP = 4-hydroxybenzophenone

B' = chromatid breaks

B'' = chromosome breaks

bw = body weight

CAs = Chromosomal Aberrations

CBPI = Cytokinesis-Block Proliferation Index

Dic = Dicentrics

EU = European Union

EFSA = European Food Safety Authority

FCS = Foetal Calf Serum

KCl = Potassium chloride

MNs = Micronuclei

OECD = Organization for Economic Co-operation and Development

R = Rings

Re = Rearrangements

TDI = Tolerable Daily Intake

TR = tri- or tetra-radials

Introduction

Ultraviolet (UV) filters are widely used in sunscreens and personal care products, such as cosmetics and shampoos, for the protection of skin and hair from UV irradiation (Chisvert et al., 2012; Asimakopoulos et al., 2014). They are also present as chemical ingredients of insecticides, agricultural chemicals and pharmaceuticals (Careghini et al., 2015), as well as they are used to coat surfaces exposed to sunlight, including some food packaging (Vione et al., 2013).

Benzophenone (BP)-type chemicals are one of the primary components in the UV-filter family, detected at high concentrations in biological fluids of different populations worldwide distributed (Calafat et al., 2008; Wang and Kannan, 2013).

The 2-Hydroxy-4-methoxybenzophenone (BP-3), also known as oxybenzone, is a compound naturally occurs in flower pigments that can adsorb sunlight in the UVA and UVB regions (French, 1992). For this reason and for its limited phototransformation, it is one of the most commonly-used chemical components in sunscreen and cosmetic products (Careghini et al., 2015; Kim and Choi, 2014), found in 59% of sunscreens in the United States (Dewalque et al., 2014). BP-3 is also employed as photostabiliser in food packaging materials, to prevent polymer photochemical degradation, in the treatment of photodermatitis, as well as in plastic surface coatings and polymers (Vione et al., 2013).

However, from ecological point of view, the increasing use of UV filters constitutes a potential risk for the environment. Indeed, these filters are often inert in traditional wastewater treatment processes, and thus have the potential to contaminate the reclaimed water system, natural water bodies and drinking water resources (Xiao et al., 2013). Moreover, these chemicals are also directly released into surface waters through swimming, bathing, leaching of land and house coatings. As final result, BP-3 and other UV-filter components were detected in several environmental matrices such as in surface and tap waters and in sediments (Balmer et al., 2005, Kameda et al., 2011; Gago-Ferrero et al., 2011). In particular, BP3 has been detected at levels of up to some μg/L in raw wastewater, at tens to several hundreds ng/L in treated wastewater, up to a hundred ng/L in lake water and at ng/g levels in solid matrices and in biota (Vione et al., 2013).

Humans are exposed to BP-type UV filters largely through dermal absorption. Because of the extensive use in personal care products, BP-3 and its metabolic derivatives, such as 2,4-dihydroxybenzophenone (BP-1), 2,2′,4,4′-tetrahydroxybenzophenone (BP-2), 2,2′-dihydroxy-4-methoxybenzophenone (BP-8), and 4-hydroxybenzophenone (4-OH-BP), were found widely in various human bodily fluids, such as urine (Calafat *et al*., 2008; Kunisue *et al*., 2012; Wolff *et al*., 2007), breast milk (Ye et al., 2008a), blood (Ye et al., 2008b), and semen (León et al., 2010). In particular, these compounds have been reported in urines of a high percentage of subjects from many countries, including United States (Calafat et al., 2008; Philippat et al., 2015; Wang and Kannan, 2013), China (Wang and Kannan, 2013; Zhang et al., 2013), and Europe (Asimakopoulos et al., 2014; Philippat et al., 2012).

To date, the reproductive and developmental toxicity of BP-type UV filters has been revealed in some animal and human studies. A few BP-type UV filters, BP-3 included, have been suspected to have endocrine disrupting effects, with consequent alterations in the reproductive system and with *in vitro* and *in vivo* estrogenic and anti-androgenic effects (Schlumpf et al., 2001; Suzuki et al., 2005). For example, BP-3 was found able to affect the reproduction of fishes (Bluthgen *et al*., 2012; Gago-Ferrero and Diaz-Cruz, 2012) and other aquatic organisms, such as the crustacean *Daphnia magna* (Fent et al., 2010) and the chlorophyte microalgae *Scenedesmus vacuolatus* (Rodil et al., 2009), as well as was found able to induce vitellogenin and impairment of reproduction in rainbow trout and in Japanese medaka (Coronado et al., 2008). Moreover, after oral administration of benzophenone, a significant increase of the incidence of mononuclear-cell leukaemia, hepatoblastoma and hepatocellular carcinoma in male mice and histiocytic sarcoma in female mice was observed (IARC, 2013).

In humans, BP-type UV filters have been linked to various endocrine and reproductive disorders, such as reducted couple fecundity and semen quality (Buck Louis et al., 2014, 2015), birth outcomes (Wolff et al., 2008), uterine leiomyoma (Pollack et al., 2015) and endometriosis (Kunisue et al., 2012).

The Council of Europe reported maximum BP-3 levels of 0.5 mg/kg for beverages and 2 mg/kg for foods (IARC, 2013). The European Food Safety Authority estimated a dietary exposure to benzophenone in the European Union (EU) and in the USA correspondent to 23 and 11 µg per capita per day, respectively (IARC, 2013). Based on published toxicological data, the EU established a temporary tolerable daily intake (TDI) and an acceptable daily intake (ADI) for BP-3 of 0.1 mg/kg bw (Conseil of Europe, 2009).

From the genetic and cytogenetic point of view, some studies evidenced that BP-type UV-filters were able to induce mutagenic effects in *Salmonella* (Zeiger et al., 1987), sister chromatid exchanges and chromosomal aberrations (CAs) in Chinese hamster ovary cells (French, 1992). However, no data are reported in literature about *in vitro* cytogenetic effects of BP-3 on human lymphocytes. For this reason, in the present study we assessed the possible *in vitro* clastogenic and aneugenic effects of BP-3 exposure on human lymphocyte cells, by CAs and micronuclei (MNs) assays.

The CAs assay allows the detection of cells carrying unstable aberrations (chromosome and chromatid breaks, deletions, fragments, rings, dicentrics and chromatid exchanges) that will lead to cell death during proliferation, an thus represents a useful test for the detection of potential clastognic effects of xenobiotics. MNs represent acentric chromosomal fragments or whole chromosomes left behind during mitotic cell division and appear in the cytoplasm of interphase cells as small additional nuclei. With respect to the CAs assay, the MNs assay allows the detection of both potential clastogenic (chromosome breakage) or aneugenic (chromosome lagging due to dysfunction of mitotic apparatus) effects of different xenobiotics. Interestingly, previous published studies provided strong evidence in support of the hypothesis that high CAs and MNs frequencies in peripheral blood lymphocytes are powerful predictors of cancer risk (Bonassi et al., 2004) and are positively associated with early events in carcinogenesis (Bonassi et al., 2011).

Methods

Chemicals and Media

The IUPAC name of Oxybenzone (CAS n. 131-57-7) is 2-Hydroxy-4-methoxybenzophenone or BP-3.

The BP-3 was dissolved in dimethyl sulfoxide (DMSO, CAS no. 67-68-5). Gibco RPMI 1640 cell culture media supplemented with L-glutamine, foetal calf serum, phytohemagglutinin (PHA), and antibiotics were purchased from Invitrogen-Life Technologies, Milan, Italy. Cytochalasin-B, Mitomycin-C (MMC), DMSO, BP-3 and Giemsa stain solution were obtained from Sigma-Aldrich, Milan, Italy. Methanol, Acetic acid, and conventional microscope slides were purchased from Carlo Erba Reagenti, Milan, Italy. Potassium chloride (KCl) and Sörensen buffer were obtained from Merck S.p.A., Milan, Italy. Vacutainer blood collection tubes were from Terumo Europe, Rome, Italy.

Subjects

Peripheral venous blood was collected from 5 healthy Italian subjects (2 males and 3 females, mean age±S.D., 31.50±14.39), non-smoking, non-alcoholic, not under drug therapy, and with no recent history of exposure to mutagens. Informed consent was obtained from all blood donors. The study was approved by the Univeristy of Turin ethics committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Blood Sample Collection and Lymphocyte cultures

Blood samples were obtained by venipuncture and collected in heparinised tubes, for genotoxicity testing. All blood samples were coded, cooled (4°C), and processed within 2 h after collection. Heparinized venous blood (0.3 mL) was cultured in 25 cm^2 flasks in 6 mL of RPMI-1640 medium supplemented with 20% foetal calf serum (FCS), 2% of the mitogenic agent PHA, L-glutamine (2 mM), antibiotics (100 IU/mL penicillin, and 100 µg/mL streptomycin). The cultures were incubated for 72 h at 37°C, under 5% of CO_2 in the air in a humidified atmosphere. After 24 h of incubation, BP-3 dissolved in DMSO (for stock solution preparation 200 µg of BP-3 were dissolved in 1 mL of DMSO) was added to the cultures to a final concentrations of 0.20 µg/mL, 0.10 µg/mL, 0.05 µg/mL, 0.025 µg/mL and 0.0125 µg/mL. The concentration at 0.10 µg/mL represents the Tolerable Daily Intake (TDI) concentration established by European Union (0.1 mg/Kg BW) for this compound (Councile of Europe, 2009); 0.20 µg/mL is a multiple of the TDI, whereas 0.05, 0.025 and 0.0125 µg/mL represent sub-multiple of this value, tested in order to determine the genotoxicity threshould limit.

Three control cultures were assessed: 1) positive control, by adding only MMC (final concentration 0.1 µg/mL culture); 2) solvent control, by adding only 0.1% of DMSO; 3) negative control, culture without both BP-3 and DMSO. Only for MNs assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 µg/mL to block cytokinesis. After 48 h (for CAs assay) and 72 h (for MNs assay) of incubation at 37°, the cells were collected by centrifugation and treated for 10 min with a pre-warmed mild hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a fresh mixture of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times. Finally, the supernatant was discarded and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect CAs and MNs by conventional staining with 5% Giemsa (pH 6.8) prepared in Sörensen buffer.

Cytokinesis-Block Micronucleus Assay

Microscopic analysis was performed at 40X magnification on a light microscope (Dialux 20, Leica, Germany). MNs were scored in 1,000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 5,000 binucleated cells per concentration), following the established criteria for MNs evaluation (Fenech et al., 2003). A total of 1,000 lymphocytes per donor per concentration were scored to evaluate the percentage of cells with 1-4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated, according to the following formula:

$[1 \times N1] + [2 \times N2] + [3 \times (N3 + N4)]/N$, where N1–N4 represents the number of cells with 1-4 nuclei, respectively, and N is the total number of cells scored.

Chromosomal Aberrations Assay

Microscope analysis was performed at 1000X magnification on a light microscope (Dialux 20, Leica, Germany). Althogh the Organization for Economic Co-operation and Development (OECD) guidelines call for 100 metaphases to be scored for each dose (OECD, 2015), in order to obtain results more significant and suitable for statistical analysis, it is the normal practice in our laboratory to score 200 metaphases *per* subject *per* dose. Thus, in the present work, for each subject and BP-3 concentration, 200 well-spread first-division complete metaphases (for a total of 1000 metaphases for each dose) were analysed for the following categories of CAs: gaps, chromatid breaks (B'), chromosome breaks (B"), dicentrics (Dic), rings (R), tri- or tetra-radials (TR), acentric fragments (AF), rearrangements (Re) and numerical aberrations. Cells containing one of more types of CAs were scored as "aberrant cell" (Ab.C).

With regard to the opportunity to include gaps in the statistical analyses, the discussion is open. Although some authors considered gaps as the appropriate indicator of genotoxic potential of chemicals (Savage, 2004), the molecular mechanism of BP-3 to induce achromatic lesion/gaps is yet to be revealed. Thus, in our statistical analyses we decided to exclude gaps. The criteria for distinguishing chromatide breaks from gaps were the acentric piece displaced with respect to the chromosome axis and the size of the discontinuity, which exceeded the width of the chromatide. A dicentric with an acentric fragment was scored as one aberration.

Statistical analysis

Comparison of mean values of the percentage of cells with MNs, CBPI and CAs between exposition levels and their controls was assessed by the non-parametric Mann-Whytney test. Statistical calculations were carried out using the SPSS software package program (version 23.0, Inc., Chicago, IL, USA). All *P* values were two tailed, and *P* values of 5% or less were considered statistically significant for all tests carried out.

Results

Effect of BP-3 on CAs formation

Table 1 shows values of CAs found in the human peripheral lymphocytes cultured in the presence of different concentrations of BP-3. BP-3 was found to induce seven types of structural CAs (gaps, chromatid and chromosome breaks, dicentric chromosomes, rings, tri- or tetraradials, acentric fragments and rearrangements). The most frequent observed aberrations were acentric fragments and chromatid aberrations, respectively, wheras no numerical aberrations were found. In figure 1 some examples of chromosomal aberrations observed at 0.1 μg/mL of BP-3 concentration were showed.

Data obtained indicated that human lymphocytes treated *in vitro* with BP-3 significantly increased the CAs and Ab.C frequencies at all tested concentrations when compared with the solvent control, including the lowest concentration of 0.0125 μg/mL that represents an eighth of the TDI value established by EU for this substance. Moreover, a dose-effect was observed since the regression analysis revealed a significant (P<0.001) correlation between the BP-3 concentrations and the level of genomic damage (Table 3).

No significant differences were found between the DMSO solvent-control and the negative control ($P = 0.142$), whereas the cultures treated with the known mutagen mitomycin-C showed a significant increase of CAs and Ab.C with respect to all BP-3 tested concentrations ($P < 0.05$), including the negative and solvent control cultures ($P = 0.008$ and $P = 0.009$, respectively).

Effect of BP-3 on MNs formation

To verify both the aneugenic and clastogenic effects of BP-3, the MN test was assessed in parallel with CAs test (Table 2). Similarly to what we already observed with the CAs assay, our results indicated that BP-3 significantly increased the MNs formation at all concentrations tested, including both 0.10 µg/mL, that represents the TDI established by EU for this substance, and 0.0125 µg/mL that represents an eighth of this limit value. Also in this case, a dose-effect was observed since the regression analysis revealed a significant ($P<0.001$) correlation between the BP-3 concentrations and the frequencies of MNs and Cells with MNs (Table 3).

The DMSO solvent-control cultures did not show any difference with the negative controls ($P = 0.126$), further confirming that at this low concentration DMSO has no cytogenetic effects evaluable by MN test. Similarly to what we observed with CAs assay, cultures treated with the mutagen MMC showed a significant increase in the MNs formation compared with the negative control ($P = 0.008$), solvent controls ($P = 0.008$) and all tested concentrations of BP-3 ($P = 0.009$ for all concentrations). Finally, after 48-h exposure, a significant reduction of the CBPI value in cultures treated with BP-3 was not observed, indicating that at the tested concentrations, BP-3 does not seem to produce effects on the proliferation/mitotic index, as confirmend by the regression analysis (Tables 2 and 3).

In Figure 2 examples of bi- and tri-nucleated cells with micronuclei, observed at 0.1 µg/mL of BP-3 concentration, were reported.

Discussion

BP-3 is widely used as sunscreen for protection of human skin and hair from damage by ultraviolet radiation. It has been found in many cosmetic products and its production and use has been rapidly increasing over the past decade. As a consequence of this increased production, BP-3 was found to have a wide presence in aquatic environments, affecting the water quality and, consequently, the human health. For these reasons, it is considered as a personal care product of emerging environmental concern and its concentration limits, in the final products for human consumption, are now regulated by the United States Food and Drug Administration and the European Commision.

Here, we discuss data obtained from our *in vitro* study conducted in order to establish the clastogenic and aneugenic potential of BP-3 on human cultured lymphocytes. The results obtained with CAs and MNs assays, evidenced a possible clastogenic effect of the BP-3 on human lymphocytes, also at the concentration of 0.0125 µg/mL that represents a value eight times lower with respect to the reference dose established for humans by EU (Conseil of Europe, 2009). This increased cytogenetic damage observed at all tested concentrations pushes towards the adoption of more safe concentration for human health with respect to the established value of 0.1 µg/mL. Indeed, increased CAs and MNs frequencies in peripheral blood lymphocytes have been positively associated with increased cancer risk and early events in carcinogenesis, respectively (Bonassi et al., 2004; 2011).

The genotoxicity of BP-type UV-filters has previously been observed by *in vivo* and *in vitro* studies using non-human organisms and cell systems (Zeiger et al., 1987; French, 1992). However, beyond these old reports, no pubblications are present in literature about the possible *in vitro* clastogenic effect of BP-3 on human lymphocytes. For this reason, we cannot compare our data with other similar data.

Notably, for BP-3 we observed a clastogenic effect also at the lower concentrations of 0.025 and 0.0125 μg/mL, whereas for the BP-A, another endocrine disruptor compound with a very similar molecular weight to that of BP-3, we failed to found it (Santovito et al., 2017). This more acute effect of BP-3 with respect of BP-A has been observed, by our group, also in *in vivo* experiments conducted using the marine polychaete *Ophryotrocha diadema.* In this case, we found an increased mortality and a lower production of eggs among polychaete treated with the same concentrations of BP-3 used in the present study, compared to those treated with identical BP-A concentrations (data not showed).

The clastogenic properties of the BP-3 could be ascribed to its demonstrated ability to generate ROS, probably by decreasing the activities of antioxidant enzymes and increasing lipid peroxidation (Wnuk et al., 2017). Indeed, Hanson et al. (2006) demonstred that BP-3 and other UV-filters are able to penetrate through the stratum corneum and to generate higly reactive oxygen species in the cytoplasm of the epidermic nucleated keratinocytes.

Moreover, the demonstrated accessibility of the BP-3 to the labile hydrogens within the DNA (Marazzi et al., 2016) could be another possible explanation of this observed clastogenic effect, as well as of the increased levels of genomic damage observed for BP-3 with respect to BP-A. This is an important issue because increased levels of genomic damage were associated with an increased risk of cancer development (Bonassi et al., 2004; 2011), and in particular BP-3 was found able to increase metastatic potential in lung cancer cells (Phiboonchaiyanan et al., 2017).

Although recent studies on BP-3 have demonstrated its cytotoxicity properties (Balázs et al., 2016**)** and its capacity to activate apoptosis (Wnuk et al., 2017), in the present study we did not observed a significant reduction of the CBPI, indicating that BP-3 does not seem to produce effects on the proliferation/mitotic index when its concentration is equal or less than 0.20 μg/mL (Tables 2 and 3).

Finally, it should be also emphasized that the observed cytogenetic damage cannot be ascribed exclusively to the direct effect of BP-3 on lymphocytes. Indeed, *in vitro* studies showed that BP-3 is converted to more hydrophilic and DNA-binding metabolites, such as BP-1, BP-2, BP-8 and 4-

OH-BP (Buck Louis et al., 2014). In this scenario, the irreversible binding of BP-3 derived compounds to the DNA could cause bases loss from the DNA strand and could be responsible for some of the BP-3 observed toxic effects. It is known that many chemicals, either directly or after conversion to reactive metabolites, covalently modify nucleosides of DNA, leading to mutational changes ans, thus, playing a role in cellular toxicity or tumorigenesis induction (Atkinson and Roy, 1995).

Conclusion

The results herein reported demonstrate, for the first time in literature, the clastogenic and aneugenic effects of BP-3 on cultured human lymphocytes, by increasing CAs and MNs frequencies also at lower concentrations with respect to those established by European Union. However, it should be emphasized that the ADI and TDI concentrations of BP-3 established by the EU were not addressed to reference limits for *in vitro* studies finalized to the assessment of a mutagenic or genotoxic potential of this compound. They were the results of *in vivo* studies conducted principally on mice, rats and rabbits analysed after oral and dermal administration of BP-3 (IARC, 2013), taking also into account the BP-3 excretion capacity of the studied organism. Moreover, it is also necessary to remark that this observed *in vitro* genotoxic potential on human lymphocytes does not seems to manifest itself in *in vivo* experiments using animal models like *Drosophila* and rat (Robison et al., 1994). Nevertheless, although simple experimental models, like mammalian and bacterial cells, cannot accurately mimic the complex kinetics of xenobiotic compounds *in vivo*, our results suggest the need for further investigations about this compound and, eventually, the adoption of more stringent measures in order to reduce the presence of this compound in the environment and to minimize the adverse effects of the BP-3 exposure on human health.

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Conflict of Interest

The authors report no conflicts of interest.

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Table 1 - Induction of chromosomal aberrations by 2-Hydroxy-4-methoxybenzophenone in human lymphocytes *in vitro*.

Test substance	**Treatment**		**Total Scored Metaphases**	**CAs**								**Total CAs**	**Total CAs + Gaps**	**Total Ab.C**	**Total Ab.C + Gaps**	**CAs/Cell ± S.E. (%)**	**Ab.C/Cell ± S.E. (%)**
	Period (h)	**Dose (µg/ml)**		**Gaps**	**B'**	**B''**	**DC**	**R**	**TR**	**AF**	**Re**						
NC	----	----	1000	5	6	0	1	0	0	8	0	15	21	15	21	1.500±0.158	1.500±0.158
0.1% DMSO	24	----	1000	10	9	0	2	0	0	8	2	21	31	21	31	2.100±0.367	2.100±0.367
MMC	24	0.10	1000	39	37	31	12	8	18	21	17	144	183	136	175	14.400±1.336 [a]	13.600±1.177 [a]
BP-3	24	0.20	1000	32	32	21	5	3	0	24	16	101	133	99	131	10.100±0.510 [b]	9.900±0.400 [b]
BP-3	24	0.10	1000	22	32	11	4	4	1	26	15	93	115	93	115	9.300±0.875 [b]	9.300±0.875 [b]
BP-3	24	0.05	1000	13	21	11	2	0	0	24	16	74	87	71	84	7.400±1.308 [b]	7.100±1.134 [b]
BP-3	24	0.025	1000	13	20	7	2	2	0	31	11	73	85	72	85	7.300±1.446 [b]	7.200±1.347 [b]
BP-3	24	0.0125	1000	10	11	5	0	1	0	17	1	35	45	35	45	3.500±0.548 [c]	3.500±0.548 [c]

CAs = chromosomal aberrations; Ab.C = aberrant cells (cells with 1 ore more aberrations); NC = Negative Control; MMC = Mitomycin-C; BP-3 = 2-Hydroxy-4-methoxybenzophenone; B': chromatid break; B'': chromosome break; DC: dicentric; R: ring; TR = tri-tetraradials; AF = acentric fragments; Re = rearrangements; S.E. = standard error.

[a] Significantly different with respect to DMSO control solvent *P* = 0.009; [b] Significantly different with respect to DMSO control solvent *P* = 0.008; [c] Significantly different with respect to DMSO control solvent *P* = 0.043

Table 2 – Induction of micronuclei by 2-Hydroxy-4-methoxybenzophenone in human lymphocytes *in vitro*.

Test substance	**Treatment**		**BNCs scored**	**Distribution of BNCs according to the number of MNs**				**MNs**	**Ab.C**	**MNs/cell ± S.E. (%)**	**Ab.C/cell ± S.E. (%)**	**CBPI ± S.E**
	Period (h)	**Dose (µg/ml)**		**1**	**2**	**3**	**4**					
NC	----	----	5000	11	0	0	0	11	11	0.220±0.000	0.220±0.000	1.732±0.025
0.1% DMSO	48	----	5000	16	0	0	0	16	16	0.320±0.000	0.320±0.000	1.811±0.062
MMC	48	0.10	5000	135	12	8	0	183	155	3.660±0.005 [a]	2.675±0.003	1.345±0.020
BP3	48	0.20	5000	69	4	0	0	77	73	1.540±0.001 [a]	1.525±0.002 [a]	1.684±0.017
BP3	48	0.10	5000	70	4	1	0	81	75	1.620±0.002 [a]	1.425±0.002 [a]	1.603±0.041
BP3	48	0.05	5000	44	1	0	0	46	45	0.920±0.001 [a]	0.850±0.001 [a]	1.690±0.033
BP3	48	0.025	5000	41	3	0	0	47	44	0.940±0.001 [b]	0.880±0.001 [b]	1.696±0.029
BP3	48	0.0125	5000	31	1	0	0	33	32	0.660±0.002 [c]	0.640±0.002 [c]	1.710±0.067

BNCs = Binucleated cells; MNs = micronuclei; Ab.C = Aberrant cells (cells with 1 or more MNs); NC = Negative Control; MMC = Mitomycin-C; BP-3 = 2-Hydroxy-4-methoxybenzophenone; S.E. = Standard Error. CBPI = Cytokinesis-Block Proliferation Index.

[a] Significantly differ with respect to DMSO control solvent P = 0.008; [b] Significantly differ with respect to DMSO control solvent P = 0.011;

[c] Significantly differ with respect to DMSO control solvent P = 0.025

Table 3 - Multiple regression analysis evaluating the relationship between BP-3 concentrations and the level of genomic damage

Biomarkers	**β-co**	***P*-value**	**95% CI** (Lower) – (Upper)
CAs	0.705	<0.001	(1.719) – (4.361)
Cells with CAs	0.723	<0.001	(1.751) – (4.209)
MNs	0.690	<0.001	(1.335) – (3.545)
Cells with MNs	0.724	<0.001	(1.332) – (3.188)
CBPI	0.227	0.274	(-0.041) – (-0.012)

CAs = Chromosomal Aberrations; MNs = micronuclei; CBPI = Cytokinesis-Block Proliferation Index; β-co = β-coefficient; CI = Confidence Interval

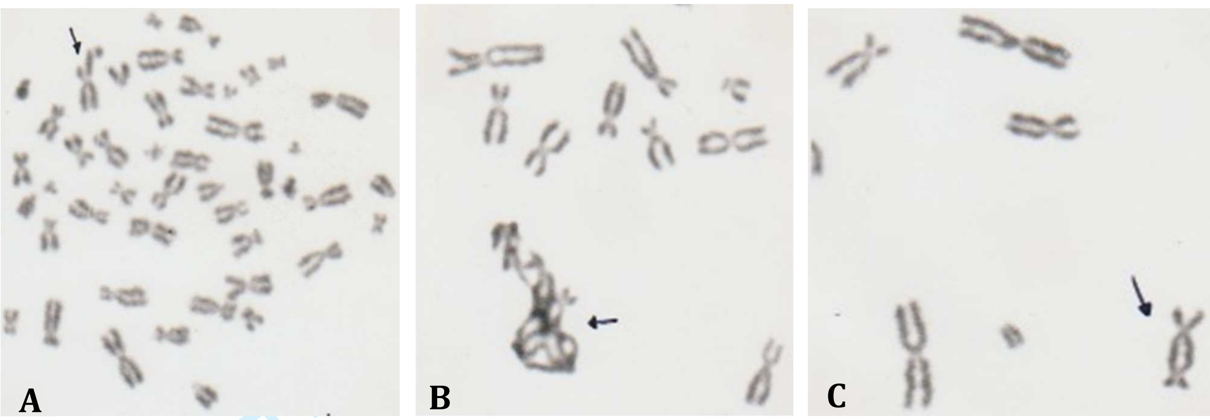

Figure 1 – Example of complete metaphase carrying a chromosomal aberration (A) and other two different metaphases details showing some examples of observed chromosomal aberrations. The arrows indicate, respectively: chromatidic break (Figure A), complex rearrangement (Figure B), dicentric chromosome (C). All these aberrations were observed at 0.1 µg/mL concentration of BP-3.

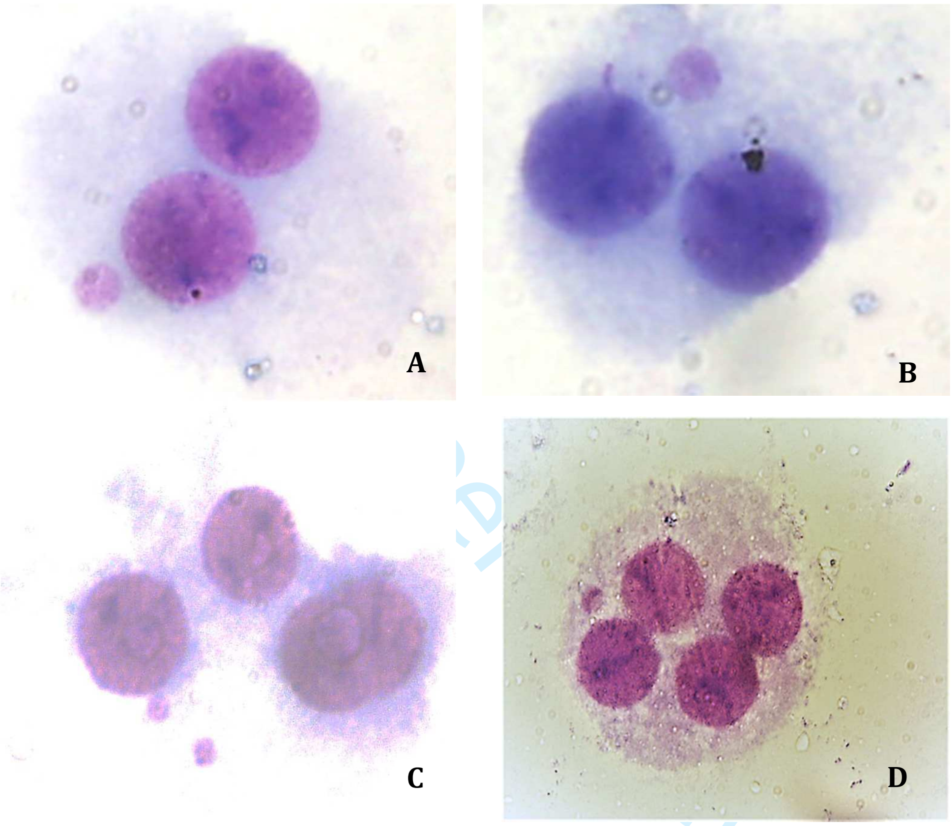

Figure 2 – Examples of observed micronuclei in bi-nucleated cells (A and B), tri-nucleated cell (C) and tetra-nucleated cell (D), observed at 0.1µg/mL concentration of BP-3. According to standardized procedures, micronuclei of tri- and tetra-nucleated cells were not scored in the evaluation of the total genomic damage.