

County Clerk

From: Joe DiNardo <jmjdinardo@aol.com>
Sent: Sunday, November 26, 2017 7:14 AM
To: IEM Committee; County Clerk
Cc: cadowns@haereticus-lab.org
Subject: Oxybenzone HEL Monograph 6 of 9
Attachments: 47 Watanabe Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes.docx; 48 Krause IntJAndrology 2012 sunscreens review.pdf; 49 Kunisue Urinary concentrations of benzophenone-type UV filters in U.S. women.docx; 50 Pollack Bisphenol A, benzophenone-type ultraviolet filters, and phthalates in relation to uterine leiomyoma.docx; 51 Wolff Environmental phenols and pubertal development in girls.docx; 52 Kunz Comparison of in vitro and in vivo estrogenic activity of UV filters in fish.docx; 53 Kinnberg Endocrine disrupting effect of benzophenone-3.docx; 54 Rodriguez Ecotoxicol Environ Saf.docx; 55 Nimrod Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens.docx; 56 Coronado Estrogenic activity and reproductive effects.docx; 57 Bluthgen Effects of the UV filter benzophenone-3.docx; 58 Silvia et al Oxybenzone metabolites.docx; 59 Chen Fighting Beta Fish Ecotoxicology.docx; 60 Paredes Chemosphere 2014 Ecotoxicological evaluation of four UV f.pdf; 61 Effects of benzophenone-3 to green algae and cyanobacteria.pdf; 62 Wolff Prenatal phenol and phthalate exposures and birth outcomes.docx; 63 Tang et al Environ Pollut 2013 prenatal exposure birth outcomes.pdf; 64 Huo Oxybenzone mechanism for causing Hirschsprung Disease in human infants.pdf; 65 Wnuk Benzophenone 3 Impairs Autophagy.pdf

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,
Craig Downs – Executive Director – Haereticus Environmental Laboratory
Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:

- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
 - 1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
 - 2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

47) *Toxicol Appl Pharmacol.* 2015 Jan 15;282(2):119-28. doi: 10.1016/j.taap.2014.12.002. Epub 2014 Dec 17.

Metabolism of UV-filter benzophenone-3 by rat and human liver microsomes and its effect on endocrine-disrupting activity.

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Abstract

Benzophenone-3 (2-hydroxy-4-methoxybenzophenone; BP-3) is widely used as sunscreen for protection of human skin and hair from damage by ultraviolet (UV) radiation. In this study, we examined the metabolism of BP-3 by rat and human liver microsomes, and the estrogenic and anti-androgenic activities of the metabolites. When BP-3 was incubated with rat liver microsomes in the presence of NADPH, 2,4,5-trihydroxybenzophenone (2,4,5-triOH BP) and 3-hydroxylated BP-3 (3-OH BP-3) were newly identified as metabolites, together with previously detected metabolites 5-hydroxylated BP-3 (5-OH BP-3), a 4-desmethylated metabolite (2,4-diOH BP) and 2,3,4-trihydroxybenzophenone (2,3,4-triOH BP). In studies with recombinant rat cytochrome P450, 3-OH BP-3 and 2,4,5-triOH BP were mainly formed by CYP1A1. BP-3 was also metabolized by human liver microsomes and CYP isoforms. In estrogen reporter (ER) assays using estrogen-responsive CHO cells, 2,4-diOH BP exhibited stronger estrogenic activity, 2,3,4-triOH BP exhibited similar activity, and 5-OH BP-3, 2,4,5-triOH BP and 3-OH BP-3 showed lower activity as compared to BP-3. Structural requirements for activity were investigated in a series of 14 BP-3 derivatives. When BP-3 was incubated with liver microsomes from untreated rats or phenobarbital-, 3-methylcholanthrene-, or acetone-treated rats in the presence of NADPH, estrogenic activity was increased. However, liver microsomes from dexamethasone-treated rats showed decreased estrogenic activity due to formation of inactive 5-OH BP-3 and reduced formation of active 2,4-diOH BP. Anti-androgenic activity of BP-3 was decreased after incubation with liver microsomes.

REVIEW ARTICLE

Sunscreens: are they beneficial for health? An overview of endocrine disrupting properties of UV-filters

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Summary

Keywords:

benzophenone-3, endocrine disruptors, homosalate, malignant melanoma, OD-PABA, PABA, sunscreens, UV-filters, 3-benzylidene camphor, 3-(4-methyl-benzylidene) camphor, 2-ethylhexyl 4-methoxy cinnamate

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Today, topical application of sunscreens, containing ultraviolet-filters (UV-filters), is preferred protection against adverse effects of ultraviolet radiation. Evidently, use of sunscreens is effective in prevention of sunburns in various models. However, evidence for their protective effects against melanoma skin cancer is less conclusive. Three important observations prompted us to review the animal data and human studies on possible side effects of selected chemical UV-filters in cosmetics. (1) the utilization of sunscreens with UV-filters is increasing worldwide; (2) the incidence of the malignant disorder for which sunscreens should protect, malignant melanoma, is rapidly increasing and (3) an increasing number of experimental studies indicating that several UV-filters might have endocrine disruptive effects. The selected UV-filters we review in this article are benzophenone-3 (BP-3), 3-benzylidene camphor (3-BC), 3-(4-methyl-benzylidene) camphor (4-MBC), 2-ethylhexyl 4-methoxy cinnamate (OMC), Homosalate (HMS), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA) and 4-aminobenzoic acid (PABA). The potential adverse effects induced by UV-filters in experimental animals include reproductive/developmental toxicity and disturbance of hypothalamic–pituitary–thyroid axis (HPT). Few human studies have investigated potential side effects of UV-filters, although human exposure is high as UV-filters in sunscreens are rapidly absorbed from the skin. One of the UV-filters, BP-3, has been found in 96% of urine samples in the US and several UV-filters in 85% of Swiss breast milk samples. It seems pertinent to evaluate whether exposure to UV-filters contribute to possible adverse effects on the developing organs of fetuses and children.

Abbreviations

3-BC, 3-benzylidene camphor; 4-MBC, 3-(4-methyl-benzylidene)-camphor; ↑, Increased; ↓, Decreased; AhR, aryl hydrocarbon receptor; AR, androgen receptor; BP-3, benzophenone-3; bw, body weight; C3, complement protein 3; Dio1, 5′deiodinase type I; EDC, endocrine disrupting chemicals; ER, oestrogen receptor; ERR1, oestrogen receptor related receptor 1; F0, parent rats; F1, 1. generation of rat offspring; FDA, Food and Drug Administration; FRTL-5, normal, non-transformed rat thyrocytes; FT3, free triiodothyronine; FT4, free thyroxine; hAR, human androgen receptor; HepG2, human hepatocarcinoma cell line; hER, human oestrogen receptor; HMS, homosalate; hPR, human progesterone receptor; HPT, hypothalamic-pituitary-thyroid axis; hrTPO, human recombinant thyroid peroxidase; IGF-I, insulin-like growth factor-I; LDH, lactate dehydrogenase; LOAEL, lowest observed adverse effect levels; M, male; MCF7, human breast cancer cells; ME, malic enzyme; MM, malignant melanoma; MPO, medial preoptic area; N-Cor, nuclear receptor co-repressor; NHANES, National Health and Nutrition Examination Survey; NOAEL, no observed adverse effect levels; OCT, 2-cyano-3,3-diphenyl acrylic acid; OD-PABA, 2-ethylhexyl 4-dimethylaminobenzoate; OMC, 2-ethylhexyl-4-methoxy cinnamate; P, plasma; ORG2058, PR agonist; PABA, 4-aminobenzoic acid; PND, post natal day; PND1, day of birth; PR, progesterone receptor; rtER, rainbow trout oestrogen receptor; SRC-1, steroid receptor coactivator-1; T3, total triiodothyronine; T4, total thyroxine; TBG, thyroxine-binding globulin; TPO, thyroid peroxidase; TR, thyroid hormone receptor; TRH, thyrotropin releasing hormone; TSH, thyroid-stimulating hormone; U2-OS, cells, human osteosarcoma cells; UV-A, ultraviolet radiation with wavelength A, 320-400 nm; UV-B, ultraviolet radiation with wavelength B, 290-320 nm; VMH, ventromedial hypothalamic nucleus- plays important role in sexual behaviour and receptivity of female rats (191); VTG, vitellogenin (oestrogen-responsive gene products in fish).

Introduction

The first commercial sunscreen was developed in the 1930s to abrogate ultraviolet-B waveband (UV-B), and thus prevent sunburn (Rebut, 1990). In 1970, sunscreens were developed further to protect against both ultraviolet-A waveband (UV-A) and UV-B (Deep, 2010), because of their suggested causal role in the development of skin cancer, in particular malignant melanoma (MM) (Wang *et al.*, 2001; Gandini *et al.*, 2005). Today it is still questionable whether this aim has been achieved. There is no doubt that sunscreens protect against sunburn, solar keratosis, and non-melanoma skin cancer (Thompson *et al.*, 1993; Green *et al.*, 1999; Dupuy *et al.*, 2005). However, the only randomized trial examining the risk of MM after regular sunscreen use, found borderline statistical significance for a reduced incidence of new primary melanoma (Green *et al.*, 2011). In addition, despite use of sunscreens with

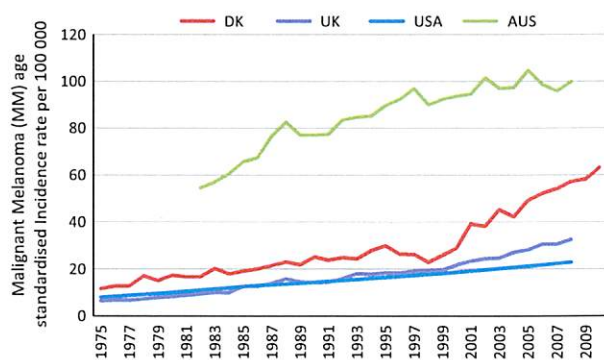


Figure 1 Age standardized MM incidence in USA, AU, NZ, UK and DK (Local cancer statistics). DK: Denmark: 1975–2000 (IARC); 2001–2010: Danish Cancer registers (Sundhedsstyrelsen, 2012). UK: United Kingdom: (Cancer Research UK). USA: (SEER). AUS: Australia: (Australian Institute of Health and Welfare [AIHW]).

Table 1 Most common UV-filters in cosmetics

	Generic name	Product name	Max concentration (%)	Spectrum of action	Approved
Chemical UV-filters	Benzophenone-3	BP-3	6 ^b –10 ^{a,c}	UV-A, UV-B	EU, US, AU
	2-Cyano-3,3-diphenyl acrylic acid	OCT	10	UV-B	EU, US, AU
	3-Benzylidene camphor	3-BC	2	UV-B	EU
	3-(4-Methyl-benzylidene) camphor	4-MBC	4	UV-B	EU, AU
	2-Ethylhexyl 4-methoxy cinnamate	OMC	7.5 ^b –10 ^{a,c}	UV-B	EU, US, AU
	Homosalate	HMS	10 ^a –15 ^{b,c}	UV-B	EU, US, AU
	2-Ethylhexyl 4-dimethylaminobenzoate	OD-PABA	8	UV-B	EU, US, AU
	4-Aminobenzoic acid	PABA	15 ^{b,c}	UV-B	US, AU
Physical UV-filter	Titanium dioxide		25	Physical	EU, US, AU
	Zinc oxide		25-no limit	Physical	US, AU

^aList of permitted UV-filters in the Council Directive of the European Committee.

^bList of permitted UV-filters in the US Food and Drug Administration monograph.

^cList of permitted UV-filters in the Australian regulatory guidelines for over-the-counter medicines (ARGOM), by the therapeutic Goods Administration.

UV-filters over decades (Figure S1), the incidence of MM is still increasing rapidly (Fig. 1) (Handel & Ramagopalan, 2010). Furthermore, an increasing number of experimental animal and in vitro studies indicated that some UV-filters might have adverse effects as endocrine disruptors.

In light of the high incidence of MM and considering the facts that the protective effect of sunscreens against MM has not been fully proven, we have reviewed the literature on possible endocrine disrupting effects of the most common chemical UV-filters used in cosmetics. Those are benzophenone-3 (BP-3), 3-benzylidene camphor (3-BC), 3-(4-methyl-benzylidene) camphor (4-MBC), 2-ethylhexyl 4-methoxy cinnamate (OMC), homosalate (HMS), 2-ethylhexyl 4-dimethylaminobenzoate (OD-PABA) and 4-aminobenzoic acid (PABA).

In vitro and in vivo adverse effects of UV-filters

A wide range of in vitro and in vivo studies have identified several UV-filters as endocrine disrupting chemicals (EDC) (European Commission) (Table S1). Table S1 lists results from in vivo and in vitro studies where possible adverse effects of the most common chemical UV-filters in cosmetics (Table 1) were examined. In the following the main results from Table S1 are summarized. A simplified version of Table S1 is presented in Table 2.

Influence on oestrogenic signalling

In vitro studies, investigating oestrogenic activity of UV-filters, varied in their design and endpoints, which may explain the diverging results. The majority of the in vitro studies reported that BP-3, 4-MBC, OMC, HMC and OD-PABA all exhibit oestrogenic activity (Schlumpf *et al.*, 2001b, 2004a; Schreurs *et al.*, 2002, 2005; Gomez *et al.*, 2005; Kunz *et al.*, 2006). However, not all of the UV-filters exhibiting in vitro oestrogenic activity were

Table 2 In vitro and In vivo effects of the most common UV-filters, used in cosmetics (increased: ↑; decreased: ↓)

Endpoint	References
BP-3	
Oestrogen activity: In vitro	
Binding to hER α	Morohoshi et al. (2005)
↑ Transactivation: ER α , ER β	Gomez et al. (2005), Kunz et al. (2006), Kunz & Fent (2006b), Morohoshi et al. (2005) and Schreurs et al. (2002)
Activation: hER β > hER α	Schreurs et al. (2005)
No Antagonism of ER β transactivation	Schreurs et al. (2002)
Antagonistic action > agonistic action on hER α	Kunz & Fent (2006b)
↑ MCF-7 cell proliferation	Schlumpf et al. (2001b)
Oestrogen activity: acute in vivo models	
Uterotrophic effect in immature rats	Schlumpf et al. (2001b)
Uterus of adult oophorectomized rats: unchanged weight, ↓ ER β , unchanged ER α , ERR1 and AhR expression	Schlecht et al. (2004)
No effect on VTG induction in juvenile fathead minnows	Kunz et al. (2006)
Androgen activity: In vitro	
Antagonism of hAR transactivation. No agonistic action	Kunz & Fent (2006b), Ma et al. (2003) and Schreurs et al. (2005)
Progesterone activity: In vitro	
Antagonism of hPR transactivation	Schreurs et al. (2005)
Reproductive organs: long-term exposure of adult animals	
Oral exposure : ↓epididymal sperm density (rat, mice), ↑abnormal spermatozoa (mice). Dermal exposure (mice): ↓epididymal sperm density	French (1992)
Dermal exposure (rats): no effect	
Oral exposure (mice); ↑oestrous cycle length, Dermal exposure (rats): no effect	French (1992)
Reproductive organs: developmental toxicity	
No data available	
Thyroid axis	
In vitro: ↑ TR transcription	Schmutzler et al. (2007b)
↓ ERR1, unchanged ER α , ER β and AhR expression in thyroid of adult oophorectomized rats (5 day)	Schlecht et al. (2004)
Additional organ toxicities and general toxicity	
↓Food consumption (2 weeks), ↓body weight, ↑ liver weight (13 weeks)	French (1992)

Table 2 Continued

Endpoint	References
BP-2	
Thyroid axis	
In vitro: ↓hTPO activity, unaltered iodide uptake	Schmutzler et al. (2007a)
adult oophorectomized rats (5 days): ↑TSH, ↓T4, altered Dio 1 activity (liver), unaltered TPO activity	
3-BC	
Oestrogen activity: In vitro	
Binding to hER β , no binding to hER α	Schlumpf et al. (2004a)
↑ Transactivation: ER α	Kunz et al. (2006) and Kunz & Fent (2006b)
Activation: hER β > hER α	Schreurs et al. (2005)
Antagonistic action > agonistic action on hER α	Kunz & Fent (2006b)
↑ MCF-7 cell proliferation	Schlumpf et al. (2001b)
Oestrogen activity: Acute in vivo models	
Uterotrophic effect in immature rats (3 days)	Schlumpf et al. (2001b)
↑ VTG induction in juvenile rainbow trouts and juvenile fathead minnows	Holbech et al. (2002) and Kunz et al. (2006)
Androgen activity: In vitro	
No antagonism of hAR transactivation	Ma et al. (2003)
Antagonism of hAR transactivation. No agonistic action	Holbech et al. (2002), Kunz & Fent (2006b) and Schreurs et al. (2005)
Progesterone activity: In vitro	
Antagonism of hPR transactivation	Schreurs et al. (2005)
Reproductive organs: Developmental toxicity	
Delayed male puberty in F1	Faass et al. (2009) and Schlumpf et al. (2008)
Oestrous cycle changes in F1	Schlumpf et al. (2008)
↓ Weight of uterus (high dose) and prostate (low dose) F1	Schlumpf et al. (2004b)
Altered gene expression in uterus and prostate in F1	
Central nervous system: Developmental toxicity	
Impaired female sexual behaviour in F1	Faass et al. (2009) and Schlumpf et al. (2008)
Additional organ toxicities and general toxicity	
Body weight in adult F1 (highest dose) (developmental study)	Schlumpf et al. (2004b)
↓ Length and weight, juvenile fathead minnows (14 days)	Kunz et al. (2006)
HMS	
Oestrogen activity: In vitro	
↑ Transactivation: hER α ,	Gomez et al. (2005)
↑ Transactivation: hER α > hER β	Schreurs et al. (2002, 2005)

Table 2 Continued

Endpoint	References
HMS	
No agonistic action: hER α , rER α	Kunz <i>et al.</i> (2006) and Kunz & Fent (2006b)
Antagonism of hER α transactivation, No antagonism at hER α or hER β	Kunz & Fent (2006b) Schreurs <i>et al.</i> (2002)
↑ MCF-7 cell proliferation	Schlumpf <i>et al.</i> (2001b)
Oestrogen activity: Acute in vivo models	
No uterotrophic effect in immature rats	Schlumpf <i>et al.</i> (2001b)
Androgen activity: In vitro	
Antagonism of hAR transactivation. No agonistic action	Ma <i>et al.</i> (2003) and Schreurs <i>et al.</i> (2005)
Agonistic and antagonistic action on hAR transactivation	Kunz & Fent (2006b)
Progesterone activity: In vitro	
Antagonism of hPR transactivation	Schreurs <i>et al.</i> (2005)
4-MBC	
Oestrogen activity: In vitro	
Binding to cytosolic ER	Tinwell <i>et al.</i> (2002) and Schlumpf <i>et al.</i> (2004a)
Binding to hER β	
No binding to hER α	Morohoshi <i>et al.</i> (2005)
↑ transactivation: hER α , hER β	Gomez <i>et al.</i> (2005) and Schreurs <i>et al.</i> (2002)
Activation hER α > hER β	Schreurs <i>et al.</i> (2005)
Activation hER α < hER β	Mueller <i>et al.</i> (2003)
No transactivation: hER α , rER α	Kunz <i>et al.</i> (2006), Kunz & Fent (2006b) and Morohoshi <i>et al.</i> (2005)
Antagonism of hER	Kunz & Fent (2006b) and Mueller <i>et al.</i> (2003)
No antagonism of hER	Morohoshi <i>et al.</i> (2005) and Schreurs <i>et al.</i> (2002)
↑ ER-mediated MCF-7 cell proliferation	Schlumpf <i>et al.</i> (2001b, 2004a) and Tinwell <i>et al.</i> (2002)
Oestrogen activity: Acute in vivo models	
Uterotrophic effect in immature rats	Schlumpf <i>et al.</i> (2001b) and Tinwell <i>et al.</i> (2002)
Uterotrophic effect in immature hairless Nu rats (dermal exposure)	Schlumpf <i>et al.</i> (2001b)
VTG induction in juvenile fathead minnows: no effect	Kunz <i>et al.</i> (2006)
No effect in transgenic juvenile zebra fish	Schreurs <i>et al.</i> (2002)
Androgen activity: In vitro	
Antagonism of hAR transactivation. No agonistic action	Kunz & Fent (2006b)
No antagonism of hAR transactivation	Ma <i>et al.</i> (2003) and Schreurs <i>et al.</i> (2005)

Table 2 Continued

Endpoint	References
4-MBC	
Progesterone activity: In vitro	
Antagonism of hPR transactivation	Schreurs <i>et al.</i> (2005)
Reproductive organs: Developmental toxicity	
Delayed puberty in males (prepubertal separation),	Durrer <i>et al.</i> (2005, 2007) and Hofkamp <i>et al.</i> (2008)
↑ prostate duct formation in F1 neonate,	
↓ prostate weight in adult F1,	
↑ testis weight in adult F1,	
↑ uterine weight	
Altered expression and sensitivity of oestrogen target genes and coactivators in prostate and uterus, no effect on onset of female puberty or oestrous cycle	Durrer <i>et al.</i> (2005, 2007) and Faass <i>et al.</i> (2009)
Reproductive organs: Long-term exposure of adult animals	
Uterus and vagina of adult oophorectomized rats	Seidlova-Wuttke <i>et al.</i> (2006)
↑ uterus weight, ↑ epithelial/ endometrial thickness, unchanged ER, PR and IGF-1 expression	
Central nervous system: Developmental toxicity	
Impaired female sexual behaviour in adult F1	Faass <i>et al.</i> (2009), Maerkel <i>et al.</i> (2005, 2007)
Altered expression and sensitivity of oestrogen target genes in sexually dimorphic brain regions	
Thyroid axis	
in vitro:	Schmutzler <i>et al.</i> (2007b)
↓ iodide uptake	
Developmental study, rats: ↑ thyroid weight in F1 (both sexes),	Maerkel <i>et al.</i> (2007)
↑ TSH and ↑ T3 in female F1	
Adult oophorectomized rats (12 weeks):	Schmutzler <i>et al.</i> (2007b)
↑ TSH, ↓ T4, ↑ T3,	
↓ Dio 1 activity (kidney),	
ME activity unchanged (liver, kidney)	
Additional organ toxicities and general toxicity	
Developmental study, rats: no effect on body weight in adult F1	Durrer <i>et al.</i> (2005, 2007) and Maerkel <i>et al.</i> (2007)
Developmental study in rats: ↓ thymus weight in adult female F1	Schlumpf <i>et al.</i> (2004b)
Adult oophorectomized rats (3 months): ↑ bone density	Seidlova-Wuttke <i>et al.</i> (2006)
↑ VTG induction and ER α gene expression in liver of male medaka (7 days)	Inui <i>et al.</i> (2003)

Table 2 Continued

Endpoint	References
OMC	
Oestrogen activity: In vitro	
No binding to hER α	Morohoshi et al. (2005)
↑ Transactivation: hER α	Gomez et al. (2005) and Schreurs et al. (2002)
No transactivation: hER α , hER β , rtER α	Kunz et al. (2006), Kunz & Fent (2006b), Morohoshi et al. (2005) and Schreurs et al. (2002)
Antagonism of hER α transactivation	Kunz & Fent (2006b) and Morohoshi et al. (2005)
↑ MCF-7 cell proliferation	Schlumpf et al. (2001b)
Oestrogen activity: Acute in vivo models	
Uterotrophic effect in immature rats	Schlumpf et al. (2001b)
Uterus of adult oophorectomized rats: ↑weight, ↑ ER β and C3 expression	Klammer et al. (2005)
↑VTG induction in male medaka	Inui et al. (2003)
VTG induction in juvenile fathead minnows: no effect	Kunz et al. (2006)
Androgen activity: In vitro	
Antagonism > agonism of hAR transactivation	Kunz & Fent (2006b)
No effect on hAR transactivation	Ma et al. (2003) and Schreurs et al. (2005)
Progesterone activity: In vitro	
Antagonism of hPR transactivation	Schreurs et al. (2005)
Reproductive organs: Developmental toxicity	
No effect on puberty in rats,	Axelstad et al. (2011)
↓ P-testosterone in F1 on PND 16,	
↓prostate weight PND 16 in adult F1,	
altered prostate histology PND 16 in adult F1,	
↓testis weight PND 16, unchanged in adult F1,	
↓ epididymal sperm count adult F1, no effect on uterus or ovary weight	
Reproductive organs: Long-term exposure of adult animals	
Adult oophorectomized rats: uterine weight unchanged or slightly ↑,	Seidlova-Wuttke et al. (2006)
↑ thickness of uterus epithelium, endometrium and myometrium, and of vagina epithelium,	
PR and IGF-1 expression in uterus and vagina	

Table 2 Continued

Endpoint	References
OMC	
Thyroid axis	
In vitro:	Schmutzler et al. (2007b)
↓ iodide uptake,	
↑ TR transactivation (high concentrations)	
Adult oophorectomized rats (5 days):	Klammer et al. (2007)
↓ TSH, ↓ T4, ↓ T3,	
↑ TSH receptor protein, TRH expression in hypothalamus unchanged,	
↓Dio 1 activity (liver)	
Adult oophorectomized rats (3 month):	Schmutzler et al. (2004, 2007b)
↓ T4; T3 and TSH unchanged,	
↓ Dio1 activity (liver, kidney),	
↑ ME activity (kidney, T3 target)	
Developmental study, rats:	Axelstad et al. (2011)
thyroid weight: ↑ in F1 PND 16, unchanged in adult F1,	
T4: ↓ in male F1 PND 16 and in dams, unchanged in female F1 PND 16 and in adult F1.	
Central nervous system: Developmental toxicity	
Developmental study, rats (gavage):	Axelstad et al. (2011)
↓ motor activity in adult female F1,	
↑ spatial learning in adult male F1	
Additional organ toxicities and general toxicity	
Developmental 2 generations study:	Schneider et al. (2005)
↓ body weight gain and ↓ adult body weight in F1 male and female rats (high dose),	
↑ liver weight in female F1	
Developmental study, rats (gavage):	Axelstad et al. (2011)
↓ birth weight and body weight gain, body weight of adult F1: males ↓, females normalized	
Adult oophorectomized rats (5 days):	Klammer et al. (2005)
↓ serum cholesterol, ↓LDL,	
↓ triglycerides and ↓ IGF-1 expression in liver (highest dose)	
OD-PABA	
Oestrogen activity: In vitro	
↑ transactivation: hER α	Gomez et al. (2005) and Schreurs et al. (2002)
↑ transactivation: hER α > hER β ,	Schreurs et al. (2005)
No agonistic action: hER α , rtER α , hER β	Kunz et al. (2006), Kunz & Fent (2006b), Morohoshi et al. (2005) and Schreurs et al. (2002)

Table 2 Continued

Endpoint	References
OD-PABA	
Antagonism of hER α transactivation	Kunz & Fent (2006b) and Morohoshi <i>et al.</i> (2005)
No antagonism: hER α or hER β	Schreurs <i>et al.</i> (2002)
↑ MCF-7 cell proliferation	Schlumpf <i>et al.</i> (2001b)
Oestrogen activity: Acute in vivo models	
No uterotrophic effect in immature rats	Schlumpf <i>et al.</i> (2001b)
Androgen activity: In vitro	
Antagonism on hAR transactivation	Kunz & Fent (2006b)
No antagonism on hAR transactivation	Ma <i>et al.</i> (2003)
No agonistic action on hAR transactivation	Kunz & Fent (2006b), Ma <i>et al.</i> (2003) and Schreurs <i>et al.</i> (2005)
Progesterone activity: In vitro	
No agonism or antagonism on hPR transactivation	Schreurs <i>et al.</i> (2005)
PABA	
Oestrogen activity: In vitro	
No binding to hER α	Morohoshi <i>et al.</i> (2005)
Antagonism of hER α transactivation	Kunz & Fent (2006b)
No antagonistic action on hER α	Morohoshi <i>et al.</i> (2005)
No agonistic actions on hER α , rER α	Kunz <i>et al.</i> (2006), Kunz & Fent (2006b) and Morohoshi <i>et al.</i> (2005)
Oestrogen activity: Acute in vivo models	
No data	
Androgen activity: In vitro	
No agonistic or antagonistic activity on hAR, yeast cells	Kunz & Fent (2006b)

oestrogenic in acute in vivo models (Schlumpf *et al.*, 2001b; Schreurs *et al.*, 2002).

Binding affinity to oestrogen receptor α (ER α) and to oestrogen receptor β (ER β) has also been examined (Mueller *et al.*, 2003; Schlumpf *et al.*, 2004a; Morohoshi *et al.*, 2005). The studies differed with respect to ER α and ER β binding preference of individual compounds, but indicate an interaction at the level of ERs. This was also demonstrated by the fact that the proliferative effect of 4-MBC on MCF-7 cells was abolished by the selective ER antagonist ICI182780 (Schlumpf *et al.*, 2001b). Additional effects on oestrogen synthesis, degradation, protein binding, receptor synthesis, etc. cannot be excluded, but have not been investigated as yet.

Interestingly, Kunz and Fent detected antagonistic activity of almost all tested UV-filters in yeast expressing human ER α (hER α): BP-3, 3-BC, 4-MBC, OMC, HMS, OD-PABA and PABA (Kunz & Fent, 2006b). Antioestrogenic activity of 4-MBC, OD-PABA and PABA was supported in a couple of other studies (Mueller *et al.*, 2003;

Morohoshi *et al.*, 2005). In contrast, BP-3, found to be the most antioestrogenic UV-filter in the Kunz and Fent study (Kunz & Fent, 2006b) had in another study only weak binding affinity to hER α , compared with 17 β -estradiol (Morohoshi *et al.*, 2005). Moreover, Schreurs and colleagues also investigated antagonistic oestrogenic activity of BP-3, 3-BC, 4-MBC, HMS, OMC and OD-PABA, but in contrast, did not report any effects of the tested compounds (Schreurs *et al.*, 2002).

These conflicting results regarding antioestrogenic activity are compatible with data from oestrogen agonist studies indicating that the agonistic activity of many UV-filters is of the partial agonist type (Schlumpf *et al.*, 2001b, 2004a). In addition, those studies differ in their type of assay, being also different in their capability to discriminate between agonistic and antagonistic effects.

The oestrogenic activity of BP-3, 3-BC, 4-MBC and OMC was confirmed by acute in vivo tests using increased uterine weight in immature rats (Schlumpf *et al.*, 2001b, 2004a; Tinwell *et al.*, 2002) or oophorectomized rats (Klammer *et al.*, 2005) as endpoint. Furthermore, elevated vitellogenin in fish, a phenotypic endpoint for the oestrogenic action, has been observed in a number of ecotoxicological studies of 3-BC, 4-MBC and OMC (Holbech *et al.*, 2002; Inui *et al.*, 2003; Kunz *et al.*, 2006). However, increased uterine weight following BP-3 exposure of immature rats conflicted with unchanged uterine weight in an adult oophorectomized rat model, indicating possible higher sensitivity of immature rats to BP-3 (Schlumpf *et al.*, 2001b; Schlecht *et al.*, 2004). In vitro oestrogenic activity of HMS and OD-PABA could not be confirmed in vivo as reported (Schlumpf *et al.*, 2001b; Schreurs *et al.*, 2002).

Influence on androgen activity

BP-3, 3-BC, 4-MBC, HMS, OMC and OD-PABA exhibited antiandrogenic activity in vitro, even though data on individual compounds were conflicting (Ma *et al.*, 2003; Schreurs *et al.*, 2005; Kunz & Fent, 2006b). In contrast to other UV-filters, which were mainly androgen antagonists, HMS exhibited both full agonistic and antagonistic androgen activity in vitro by producing full dose-response curve binding to the human androgen receptor (hAR) and inhibiting the action of dihydrotestosterone (DHT) (Kunz & Fent, 2006b). In addition to in vitro antiandrogenic activity, OMC caused a decrease in serum-testosterone among immature offspring in a developmental study in rats (Axelstad *et al.*, 2011). For the remaining compounds in Table 1, antiandrogenic activity of UV-filters has not yet been investigated in vivo.

Influence on progesterone activity

3-BC, 4-MBC, BP-3, OMC and HMS were all tested using a progesterone receptor (PR) CALUX bioassay and all

found to exhibit antagonistic action on the PR in U2-OS cells (Schreurs *et al.*, 2005). The action of those UV-filters could be reversed by the PR agonist ORG2058, indicating a PR mediated action. OD-PABA was also tested, but did not exhibit progesterone activity in vitro (Schreurs *et al.*, 2005).

OMC and 4-MBC were examined for progesterone effect in vivo (Seidlova-Wuttke *et al.*, 2006; Axelstad *et al.*, 2011). Only exposure to OMC in vivo confirmed progesterone activity observed in vitro, resulting in a decrease in the plasma-progesterone concentration in a developmental study in rats (Axelstad *et al.*, 2011) and in altered transcription of PR in uterus and vagina among oophorectomized Sprague–Dawley rats orally exposed for 3 months (Seidlova-Wuttke *et al.*, 2006). In vitro progesterone activity of 4-MBC was not confirmed in the later study performed on oophorectomized rats (Seidlova-Wuttke *et al.*, 2006). This finding does not rule out the possibility of 4-MBC's interference with progesterone signalling in vivo among immature rats, if their sensitivity is higher compared with oophorectomized rats, just as it was the case for oestrogenic activity of BP-3 (Schlumpf *et al.*, 2001b; Schlecht *et al.*, 2004).

Effects on reproductive organs and development

Studies on reproductive and developmental toxicity have been published for only three of the endocrine active UV-filters, namely 4-MBC, 3-BC and OMC. Delay of male puberty and reduced prostate weight were the most sensitive variables for reproductive toxicity following exposure to 3-BC and 4-MBC in extended one generation developmental studies, where Long Evans rats were orally exposed for 10 weeks before mating, during pregnancy and lactation and then their offspring continued oral exposure until adulthood (Schlumpf *et al.*, 2004b; Durrer *et al.*, 2007). Those effects were seen at a dose of 0.24 mg/kg bw/day for 3-BC and of 7 mg/kg bw/day for 4-MBC. In contrast, the reproductive toxicity two generation study with OMC reported only delayed male and female puberty at the highest dose of 1000 mg/kg bw/day, which was not attributed to the compound, but rather to a natural variation within the 'historical control range', in spite of the statistically significant difference compared with control animals in the study (Schneider *et al.*, 2005). Axelstad *et al.* (2011) did not find any effect of OMC on the time of puberty in a one generation developmental study either.

Several other adverse effects on the reproductive system were observed in extended one generation developmental studies after exposure to BP-3, 3-BC, 4-MBC and OMC comprising alteration in weight and histology of reproductive organs in both sexes (Schlumpf *et al.*, 2004b; Durrer *et al.*, 2007; Hofkamp *et al.*, 2008; Axelstad *et al.*, 2011). Developmental studies with 3-BC and 4-MBC (Schlumpf

et al., 2004b; Durrer *et al.*, 2005, 2007) and acute and long-term BP-3 and OMC studies in adult oophorectomized rats (Klammer *et al.*, 2005; Schlecht *et al.*, 2004; Seidlova-Wuttke *et al.*, 2006) found alterations in proteins and gene expression of ER, AR, PR, insulin-like growth factor-I (IGF-1), complement protein 3 (C3), nuclear receptor co-repressor (N-Cor), steroid receptor coactivator-1 (SRC-1) in uterus and prostate. Those findings indicate possible mechanisms of action behind the reproductive toxicity. Alterations in oestrogen target gene expression following peri- and postnatal exposure to 3-BC and 4-MBC also occurred in brain regions important for rats' sexual behaviour (Ventromedial Hypothalamic nucleus (VMH) and Medial Preoptic area (MPO)) (Maerkel *et al.*, 2005, 2007; Faass *et al.*, 2009). This was supported by observed changes in female sexual behaviour, such as reduction in proceptive behaviour, altered attractive behaviour resulting in a decreased number of mounts, impaired receptive behaviour and episodes of rejection following exposure to 3-BC and 4-MBC in an extended one generation developmental study (Faass *et al.*, 2009).

In addition, a 90-day BP-3 study in adult mice (French, 1992) and a 3-BC extended one generation developmental study in rats (Faass *et al.*, 2009) resulted in changes in the oestrous cycle.

Fertility in males was affected in a 90-day study with BP-3, where sperm density decreased in a dose-related manner following dermal exposure in mice and at the highest dose following oral exposure in mice and rats (French, 1992). In addition, at the same dose level an increased number of abnormal spermatozoa was observed in mice. Perinatal and early postnatal exposure to OMC in rats also resulted in decreased sperm count (Schneider *et al.*, 2005; Axelstad *et al.*, 2011).

Reduction of litter size and survival rate in offspring were seen after exposure of dams during pregnancy to higher doses of 3-BC (above 2.4 mg/kg bw/day) and 4-MBC (above 24 mg/kg bw/day) (Schlumpf *et al.*, 2001a, 2004b). The mechanisms behind this perinatal toxicity have not been clarified, but involvement of the immune system and metabolism of the compounds are suspected because the same doses of 4-MBC caused decrease in thymus weight of offspring and increase in weight of thyroid in dams (Schlumpf *et al.*, 2004b).

Effects on hypothalamic–pituitary–thyroid axis

A wide range of in vitro and in vivo studies support that 4-MBC, BP-3 and OMC may interfere with the hypothalamic–pituitary–thyroid axis (HPT).

An in vitro and a 5-day in vivo study with BP-3 have shown that this compound interacts with thyroid function by an agonistic effect on the thyroid receptor (TR) in HepG2 cells (Schmutzler *et al.*, 2007b) and by decreasing

the expression of the *ERR1* gene in the thyroid gland (Schlecht *et al.*, 2004). Whether those findings result in any adverse effect on the thyroid axis have not been examined and further investigations on BP-3 in long-term studies seem indicated.

An adverse effect on the thyroid axis indicated by alterations in the concentrations of thyroid hormones following exposure to 4-MBC and OMC was found in 90 days toxicological studies (Schmutzler *et al.*, 2004, 2007b). An extended one generation developmental study in rats confirmed alterations in thyroid-stimulating hormone (TSH) and total triiodothyronine (T3) following 4-MBC exposure, supplemented with increased thyroid weight in offspring (Maerker *et al.*, 2007). A developmental study of OMC resulted in decreased total thyroxine (T4) in dams and in juvenile male offspring and in increased weight of thyroid gland in juvenile rats of both sexes (Axelstad *et al.*, 2011). A sex difference was noted: female offspring were less sensitive to OMC exposure, resulting in unaltered T4 (Axelstad *et al.*, 2011).

The mechanisms behind the adverse effects observed in the thyroid axis following *in vivo* exposure to 4-MBC and OMC could be partially explained by a decrease in Dio 1 activity, an enzyme promoting both activation and inactivation of thyroid hormones and by decreased iodide uptake in FRTL-5 cells (Klammer *et al.*, 2007; Schmutzler *et al.*, 2007b). In addition, OMC exhibited agonistic action on TR in the HepG2 cell line (Schmutzler *et al.*, 2007b). In contrast, thyroid peroxidase (TPO) activity was not affected neither following OMC exposure nor 4-MBC exposure (Schmutzler *et al.*, 2004; Klammer *et al.*, 2007).

The UV-filter benzophenone-2 (BP-2) is not allowed to be used in cosmetics in EU (European Commission), but is still used in USA to protect cosmetic products against UV-rays (Food and Drug Administration [FDA], 2012). This is of concern as it has been shown to disturb thyroid function in an acute toxicity study on oophorectomized Sprague Dawley rats, altering TSH, T4 and Dio 1 activity and to decrease human receptor TPO (hrTPO) activity *in vitro* (Schmutzler *et al.*, 2007a).

Several UV-filters, including 3-BC, HMS, OD-PABA or PABA seem not to have been examined for their possible effects on the thyroid axis.

General toxicity

In mammalian long-term exposure models, general toxicity evaluated by alterations of food consumption and in body and liver weights was found in the higher dose range after exposure to BP-3 (French, 1992), 3-BC (>2.4 mg/kg bw/day) (Schlumpf *et al.*, 2004b) and OMC (>500mg/kg bw/day) (Schneider *et al.*, 2005; Axelstad *et al.*, 2011). Liver and kidney weights were affected after both dermal and oral exposure to BP-3 (French, 1992).

In contrast, histological alterations in liver and kidney were observed only after oral exposure to BP-3 in the latter study. 4-MBC reduced body weight only transiently in postnatal 4-MBC exposed F1 offspring. Body weights were again normal at puberty and in adulthood also in the higher dose groups, and no signs of general toxicity were observed in the parent animals (F0) (Durrer *et al.*, 2007; Maerker *et al.*, 2007). Effects on reproductive organs and development were also present at doses devoid of general toxicity. Indications of general toxicity were further observed in experiments on fish exposed to 3-BC and 4-MBC, where body weight decreased in a dose-dependent manner (Kunz *et al.*, 2006). A few deaths were observed by Schneider and colleges in a two generation study in rats, but were not considered to be related to OMC exposure (Schneider *et al.*, 2005).

Human exposure to sunscreens

Table 3 summarizes prevailing data on human exposure to chemical UV-filters used in cosmetics. Experimental studies showed that BP-3, 4-MBC and OMC rapidly permeated intact skin (Gustavsson *et al.*, 2002; Janjua *et al.*, 2004, 2008; Gonzalez *et al.*, 2006) and could be detected in plasma after 1–2 h following application (Fig. 2) (Janjua *et al.*, 2008). Interestingly, the concentrations of these compounds in the same experimental study in male urine and plasma were higher than in female samples (Janjua *et al.*, 2004), indicating a gender difference in the metabolism, distribution and possibly also in the accumulation of UV-filters in adipose tissue.

Furthermore, Table 3 shows a substantial exposure of the general population to UV-filters. BP-3, the most common UV-filter in the USA, was found in more than 96% of 2517 urine samples collected throughout 1-year (2003–2004) from the general US population in the NHANES study (Calafat *et al.*, 2008). BP-3 was also detected in all urine samples collected from 129 Danish children and adolescents in the month of November, even though days are short and sun protection is not needed at that time of year (H. Frederiksen, O. Nielsen, L. Aksglaede, K. Sorensen, T. H. Lassen, N. E. Skakkebaek, K. Main, A. Juul & A. Andersson, unpublished data, 2012).

Breastfed babies are exposed to UV-filters through breast milk (Schlumpf *et al.*, 2010). One or more UV-filters were present in 85% of Swiss human milk samples (Schlumpf *et al.*, 2010). Bisphenol A with a similar chemical structure to BP-3, were shown to pass the blood-placenta barrier (Schonfelder *et al.*, 2002; Lee *et al.*, 2008). Thus in theory, chemicals like BP-3 may also pass the blood-placenta barrier. Studies investigating amniotic fluid are required to investigate whether perinatal exposure to UV-filters, which are found in the urine of pregnant women occurs (Wolff

Table 3 Human exposure to UV-filters

	Study design	Number of test subjects	Sample type	UV-filter	Reported use(%)	Positive ^d samples(%)	Concentrations	Ref.		
Observational studies	USA: NHANES study 2003–2004 based on US general population > 6 years of age	2517 people	Urine	BP-3	–	96.8	22.9 (18.1–28.9) µg/L ^c	Calafat et al. (2008)		
	USA: The Children's Environmental Health study 1998–2002: Multiethnic prospective cohort of pregnant women-infant pairs in New York during 3. trimester	404 pregnant women	Urine	BP-3	–	97.8	7.5 µg/L ^a	Wolff et al. (2008)		
	France: Eden Mother-Child cohort recruited before gestational week 28 in 2003–2006	191 pregnant women	Urine	BP-3	–	80.5	1.3 µg/L ^a	Philippat et al. (2012)		
	Switzerland: Cohorts 2004–2006 Mothers who gave birth to a single child at the University Women's Hospital Basel	54 women	Human breast milk	BP-3	13.21	12.96	52.23 ± 50.69 ng/g lipid ^a	Schlumpf et al. (2010)		
				4-MBC	26.42	20.37	22.12 ± 12.80 ng/g lipid ^a			
				OMC	66.04	77.78	27.50 ± 22.15 ng/g lipid ^a			
				HMS	15.09	5.56	29.37 ± 27.64 ng/g lipid ^a			
			OCT	43.40	66.67	30.18 ± 24.51 ng/g lipid ^a				
			OD-PABA	1.89	1.85	49.00 ng/g lipid ^c				
			3-BC	0	0	–				
Experimental studies	Denmark: Single blinded experimental study 1 week with UV-filters free lotion + 1 week with Daily whole-body application of sunscreen 2 mg/cm ² with BP-3, 4-MBC and OMC Concentration 10% of each	32: 15 males + 17 postmenopausal females	Plasma	BP-3	100	100	Female (ng/ml)	Male (ng/ml)	Janjua et al. (2004)	
				4-MBC			200 ^b	300 ^b		
				OMC			20 ^b	20 ^b		
				Urine			BP-3	60 ^b		140 ^b
				4-MBC			5 ^b	7 ^b		
	Sweden: Experimental study 1x whole-body application of sunscreen with BP-3 2mg/cm ² Concentration 4% Sweden: Experimental study: hole-body application of 2 mg/cm ² sunscreen SPF14 with 4% BP-3 twice a day for 5 days One half of participants was daily irradiated with UVA+UVB according to Fitzpatrick skin type	11: 7 males + 4 females 25: 16 women and 9 men	Urine	BP-3	100	100	187 ^b	238 ^b	Janjua et al. (2008)	
				4-MBC			16 ^b	18 ^b		
				OMC			7 ^b	16 ^b		
				Urine			BP-3	44 ^b		81 ^b
				4-MBC			4 ^b	4 ^b		
			OMC	6 ^b	4 ^b					
			BP-3	100	100	9.8 mg = 0.5% of the applied amount		Gustavsson et al. (2002)		
			BP-3	100	100	3.7% of applied amount		Gonzalez et al. (2006)		

^aMedian concentration.^bMaximum median concentration.^cGeometric mean concentration.^dPercentage of samples with contest of UV-filters above analytical detection limit.

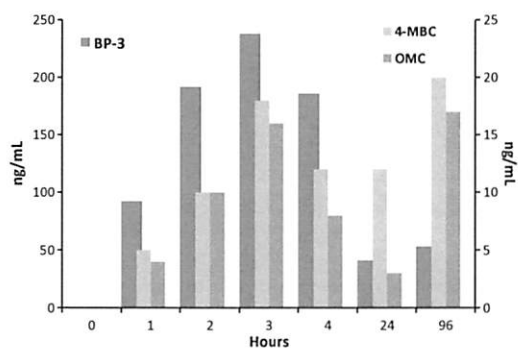


Figure 2 Plasma concentration of UV-filters BP-3, 4-MBC and OMC among males after one dermal application daily for four days (Janjua *et al.*, 2008).

et al., 2008). High concentrations of BP-3 in mothers' urine were associated with decreased birth weight in girls and increased birth weight and head circumference in boys (Wolff *et al.*, 2008; Philippat *et al.*, 2012). Human studies with genital malformations in infants as an endocrine specific endpoint are required to clarify a possible endocrine disrupting effect of UV-filters on the human foetus. However, developmental animal studies where rats were exposed to UV-filters, did not report any reduction of the anogenital distance (ADG) or increased rate of genital malformations.

In spite of the wide human exposure to UV-filters only few studies have examined the effects of UV-filters on humans (Janjua *et al.*, 2004, 2007; Jannesson *et al.*, 2004; Wolff *et al.*, 2008; Philippat *et al.*, 2012). A double blinded clinical trial measuring the gingival index, that relate to the severity and location of periodontal disease, showed that dentifrice containing BP-3 reduced periodontal disease by 25% (Jannesson *et al.*, 2004), which supported the *in vitro* data suggesting BP-3 to be an inhibitor of PG synthesis (Jannesson *et al.*, 2004; Kristensen *et al.*, 2011).

Janjua and colleagues examined the effects on reproductive (Janjua *et al.*, 2004) and thyroid (Janjua *et al.*, 2007) hormones following dermal application of a mixture of BP-3, 4-MBC and OMC. A significant increase in inhibin B and a decrease in free triiodothyronine (FT3), free thyroxine (FT4), T3, T4, thyroxine-binding globulin (TBG) and testosterone were seen, but were not considered to be related to the application of a sunscreen mixture, but rather to biological variation (Janjua *et al.*, 2007). However, the duration of those studies was too short to be conclusive.

Discussion

As summarized in this review, a large number of *in vivo* animal studies and *in vitro* studies have shown that there are numerous potential adverse effects of UV-filters pres-

ent in sunscreens and cosmetics. The effects include developmental and reproductive effects, apparently caused by endocrine disrupting actions of these chemicals. Other studies could not find such adverse effects. However, because of the wide human exposure in combination with the clear endocrine disruptive effects observed in a large number of well designed studies, the UV-filters BP-3, 4-MBC and OMC can be considered as substances of high concern in relation to human risk.

Importantly, most of the studied adverse effects of UV-filters have been evaluated after oral exposure. However, the primary exposure of humans to UV-filters via cosmetics occurs through dermal application. Therefore, the UV-filters enter the systemic circulation directly without first being metabolized by passage through the liver, thereby leading to a greater risk of the compounds reaching all tissues of the body unaltered, as was observed in rats following dermal exposure to 3-BC (Søeborg *et al.*, 2006). In addition, a three-fold greater oestrogenic effect of 4-MBC in rats was observed after topical application compared with oral exposure indicating higher bioavailability of the compound (Schlumpf *et al.*, 2001b).

Another challenge in studies of sunscreens in cosmetics is that the products often contain several UV-filters in combination. The total effect of these mixtures are poorly examined although a few existing studies have shown that mixtures of chemicals, including UV-filters, might act additively and exhibit toxic activity, even at the no observed adverse effect level (NOAEL) of the individual compounds (Heneweer *et al.*, 2005; Kunz & Fent, 2006a; Kortenkamp *et al.*, 2007).

Humans are not only exposed to UV-filters when the agents are used for sun protection of the skin. Exposure apparently occurs from various sources. Almost all samples in NHANES study (Calafat *et al.*, 2008) and all samples in a Danish children cohort (H. Frederiksen, O. Nielsen, L. Aksglaede, K. Sorensen, T. H. Lassen, N. E. Skakkebaek, K. Main, A. Juul & A. Andersson, unpublished data, 2012), contained BP-3, indicating year round exposure independent of sunscreen use. The presence of UV-filters in milk of Swiss mothers was correlated with use of sunscreens in 55% of the cases; in 60% of the cases, the presence of these compounds in milk was related to the use of other cosmetic products containing UV-filters (Schlumpf *et al.*, 2010). The likely sources may be hair spray, lipsticks, shampoo, make-up, perfumes, skin care products as well as non-cosmetic products, such as carpets, furniture, clothing and washing powder (Schlecht *et al.*, 2004; Morohoshi *et al.*, 2005; Kunz & Fent, 2006b; Schlumpf *et al.*, 2010). Here, the UV-filters are used to protect the products from effects of UV-radiation. Considering these findings, it cannot be ruled out that a considerable part of the total human exposure to UV-filters might occur via products other than sunscreens.

It is of particular concern that human babies are exposed to UV-filters through breast milk (Schlumpf *et al.*, 2010). The highest concentration of 4-MBC found in human milk was 48.37 ng/g lipid (Schlumpf *et al.*, 2010), which was only 4.3 times lower than the concentration of 4-MBC in rat milk (208.6 ng/g lipid) (Schlumpf *et al.*, 2008) following oral exposure to 4-MBC at the lowest observed adverse effect level (LOAEL) (7 mg/kg/day), with delay of male puberty and prostate weight as endpoints (Durrer *et al.*, 2007).

In conclusion, it is of concern that (1) a large number of in vitro and in vivo animal studies have shown endocrine disrupting effects of UV-filters present in sunscreens, although other studies failed to find such effects and (2) application of cosmetics with UV-filters to the skin can result in absorption of UV-filters into the human systemic circulation and subsequently might result in exposure of all tissues in the body. Considering these facts together with the wide and increasing use of sunscreens and the increasing incidence of malignant melanoma, for which UV-filters are assumed to protect, it seems pertinent to investigate whether sunscreen use in humans on balance is beneficial for human health.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Purchase of sunscreen products by volume per head over time in the US, UK, AUS and DK.

Table S1. In vitro and in vivo effects of UV-filters in animals (Presence of effect: +; Absence of effect: –; Increased: ↑; Decreased: ↓).

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49) Environ Sci Technol. 2012 Apr 17;46(8):4624-32. doi: 10.1021/es204415a. Epub 2012 Mar 29.

Urinary concentrations of benzophenone-type UV filters in U.S. women and their association with endometriosis.

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Abstract

Benzophenone (BP)-type UV filters are widely used in a variety of personal care products for the protection of skin and hair from UV irradiation. Despite the estrogenic potencies of BP derivatives, few studies have examined the occurrence of these compounds in human matrices. Furthermore, associations among exposure to these compounds and estrogen-dependent diseases (such as endometriosis) have not been examined previously. In this study, we determined the concentrations of five BP derivatives, 2-hydroxy-4-methoxybenzophenone (2OH-4MeO-BP), 2,4-dihydroxybenzophenone (2,4OH-BP), 2,2'-dihydroxy-4-methoxybenzophenone (2,2'OH-4MeO-BP), 2,2',4,4'-tetrahydroxybenzophenone (2,2',4,4'OH-BP), and 4-hydroxybenzophenone (4OH-BP), in urine collected from 625 women in Utah and California, using liquid chromatography (LC)-tandem mass spectrometry (MS/MS). The association of urinary concentrations of BP derivatives with an increase in the odds of a diagnosis of endometriosis was examined in 600 women who underwent laparoscopy/laparotomy (n = 473: operative cohort) or pelvic magnetic resonance imaging (n = 127: population cohort), during 2007-2009. 2OH-4MeO-BP, 2,4OH-BP, and 4OH-BP respectively were detected in 99.0%, 93.3%, and 83.8% of the urine samples analyzed, whereas the detection rates for 2,2',4,4'OH-BP and 2,2'OH-4MeO-BP were below 6.0%. Significant regional differences (higher concentrations in California) and monthly variations (higher concentrations in July and August) were found for urinary concentrations of 2OH-4MeO-BP and 2,4OH-BP. In addition, urinary concentrations of 2OH-4MeO-BP and 2,4OH-BP tended to be higher in more affluent, older, and leaner women. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated for the urinary concentrations of BP derivatives and the odds of an endometriosis diagnosis; ORs increased across quartiles of 2OH-4MeO-BP and 2,4OH-BP concentrations, but a significant trend was observed only between 2,4OH-BP and the odds of an endometriosis diagnosis in the operative cohort (OR = 1.19; 95% CI = 1.01, 1.41). When women in the highest quartile of 2,4OH-BP concentrations were compared with women in the first three quartiles, the OR increased considerably (OR = 1.65; 95% CI = 1.07, 2.53). Given that 2,4OH-BP possesses an estrogenic activity higher than that of 2OH-4MeO-BP, our results invite the speculation that exposure to elevated 2,4OH-BP levels may be associated with endometriosis.

50) Environ Res. 2015 Feb;137:101-7. doi: 10.1016/j.envres.2014.06.028. Epub 2014 Dec 19.

Bisphenol A, benzophenone-type ultraviolet filters, and phthalates in relation to uterine leiomyoma.

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Abstract

Bisphenol A, benzophenone-type UV filters, and phthalates are chemicals in high production and use including in a range of personal care products. Exposure of humans to these chemicals has been shown to affect endocrine function. Although short-lived, widespread exposure may lead to continual opportunity for these chemicals to elicit health effects in humans. The association of these chemicals with incident uterine leiomyoma, an estrogen sensitive disease, is not known. Urinary concentrations of bisphenol A (BPA), five benzophenone-type UV filters (2-hydroxy-4-methoxybenzophenone (2OH-4MeO-BP), 2,4-dihydroxybenzophenone (2,4OH-BP), 2,2'-dihydroxybenzophenone (2,2'OH-4MeO-BP), 2,2',4,4'-tetrahydroxybenzophenone (2,2',4,4'OH-BP), and 4-hydroxybenzophenone (4OH-BP), and 14 phthalate monoesters were quantified in 495 women who later underwent laparoscopy/laparotomy at 14 clinical sites for the diagnosis of fibroids. Significantly higher geometric mean creatinine-corrected concentrations of BPA, 2,4OH-BP, and 2OH-4MeO-BP were observed in women with than without fibroids [BPA: 2.09 μ g/g vs. 1.46 μ g/g p=0.004; 2,4OH-BP:11.10 μ g/g vs. 6.71 μ g/g p=0.01; 2OH-4MeO-BP: 11.31 μ g/g vs. 6.10 μ g/g p=0.01]. Mono-methyl phthalate levels were significantly lower in women with than without fibroids (1.78 μ g/g vs. 2.40 μ g/g). However, none of the exposures were associated with a significant odds ratio even when adjusting for relevant covariates. There was a lack of an association between select nonpersistent chemicals and the odds of a fibroid diagnosis.

51) Environ Int. 2015 Nov;84:174-80. doi: 10.1016/j.envint.2015.08.008. Epub 2015 Aug 31.

Environmental phenols and pubertal development in girls.

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Abstract

Environmental exposures to many phenols are documented worldwide and exposures can be quite high (>1 μM of urine metabolites). Phenols have a range of hormonal activity, but knowledge of effects on child reproductive development is limited, coming mostly from cross-sectional studies. We undertook a prospective study of pubertal development among 1239 girls recruited at three U.S. sites when they were 6-8 years old and were followed annually for 7 years to determine age at first breast or pubic hair development. Ten phenols were measured in urine collected at enrollment (benzophenone-3, enterolactone, bisphenol A, three parabens (methyl-, ethyl-, propyl-), 2,5-dichlorophenol, triclosan, genistein, daidzein). We used multivariable adjusted Cox proportional hazards ratios (HR (95% confidence intervals)) and Kaplan-Meier survival analyses to estimate relative risk of earlier or later age at puberty associated with phenol exposures. For enterolactone and benzophenone-3, girls experienced breast development 5-6 months later, adjusted HR 0.79 (0.64-0.98) and HR 0.80 (0.65-0.98) respectively for the 5th vs 1st quintiles of urinary biomarkers ($\mu\text{g}/\text{g}$ -creatinine). Earlier breast development was seen for triclosan and 2,5-dichlorophenol: 4-9 months sooner for 5th vs 1st quintiles of urinary concentrations (HR 1.17 (0.96-1.43) and HR 1.37 (1.09-1.72), respectively). Association of breast development with enterolactone, but not the other three phenols, was mediated by body size. These phenols may be antiadipogens (benzophenone-3 and enterolactone) or thyroid agonists (triclosan and 2,5-dichlorophenol), and their ubiquity and relatively high levels in children would benefit from further investigation to confirm these findings and to establish whether there are certain windows of susceptibility during which exposure can affect pubertal development.

52) Toxicol Sci. 2006 Apr;90(2):349-61. Epub 2006 Jan 10.

Comparison of in vitro and in vivo estrogenic activity of UV filters in fish.

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Abstract

In this work, we evaluate whether in vitro systems are good predictors for in vivo estrogenic activity in fish. We focus on UV filters being used in sunscreens and in UV stabilization of materials. First, we determined the estrogenic activity of 23 UV filters and one UV filter metabolite employing a recombinant yeast carrying the estrogen receptor of rainbow trout (rtERalpha) and made comparisons with yeast carrying the human hERalpha for receptor specificity. Benzophenone-1 (BP1), benzophenone-2 (BP2), 4,4-dihydroxybenzophenone, 4-hydroxybenzophenone, 2,4,4-trihydroxybenzophenone, and phenylsalicylate showed full dose-response curves with maximal responses of 81-115%, whereas 3-benzylidene camphor (3BC), octylsalicylate, benzylsalicylate, benzophenone-3, and benzophenone-4 displayed lower maximal responses of 15-74%. Whereas the activity of 17beta-estradiol was lower in the rtERalpha than the hERalpha assay, the activities of UV filters were similar or relatively higher in rtERalpha, indicating different relative binding activities of both ER. Subsequently, we analyzed whether the in vitro estrogenicity of eight UV filters is also displayed in vivo in fathead minnows by the induction potential of vitellogenin after 14 days of aqueous exposure. Of the three active compounds in vivo, 3BC induced vitellogenin at lower concentrations (435 microg/l) than BP1 (4919 microg/l) and BP2 (8783 microg/l). The study shows, for the first time, estrogenic activities of UV filters in fish both in vitro and in vivo. Thus we propose that receptor-based assays should be used for in vitro screening prior to in vivo testing, leading to environmental risk assessments based on combined, complementary, and appropriate species-related assays for hormonal activity.

53) Environ Toxicol Chem. 2015 Dec;34(12):2833-40. doi: 10.1002/etc.3129. Epub 2015 Oct 19.

Endocrine-disrupting effect of the ultraviolet filter benzophenone-3 in zebrafish, *Danio rerio*.

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Abstract

The chemical ultraviolet (UV) filter benzophenone-3 (BP-3) is suspected to be an endocrine disruptor based on results from in vitro and in vivo testing. However, studies including endpoints of endocrine adversity are lacking. The present study investigated the potential endocrine-disrupting effects of BP-3 in zebrafish (*Danio rerio*) in the Fish Sexual Development Test (Organisation for Economic Co-operation and Development TG 234) and a 12-d adult male zebrafish study. In TG 234, exposure from 0 d to 60 d posthatch caused a monotone dose-dependent skewing of the phenotypic sex ratio toward fewer males and more female zebrafish (no observed effect concentration [NOEC]: 191 µg/L, lowest observed effect concentration [LOEC]: 388 µg/L). Besides, gonad maturation was affected in both female fish (NOEC 191 µg/L, LOEC 388 µg/L) and male fish (NOEC 388 µg/L, LOEC 470 µg/L). Exposure to BP-3 did not affect the vitellogenin concentration in TG 234. After 12 d exposure of adult male zebrafish, a slight yet significant increase in the vitellogenin concentration was observed at 268 µg/L but not at 63 µg/L and 437 µg/L BP-3. Skewing of the sex ratio is a marker of an endocrine-mediated mechanism as well as a marker of adversity, and therefore the conclusion of the present study is that BP-3 is an endocrine-disrupting chemical in accordance with the World Health Organization's definition.

54 *Ecotoxicol Environ Saf.* 2015 May;115:14-8. doi: 10.1016/j.ecoenv.2015.01.033.
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Evaluation of the estrogenic and oxidative stress effects of the UV filter 3-benzophenone in zebrafish (*Danio rerio*) eleuthero-embryos.

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Abstract

Personal care products have been detected in superficial waters, representing an environmental risk to the biota. Some studies indicated that 3-benzophenone (3BP) alters hormones, inducing vitellogenesis and having adverse effects on fish reproduction. Other studies have reported generation of free radicals and changes in antioxidant enzymes. Therefore, the aim of the present study was to test acute exposure to 3BP at concentrations within and beyond that found environmentally to provide important toxicological information regarding this chemical. We evaluated the effect of 3BP on vitellogenin 1 (VTG1) gene expression and the transcription of the enzymes catalase (CAT), superoxide dismutase (SOD) or glutathione peroxidase (GPx), which are involved in cellular redox balance. Zebrafish eleuthero-embryos (168hpf) were exposed to 1, 10, 100, 1000 µg/L 3BP, in addition to a negative control and a 0.1% ethanol control for 48h. The results of our study indicated a positive significant correlation between exposure concentrations and VTG1 expression ($r=0.986$, $p=0.0028$) but only 1000 µg/L 3BP produced a significant increase from control. Acute exposure showed no significant differences in transcription levels of CAT, SOD or GPx at the tested conditions. Nevertheless, a trend toward increase in GPx expression was observed as a positive significant correlation ($r=0.928$, $p=0.017$) was noted.

55) Aquatic Toxicology Volume 44, Issues 1-2, December 1998, Pages 141-156

Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens

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Abstract

Japanese medaka were exposed to environmentally-relevant concentrations of environmental estrogens: nonylphenol (NP, 0.5, 0.8 and 1.9 $\mu\text{g l}^{-1}$), methoxychlor (MXC, 0.2, 0.6 and 2.3 $\mu\text{g l}^{-1}$) and estradiol (E2, 0.01, 0.12 and 1.66 $\mu\text{g l}^{-1}$). Exposure occurred throughout the first month following hatch. E2 survival ratios following the exposure period were significantly altered compared to control groups. Following a month period of 'growout' in dilution water only, sex ratios were measured and reproductive capabilities assessed. No alteration in sex ratios was observed following treatment with NP or MXC. All three concentrations of E2 were sufficient to produce exclusively female populations. There was no depreciation in reproductive capability in the NP or MXC-treated fish as measured by fecundity, viability of eggs, or hatchability of eggs. E2-treated female fish had a lower fecundity in the highest concentration.

Keywords

Reproductive toxicity Medaka Xenoestrogens Estradiol Nonylphenol Methoxychlor

56) *Aquat Toxicol.* 2008 Nov 21;90(3):182-7. doi: 10.1016/j.aquatox.2008.08.018.
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Estrogenic activity and reproductive effects of the UV-filter oxybenzone (2-hydroxy-4-methoxyphenyl-methanone) in fish.

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Abstract

Previous studies in extracts of sediments surrounding municipal outfalls off the coast of California, USA and effluents of New York City, NY, USA indicated the UV-filtering agent, oxybenzone (CAS# 131-57-7; benzophenone-3) as a potential estrogen. The effects of oxybenzone on estrogenic activity and reproduction were evaluated using a 14-day juvenile rainbow trout assay for plasma vitellogenin and a subsequent 21-day Japanese medaka reproduction assay. Significant induction of vitellogenin was observed in the rainbow trout at the 1000 microg/L nominal concentration (749 microg/L median measured value) of oxybenzone which was approximately 75 times greater than the concentrations observed in previous wastewater effluent. Vitellogenin induction was also observed in the 1000 microg/L nominal concentration (620 microg/L median measured) of oxybenzone in male Japanese medaka (*Oryzias latipes*) after 21 days of exposure. The number of eggs produced per female per day exposed to the same concentration (620 microg/L) were significantly lower after 7 days, but returned to control values after 21 days. Fertilized eggs were then monitored for 20 days to assess hatching success. The overall percentage of fertilized eggs collected during the 21-day exposure that hatched was significantly lower in the 620 microg/L oxybenzone concentration. There was also a temporal effect at this concentration as egg viability (percentage of fertilized eggs that hatched) was diminished 13-15 days after eggs were collected. All three oxybenzone concentrations (16, 132, and 620 microg/L) and the 50 ng/L estradiol positive control showed reduced hatching of eggs at day 15, and the 132 and 620 microg/L oxybenzone concentrations diminished the percentage of eggs that hatched on days 13-15. These data indicate that the UV-filter oxybenzone alters endocrine or reproduction endpoints in two fish species, but at concentrations significantly higher than those measured in the environment.

57) *Toxicol Appl Pharmacol.* 2012 Sep 1;263(2):184-94. doi: 10.1016/j.taap.2012.06.008. Epub 2012 Jun 18.

Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*).

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Abstract

Organic UV filters including benzophenone-3 (BP-3) are widely used to protect humans and materials from damage by UV irradiation. Despite the environmental occurrence of BP-3 in the aquatic environment, little is known about its effects and modes of action. In the present study we assess molecular and physiological effects of BP-3 in adult male zebrafish (*Danio rerio*) and in eleuthero-embryos by a targeted gene expression approach focusing on the sex hormone system. Fish and embryos are exposed for 14 days and 120 hours post fertilization, respectively, to 2.4-312 µg/L and 8.2-438 µg/L BP-3. Chemical analysis of water and fish demonstrates that BP-3 is partly transformed to benzophenone-1 (BP-1) and both compounds are accumulated in adult fish. Biotransformation to BP-1 is absent in eleuthero-embryos. BP-3 exposure leads to similar alterations of gene expression in both adult fish and eleuthero-embryos. In the brain of adult males *esr1*, *ar* and *cyp19b* are down-regulated at 84 µg/L BP-3. There is no induction of vitellogenin expression by BP-3, both at the transcriptional and protein level. An overall down-regulation of the *hsd3b*, *hsd17b3*, *hsd11b2* and *cyp11b2* transcripts is observed in the testes, suggesting an antiandrogenic activity. No histological changes were observed in the testes after BP-3 treatment. The study leads to the conclusion that low concentrations of BP-3 exhibit similar multiple hormonal activities at the transcription level in two different life stages of zebrafish. Forthcoming studies should show whether this translates to additional physiological effects.

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Organic UV filters and their photodegradates, metabolites and disinfection by-products in the aquatic environment

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Abstract

Organic ultraviolet (UV) filters are compounds used to absorb UV radiation and are increasingly being used as a result of growing concern about UV radiation and skin cancer. Their growing use may increase environmental contamination and exposure through the food chain. There is therefore major concern about the environmental fate and potential effect of organic UV filters used in beauty creams, hair sprays, shampoos, and other personal-care products, as well as those added to plastics and other materials to prevent degradation of polymers and pigments.

This review describes the processes undergone by these compounds once released into the environment and the instrumental methods based on chromatography and mass spectrometry reported in the literature for their determination in environmental samples. We include concentrations found in the environment (e.g., water, soil, sediments, sludge and biota). The main focus is on metabolites, photodegradates and by-products of wastewater treatment.

Keywords

Chromatography Environmental contamination Environmental analysis Mass spectrometry Metabolite Organic UV filter Personal-care product Photodegradation Sunscreen Wastewater treatment

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UV-filter benzophenone-3 inhibits agonistic behavior in male Siamese fighting fish (*Betta splendens*).

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Abstract

Benzophenone-3 (BP-3) is a widely used organic UV-filter compound. Despite the frequent occurrence of BP-3 in aquatic environments, little is known about its effect on fish behavior. The aim of this study was to investigate the endocrine disrupting effects of BP-3 in male fighting fish (*Betta splendens*) with a focus on agonistic behavior. Male fighting fish were exposed to 10, 100, and 1000 µg/L BP-3, as well as a solvent control (0.1% ethanol) and a positive control (100 ng/L 17α-ethynylestradiol, EE2), for 28 days. At the beginning and the end of exposure, standard length and body mass of the fish were measured for calculating the condition factor (CF). In addition, spontaneous swimming activity (total distance moved) and agonistic behavior (maximum velocity and duration of opercular display in front of a mirror) were also quantified. At the end of exposure, the fish gonads were sampled for gonadosomatic index (GSI) measurement and histology. After the exposure, CF was significantly decreased in the 1000 µg/L BP-3 groups. Spontaneous swimming activity was not affected. However, maximum velocity was significantly reduced in the EE2 and 1000 µg/L BP-3 treatments; duration of opercular display was significantly decreased in the EE2 and 10 and 1000 µg/L BP-3 treatments. GSI was not significantly different between groups. There was a slight but statistically significant decrease of relative proportion of mature spermatozoa in testicular tissue in the 100 µg/L BP-3 treatment. Collectively, our results demonstrate that BP-3 can disrupt agonistic behavior of male fighting fish, indicating the endocrine disrupting activity of this compound.

KEYWORDS:

Aggression; Behavior; Benzophenone-3; *Betta splendens*; Gonad



Ecotoxicological evaluation of four UV filters using marine organisms from different trophic levels *Isochrysis galbana*, *Mytilus galloprovincialis*, *Paracentrotus lividus*, and *Siriella armata*



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HIGHLIGHTS

- We assessed the risk by input of UV filters into marine recreational waters.
- UV filter concentrations measured in beach water ranged between 35 and 164 ng L⁻¹.
- 4-MBC and BP-3 toxicity thresholds to marine organisms were 4–5 µg L⁻¹.
- EHMC and BP-4 were less toxic.
- There is a potential risk to coastal environment for UV filters BP-3 and 4-MBC.

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ABSTRACT

Due to the concern about the negative effects of exposure to sunlight, combinations of UV filters like 4-Methylbenzylidene-camphor (4-MBC), Benzophenone-3 (BP-3), Benzophenone-4 (BP-4) and 2-Ethylhexyl-4-methoxycinnamate (EHMC) are being introduced in all kind of cosmetic formulas. These chemicals are acquiring a concerning status due to their increasingly common use and the potential risk for the environment. The aim of this study is to assess the behaviour of these compounds in seawater, the toxicity to marine organisms from three trophic levels including autotrophs (*Isochrysis galbana*), herbivores (*Mytilus galloprovincialis* and *Paracentrotus lividus*) and carnivores (*Siriella armata*), and set a preliminary assessment of potential ecological risk of UV filters in coastal ecosystems. In general, EC₅₀ results show that both EHMC and 4-MBC are the most toxic for our test species, followed by BP-3 and finally BP-4. The most affected species by the presence of these UV filters are the microalgae *I. galbana*, which showed toxicity thresholds in the range of µg L⁻¹ units, followed by *S. armata* > *P. lividus* > *M. galloprovincialis*. The UV filter concentrations measured in the sampled beach water were in the range of tens or even hundreds of ng L⁻¹. The resulting risk quotients showed appreciable environmental risk in coastal environments for BP-3 and 4-MBC.

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1. Introduction

Emerging pollutants are chemical compounds not usually considered of interest in routine environmental analysis but that are becoming of environmental concern because of their increasingly common use and the potential risk to human and ecosystem health, including: pharmaceuticals and personal care products, endocrine disruptors, nanoparticles, industrial chemicals, biological metabolites and toxins among these substances (Richardson, 2010; Rosenfeld and Feng, 2011). Due to the increasing concern

about the negative effects of extended exposure to sunlight, combinations of several chemicals – what we refer as UV filters – are being introduced not only into sunscreens but also into all kinds of cosmetic formulas (creams, shampoos). UV filters are aromatic compounds such as 4-Methylbenzylidene-camphor (4-MBC), Benzophenone-3 (BP-3), Benzophenone-4 (BP-4) and 2-Ethylhexyl-4-methoxycinnamate (EHMC) which are extensively used in common personal care products because they adsorb UV radiation. Composition of cosmetic products in Europe is regulated by Directive 76/768/CEE (EC, 1976), and the maximum content of the above compounds in commercial products is by law 4% 4-MBC, 10% BP-3, 5% BP-4 and 10% EHMC. In USA the composition is regulated by US Food and Drug Administration (US Food 2013) and

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stipulates slightly different values for the maximum content of UV filters, namely: 6% BP-3, 10% BP-4 and 10% EHMC, the use of 4-MBC is not allowed as a sunscreen component in USA. Some UV filters are also considered chemicals of concern because they have shown estrogenic activity in *in vitro* laboratory tests on fish and other aquatic organisms (Balmer et al., 2005; Kunz and Fent, 2006; Kunz et al., 2006; Brausch and Rand, 2011).

To be effective, UV filters must be stable on exposure to UV radiation, but several studies have reported degradation by photolysis (Diaz-Cruz et al., 2008; Rodil et al., 2008; Richardson, 2010) and little is known about the toxicity of the degradation products. UV filters enter the marine environment from discharges of lakes and rivers (Balmer et al., 2005), from sewage sludge and from direct washing in recreational locations and beaches (Plagellat et al., 2006; Rodil et al., 2008; Fent et al., 2009). Their use is so widespread that they are commonly detected in wastewaters, swimming-pool waters, rivers, lakes and the oceanic superficial microlayer. However most information about the detection, concentration and effects on living organisms comes mainly from rivers and lakes where their high environmental concentration is increasingly worrying (Balmer et al., 2005; Buser et al., 2006; Fent et al., 2010). In contrast, data available from marine/coastal ecosystems are very scarce (Langford and Thomas, 2008; Rodil et al., 2012).

The aim of this study is to assess the behaviour in seawater and the toxicity to marine organisms of four of the most commonly used UV filters, 4-MBC, BP-3, BP-4 and EHMC. For the toxicity assessment four representative test organisms from three different trophic levels were used. These experiments provide a preliminary assessment of potential ecological risk of UV filters in coastal ecosystems, by following risk characterization procedures based on both the presence of these compounds in the marine environment and the inherent toxicity of these chemicals tested in laboratory bioassays. In our knowledge, this is the first risk assessment study of UV filters conducted in the marine environment.

2. Materials and methods

2.1. Biological material and toxicity tests

All biological materials were provided by ECIMAT (Toralla Marine Station, University of Vigo), either cultured in the Marine Station or collected from the most oceanic part of Ria de Vigo (Galicia, NW Iberian Peninsula), and conditioned in running seawater facilities.

A non-axenic culture of *Isochrysis galbana* provided by ECIMAT was grown in a 10-fold diluted EDTA-free f/2 medium, and kept in an incubator at 20 °C with 24 h light cycle (salinity 34 ppt, $\geq 8 \text{ mg L}^{-1}$ dissolved oxygen, pH ~ 8) until exponential growth was reached (5–6 days). Increasing concentrations of each one of UV filters were tested following the procedure proposed by Pérez et al. (2010a) in 250 mL borosilicate Erlenmeyer flasks in triplicate and three additional flasks were used as control cultures. Algae inoculum was added from the 5 L Erlenmeyer flask to an initial cell density of 7000 cells mL^{-1} . Cell density, was measured after the 72 h of exposure and cell counts were carried out with a Z2 Coulter Counter particle size analyser (Beckman-Coulter Particle Count and size analyzer USA). Growth rate (GR) was calculated as described by Pérez et al. (2010b); $\text{GR} (\text{day}^{-1}) = [\ln (\text{initial number cells}) - \ln (\text{final number cells})]/t$.

Sea urchin (*Paracentrotus lividus*) embryo-larval test was performed as described by Saco-Alvarez et al. (2010). Gamete maturity was checked under the microscope and only batches of eggs that were spherical and undamaged, and mobile sperm were used for the experiments. Fertilized eggs (density 40 mL^{-1}) were

transferred to vials containing the experimental solutions and were incubated at 20 °C for 48 h until larvae reached the 4-arm-pluteus stage. Vials were then fixed with two drops of 40% formalin and observed under an inverted microscope. Measurement of sea urchin larval growth ($n = 35$) were recorded. Larval growth was assessed by measuring the maximum dimension in the first 35 individuals per vial (including embryos), and subtracting the average diameter of the fertilized eggs.

Mussels, *Mytilus galloprovincialis*, were induced to spawn by thermal stimulation in separated beakers with 0.2 μm -filtered seawater. Gametes were examined under the microscope, checking shape and color for eggs and motility for sperm (Beiras and His, 1995). After fertilization 40 eggs mL^{-1} were transferred to the experimental vials that were incubated at 20 °C for 48 h until the second larval stage (D-veliger larvae) is reached (Bellas et al., 2005). The biological response for mussel bioassay is percentage of normal D-larvae ($n = 100$).

Swarms of *Siriella armata* were maintained in 100 L plastic tanks. The organisms were fed every two days with nauplii or metanauplii of *Artemia salina*. One day before the start of the test, mature females bearing embryos in the last stage of development, i.e. in the late post-nauplioid phase (Cuzin-Roudy and Tchernigovtzeff, 1985) were separated. The neonates released within 24 h were used in the tests. Incubations were conducted in 20 mL glass vials, a single individual per vial were delivered by plastic pipette in each compartment. A total of twenty individuals were used for each concentration. Glass vials were incubated in an isothermal room at 20 °C and 16 h light: 8 h dark photoperiod for 96 h. Neonates were fed daily between 10 and 15 nauplii of 24–48 h. post-hatch *Artemia salina*. Dead mysids were counted after 24, 48, 72 and 96 h. Exposure time was set to 96 h as described in Pérez and Beiras (2010).

2.2. Experimental solutions

4-Methylbenzylidene-camphor (4-MBC), Benzophenone-3 (BP-3), Benzophenone-4 (BP-4) and 2-Ethylhexyl-4-methoxycinnamate (EHMC) were obtained from Aldrich (Milwaukee, WI, USA) and Merck (Darmstadt, Germany). UV solution stocks were prepared in Dimethyl sulfoxide (Me_2SO) and stored in cold and darkness. Nominal solutions were prepared from stocks in Artificial Sea Water (ASW) and maintained in darkness during the whole process. Me_2SO in the experimental solutions was always under 0.1% (v/v).

2.3. Chemical analysis

Analyses of the stock solutions were performed in order to identify the stability of the UV filters in solution along the experimental conditions for each of the bioassays. Some UV filter solutions were made up again and the whole bioassay procedures were replicated. T_0 samples as well as final samples (48 h for sea urchin and mussel, 24 and 72 h for microalgae and 96 h later for crustacean) were analyzed within the next 24 h. UV filters determination was performed by LC–MS, using a LC–QTOF–MS instrument acquired from Agilent (Wilmington, DE, USA). The LC was an Agilent 1200 Series, consisting of a vacuum degasser unit, an auto sampler, two isocratic high pressure mixing pumps and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source. A sample volume of 100 μL was injected into a Ascentis Express C18 column (50 mm \times 2.1 mm, 2.7 μm particle diameter; 100 Å pore size) (Supelco) maintained at a constant temperature of 35 °C. The target compounds were separated at a flow rate of 0.2 mL min^{-1} using 5 mM of ammonium acetate in both, Milli-Q water (A) and MeOH (B). The following binary gradient was applied: 0–1 min, 5% B; 1–10 min, linear gradient to 100%

B; 10–12 min, 100% B and finally 12–22 min, 5% B. Nitrogen (99.999%), used as nebulizing (42 psi) and drying gas (350 °C, 9 L min⁻¹) in the dual ESI source, was provided by a high purity generator (ErreDue srl, Livorno, Italy). The QTOF instrument was operated in the 2 GHz (Extended Dynamic Range) mode and compounds were ionized in positive ESI, applying capillary and fragmentor voltages of 4000 and 150 V, respectively. The ions with *m/z* ratios 229.0859 for BP-3, 255.1743 for 4-MBC and 291.1955 for EHMC were considered for quantification. A reference calibration solution (Agilent calibration solution A) was continuously sprayed in the source of the QTOF system, through a second nebulizer. The ions with *m/z* ratios of 121.050873 and 922.009798 were considered for recalibrating the mass axis, ensuring the accuracy of mass assignments throughout the chromatographic run. The Mass Hunter Workstation software was used to control all the acquisition parameters of the LC–ESI–QTOF–MS system and also to process the obtained data. The method afforded quantification limits of 2, 1 and 2 ng mL⁻¹ for BP-3, 4-MBC and EHMC, respectively.

2.4. Risk assessment

Following the standard steps of quantitative risk assessment (US EPA, 2012, 2013), we have identified a hazardous group of substances used as UV filters, assessed the exposure levels by measuring the environmental concentrations (MEC) in coastal environments, and quantified their toxicity for marine organisms representative of three trophic levels using toxicity tests with sensitive early life stages in order to calculate the PNEC (Predicted No Effect Concentration).

In order to measure the MEC in coastal environments, samples of sea water from two touristic beaches, Coira and Toralla, from the Galician rias (NW Iberian Peninsula), were taken in July 2012 and the concentrations of our target compounds were determined following the procedures detailed elsewhere (Rodil et al., 2009b). In brief, 200–500 mL of filtered samples were subjected to solid-phase extraction (SPE) on an Oasis HLB 200 mg cartridge and eluted with 3 × 10 mL of MeOH. This extract was evaporated and reconstituted in 1 mL of MeOH/water (1/1) and 10 µL were injected and analyzed by LC–ESI–QTOF–MS as described previously. The method afforded quantification limits of 10, 9 and 10 ng L⁻¹ for BP-3, 4-MBC and EHMC, respectively. Water was sampled with a previously acid washed (10% H₂SO₄) and distilled water cleaned Pyrex bottle at 30 cm depth avoiding the surface microlayer. All samples were maintained in cold and darkness until analysed.

In order to assess the toxicity of the UV filters for marine organisms we conducted the toxicity tests described above and calculated the toxicity parameters NOEC (no observed effect concentration), LOEC (lowest observed effect concentration), EC₅₀ (concentration causing a 50% reduction in the endpoint) and EC₁₀ (concentration causing a 10% reduction in the endpoint) from dose–response curves, following the statistical methods described below.

Finally risk was assessed by comparing the EC₁₀ values of the most sensitive test species for each compound (the so called critical values) with the measured environmental concentrations (Meister and Van der Brink, 2000; Beiras and Bellas, 2008). An assessment/uncertainty factor of 100 that takes into account inter and intraspecific variability of responses to the compounds analysed (Fent et al., 2010; US EPA, 2013) was applied to the EC₁₀ threshold to calculate the PNEC: PNEC = EC₁₀/100. The Risk quotient (R) was then calculated as the ratio R = MEC/PNEC.

2.5. Statistical analysis

Statistical analyses were conducted using SPSS version 19.0 and Statistica version 6.0 software. *I. galbana* cell growth, percentage of

normal D-larvae for mussel, larval size for sea urchin and survival for the mysids were the endpoints analysed. All data were corrected by the control response. Normal distribution and homoscedasticity of data was checked using the Shapiro–Wilk and the Levene tests respectively. Angular transformation was required for the percentage of normal D-larvae to achieve normal distribution. When significant differences (*p* < 0.05) among groups were found using ANOVA then each treatment was compared to the control using Dunnett's post hoc test to calculate NOEC and LOEC.

In the case of *S. armata* NOEC and LOEC values were calculated with the Kruskal–Wallis and the Mann–Whitney U non-parametric tests because data did not meet the requirements for parametric tests. The EC₅₀ and EC₁₀ values and their 95% confidence intervals were calculated by fitting the survival data to a modified Weibull dose–response model (Murado et al., 2002).

3. Results

3.1. Stability of the chemicals in solution

Concerning their stability in aqueous solution, the UV filters tested showed different behaviour depending on the substance and the experimental set-up (Table 1). In the testing conditions used for the *Paracentotus lividus* and *M. galloprovincialis* bioassays, BP-3 and 4-MBC showed remarkable stability, with an average drop of 23% for BP-3 and 27% for 4-MBC after 48 h. In contrast, EHMC was very instable and its measured concentration showed a 72% reduction after 48 h in solution. In the testing conditions used for *S. armata* the stability was again high for BP-3 but lower for 4-MBC (32.5% and 75.5% reduction respectively), while EHMC initial concentration measured was on average 80% less than the initial nominal concentration, and virtually disappeared from solution after the 96 h incubation period. Finally in the *I. galbana* bioassay the same stability pattern is reproduced; BP-3 was remarkably stable (15% reduction at 72 h), and both 4-MBC and EHMC actual concentrations dropped throughout the incubation period (82% and 99% reduction in average). Therefore, when interpreting the toxicity data presented below, calculated on a nominal concentration basis, it must be taken into account that the EHMC and (in the cases of *I. galbana* and *S. armata*) 4-MBC EC_x values are overestimated. This overestimation concerns only the description of the theoretical toxicity parameters of the isolated compounds in ideal laboratory conditions, since the actual toxicity of those compounds in the environment, where degradation is expected, is more accurately described by the toxicity parameters here presented, calculated on a nominal concentration basis.

3.2. Toxicity tests

For the microalgae *I. galbana* (Fig. 1a), the most toxic compound was BP-3 (EC₅₀ 13.87 µg L⁻¹) followed by EHMC (EC₅₀ 74.73 µg L⁻¹) and 4-MBC (EC₅₀ 171.45 µg L⁻¹). In contrast, for *M. galloprovincialis* (Fig. 1b) the most toxic UV filter resulted to be 4-MBC (EC₅₀ 587.17 µg L⁻¹), then with similar toxicity EHMC (EC₅₀ 3118.19 µg L⁻¹) and finally BP-3 (EC₅₀ 3472.59 µg L⁻¹). *P. lividus* (Fig. 1c) results showed that sea urchin larvae are more affected by the exposure to EHMC (EC₅₀ 284 µg L⁻¹) and 4-MBC (EC₅₀ 854 µg L⁻¹) than to BP-3 (EC₅₀ 3280 µg L⁻¹).

The crustacean *S. armata* (Fig. 1d) was similarly affected by the exposure to 4-MBC (EC₅₀ 192.63 µg L⁻¹) and EHMC (EC₅₀ 199.43 µg L⁻¹) and less affected by BP-3 exposure (EC₅₀ 710.76 µg L⁻¹). The EC₅₀ and EC₁₀ values with their 95% confidence intervals for all species and UV filters, as well as the NOEC and LOEC values are shown in Table 2.

Table 1

Nominal (first value) and measured (second value) chemical concentrations ($\mu\text{g L}^{-1}$) in the exposure water along the incubation time (h) for the different tests conducted. Percentages indicate measured: nominal concentration ratios. Incubation conditions for the *P. lividus* and *M. galloprovincialis* tests were the same.

UV filter	<i>P. lividus</i> and <i>M. galloprovincialis</i>		<i>S. armata</i>		<i>I. galbana</i>		
	0 h (%)	48 h (%)	0 h (%)	96 h (%)	0 h (%)	24 h (%)	72 h (%)
BP-3	300–325–108	300–369–102.5	345–349–101.2	345–393–114	5–4.3–86	5–3.9–78	5–3.5–70
	3000–2981–99.4	3000–2900–96.7	750–672–89.6	750–604–80.7	15–18.2–121.3	15–14.9–99.3	15–17.2–114.7
	30000–24063–80.2	30000–10138–33.8	1500–1488–99.2	1500–328–21.9			
4-MBC	300–294–98	300–227–75.7	37.04–31.3–84.5	37.04–5.5–14.8	10–1.8–18	10–2.2–22	10–2.1–21
	600–661–110.2	600–387–64.5	185–118–63.8	185–55–29.7	30–4–13.3	30–4.8–16	30–4.3–14.3
	1200–1212–101	1200–951–79.3	333.3–204–61.2	333.3–97–29.1	100–30–30	100–20–20	100–17.9–17.9
EHMC	50–39–78	50–23–46	50–15–30	50–0–0	10–15.1–151	10–11.4–114	10–0–0
	200–111–55.5	200–44–22	200–27–13.5	200–0–0	30–43.8–146	30–12.9–43	30–0.3–1
	600–463–77.2	600–93–15.5	800–126–15.8	800–18–2.3	100–70.3–70.3	100–24.7–24.7	100–1.9–1.9

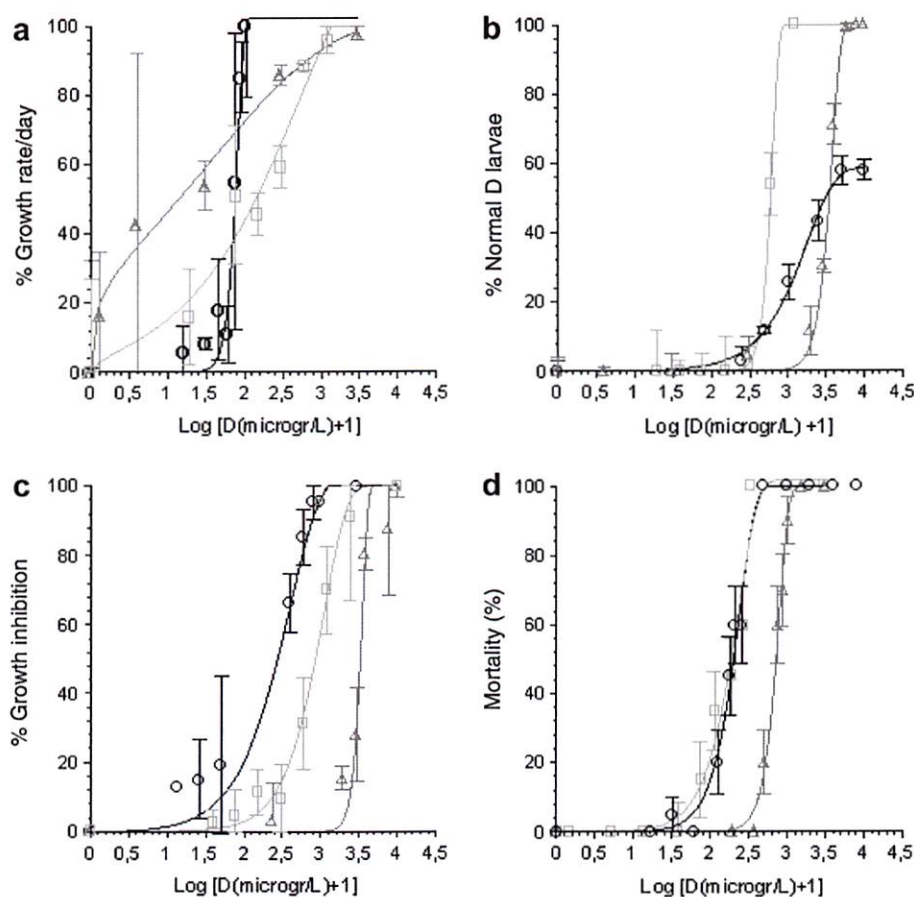


Fig. 1. Percentage of growth per day of *Isochrysis galbana* at 72 h (a), percentage of normal D-larvae *Mytilus galloprovincialis* at 48 h (b), percentage of growth inhibition of *Paracentrotus lividus* at 48 h (c) and percentage of dead neonates of *Siriella armata* at 96 h in each concentration (d), represented as $\log(\mu\text{g L}^{-1} + 1)$ for BP-3 (triangles), 4-MBC (squares), EHMC (circles).

BP-4 was not toxic for any of the organisms tested, with EC_{50} values over $10000 \mu\text{g L}^{-1}$.

3.3. Environmental concentrations and risk assessment

In order to estimate the environmental concentration of UV filters caused by direct wash from swimmers in recreational locations and beaches, samples of sea water from two touristic places were analyzed. Fig. 2 shows the results of these analyses, along with concentrations of UV filters measured in other natural coastal waters taken from the literature. The values found ranged in general between 10 and 300 ng L^{-1} (Fig. 2). The substance present in the highest concentration was BP-4 (164.4 ng L^{-1} in Coira beach water and 58.8 ng L^{-1} in Toralla), followed by 4-MBC that could only be identified in Coira (84.6 ng L^{-1}). The concentration of

BP-3 was twofold in Coira (68.6 ng L^{-1}) compared to Toralla (21.7 ng L^{-1}), and finally the concentration of EHMC was 52.5 ng L^{-1} in Coira and 35.7 ng L^{-1} in Toralla.

Table 3 reflects the resulting Risk quotient (R) values for the four UV-filters studied, and for BP-3 and 4-MBC $R > 1$, therefore, there is a potential threat to the coastal environments caused by these chemicals.

4. Discussion

Based on the EC_{50} values, EHMC and 4-MBC are the most toxic UV filters for marine invertebrates, whereas BP-3 was selectively toxic for the microalgae *I. galbana* at concentrations as low as ng mL^{-1} units. Finally BP-4 was found to have the lowest toxicity

Table 2
EC₅₀, EC₁₀ for UV filters (μg L⁻¹). The 95% confidence intervals are given in brackets No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) levels for all the compounds. In the table, nc means “not calculated” and nm “not measured”.

		<i>I. galbana</i>	<i>M. galloprovincialis</i>	<i>P. lividus</i>	<i>S. armata</i>
BP-3	EC ₅₀	13.87 (0–41.69)	3472.59 (3359.65–3585.52)	3280 (2507.85–3951.66)	710.76 (672.46–749.05)
	EC ₁₀	3.69 (n.c.)	2146 (1937.84–2354.67)	2423.22 (1247.69–3586.74)	421.1 (361.15–481.05)
	NOEC	30	30	1920	375
	LOEC	300	300	3840	500
BP-4	EC ₅₀	>10000	>10000	>10000	>10000
	EC ₁₀	n.m.	n.m.	n.m.	n.m.
	NOEC	n.m.	n.m.	n.m.	n.m.
	LOEC	n.m.	n.m.	n.m.	n.m.
4-MBC	EC ₅₀	171.45 (0–421.72)	587.17 (586.94–587.41)	853.74 (620.35–980.82)	192.63 (156.18–229.10)
	EC ₁₀	5.4 (0–14.84)	410.61 (409.31–411.92)	238.94 (149.68–343.43)	71.63 (38.58–104.68)
	NOEC	18	300	300	37.04
	LOEC	75	600	600	74
EHMC	EC ₅₀	74.72 (64.45–84.99)	3118.18 (n.c.)	283.69 (159.87–407.34)	199.43 (184.47–214.39)
	EC ₁₀	51.506 (38.84–64.62)	430.648 (147.69–38.09)	48.84 (1.54–99.04)	80.79 (59.80–101.78)
	NOEC	15	500	600	62.5
	LOEC	30	1000	800	125

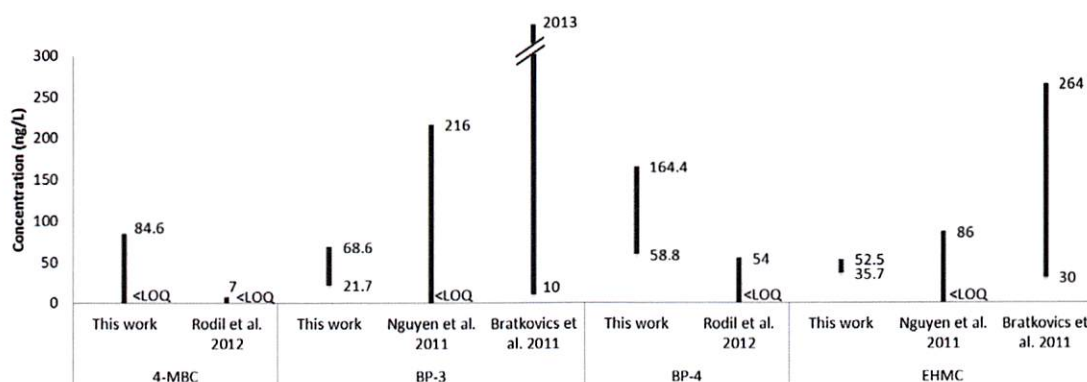


Fig. 2. Environmental concentration of UV-filter compounds measured in coastal sea water samples from two touristic beaches in NW Iberian Peninsula (this work) along with values from the literature.

for all test species. This ranking of toxicity is consistent with previous results on freshwater species, such as the crustacean *D. magna* (Fent et al., 2009) where EHMC was more toxic than 4-MBC, followed by BP-3 and finally BP-4. These aromatic compounds have a moderate to high toxicity according to the EPA criteria (Callow, 1998) that considers highly toxic those compounds with an EC₅₀ < 1 mg L⁻¹ and intermediate-low toxicity those with EC₅₀ among 1–100 mg L⁻¹.

By species, the most affected by the presence of these UV filters are the unicellular alga *I. galbana* followed by the crustacean *S. armata*, the sea-urchin *P. lividus* and the bivalve *M. galloprovincialis*, in this order.

The results obtained in the present study show that, according to their EC₅₀ values for the same test species using the same standard bioassays, the toxicity of the UV filters BP-3, EHMC and 4-MBC is similar to that of the most toxic trace metals Copper, Mercury, Cadmium, Lead or Zinc (Fernández and Beiras, 2001; Beiras and Albentosa, 2004; Pérez and Beiras, 2010).

When assessing environmental risk, it should be also taken into account that the stability of these products in solution was quite different. EHMC was the most unstable, with a reduction from 72% to almost 100% for the three different bioassay methodologies. 4-MBC and especially BP-3 were more stable in solution, but 4-MBC concentrations decreased with time in the *Siriella* and *Isochrysis* test conditions. 4-MBC proved to be quite stable when the toxicity test was conducted in darkness (27% reduction for *P. lividus* and *M. galloprovincialis* bioassay) but unstable when conducted in the presence of light (75.5% and 82% reduction in the experimental conditions used for the *S. armata* and *I. galbana* tests, due to the need of light for those species). This phenomena might be caused by the photodegradation into phototransformation products (Rodil et al., 2009a), but there might be also uptake by the organisms because the reduction in the concentrations along time (note also that test duration is different for both species) takes place also in those toxicity tests that were conducted in darkness, though at a lower extent.

Table 3
UV filters Risk Assessment in coastal environments. R: risk quotient. No Observed Effect Concentration (NOEC) of the most sensitive species, Predicted No Effect Concentration (PNEC = NOEC/Assessment factor) and Measured Environmental Concentration (highest concentration measured) (MEC).

UV filter	EC ₁₀ (μg L ⁻¹)	Assessment factor	PNEC (ng L ⁻¹)	MEC (ng L ⁻¹)	R MEC/PNEC	Environmental risk
BP-3	3.69	100	36.9	68.6	1.86	Yes
4-MBC	5.40	100	54.0	84.6	1.57	Yes
EHMC	48.84	100	488.4	52.5	0.11	No

The reduction in the concentrations in the test media of some of the UV filters with exposure time indicates that EC₅₀, EC₁₀, NOEC and LOEC values shown in Table 2 can be overestimated. However, nominal concentrations were still preferred to measured concentrations for calculation of toxicity parameters for two reasons. First, it is likely that degradation products, not detected in the analytical checking of actual concentrations, show similar toxicity than parental compounds. Secondly, the same photodegradation and chemical degradation processes that take place in the test chambers are likely to take place in the natural environment also. The actual chemical processes that are taking place on the UV filters dissolved in natural sea water along time, the role of microbial degradation, potential enhancement or reduction of their toxicity due to phototransformation, and the kinetics of the bioaccumulation processes are worth further study.

The present study focused on the environmental risk caused by direct input of UV filters from cosmetics into recreational waters. Levels of these compounds in beaches were in the range of ng L⁻¹ (see also Bratkovics and Sapozhnikova, 2011, Brauch et al., 2011, Richardson, 2010; Nguyen et al., 2011; Rodil et al., 2012) and there is a potential risk to the environment for two of the UV filters tested, namely BP-3 and 4-MBC (Table 3). However other sources of UV filters such as urban waste waters were not considered in this study. UV filter concentrations in that case may be orders of magnitude higher (µg L⁻¹) in areas close to sewage sludge discharges (Giokas et al., 2007; Brausch and Rand, 2011). Therefore in that scenario the risk will be even higher.

The quantification of risk using risk quotients is highly dependent on the choice of the Assessment Factor, a debatable topic beyond the scope of the present study. We have followed international standard procedures (US EPA, 2012, 2013) that recommend for our case an AF value of 100. This recommendation is similar to that from the EC Guidance Document for derivation of Environmental Quality Standards for the case of studies including not only short term results from three trophic levels but also results from one long term test from the most sensitive organism in the acute tests (EC, 2011). In our case the embryo tests and algal growth tests may both be considered as chronic tests, since they include exposure during the full duration of that particular life stage. Therefore, when algae, sea urchin or mussel embryos provide the critical values, the AF should not be higher than 100, otherwise risk estimation would be overconservative.

This research supports the need of establishing environmental quality standards (EQS) for UV filters based on toxicity testing with key aquatic organisms, as well as identifying and reducing the different input sources to the environment. The establishment of meaningful EQS for UV filters that should not be exceeded, based on their biological toxicity, in combination to regulation on maximum input of these products to the marine environment, will help us to control these emerging pollutants before critical levels are reached in the environment.

Acknowledgements

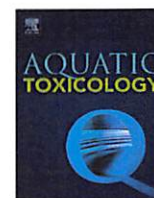
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Effects of benzophenone-3 on the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Microcystis aeruginosa*



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ABSTRACT

Effects of benzophenone-3 (BP-3) on the green alga, *Chlamydomonas reinhardtii*, and the cyanobacterium, *Microcystis aeruginosa*, were investigated. The tested organisms were exposed to environmental levels of BP-3 for 10 days, at nominal concentrations from 0.01 to 5000 $\mu\text{g L}^{-1}$. Specific growth rate and photosynthetic pigments were employed to evaluate the toxic responses. The two tested algae had distinct toxic responses towards BP-3 stress, with the green alga *C. reinhardtii* being more sensitive than the cyanobacterium *M. aeruginosa*, based on EC_{20} and EC_{50} values. Uptake of BP-3 from the medium occurred in both species, with *M. aeruginosa* showing greater overall uptake (27.2–77.4%) compared to *C. reinhardtii* (1.1–58.4%). The effects of BP-3 on *C. reinhardtii* were variable at concentrations lower than 100 $\mu\text{g L}^{-1}$. At higher concentrations, the specific growth rate of *C. reinhardtii* decreased following a reduction in chlorophyll *a* (chl-*a*) content. Further experiments showed that BP-3 regulated the growth of *C. reinhardtii* by affecting the production of chl-*a*, chlorophyll *b* and carotenoids. In *M. aeruginosa*, specific growth rate was only moderately affected by BP-3. Additionally, the production of chl-*a* was significantly inhibited over the different exposure concentrations, while the production of carotenoids was stimulated. These results indicate a potential detrimental effect on prokaryotes and eukaryotes and that the mechanism of action varies with species.

1. Introduction

As primary producers, phytoplankton organisms are the base of the trophic chain in aquatic ecosystems. Cyanobacteria and green algae are major constituents of the phytoplankton community in aquatic ecosystems. Any alterations in these photoautotrophic populations may result in significant bottom-up effects on organisms at higher trophic levels (Nie et al., 2013). Studies have shown that the growth of these photoautotrophs can be affected by numerous environmental factors. However, most of these studies focused mainly on traditional factors, such as nutrients, temperature, pH, light, salinity and metals, with limited attention on emerging organic contaminants (Chen et al., 2015; Liu et al., 2015; Pillai et al., 2014).

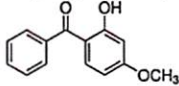
Benzophenone-3 (BP-3), a widely used benzophenone type UV filter, has been available as a sunscreen ingredient for over 40 years. BP-3 is permitted in sunscreen products at levels of up to 5–6% in Japan, Korea and U.S.A., and up to 10% in Australia, China and Europe (Sánchez-Quiles and Tovar-Sánchez, 2015). BP-3 is also added into

other cosmetic products (body lotions, hair sprays, and shampoos) as well as insecticides, agricultural chemicals and plastic bags because it can prevent polymers from degradation or pigmentation under sunlight (Jeon et al., 2006; Zhang et al., 2011a, 2011b). Widespread use of BP-3 results in its constant release into ambient environments. In environmental waters, reported BP-3 concentrations are normally relatively low (at ng L^{-1} level), while in wastewater effluents or recreational water bodies affected by swimmers, the concentrations can reach up to $\mu\text{g L}^{-1}$ or mg L^{-1} (Downs et al., 2015; Loraine and Pettigrove, 2006). Therefore, BP-3 can be present at high concentrations in environmental waters, leading to possible toxic effects in aquatic organisms.

Several studies have reported that BP-3 can affect the growth of algae (Pablos et al., 2015; Petersen et al., 2014; Rodil et al., 2009). A study assessing the toxicity of BP-3 on the green alga, *Scenedesmus vacuolatus*, reported a 24 h IC_{50} of 0.36 mg L^{-1} (Rodil et al., 2009). Another study showed a 72-h IC_{10} of 0.61 mg L^{-1} and 72-h IC_{50} of 0.96 mg L^{-1} in the green alga, *Desmodesmus subspicatus* (Sieratowicz et al., 2011). However, studies on evaluating the toxicity of BP-3

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Table 1
Structure and some physico-chemical properties of benzophenone-3.

Properties	Benzophenone-3
Synonym	2-Hydroxy-4-methoxybenzophenone, Oxybenzone
Chemical structure	
Molecular formula	C ₁₄ H ₁₂ O ₃
CAS. number	131-57-7
Molecular weight (g mol ⁻¹)	228.24
Water solubility (mg L ⁻¹)	2295.40 ^a
logK _{ow}	3.79 ^a
pKa	7.56 ^b
Half-life in surface water (h)	502.00 ^a

^a Values for boiling point, water solubility, octanol-water partition coefficient (logK_{ow}) and surface water half-life were obtained by Estimation Programs Interface (EPI) Suite developed by US EPA and Syracuse Research Corp. (SRC).

^b Acid dissociation constant (pKa) was obtained from (Kunz et al., 2006).

focused solely on green algae, with no information with regards to cyanobacteria.

The objective of this work therefore, was to further evaluate the uptake and effects of the emerging contaminant BP-3 at environmentally relevant levels in two common freshwater phytoplankton primary producers, representing prokaryotic and eukaryotic species. This was carried out with particular reference to ascertaining the effects of BP-3 on production of photosynthetic pigments. This is the first study assessing the uptake and toxicity of BP-3 on cyanobacteria and is also one of the few reports regarding its effects in green algae.

2. Materials and methods

2.1. Test substance

BP-3 was purchased from Sigma-Aldrich (Sigma-Aldrich, Singapore, product number H36206). The Chemical Abstract Services (CAS) number and physico-chemical properties are listed in Table 1. Stock solution of BP-3 (5000 mg L⁻¹) was prepared in methanol (HPLC grade, Sigma-Aldrich, Singapore) and stored at -20 °C in the dark.

2.2. Preparation of the algal culture

The green alga, *C. reinhardtii* (strain NIES 2463), and the cyanobacterium, *M. aeruginosa* (strain NIES 843), were both purchased from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES Collection), Japan. These two species were selected because of their widespread distribution, ecological relevance and importance in ecotoxicological studies (Chen et al., 2015; Pillai et al., 2014). Following Organization of Economic Co-Operation and Development test guidelines (OECD, 2001), the axenic strains were individually maintained in 500 ml sterilized McBride *Listeria* agar (MLA) medium at an initial inoculation of 10% concentration ($V_{inoculum}/V_{medium}$) in several 1 L Erlenmeyer flasks (Bolch and Blackburn, 1996). The cultures were incubated in a constant temperature room (28 ± 1 °C) under cool white fluorescent lamps with a light intensity of 25 ± 2 μmol photons m⁻² s⁻¹ (12/12 h light/dark photoperiod). To ensure homogenous cell growth, flasks were shaken and randomly relocated two or three times a day to eliminate the variations in light intensity at different positions. After reaching the mid-logarithmic growth stage, the cells were harvested by centrifugation (5000g, 4 °C, 15 min). The cell pellets were then washed three times with sterilized MLA medium. The concentrated cell mass was stored at 4 °C in the dark for a maximum of 7 days before downstream experiments.

2.3. Bio-assays

The growth inhibition assay was performed according to OECD (2001). In brief, 1 ml of the concentrated cell mass was inoculated in a clean 500 ml Erlenmeyer flask containing 200 ml MLA medium, reaching an initial cell density of approximately 5 × 10⁵ cells ml⁻¹ for *C. reinhardtii* and 1 × 10⁶ cells ml⁻¹ for *M. aeruginosa*. The medium was spiked with varying concentrations of BP-3 (0.01, 0.1, 1, 10, 100, 1000, 2000, 3000, 4000 and 5000 μg L⁻¹) prior to inoculation. The two lowest concentrations represent the most environmentally relevant concentrations. In all BP-3-treated groups, an appropriate volume of methanol was added to the medium to make the final methanol concentration to 0.1% (v/v). To check the effects of methanol on the growth of the tested strains, a solvent control was used by adding methanol to the medium to a final concentration of 0.1% (v/v). The abiotic removal of BP-3 was monitored by supplementing the culture flasks with an initial BP-3 concentration of 10 μg L⁻¹ without cells. The cultures were incubated for 10 days under the conditions as described previously. All the experiments were conducted in triplicate. Optical density (OD₆₈₀ for *C. reinhardtii*, OD₇₅₀ for *M. aeruginosa*) was monitored every two days. Chl-a concentration was measured on the last day (day 10) of the experiments.

Similar to the growth inhibition assay, another set of experiments was conducted to evaluate the effects of BP-3 on the production of photosynthetic pigments in the two tested algae with some modifications (hereinafter referred to as the confirmation assay). To provide a better growth environment for the tested algae, a TPP tissue culture flask (growth surface: 300 cm²; with filter; TPP Techno Plastic Products AG, Switzerland) was used. The MLA medium was spiked with BP-3 at concentrations of 0.01, 1 and 1000 μg L⁻¹. The three concentrations were selected based on the results of the previous growth inhibition experiment. The cultures were incubated for 7 days under the conditions described previously. All the experiments were conducted in triplicate. Optical density was monitored every two days. At days 1, 4 and 7, an aliquot of cell suspension was collected for measuring photosynthetic pigments.

2.4. Measurement of cell growth and photosynthetic pigments

Cell growth was monitored both for the growth inhibition assay and confirmation assay. The obtained OD values were converted to cell density based on the developed linear relationship between OD and cell density ($\frac{1}{2}(OD_{680} + 0.0059) \times 10^7$ cells ml⁻¹ for *C. reinhardtii* and $(OD_{750} + 0.0005) \times 10^7$ cells ml⁻¹ for *M. aeruginosa*, R² > 0.99). The specific growth rate (μ) was then calculated by fitting the cell number to the following exponential function (Fent et al., 2010):

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}$$

where N_1 and N_2 are the cell numbers at time t_1 and t_2 , respectively.

Apart from cell growth, chl-a concentration was measured on the last day (day 10) of the experiments for the growth inhibition assay. Chl-a variation (%) was then calculated by comparing chl-a content of the BP-3 treated groups with that of the controls (de Morais et al., 2014). A low variation value indicates a high inhibition effect.

For the confirmation assay, two lipid-soluble pigments (i.e., chl-a and carotenoid) and three water-soluble pigments (i.e., phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE)) were measured for *M. aeruginosa*. Three lipid-soluble pigments (i.e., chl-a, chlorophyll b (chl-b), and carotenoid) were measured for *C. reinhardtii*. Detailed analytical processes on these photosynthetic pigments can be found in Supporting Information (SI).

2.5. BP-3 measurement

Initial BP-3 concentrations in the culture medium were measured

before inoculation and residual BP-3 concentrations were determined on the last day of the experiment. BP-3 concentration was determined by a previously developed solid phase extraction (SPE) method coupled with high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) (You et al., 2015).

To enable the detection of BP-3, 50 ml of the cell suspension was collected from the Erlenmeyer flasks. All aliquots collected were centrifuged (5000 rpm, 15 min) and the supernatant (50 ml) was then subjected to SPE clean-up with some modifications, i.e., dilution was needed for concentrations higher than $1000 \mu\text{g L}^{-1}$. Briefly, the supernatant was first spiked with 20 ng of the isotopically-labeled standard. The mixture was then passed through an Oasis hydrophilic-lipophilic balance (HLB) C₁₈ SPE cartridge (200 mg, 6 ml, Waters, USA) which was pre-conditioned with 5 ml of methanol followed by 5 ml of Milli-Q water. The mixture was loaded onto the cartridge at a velocity of about 1 ml min^{-1} . The cartridge was washed with 5 ml Milli-Q water after loading, followed by vacuum drying for 30 min. The target chemical was eluted with 5 ml of methanol and another 5 ml of methanol/acetone (1/1, v/v). The extract was concentrated under a gentle stream of high-purity nitrogen (Air Products, Singapore) to less than 0.5 ml, and then reconstituted to 0.5 ml.

The target chemical (BP-3) was quantified by HPLC–MS/MS (Dionex Ultimate 3000, Dionex, USA) coupled with electro-spray ionization (ESI) tandem mass spectrometry (AB Sciex Qtrap 5500, Toronto, Canada) equipped with an Agilent Poroshell 120 EC-C18 reverse phase column (100 mm length \times 4.6 mm internal diameter; $2.7 \mu\text{m}$ particle size, Agilent, USA) connected with a guard column ($20 \times 2.1 \text{ mm}$, $5 \mu\text{m}$; Thermo Electron Corporation, Bellefonte, PA, USA), at a flow rate of 0.4 ml min^{-1} . The mobile phases were 5 mM ammonium acetate in Milli-Q water (mobile phase A) and acetonitrile/methanol (1/1, V/V) (mobile phase B). The gradient elution started with 10% B at 0 min, held for 0.5 min; linearly increased to 50% B at 0.5–1.5 min, then linearly increased to 95% B at 1.5–9 min, held for 6 min to 15 min, then re-equilibrate the column to initial conditions at 15.1 min and stabilized for 2 min. The overall running time was 17 min. The injection volume was $10 \mu\text{l}$ and the column temperature was 25°C .

The uptake of BP-3 from the culture medium was calculated as the difference between the concentrations on the first day and last day of the experiment divided by initial exposure concentrations.

2.6. Data analysis and statistical evaluation

Effective concentrations (EC₂₀ and EC₅₀) were calculated following OECD (2001) protocol, using actual initial exposure concentrations of BP-3 (expressed as the average of the triplicate measurements). Variations in growth were assessed by comparing the chl-a contents and specific growth rates between BP-3-treated groups and corresponding solvent (methanol) controls. The data were analyzed using a one-way analysis of variance (ANOVA) by OriginPro 2016 software (OriginLab Corporation). A Student's *t*-test was carried out to compare the differences in algal growth among the different BP-3 treatments.

3. Results

3.1. Uptake of BP-3 by the green alga *C. reinhardtii*

The abiotic control experiment (MLA spiked with BP-3 without algae) suggested that the removal of BP-3 from MLA medium due to abiotic factors (i.e., sorption to side wall of the flask, volatilization, and photolysis) under the same conditions was negligible (Fig. S1).

For the green alga, *C. reinhardtii*, the actual measured mean exposure concentrations were 0.02, 0.11, 1.04, 10.9, 102, 1034, 2110, 2996, 4211 and $5137 \mu\text{g L}^{-1}$ for the nominal concentrations of 0.01, 0.1, 1, 10, 100, 1000, 2000, 3000, 4000 and $5000 \mu\text{g L}^{-1}$, respectively (Fig. 1A). After 10 days of exposure, part of the BP-3 in the medium was taken up by the tested green alga. At the lowest exposure concentration

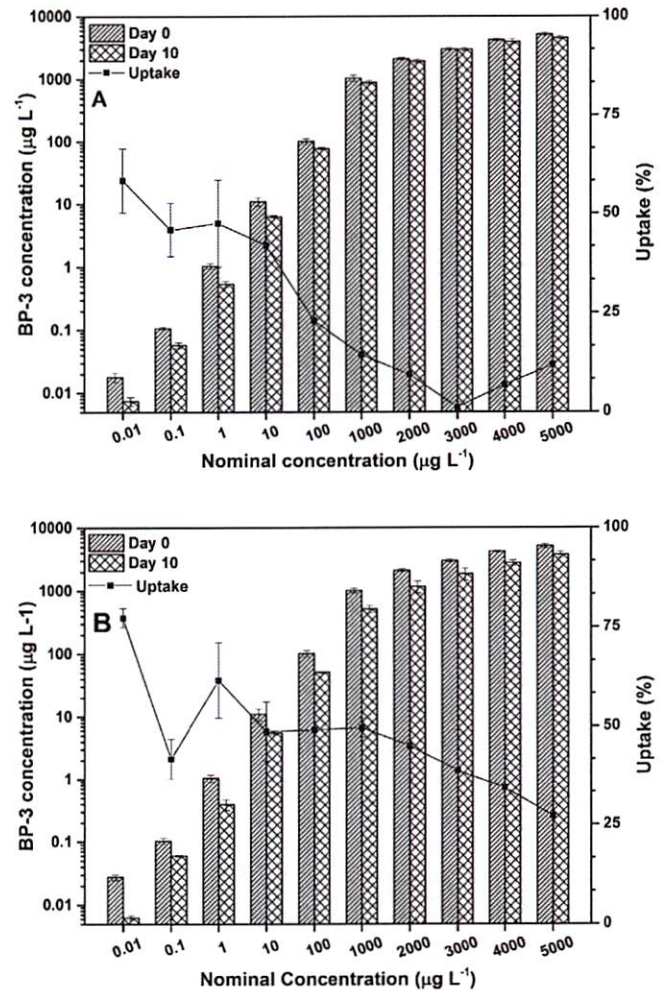


Fig. 1. Measured BP-3 concentrations ($\mu\text{g L}^{-1}$) in the first day and the last day of the 10-day exposure experiment (column) and uptake ratio (%) of BP-3 (plot) as a function of nominal concentrations, in the culture medium of (A) *C. reinhardtii* and (B) *M. aeruginosa*. Mean and standard deviation of triplicates are shown.

($0.01 \mu\text{g L}^{-1}$), the tested alga could take up 58.4% of the BP-3 in the medium, i.e., the highest uptake percentage among all the exposure concentrations. The uptake ratio decreased following increased exposure concentrations up to $3000 \mu\text{g L}^{-1}$, at which the lowest uptake rate (1.1% of the compound taken up in 10 days) was observed. It is worthwhile mentioning that the tested green alga tended to take up more BP-3 from the medium when the exposure concentrations were higher than $3000 \mu\text{g L}^{-1}$, i.e., 4000 and $5000 \mu\text{g L}^{-1}$.

3.2. Uptake of BP-3 by the cyanobacterium *M. aeruginosa*

For the nominal concentrations of 0.01, 0.1, 1, 10, 100, 1000, 2000, 3000, 4000 and $5000 \mu\text{g L}^{-1}$ for cyanobacterium *M. aeruginosa*, the actual initial exposure concentrations were measured to be 0.03, 0.10, 1.04, 11.0, 102, 1017, 2110, 2996, 4212 and $5137 \mu\text{g L}^{-1}$, respectively (Fig. 1B). Similar to *C. reinhardtii*, the tested cyanobacterium, *M. aeruginosa*, could also take up BP-3 from the medium, which was generally characterized by a higher uptake ratio at low exposure concentrations and a lower uptake ratio at high exposure concentrations. The highest uptake in 10 days (77.4%) was observed at $0.01 \mu\text{g L}^{-1}$ of BP-3, and the lowest (27.2%) was at $5000 \mu\text{g L}^{-1}$. It was clear that *M. aeruginosa* could take up more BP-3 in ten days than *C. reinhardtii*.

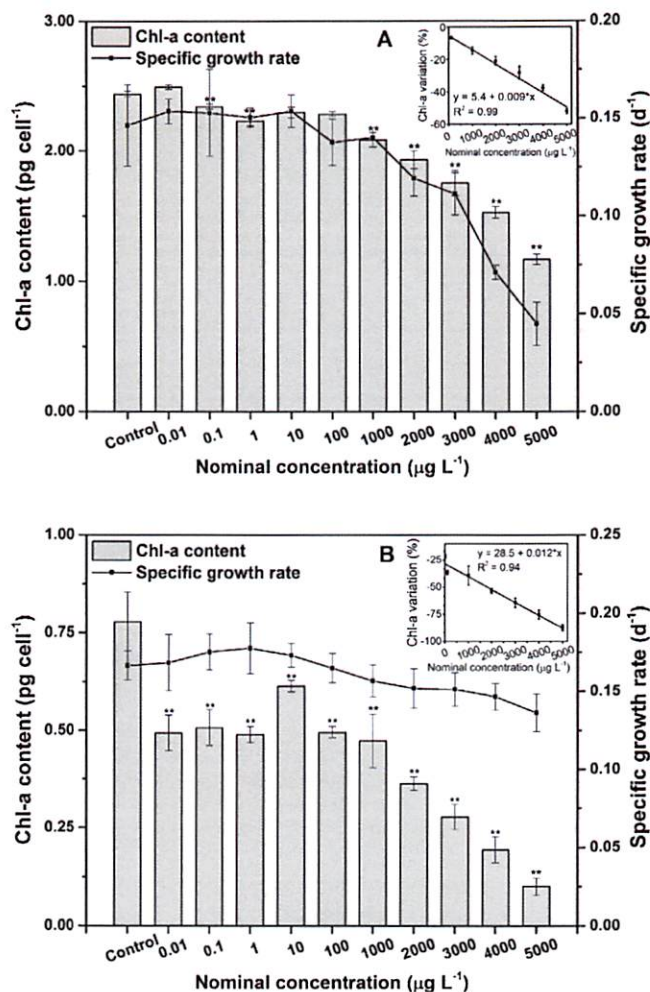


Fig. 2. Effects of BP-3 on chl-a content (column) and specific growth rate (plot) on (A) *C. reinhardtii* and (B) *M. aeruginosa* during 10 days of cultivation. Insets indicate the concentration dependency based on chl-a variation. A low variation value means large reduction of chl-a content. Mean and standard deviation of triplicates are shown. Columns with asterisks denote significant difference between control and treatment (** < 0.01).

3.3. Effects of BP-3 on the growth of *C. reinhardtii*

Fig. 2A shows the effects of varying BP-3 concentrations on chl-a content and specific growth rate of the green alga, *C. reinhardtii*. Except for three nominal concentrations (i.e., 0.01, 10 and 100 μg L⁻¹), chl-a content was significantly decreased when compared to respective controls (one-sided *t*-test, *p* < 0.01). In addition, the decrease of chl-a content was concentration-dependent when exposure concentrations were higher than 100 μg L⁻¹ (Fig. 2A, inset), leading to EC₂₀ and EC₅₀ values of 0.64 ± 0.03 and 1.85 ± 0.41 mg L⁻¹, respectively.

Regarding specific growth rate, the growth of *C. reinhardtii* was not affected at concentrations lower than 10 μg L⁻¹. However, beyond this concentration, concentration-dependent growth inhibition was observed (Fig. 2A). This concentration dependency corroborates the chl-a results, where a stronger inhibition was observed at higher concentrations of BP-3.

3.4. Effects of BP-3 on the growth of *M. aeruginosa*

For the cyanobacterium (*M. aeruginosa*), results for growth variations as assessed by chl-a were variable. The chl-a content was decreased along the entire exposure concentration range (Fig. 2B). Exposing to 5000 μg L⁻¹ saw the highest decrease (87.1 ± 2.9%) and 10 μg L⁻¹ the lowest (21.2 ± 1.9%). The tested alga produced similar

Table 2

Specific growth rate and production rates of photosynthetic pigments in tested algae in a 7-day BP-3 exposure experiment (n = 3).

Treatment	Control	Treatment A	Treatment B	Treatment C
<i>C. reinhardtii</i>				
μ	0.38 ± 0.01	0.38 ± 0.005	0.35 ± 0.01	0.34 ± 0.01
R _{Chl-a}	1.38 ± 0.02	1.40 ± 0.01	1.18 ± 0.03	0.79 ± 0.04
R _{Chl-b}	0.37 ± 0.01	0.34 ± 0.06	0.26 ± 0.05	0.16 ± 0.03
R _{Carotenoids}	0.68 ± 0.01	0.63 ± 0.03	0.52 ± 0.03	0.37 ± 0.01
<i>M. aeruginosa</i>				
μ	0.26 ± 0.001	0.28 ± 0.01	0.27 ± 0.01	0.25 ± 0.01
R _{Chl-a}	0.17 ± 0.004	0.12 ± 0.01	0.11 ± 0.01	0.11 ± 0.01
R _{Carotenoids}	0.14 ± 0.02	0.16 ± 0.004	0.15 ± 0.02	0.20 ± 0.01

Note:

Treatment A: algae exposed to BP-3 concentration of 0.01 μg L⁻¹.

Treatment B: algae exposed to BP-3 concentration of 1 μg L⁻¹.

Treatment C: algae exposed to BP-3 concentration of 1000 μg L⁻¹.

μ: specific growth rate, d⁻¹.

R: pigment production rate, μg ml⁻¹ d⁻¹.

amounts of chl-a in exposure concentrations below 10 μg L⁻¹. When concentrations were higher than 10 μg L⁻¹, the inhibition of production was concentration-dependent (Fig. 2B, inset). Based on chl-a production, EC₂₀ and EC₅₀ values were calculated to be 1.17 ± 0.42 and 2.46 ± 0.34 mg L⁻¹, respectively.

BP-3 did not affect the specific growth rate of *M. aeruginosa* at concentrations less than 10 μg L⁻¹ (Fig. 2B). However, above this concentration, growth inhibition was concentration-dependent. The overall inhibition was moderate, with a maximum inhibition of 18.2% at the concentration of 5000 μg L⁻¹.

3.5. Effects of BP-3 on photosynthetic pigments in *C. reinhardtii*

According to the results from the growth inhibition assay, at concentrations higher than 10 μg L⁻¹, the specific growth rate of *C. reinhardtii* decreased following a reduction in chl-a content, a phenomenon different from *M. aeruginosa* (Fig. 2). Therefore, we further explored this phenomenon by performing a new set of experiments, assessing other important photosynthetic pigments (e.g. carotenoids). This allowed for the calculation of the specific growth rate and pigment production rates over the entire exposure period (Table 2).

The growth rates of *C. reinhardtii* decreased at exposure levels of 1 and 1000 μg L⁻¹ (one-sided *t*-test, *p* < 0.05). Higher specific growth rates (0.34–0.38 d⁻¹) were obtained in this new set of experiments compared to the results obtained from the previous experiment (Table 2 and Fig. 2A). This may be due to differences in culture flasks (TPP flask and Erlenmeyer flask) as TPP flasks provide a better growth environment (i.e., bigger growth surface and better air exchange,) than Erlenmeyer flasks. It was clear that chl-a content in *C. reinhardtii* was significantly decreased by 1 and 1000 μg L⁻¹ of BP-3, with a decrease of 13–38% on day 7 relative to the controls (Fig. 3A, one-sided *t*-test, *p* < 0.05). Similar effects were observed for chl-b and carotenoid contents in *C. reinhardtii*, but with higher percentage decrease on day 7 (23–42% for chl-b and 22–43% for carotenoids) (Fig. S2 and Fig. 3B).

3.6. Effects of BP-3 on photosynthetic pigments in *M. aeruginosa*

As for *M. aeruginosa*, BP-3 did not affect the growth rates at exposure levels of 0.01, 1 and 1000 μg L⁻¹ (one-sided *t*-test, *p* > 0.05), agreeing with our previous results (Table 2, Fig. 2B). The chl-a content was significantly decreased by BP-3 over the entire range of tested concentrations during the exposure period, with a decrease of 15–36% relative to the control (Fig. 4A, one side *t*-test, *p* < 0.01). There was no significant difference in chl-a content among the three treatments (i.e., 0.01, 1 and 1000 μg L⁻¹) (one-sided *t*-test, *p* > 0.05). In contrast, carotenoid content showed a significant increase (one-sided *t*-test,

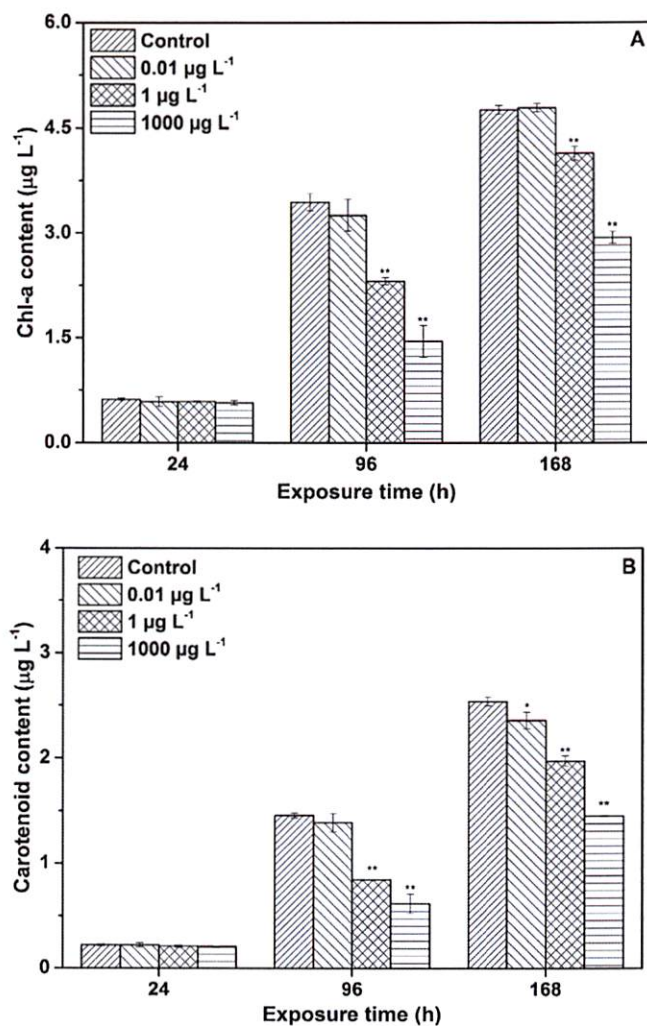


Fig. 3. Effects of BP-3 (initial concentrations: 0.01, 1 and 1000 µg L⁻¹) on (A) chl-a content and (B) carotenoid content in *C. reinhardtii* after 1, 4 and 7 days of exposure. Mean and standard deviation of triplicates are shown. Columns with asterisks denote significant difference between control and treatment (* < 0.05, ** < 0.01).

$p < 0.05$) in response to BP-3 at all tested concentrations (Fig. 4B). Additionally, *M. aeruginosa* produced more carotenoids under higher concentrations of BP-3, corroborating the results for carotenoid production rate (Table 2, Fig. 4B). The highest carotenoid content was observed at 1000 µg L⁻¹ of BP-3-treated group after 7-day exposure being 1.5-fold that of the control. No significant change was observed for the three water-soluble pigments (i.e., PC, APC and PE), except that PE content was reduced in 1000 µg L⁻¹ of BP-3 at days 4 and 7 (Fig. S3).

4. Discussion

4.1. Comparison of BP-3 uptake by *C. reinhardtii* and *M. aeruginosa*

With a relatively high logK_{ow} value of 3.79 (Table 1), BP-3 was readily bioaccumulated in aquatic phytoplankton as they are naturally rich in lipid contents (Maes et al., 2014). Bioaccumulation of BP-3 in algae is an important step for exerting toxic effects. Therefore, it is important to evaluate the uptake of BP-3 when assessing the toxic effects in algae. In this study, we evaluated the uptake by comparing the differences in concentration before and after the exposure experiment.

Uptake of BP-3 by green algae or cyanobacteria has rarely been studied. Our results demonstrated that the two tested species were able to take up BP-3 from the medium and the cyanobacterium generally

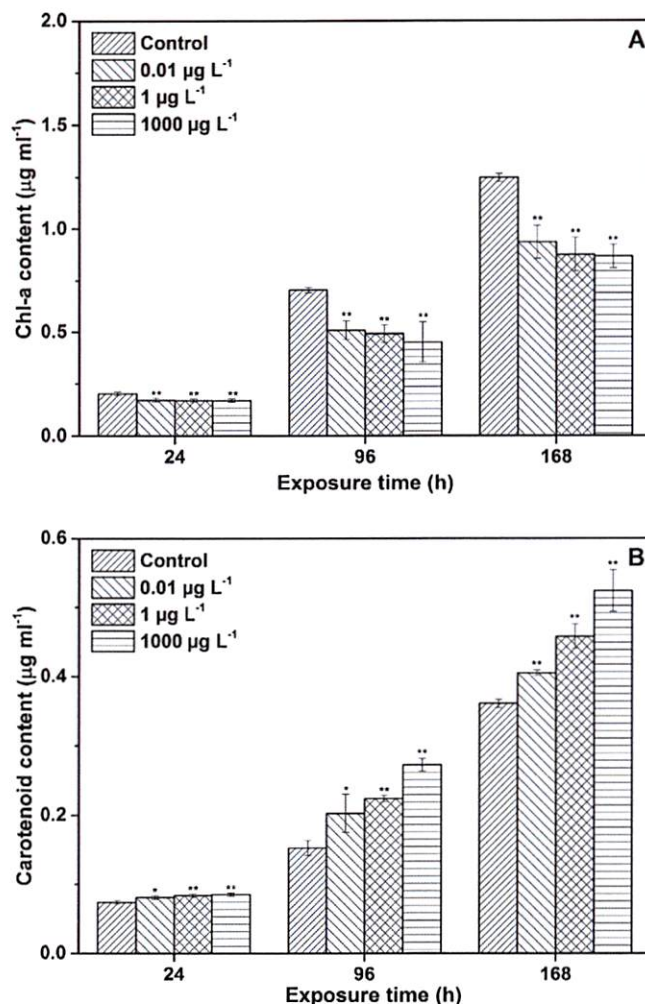


Fig. 4. Effects of BP-3 (initial concentrations: 0.01, 1 and 1000 µg L⁻¹) on (A) chl-a content and (B) carotenoid content in *M. aeruginosa* after 1, 4 and 7 days of exposure. Mean and standard deviation of triplicates are shown. Columns with asterisks denote significant difference between control and treatment (* < 0.05, ** < 0.01).

had a higher ability than green algae over the entire suite of exposure concentrations (Fig. 1). The potential mechanism could be explained by passive diffusion, where fast adsorption to the cell surface is followed by slow diffusion into the cell interior (internalization) through a partitioning-like process, a phenomenon already observed before (Diepens et al., 2014; Maes et al., 2014). Baptista et al. (2009) demonstrated that the cyanobacterium, *M. aeruginosa*, not only can uptake octylphenol from the medium, but can also metabolize it. Xiong et al. (2017) reported that the green alga, *Chlamydomonas mexicana*, could remove 13% of ciprofloxacin (initial exposure concentration: 2 mg L⁻¹) after 11 days of cultivation due to biosorption, bioaccumulation and biodegradation.

At low exposure levels, chl-a in *M. aeruginosa* was more sensitive to BP-3 than in *C. reinhardtii* (Fig. 2), agreeing with the observation that *M. aeruginosa* tends to take up more BP-3 from the medium than *C. reinhardtii* (Fig. 1). This is reasonable because arriving at the site of action is the prerequisite for a contaminant to trigger a toxic response in organisms. Similarly, it was reported that when exposed to pentachlorophenol (exposure level as low as 0.01 µg L⁻¹), *M. aeruginosa* was more sensitive and took up more pentachlorophenol compared to *Chlorella vulgaris*, a green alga (de Morais et al., 2014). At exposure levels higher than 100 µg L⁻¹, uptake of BP-3 from the medium decreased followed by reduced specific growth rate and chl-a production (Fig. 2A). This suggests that an elevated amount of BP-3 was present at the action site for higher exposure concentrations, corroborating the

high inhibition effects. For the highest two tested concentrations, the uptake for *M. aeruginosa* decreased, whereas *C. reinhardtii* was found to take up more BP-3 (Fig. 1A). There may be several mechanisms responsible for this. First, the dead and live cells together likely contributed to the overall removal of BP-3 from the medium, as dead green algae can also act as sorbent for other hydrophobic contaminants (Tam et al., 2002). Second, cells exposed to higher levels of BP-3 may produce other extracellular polymeric substances (EPS) which could be involved in the adsorption process (Vandevivere and Kirchman, 1993). In addition, cells at these high BP-3 levels may contain different amounts of lipid, protein and polysaccharide, affecting the overall biosorption process (Swackhamer and Skoglund, 1993).

4.2. Toxicity of BP-3 in *C. reinhardtii*

In this study, the effects of BP-3 on chl-a content at exposure concentrations lower than $100 \mu\text{g L}^{-1}$ were variable (Fig. 2A). When exposed to environmentally relevant concentrations (i.e., 0.1 and $1 \mu\text{g L}^{-1}$), chl-a in *C. reinhardtii* was inhibited, suggesting that BP-3 can perturb the physiological processes in the tested green alga at low concentrations. As BP-3 is a potential endocrine disrupting chemical (EDC), it may induce endocrine disrupting effects on organisms at very low concentrations, i.e., ng L^{-1} (de Morais et al., 2014; Li et al., 2010). Additionally, some trace organic contaminants can also negatively affect photosynthetic processes in algae by disrupting the energy flow through photosystem II (PSII) (Perron and Juneau, 2011). For example, the synthesis of protoporphyrin IX, a precursor for chlorophyll synthesis, is retarded under atrazine stress (Mofeed and Mosleh, 2013). An additional study also indicated that chl-a production in a green alga, *Chlorella vulgaris*, was inhibited at low concentrations of pentachlorophenol, another potential EDC (de Morais et al., 2014).

At the organismic level, for exposure levels lower than $10 \mu\text{g L}^{-1}$, no effects on specific growth rate were observed although the chl-a content was reduced (Fig. 2A). The fact that the tested green alga kept growing under low exposure levels suggested that the alga was trying to reduce the toxic effects by growth dilution since a higher cell number could dilute the concentration of contaminants in the organisms (Skoglund and Swackhamer, 1994; Tikoo et al., 1997). The observation that reduced chl-a content did not affect the growth rate of alga is not unique. A study focused on the effects of herbicides on phytoplankton revealed that even when photosynthesis was reduced by 30%, the growth rate remained constant (Lay et al., 1984). Plumley and Davis (1980) reported that cell division rates in three estuarine algal species were not affected when chlorophyll production was reduced. Another study demonstrated that pentachlorophenol did not affect the growth of phytoplankton, while it had a negative effect on chl-a production (El-Dib et al., 2000). Our results further showed that both chl-a content and specific growth rate decreased with increasing BP-3 concentration at levels higher than $10 \mu\text{g L}^{-1}$ (Fig. 2A). Additionally, the simultaneous decrease in chl-a, chl-b and carotenoid contents (Fig. 3 and Fig. S2) suggests that BP-3 influenced the growth of *C. reinhardtii* by regulating the production of photosynthetic pigments, thus affecting photosynthesis by which algae convert light energy to chemical energy used for growth. Similarly, both chl-a and carotenoid contents were reduced in *Scenedesmus quadricauda* (a green alga) after exposure to $100 \mu\text{g L}^{-1}$ of naproxen (Ding et al., 2017). Studies focused on the toxicity of various contaminants to green algae have showed that the production of chl-a was positively correlated with biomass (Kabra et al., 2014; Zhang et al., 2011a, 2011b) and that reduced total carotenoid content could lead to reduced algal growth rate (Melegari et al., 2013; Zamani et al., 2014).

EC_{50} for *C. reinhardtii* was calculated to be $1.85 \pm 0.41 \text{ mg L}^{-1}$. According to previous studies, the 72-h IC_{50} values of BP-3 for other green algae were estimated to be 0.25 mg L^{-1} for *Skeletonema pseudocostatum* (Petersen et al., 2014), 0.96 mg L^{-1} for *Desmodesmus subspicatus* (Sieratowicz et al., 2011), and 22.4 mg L^{-1} for *Chlorella vulgaris* (Pablos et al., 2015). These results showed that the EC_{50} values for BP-3

varied considerably among different algal species. However, part of the differences may lie in the differences in exposure time, endpoints, culture medium, and algal species. Nevertheless, the EC_{50} value obtained from the present study lies within the range of reported literature data.

The observed lag phases varied for the different exposure concentrations from 48 to 96 h. A longer lag phase may indicate a higher toxicity of BP-3. As BP-3 concentrations reached up to $4000 \mu\text{g L}^{-1}$, a longer lag phase (almost 96 h) was observed. The lag phase has been attributed to the physiological adaptation of cell metabolism to growth, such as the increase in levels of enzymes and metabolites involved in cell division and carbon fixation. This adaptation may also be related to detoxification mechanisms before the cell can resume growth (Olivier et al., 2003). Indeed, a similar longer lag phase has been reported for *Chlorella* species when exposed to high concentrations of nonylphenol (Gao et al., 2011).

4.3. Toxicity of BP-3 in *M. aeruginosa*

The chl-a content in *M. aeruginosa* was significantly reduced over the entire tested concentration range, suggesting that chl-a was sensitive to BP-3 stress (Fig. 2B). The reduction ranged from 33.8% to 82.8%, with a median value of 43.5%. When the exposure levels were lower than $10 \mu\text{g L}^{-1}$, the inhibition variations were small (Fig. 2B), while the inhibition was concentration-dependent when concentrations were higher than $10 \mu\text{g L}^{-1}$. These results implied that the production of chl-a in *M. aeruginosa* may be inhibited at environmentally relevant levels (low ng L^{-1} levels). Since this is the first study on the toxic effects of BP-3 in cyanobacteria, no literature data could be cited for comparison. However, the potential endocrine disrupting property of BP-3 enabled us to evaluate the toxicity of this compound compared to other EDCs. Other EDCs were found to be toxic to cyanobacteria at very low concentrations, i.e., $\mu\text{g L}^{-1}$ or lower, as they are known to initiate endocrine disrupting effects (Li et al., 2010), or to interrupt photosynthetic electron flow (Perron and Juneau, 2011). It has been further shown that trace organic contaminants, such as antibiotics and EDCs, can reduce chl-a accumulation in cyanobacteria (Chen and Guo, 2012; de Morais et al., 2014).

Although chl-a content in *M. aeruginosa* was reduced over the concentration range tested, specific growth rates were not affected by BP-3 at concentrations lower than $10 \mu\text{g L}^{-1}$, and only a slight decrease was observed at higher concentrations (Fig. 2B). This was possibly due to the elevated production of carotenoids (Fig. 4B), which are important photosynthetic pigments. Therefore, for the tested cyanobacterium, the reduced photosynthesis in chl-a may have been compensated for by carotenoids and thus, the overall growth of the cyanobacterium remained unaffected (Fig. 4, Table 2). In addition to their role in photosynthesis, carotenoids also play a part in protecting chlorophyll, acting as antioxidants to scavenge excessive reactive oxygen species (ROS) formed when cells are under stresses (Flores-Rojas et al., 2015). This may explain the observation that chl-a contents did not vary significantly among different treatment groups (Fig. 4A). Further investigation is necessary to clarify the mechanism of action in terms of the exposure of *M. aeruginosa* to BP-3.

Our result suggest that chl-a content was more susceptible to BP-3 stress than carotenoids and cell growth. The different sensitivities of algal growth and production of photosynthetic pigments to environmental stresses have attracted significant interest, leading to controversial results in different studies. Some have reported that the growth of algae was more sensitive (Perales-Vela et al., 2007), while others have claimed that chlorophyll production is more sensitive to environmental stress (Wang et al., 2012). The observation that algal cells can continue to multiply even with a reduced production of chl-a has been previously reported (de Morais et al., 2014; El-Dib et al., 2000; Wang et al., 2012). As photosynthetic pigments in cyanobacteria have different sensitivities to environmental stressors, photosynthesis carried

out by other less sensitive pigments may compensate for the loss caused by the sensitive ones. For example, carotenoid concentration in *M. aeruginosa* was found to be less sensitive to arsenic than chl-a concentration (Wang et al., 2012), while it was more sensitive when exposed to β -ionone (Shao et al., 2011). This strengthens the premise that the physiological mechanism of each contaminant is different.

At the most environmentally relevant levels of BP-3 (i.e., 0.01 and $1 \mu\text{g L}^{-1}$), chl-a production in *M. aeruginosa* was more sensitive compared to that in *C. reinhardtii*. Several factors may be responsible for this. First, the fact that cyanobacteria have higher surface/volume ratios could make them more susceptible to absorb contaminants from the culture medium into the cell. Second, photosynthesis and phosphorylation occur in the cytoplasm in prokaryotic cyanobacteria, while in eukaryotic green algae, they occur in chloroplasts and mitochondria. Thus, to induce toxic effects for chl-a production in green algae, xenobiotics must pass a longer route and an additional double membrane.

5. Conclusion

Effects of BP-3 on cell growth and chl-a content of *C. reinhardtii* were variable at levels lower than $100 \mu\text{g L}^{-1}$, while significant inhibition was observed above this level. The simultaneous decrease in chl-a, chl-b and carotenoid contents suggested that BP-3 regulated the growth of *C. reinhardtii* by affecting the photosynthesis process. For *M. aeruginosa*, chl-a contents decreased over the tested concentration range, while the inhibition in cell growth was moderate in comparison to *C. reinhardtii*. The carotenoid content increased in *M. aeruginosa* when exposed to BP-3, indicating an alternative protective photosynthetic mechanism in this organism. Thus, the effects of BP-3 on chl-a in the cyanobacterium may have a lower effect on their cell growth than in the green alga. These differences may also reflect possibly different modes of toxic action in the two algae. Both species were able to uptake BP-3 from the medium, with *M. aeruginosa* showing greater overall uptake (27.2–77.4%) compared to *C. reinhardtii* (1.1–58.4%).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.09.029>.

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Prenatal phenol and phthalate exposures and birth outcomes.

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Abstract

BACKGROUND:

Many phthalates and phenols are hormonally active and are suspected to alter the course of development.

OBJECTIVE:

We investigated prenatal exposures to phthalate and phenol metabolites and their associations with body size measures of the infants at birth.

METHODS:

We measured 5 phenol and 10 phthalate urinary metabolites in a multiethnic cohort of 404 women in New York City during their third trimester of pregnancy and recorded size of infants at birth.

RESULTS:

Median urinary concentrations were > 10 microg/L for 2 of 5 phenols and 6 of 10 phthalate monoester metabolites. Concentrations of low-molecular-weight phthalate monoesters (low-MWP) were approximately 5-fold greater than those of high-molecular-weight metabolites. Low-MWP metabolites had a positive association with gestational age [0.97 day gestational age per ln-biomarker; 95% confidence interval (CI), 0.07-1.9 days, multivariate adjusted] and with head circumference. Higher prenatal exposures to 2,5-dichlorophenol (2,5-DCP) predicted lower birth weight in boys (-210 g average birth weight difference between the third tertile and first tertile of 2,5-DCP; 95% CI, 71-348 g). Higher maternal benzophenone-3 (BP3) concentrations were associated with a similar decrease in birth weight among girls but with greater birth weight in boys.

CONCLUSIONS:

We observed a range of phthalate and phenol exposures during pregnancy in our population, but few were associated with birth size. The association of 2,5-DCP and BP3 with reduced or increased birth weight could be important in very early or small-size births. In addition, positive associations of urinary metabolites with some outcomes may be attributable partly to unresolved confounding with maternal anthropometric factors.



Associations of prenatal exposure to phenols with birth outcomes



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ABSTRACT

Many phenols are known to mimic or antagonize hormonal activities and may adversely affect fetal growth. A study of 567 pregnant women was conducted to investigate the relationship between prenatal phenol exposure and birth outcomes, including birth weight, length, and gestational age. We measured the concentrations of bisphenol A, benzophenone-3, 4-*n*-octylphenol and 4-*n*-nonylphenol in maternal urine and examine their association with birth outcomes. Categories of urinary benzophenone-3 concentration were associated with decreased gestational age in all infants (p for trend = 0.03). Between middle and low exposure groups, we also found bisphenol A was negatively associated with gestational duration ($\beta_{\text{adjusted}} = -0.48$ week; 95% confidence interval: $-0.91, -0.05$). After stratification by gender, we found the consistent results in infant boys with those in all infants, but we did not observe significant association for girls. In conclusion, we found prenatal phenol exposure was sex-specifically related to birth outcomes.

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1. Introduction

Phenols are extensively used as a major component in a wide spectrum of products in industry, agriculture and daily life. Bisphenol A (BPA) has been used for over 50 years in the manufacture of polycarbonate plastics, epoxy resins and thermal paper (Centers for Disease Control and Prevention, 2009). The sunscreen benzophenone-3 (BP-3) is commonly used in lotions, conditioners, cosmetics and plastic surface coatings (Centers for Disease Control and Prevention, 2009). Alkylphenols (APs) [octylphenols (OPs) and nonylphenol (NPs)] are degradation products of the corresponding alkylphenol ethoxylates (APEs) which are surfactants used in detergents, industrial cleaners and emulsifiers (Bonfeld-Jorgensen

et al., 2007; Centers for Disease Control and Prevention, 2009). The huge volume production and widespread application of the above compounds lead to the exposure to human. Recent studies in the fields of exposure science and analytical chemistry have documented that phenols can be detected in human urine, plasma, breast milk, amniotic fluid, placental tissue and umbilical cord blood in many countries (Chen et al., 2010; Schlumpf et al., 2010; Tan and Ali Mohd, 2003; Vandenberg et al., 2012; Vela-Soria et al., 2011; Woodruff et al., 2011).

There is a heightened awareness of the potential role of environment factors in child development. During critical periods of development, including the prenatal period, exposure to a number of environmental chemical contaminants may adversely affect the growth, reproduction, and development in wildlife and humans, causing morphologic and functional alterations (Schonfelder et al., 2002). Many of these chemicals are known to impact the endocrine system of animals and humans. Several researches indicated that compounds with endocrine disrupting effects were associated with adverse birth outcomes (Govarts et al., 2012; Longnecker et al., 2001; Meeker et al., 2009). BPA, BP-3 and APs are all known as endocrine disrupting chemicals (EDCs) (Isidori et al., 2010; Li et al., 2012; Schlumpf et al., 2004), therefore, their effect on birth outcomes needs particular attention. So far, there is still limited

Abbreviations: BPA, bisphenol A; BP-3, benzophenone-3; APs, alkylphenols; 4-*n*-OP, 4-*n*-octylphenol; 4-*n*-NP, 4-*n*-nonylphenol; CR, creatinine; CI, confidence interval; LOD, limits of detection; UPLC–MS/MS, ultra high performance liquid chromatography–tandem mass spectrometry; BMI, body mass index; EDCs, endocrine disrupting chemicals.

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evidence for adverse birth outcomes in association with phenol exposure in animal studies, and most of them mainly focus on BPA (Kim et al., 2001; Rubin et al., 2001; Savabieasfahani et al., 2006). Aside from BPA (Cantonwine et al., 2010; Miao et al., 2011; Padmanabhan et al., 2008), the epidemiologic studies regarding prenatal exposure to other phenols are also lacking. In addition, currently, little is known about the extent of exposure to these agents and their potential toxic effects on development in the general Chinese population.

Phenols are mainly excreted into urine (Ye et al., 2005). Therefore, total (conjugated and free) phenols measured in human urine can reflect individual internal exposure level (Ye et al., 2005) and has been widely used in exploring the relationships between exposure to those compounds and diseases as well as laboratory abnormalities (Chevrier et al., 2013; Kim and Park, in press; Lang et al., 2008).

Therefore, we investigated urinary levels of BPA, BP-3, 4-*n*-octylphenol (4-*n*-OP), 4-*n*-nonylphenol (4-*n*-NP) in pregnant women, and examined their relationship with birth outcomes, including birth weight, length and gestational age.

2. Material and methods

2.1. Study population

Pregnant women were recruited from hospitals affiliated to Nanjing Medical University between September 2010 and April 2012. Eligible women with singleton pregnancy were ≥ 18 years old, and they reported no assisted reproduction and medical complications (e.g., gestational or preexisting diabetes, hypertension, HIV infection or AIDS) (Wang et al., 2012). Newborn infants with severe neonatal illness [e.g., very premature births (delivery at < 32 completed gestational weeks or birth weight < 1500 g), genetic abnormalities or malformations] were further excluded (Wolff et al., 2008). A total of 592 women met the eligibility criteria. Of these, 567 women agreed to participate in this study (response rate: 95.8%). The study was approved by the Institutional Review Board of Nanjing Medical University. All participants provided written informed consent prior to the study.

2.2. Face-to-face interview

A questionnaire was conducted with each participant by face-to-face interview, collecting information as follows: demographic and socioeconomic information (maternal age, height, end-of-pregnancy weight, education level, household income, and address), maternal characteristics (dietary habits, any smoking or alcohol use during pregnancy), occupational history, exposures to environmental hazards and reproduction status. Maternal BMI in late pregnancy was calculated as end-of-pregnancy weight (kg) divided by the height (m) squared. Additionally, information about previous pregnancies, medications, medical conditions, complications during the current pregnancy and self-reported last menstrual period (LMP) was also obtained by interview and confirmed by medical records.

2.3. Measurement of fetal growth

Infant sex, birth date, parity, weight, and crown–heel length were obtained from hospital delivery logs and medical records. Date on clinical estimate of gestational age (ultrasound) was also collected. Gestational age was estimated based on the onset of LMP; if the LMP was unreliable or if there was a significant discordance between the clinical estimate and LMP (> 2 weeks), the first clinical estimation of gestational age was used (Cheng et al., 2007; Eskenazi et al., 2004). Low birth weight was defined as < 2500 g. Preterm delivery was defined as birth at less than 37 completed weeks of gestation.

2.4. Urinary phenols determination

Urine samples were collected from each subject during hospital admission for delivery, and were frozen at -20 °C until analysis. The total urinary concentrations of BPA, BP-3, 4-*n*-OP and 4-*n*-NP were measured with a sensitive method as previously described (Chen et al., 2012). Briefly, 4 ml of urine samples were incubated with beta-glucuronidase/sulfatase at 37 °C overnight, then the hydrolyzed compounds in the urine was purified by solid phase extraction. After dryness, the residue was redissolved in methanol. The solution was analyzed by ultra high performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS, Waters, USA). The limits of detection (LODs) for these chemicals were based on a signal to noise ratio (*S/N*) of three, LODs were 0.36 $\mu\text{g/L}$ (BPA), 0.04 $\mu\text{g/L}$ (BP-3), 0.02 $\mu\text{g/L}$ (4-*n*-OP) and 0.02 $\mu\text{g/L}$ (4-*n*-NP). Quality control (QC) samples which were prepared from

spiked pooled urine were analyzed along with standards, blanks and unknown samples. Urinary creatinine (CR) concentrations were used to correct the metabolite concentrations for variable urine dilutions. CR concentrations were determined using an automated chemistry analyzer (7020 Hitachi, Tokyo, Japan).

2.5. Statistical analysis

Statistical analyses were performed using STATA statistical package (Version 9.2, Stata Corp, LP). Because of the high proportion of samples with target compounds that had levels below the detection limit, a three-level ordinal variable was formed: all samples with concentrations $< \text{LOD}$ were assigned to the lowest group, and two equally sized groups were formed among the samples with detectable concentrations to form the middle- and high-exposure groups (Meeker et al., 2011).

Multivariate linear regression model was used to examine relationship between prenatal exposure to phenols and birth outcomes. Inclusion of covariates was based on biological and statistical considerations. Covariates were included in the final models if they showing associations ($p < 0.1$) with birth outcomes or biomarkers by univariate analysis (Wolff et al., 2008). We included the set of covariates in models as follows: urinary CR, gestational age at birth (not included in models predicting gestational age), maternal age, body mass index (BMI) in late pregnancy and parity. Urinary CR was included as continuous variables to adjust for urinary dilution (Meeker et al., 2011). Maternal age, gestational age and BMI in late pregnancy were also modeled as continuous variables, whereas parity (0, or ≥ 1) were treated as a binary variable. Smoking and alcohol were not included in the final models because very few women reported use. Considering that the association between exposure and birth outcomes might differ by infant sex, we also performed stratified analysis for boys and girls. Tests for trend were performed for ordinal categories of target compounds in the adjusted regression models with integer values (0, 1, 2) (Meeker et al., 2011); a p -value of < 0.05 indicated statistical significance.

3. Results

3.1. Main demographic characteristics of study participants

Maternal and infant characteristics in this study were presented in Table 1. Maternal age ranged from 18 to 45 years of age with mean (SD) of 27.8 (4.7). Average (SD) maternal BMI in late pregnancy was 27.6 (4.4) kg/m^2 . Approximately three-fourths of the women were primiparous and over half (58.9%) had graduated from high school or college. Very few mothers reported tobacco smoking (4 mothers) and regular alcohol use (9 mothers) during pregnancy. For the newborns, 50.6% of them were male. The mean (SD) birth weight, length and gestational age were 3370 (556) g, 50.2 (2.4) cm, and 39.1 (1.9) weeks, respectively. A total of 5.7%

Table 1
Characteristics of mothers and newborns in this study ($n = 567$).

	<i>n</i> (%) or mean
Maternal characteristics	
Maternal age (year, mean \pm SD)	27.8 \pm 4.7
Parity (%)	
0	73.1
≥ 1	26.9
Education (years, %)	
≤ 9	41.9
10–12	22.2
≥ 13	35.9
Maternal height (cm, mean \pm SD)	161.7 \pm 4.3
End-of-pregnancy weight (kg, mean \pm SD)	72.2 \pm 11.8
BMI in late pregnancy (kg/m^2 , mean \pm SD) ^a	27.6 \pm 4.4
Smoking during pregnancy (%)	0.7
Drinking during pregnancy (%)	1.6
Newborn characteristics	
Sex (%)	
Male	50.6
Female	49.4
Birth weight (g, mean \pm SD) ^b	3370 \pm 556
Body length (cm, mean \pm SD) ^c	50.2 \pm 2.4
Length of gestation (week, mean \pm SD) ^d	39.1 \pm 1.9

^a Values are end-of-pregnancy weight (kg) divided by the height (m) squared.

^b Newborns with missing data, $n = 3$.

^c Newborns with missing data, $n = 10$.

^d Newborns with missing data, $n = 16$.

Table 2
Distribution of urinary phenol concentrations of pregnant women ($n = 567$).^a

Analyte	Detection limit (ng/ml)	Detection rate (%)	GM (ng/ml)	Selected percentiles				Maximum (ng/ml)
				50th	75th	90th	95th	
BPA	0.36	60%	0.91	0.67	2.73	10.76	28.42	355.33
BP-3	0.04	61.6%	0.08	0.08	0.16	0.42	0.77	400.72
4- <i>n</i> -OP	0.02	14.8%	^b	<LOD	<LOD	0.03	0.04	2.53
4- <i>n</i> -NP	0.02	14%	^b	<LOD	<LOD	0.02	0.03	1.02

Abbreviations: BPA, bisphenol A; BP-3, benzophenone-3; 4-*n*-OP, 4-*n*-octylphenol; 4-*n*-NP, 4-*n*-nonylphenol; GM: geometric mean.

^a Samples with concentrations <LOD were given a value equal to LOD/2 for the statistical calculations.

^b Not calculated: proportion of results below LOD was too high to provide a valid result.

($n = 32$) of infants were born of low birth weight, and 4.9% ($n = 27$) were preterm births.

3.2. Maternal urine phenols exposure levels

Table 2 showed urinary concentrations of phenols in the present subjects along with the detection limit for those compounds. Among those compounds, BPA and BP-3 exhibited relatively higher detection frequencies. BPA showed highest urinary levels, followed by BP-3, 4-*n*-OP and 4-*n*-NP.

3.3. Relationship between prenatal phenols exposure and birth outcomes

Crude and adjusted regression coefficients (β) and 95% confidence intervals (CI), based on the entire sample, for the relationship between phenol exposure levels and birth outcomes are presented in Table 3. The crude and adjusted results were similar. Categories of urinary BP-3 concentration were associated with a decrease in gestational age (p -value for trend = 0.03). Between middle and low exposure groups, we also found that BPA was negatively associated with gestational duration ($\beta_{\text{adjusted}} = -0.48$ week; 95% CI: -0.91 to -0.05). No significant relationships were observed between exposure to APs and any of birth outcomes.

In Fig. 1, Table S1 and S2 (Supplementary material), results of stratification analyses performed for boys and girls revealed possible sex-specific association of prenatal exposure to BP-3 and BPA with gestational age. We found that, in infant boys, BP-3 exposure categories were related to decreased length of gestation [p -value for

trend = 0.006; Fig. 1], and BPA showed significantly negative relationship with gestational duration between middle and low exposure groups [$\beta_{\text{adjusted}} = -0.78$ week; 95% CI: -1.44 to -0.11 ; Table S1 and Fig. 1]. Results in boys were consistent with those in all infants, while we did not observe significant association for girls.

4. Discussion

As shown in many studies, a number of chemicals, such as PCBs, organochlorine pesticides and phthalates can pass through the placenta (Correia Carreira et al., 2011; Mose et al., 2007; Waliszewski et al., 2000) and prenatal exposure to them have been demonstrated to associate with decreased gestational duration (Meeker, 2012; Meeker et al., 2009; Wigle et al., 2008). Among phenols, BPA has been confirmed to easily cross the blood–placenta barrier at low environmentally relevant levels (Balakrishnan et al., 2010; Schonfelder et al., 2002; Wan et al., 2010) and BP-3, with a similar chemical structure to BPA, may also pass the blood–placenta barrier (Krause et al., 2012). Additionally, the rate of clearance of phenols, such as BPA, is slower in the fetus than in maternal blood (Takahashi and Oishi, 2000), because most uridinediphosphate–glucuronosyltransferase (UDPGT) isoenzymes which involved in biotransformation and elimination of a wide variety of xenobiotic phenols did not appear until after birth (Coughtrie et al., 1988). Thus, the fetus is especially vulnerable to these phenols and prenatal exposure to them might adversely affect fetal growth and gestational duration.

Among numerous chemicals, EDCs are especially of concern because once inside the body, they can affect the endocrine and

Table 3
Crude and adjusted linear regression coefficients for change in birth outcomes associated with categories of urinary phenols.

Analyte		Length of gestation (weeks) ($n = 551$)		Birth weight (g) ($n = 564$)		Body length (cm) ($n = 557$)	
		β (95% CI)		β (95% CI)		β (95% CI)	
		Crude	Adjusted ^a	Crude	Adjusted ^b	Crude	Adjusted ^b
BPA	Low	0	0	0	0	0	0
	Middle	-0.38 (-0.81,0.04)*	-0.48 (-0.91,-0.05)**	25.25 (-91.72,142.21)	82.14 (-26.36,190.64)	-0.18 (-0.7,0.35)	0.04 (-0.49,0.57)
	High	-0.23 (-0.65,0.2)	-0.31 (-0.74,0.13)	73.46 (-43.51,190.43)	78.75 (-30.81,188.3)	0.09 (-0.44,0.61)	0.11 (-0.43,0.64)
	p -Value for trend	0.23	0.11	0.22	0.13	0.82	0.7
BP-3	Low	0	0	0	0	0	0
	Middle	0.18 (-0.21,0.57)	0.11(-0.3,0.53)	-17.89 (-129.21,93.42)	-21.29 (-129.34,86.76)	-0.12 (-0.6,0.36)	-0.17 (-0.68,0.33)
	High	-0.39 (-0.77,0)**	-0.45 (-0.87,-0.04)**	27.5 (-83.99,138.99)	57.99 (-49.92,165.89)	0.02 (-0.46,0.5)	0.16 (-0.34,0.68)
	p -Value for trend	0.06	0.03	0.65	0.28	0.95	0.52
4- <i>n</i> -OP	Low	0	0	0	0	0	0
	Middle	0.09 (-0.53,0.71)	0.06 (-0.59,0.71)	15.22 (-162.48,192.92)	-16.73 (-181.89,148.42)	0.13 (-0.64,0.91)	-0.07 (-0.86,0.72)
	High	0.21 (-0.43,0.85)	0.07 (-0.59,0.73)	84.44 (-99.61,268.49)	79.45 (-89.16,248.06)	-0.03 (-0.82,0.77)	-0.11 (-0.91,0.68)
	p -Value for trend	0.49	0.8	0.39	0.46	0.94	0.75
4- <i>n</i> -NP	Low	0	0	0	0	0	0
	Middle	0.09 (-0.54,0.72)	0.11 (-0.53,0.75)	-48.02 (-229.51,133.47)	-59.161 (-222.43,104.1)	-0.45 (-1.23,0.33)	-0.4 (-1.17,0.36)
	High	0.15 (-0.46,0.77)	0.15 (-0.48,0.79)	150.1 (-23.4320.61)	76.04 (-86.96,239.04)	-0.37 (-1.12,0.39)	-0.49 (-1.25,0.27)
	p -Value for trend	0.58	0.58	0.2	0.59	0.2	0.13

Abbreviations: BPA, bisphenol A; BP-3, benzophenone-3; 4-*n*-OP, 4-*n*-octylphenol; 4-*n*-NP, 4-*n*-nonylphenol; CI: confidence interval.

* $p < 0.1$, ** $p < 0.05$.

^a Models adjusted for maternal age, BMI in late pregnancy, parity and CR.

^b Models adjusted for maternal age, gestational age, BMI in late pregnancy, parity and CR.

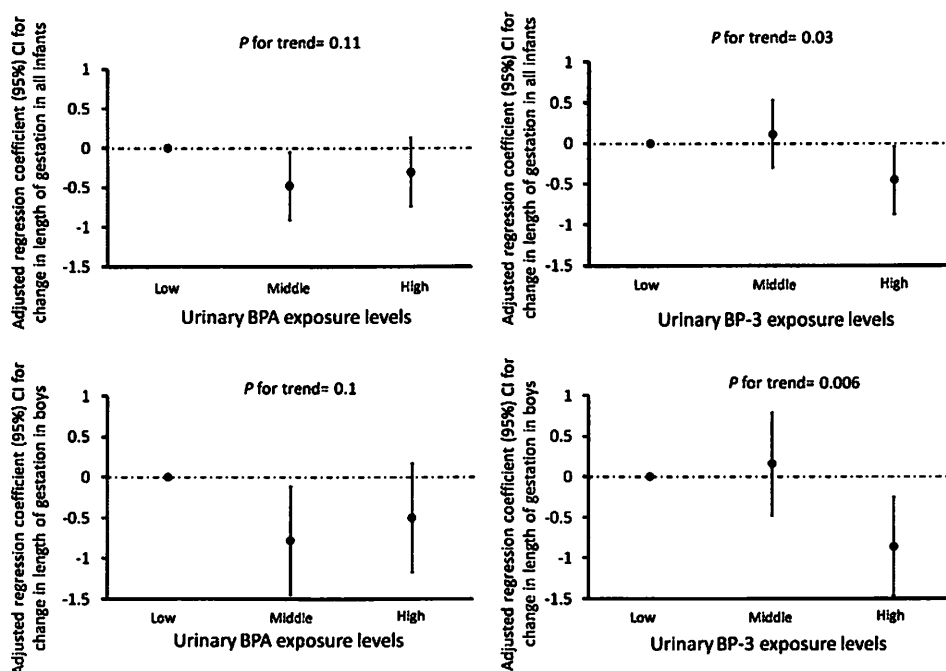


Fig. 1. Adjusted regression coefficients for change in length of gestation associated with prenatal BPA and BP-3 exposure categories in all infants and boys (adjusted for maternal age, BMI in late pregnancy, parity and CR). Abbreviations: BPA, bisphenol A; BP-3, benzophenone-3.

reproductive system along multiple points including the hypothalamus and the gonad (Robins et al., 2011). Thus, the maternal–placental–fetal unit, which is now accepted to represent the interaction of three endocrine systems changing throughout pregnancy in mammals (Biggsby et al., 1999), can also be the target of the EDCs. Indeed, prenatal exposures to EDCs, such as PCBs, organochlorine pesticides and phthalates, have been indicated to be related to shortened length of gestation in many epidemiological studies (Meeker, 2012; Meeker et al., 2009; Wigle et al., 2008). Both BP-3 and BPA exhibited estrogenic activities and were possessed of binding affinity to estrogen receptor α ($ER\alpha$), estrogen receptor β ($ER\beta$) and some other steroid receptor, such as estrogen receptor-related receptors (ERRs) (Gomez et al., 2005; Lee et al., 2012; Li et al., 2012; Matsushima et al., 2007; Schlecht et al., 2004; Schreurs et al., 2005). Evidence for decreased gestational duration in relation to BPA has also been reported (Cantonwine et al., 2010). Recently, a proposed molecular biology of endocrine disruption lead to adverse birth outcomes was that, the placenta is central to successful pregnant outcomes and the estrogen-related receptor gamma ($ERR\gamma$) protein, exhibited a strong affinity for BPA, has been described as highly expression in the placenta (Poidatz et al., 2012), so that EDCs like BPA may exert its adverse influence on pregnant outcomes through binding and activation of this receptor and its downstream signaling (Robins et al., 2011). Therefore, our results that shortened gestational age was associated with the categories of BP-3 exposure and with BPA concentration between middle and low exposure group (Table 3) may be consisted with their endocrine disrupting activities. Our data provides a new evidence of potential developmental risk of prenatal exposure to phenols on gestational duration.

In the present study, stratified analysis for infant sex revealed the sex-specific association of BP-3 and BPA exposure with gestational age. The phenomenon of sex-specific association was also present in many other researches regarding effects of exposure to environmental chemical contaminants on birth outcomes (Chou et al., 2011; Philippat et al., 2012; Wang et al., 2012; Wolff et al., 2008). However, these associations still require further confirmation.

APs, which are also the commonly mentioned phenolic endocrine disruptors (Bonefeld-Jorgensen et al., 2007; Chen et al., 2010), showed no association with any of birth outcomes in this study. It may occur because the detection rate of 4-n-OP and 4-n-NP were too low to examine the statistical associations and their relatively lower exposure levels may not elicit biological effects.

To the best of our knowledge, we present the first human study in China to examine the relationship between prenatal exposures to various phenols and birth outcomes. However, as in many studies (Philippat et al., 2012; Wang et al., 2012; Wolff et al., 2008), we measured urinary concentrations of these compounds at a single time point exclusively. We are limited in our ability to understand the average cumulative dose from different sources and to what extent these measurements accurately reflected the exposure throughout the entire critical period of fetal growth. Future research should increase the number of urine samples collected during the whole gestation and repeatedly measure those samples to provide a more accurate estimate of the average exposure.

5. Conclusions

In summary, we investigated the relationship of prenatal exposure to phenols with fetal growth and gestational duration in China. Prenatal BP-3 and BPA exposures were found to be associated with shortened gestational duration. Further study may be needed to confirm these results and identify potential mechanisms.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2013.03.023>.

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The relationship between prenatal exposure to BP-3 and Hirschsprung's disease

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HIGHLIGHTS

- We examined the association between maternal BP-3 exposure and the offspring's HSCR risks.
- BP-3 suppressed cell migration and regulated RET, miR-218, PLAG1, SLIT2 and ROBO1 expressions.
- SLIT2/ROBO1-miR-218-RET/PLAG1 pathway was involved in the pathogenesis of HSCR induced by BP-3.

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ABSTRACT

Hirschsprung's disease (HSCR) is neonatal intestinal abnormality which derived from the failure of enteric neural crest cells migration to hindgut during embryogenesis from 5 to 12 weeks. Currently, the knowledge of environmental factors contributing to HSCR is still scarce. Benzophenone-3 (BP-3) is one of the most widely used UV filters, and has weak estrogen and strong anti-androgenic effects. In order to examine the effect of maternal BP-3 exposure on development of offspring and explore the potential mechanism, we conducted case and control study and in vitro study. In this work, BP-3 concentrations in maternal urine was detected by ultra-high performance liquid chromatography. Besides, we investigated the cytotoxicity and receptor tyrosine kinase (RET) expression in cells exposed to BP-3. The results showed that maternal BP-3 exposure was associated with offspring's HSCR in the population as well as inhibited migration of 293T and SH-SY5Y cells. What's more, we discovered dose-response relationship between RET expression and BP-3 exposure dose, and miR-218 and some other genes involved in SLIT2/ROBO1-miR-218-RET/PLAG1 pathway were also related to BP-3 exposure. Therefore, we deduced that BP-3 influenced cell migration via SLIT2/ROBO1-miR-218-RET/PLAG1 pathway. Our study firstly revealed the relationship between maternal BP-3 exposure and HSCR as well as its potential mechanism.

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1. Introduction

Hirschsprung's disease (HSCR) is a complex congenital disease caused by gene-environment interaction and can lead to intestinal

obstruction and chronic constipation (Goldberg, 1984; Amiel et al., 2008). The incidence of HSCR is 1:2000–1:5000 in live births while males are 4 times more susceptible than females (Parisi et al., 2002). HSCR results from enteric neural crest cells' failing in migrating to certain segments of colon during embryonic development from 5 to 12 weeks (Nishiyama et al., 2012; McKeown et al., 2013; Takahashi et al., 2013). Genetic studies have found that various gene mutations are associated with HSCR, such as receptor tyrosine kinase (RET), EDNRB, GDNF and SOX10. In particular, RET, which encodes a tyrosine-kinase receptor, is the most frequently mutated gene (Angrist et al., 1996; Paratore et al., 2002; Iwashita et al., 2003; Miao et al., 2010). However, the knowledge of environmental factors contributing to HSCR is still quite limited (Gershon, 2010; Lake and Heuckeroth, 2013).

Abbreviations: HSCR, Hirschsprung's disease; BP-3, Benzophenone-3; RET, receptor tyrosine kinase; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; DEPC, diethylpyrocarbonate; DMEM, Dulbecco minimal essential medium; FBS, fetal bovine serum; CCK-8, cell counting kit-8; LODs, limits of detection; CR, creatinine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BMI, body mass index; ORs, odds ratios; CIs, confidence intervals.

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The toxic potential of UV filters that used in a variety of sunscreen and personal care products to attenuate the effects of harmful UV radiation on skin and hair, has attracted public concerns (Okereke et al., 1995). Benzophenone-3 (BP-3) occurs naturally in flower pigments and is synthesized for use in sunscreens, various cosmetic products, and plastic surface coatings and polymers. The application of some personal care products that contain UV filters on the skin can increase the systemic absorption (Gustavsson Gonzalez et al., 2002; Liao and Kannan, 2014). In some cases, as much as 10% of the applied dermal dose was absorbed into the systemic circulation (Janjua et al., 2004). Because of the extensive use of BP-3 in personal care products, human exposure to this compound is widespread. BP-3 was found in >95% of urine samples collected from the U.S. general population, at concentrations ranging from 0.4 to 21,700 ng/mL (Calafat et al., 2008). It is also worth noting that exposure to BP-3 in women is much higher than men (Chen et al., 2012).

BP-3 is a bioactive chemical which is established to be weakly estrogenic and exhibits both intensely anti-estrogenic and anti-androgenic activities in vitro (Schlumpf et al., 2001; Schreurs et al., 2002, 2005). It is reported that BP-3 altered embryo development in insects (Ozaez et al., 2014). Previous study have found prenatal BP-3 exposure is associated with adverse birth outcomes (Wolff et al., 2008; Tang et al., 2013). Therefore, the effects of BP-3 on fetal development need particular concerns.

miR-218 has been extensively studied in pathologies. And it can markedly suppresses cell motility, invasion, and proliferation (Tu et al., 2013; Heckmann et al., 2014). According to a previous report, miR-218 suppresses tumor cell migration through SLIT2-ROBO1 pathway (Alajez et al., 2011). In this study, we conducted a population study to explore the potential effects of BP-3 exposure on HSCR and underlying mechanism in relation to miR-218-mediated pathways was further studied in vitro.

2. Materials and methods

2.1. Study population

We recruited 101 HSCR patients' mothers and 322 mothers as controls in affiliated hospitals of Nanjing Medical University from October 2009 to May 2014 (Nanjing Medical University Birth Cohort, NJMU Birth Cohort). The Institutional Review Board of Nanjing Medical University approved the protocols. After detailed explanation of the study procedures and clarification of questions raised, the total 423 women signed informed consent forms for the questionnaire interview and sample collections. A complete physical examination was performed on them and questionnaires were used to collect information including personal background, lifestyle factors, occupational and environmental exposures, genetic risk factors, sexual and reproduction status, medical history and physical activity. As far as we were aware, all participants in our study had not changed their eating habits, life styles or environments for several months prior to the sample collection. Spot urines samples of HSCR mothers as well as 322 controls were collected for BP-3 detection and were frozen at -20°C until analysis. Besides, 101 HSCR patients were diagnosed by pathological analysis after surgery. All the HSCR patients were diagnosed by barium enema and anorectal manometry evaluation before surgical procedures. From the surgical pathology perspective, HSCR is confirmed none ganglion cells in colorectum which is identified by the submucosa of a distal rectal biopsy. In the meantime, the 322 controls' children were divided into two groups, i.e. 103 infants received surgery because of intussusception or incarcerated and strangulated inguinal hernia without ischemia or necrosis as controls which got rid of HSCR or other congenital malformations, and the other 219 children who received non-surgical treatment.

Finally, 101 HSCR and 103 control colon tissues were obtained. The tissues were immediately frozen and stored at -80°C after surgery. All the studies involving human subjects were done under full compliance with government policies and the Helsinki Declaration.

2.2. Chemicals and reagents

BP-3 (CAS NO. 131-57-7, 99.5% purity) was purchased from Dr. Ehrenstorfer GmbH (Bürgermeister-Schlosser, Germany). β -glucuronidase/sulfatase type H-1 from *Helix pomatia*, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), diethylpyrocarbonate (DEPC) were obtained from Sigma-Aldrich (St. Louis, USA). BP-3 was first dissolved in DMSO and added to the Dulbecco minimal essential medium (DMEM) to final concentrations of $100\ \mu\text{M}$ which was further diluted to lower concentrations for testing. The cells treated with 0.1% DMSO served as controls.

2.3. Measurement of urinary phenols

We measured total (free and conjugated) urinary concentrations of phenols using a sensitive method as previously described (Chen et al., 2012). Briefly, urine samples were incubated in 1 M ammonium acetate buffer solution ($\text{pH} = 5.0$) for hydrolyzation with β -glucuronidase/sulfatase ($20,000\ \text{units mL}^{-1}$) overnight. After hydrolysis, the phenols were extracted and preconcentrated with solid phase extraction ($500\ \text{mg}/3\ \text{mL}$, Supelclean, USA), and further detected using ultra-high performance liquid chromatography (Waters, USA)-electrospray ionization tandem mass spectrometry (Waters, USA). The detection was done in the negative ion mode by multiple reaction monitoring. The limits of detection (LODs) were $0.04\ \text{ng mL}^{-1}$. Strict quality control was conducted during the analysis. CR data were collected using an automated chemistry analyzer (7020 Hitachi, Japan), which were obtained for correcting the phenol concentrations caused by urine concentration and dilution.

2.4. Cell culture and BP-3 treatment

Human 293T and SH-SY5Y cells were widely used as cell models in research on mechanisms of HSCR (Kawamoto et al., 2003; Vargiolu et al., 2009). Human 293T cells and SH-SY5Y cells were obtained from American Type Culture Collection (USA). These cells were cultivated in complete growth medium DMEM (Hyclone, USA), supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 $\mu\text{g mL}^{-1}$ streptomycin at 37°C , 5% CO_2 . For chemical treatment, 293T cells and SH-SY5Y cells were plated in 6-well, 24-well or 96-well plates and then treated with BP-3 (0.1 μM , 1 μM , 10 μM , and 100 μM) dissolved in DMSO for 24 h. As a negative control, cells were also exposed to 0.1% DMSO alone. 293T cells and SH-SY5Y cells were collected and used for gene and protein expression analysis on d 2.

2.5. Cell proliferation assays

Cell viability was assessed by cell counting kit-8 (CCK-8 kit, Beyotime Institute of Biotechnology, China). 293T cells or SH-SY5Y cells were seeded at 5000 cells/well in 96-well plates. After 24 h incubation, the cells were treated with BP-3 at concentrations of 0, 0.1, 1, 10, 100 and 1000 μM . After 24 h, 100 μL medium solution (content 10% CCK-8) was added and incubated at 37°C for 1 h. Finally, absorbance was measured on DU-800 Nucleic Acid and Protein Analyzer (Beckman, USA) at 450 nm. The experiment was repeated three times.

2.6. Cell cycle and apoptosis analysis

To estimate if BP-3 could affect the cell cycle and induce apoptosis in 293T cells and SH-SY5Y cells, flow cytometric analysis was used to determine the state of cell cycle and the DNA fragmentation. 293T cells and SH-SY5Y cells were seeded on 6-well plates at a density of about 1×10^6 cells per well. Cells were incubated overnight and subsequently exposed to BP-3 (0.1 μM , 1 μM , 10 μM and 100 μM) and control medium containing 0.1% DMSO. After 24 h, cells were washed with PBS and harvested with trypsin. Cells were fixed in 70% ethanol for 2 h or washed in cold PBS, then stained with propidium iodide and annexin V for 30 min protected from light. The fixed/stained cells were analyzed by FACS Calibur Flow Cytometry (BD Biosciences, USA) to quantify cell cycle or cell apoptosis.

2.7. Cell transwell assays

For those cells treated with BP-3 (0, 0.1, 1, 10 and 100 μM), after 24 h, cells (1×10^4) were seeded in the upper chamber with serum-free medium. DMEM containing 10% FBS was added to the lower chamber and followed by incubation for 1 d. Cells in upper chamber were stained with crystal violet staining solution (Beyotime Institute of Biotechnology, China), and then counted and photographed under $40 \times$ magnification (five views per well). Migrated cells were counted using Image-pro Plus 6.0 while cell numbers of normal control group were normalized to 1. All experiments were performed in triplicate.

2.8. RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA pellets were dissolved in ddH₂O containing 0.1% DEPC, and quantified by measuring the absorbency at 260 nm by DU-800 Nucleic Acid and Protein Analyzer (Beckman, USA). We manipulated all real-time PCR reactions on ABI7900 Fast Real-Time System (Applied Biosystems, USA) according to the manufacturer's instructions for quantification of gene expression. cDNA synthesis for coding genes was performed with 1 μg of total RNA according the manufacturer's instructions (Takara, Japan). TaqMan[®]MicroRNA Assays (Applied Biosystems, USA) were used as the probe for hsa-miR-218 and U6 which acted as an internal control. mRNA levels of RET and other genes were measured using SYBR PCR Master Mix reagent kits (Takara, Japan). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. All primer sequences are given in Table S1. The experiments were carried out three times.

2.9. Protein analysis

Total proteins were isolated from cultured cells using RIPA buffer containing protease inhibitors complete, ULTRA, Mini, ethylenediaminetetraacetic acid-free, EASY pack (Roche, Switzerland), while the membrane proteins were extracted from tissues by Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Scientific, USA). The protein samples (100 μg from each group) were separated with 7.5%/12.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. Primary polyclonal antibodies included RET antibody (SC167, Santa Cruz, USA), PLAG1 antibody (BP11647a, Abgent, USA), SLIT2 antibody (ab134166, Abcam, UK) and ROBO1 antibody (MAB7118, R&D, USA). The secondary antibodies with horse radish peroxidase were anti-rabbit, anti-goat and anti-mouse HRP-linked (Beyotime Institute of Biotechnology, China). The bands were visualized using ECL reagent (Millipore, USA). Equal amount of protein loading in

each lane was confirmed using GAPDH antibody. The experiment was replicated thrice for each protein.

2.10. Statistical analysis

The data analysis was performed using Stata 9.2 statistical software package (Stata Corp, LP). T test was used to compare the mean age, body mass index (BMI) between case and control groups; if the variances were far from equal, the Wilcoxon signed rank test was used. The chi-squared test was used to evaluate the differences in smoking status and drinking status between case and control groups. All urine samples were divided into three-level variable for statistical analysis. The samples with concentrations <LOD were assigned to the low exposure group, and the remaining samples were divided into 2 groups approximately as the median exposure group and the high exposure group. The samples between detectable concentrations and 0.10 ng mL⁻¹ were assigned to the median exposure group. The remaining samples were assigned to the high exposure group. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for the estimation of the relationship between HSCR and urinary BP-3 levels by unconditional logistic regression analysis adjusted by BMI which was significantly different between case and control groups. Additionally, we employed urinary creatinine (CR) as a continuous variable to adjust for the urinary dilution.

Statistically significant differences between the treatments and the control were determined by one-way analysis of variance, followed by Dunnett's multiple comparison test. All tests of statistical significance were two-sided, and the statistical significance was set at $p < 0.05$.

3. Result

3.1. The association between BP-3 and HSCR in the populations

All of the 423 women participants were ethnic Han-Chinese, including 101 cases and 322 controls. Age, weight and BMI of mother were possible confounders which were related with birth outcome (Fraser et al., 1995; Cnattingius et al., 1998; Kiel et al., 2007). As shown in Table 1, there were significant differences in weight and BMI between cases and controls, which could be due to higher weight of the controls in pregnancy. While no significant differences were identified in smoking status and alcohol consumption. Therefore, we matched age in mothers of HSCR and controls in our study. Weight and BMI were significant in the case and control groups and BMI was adjusted to calculate ORs and CIs between the case and control groups. The distributions of urinary concentrations of BP-3 in the 423 participants are presented in Table 2. BP-3 were detectable in over 50% samples and adjusted ORs for the relationship between phenol exposure levels and HSCR are presented in Table 2. The maximum detection concentration was 400.72 ng mL⁻¹ and percentiles at 50th, 75th, 90th, 95th were 0.08, 0.17, 0.54, 1.10 ng mL⁻¹. Compared with women in the lowest exposure group, women in both the median and high BP-3 exposure groups were more likely to give birth of HSCR children, [for BP-3: adjusted ORs for increasing exposure levels = 2.39 (95% CI, 1.10–5.21), 2.61 (95% CI, 1.15–5.92), p -value for trend <0.05] which indicated that maternal BP-3 exposures were associated with the offspring's HSCR.

The age of 101 HSCR and 103 control groups were 3.62 ± 0.21 and 3.67 ± 0.25 months old, respectively. The body weight were 5.13 ± 0.14 and 5.35 ± 0.12 kg, respectively. There was no statistical difference between cases and controls in age and body weight. The gender rate (Male/Female) of HSCR and control was 79/22 and 83/20, respectively, which matched the common gender rate of this disease in human beings.

Table 1
Characteristics of participants in this study.

	HSCR	Control	P
Mother characteristics (n = 423)			
Maternal age (year, mean ± SD)	28.88 ± 4.30	27.48 ± 4.65	0.07 ^a
Parity (%)			0.54 ^b
0	65.28	68.96	
≥1	34.72	31.04	
Education (year, %)			0.38
≤9	67.60	69.25	
10–12	32.40	30.75	
Maternal height (cm, mean ± SD)	160.41 ± 3.71	161.19 ± 4.03	0.23
Maternal weight (kg, mean ± SD)	56.27 ± 8.19 ^c	71.32 ± 10.17 ^d	0.01*
BMI	21.86 ± 3.01	28.57 ± 4.80	0.01*
Smoking in pregnancy (%)	0	0	
Drinking in pregnancy (%)	6.90	6.73	0.56
Children characteristics (n = 204)			
Age (month, mean, SD)	3.62 ± 0.21	3.67 ± 0.25	0.47
Sex (%)			
Male	79 (78.22)	83 (80.58)	0.58
Female	22 (21.78)	20 (19.42)	
Weight (kg, mean, SD)	5.13 ± 0.14	5.35 ± 0.12	0.39

There was significant difference both at maternal weight and BMI variables between HSCR mothers and the controls' mother. In this case our results of BP-3 detection in urine was corrected by BMI.

*P < 0.05 indicates significant difference compared with the control group.

^a Student t-test.

^b Two-sided χ^2 test.

^c Values are usual weight (kg) divided by the height (m) squared.

^d Values are end-of-pregnancy weight (kg) divided by the height (m) squared.

3.2. The cytobiology change after BP-3 exposure in 293T and SH-SY5Y cell lines

To examine the effect of BP-3 on cell viability and morphology, 293T cells and SH-SY5Y cells were exposed to various concentrations of BP-3 for 24 h. After that, a significant decrease in viability was observed at the dose of 1000 μ M (Fig. S1A). Since cytotoxic effects were not observed during 0.1 μ M, 1 μ M, 10 μ M, 100 μ M of treatment, cells were treated with BP-3 at these concentrations in all of the following experiments.

As abnormal migration is the major cause of HSCR, the migration of 293T and SH-SY5Y cell lines after BP-3 treatment were tested with transwell assay. A suppressive effect was observed in both 293T and SH-SY5Y cell lines at the dose of 100 μ M (Fig. 1A). The number of migrated cells were significantly lower in 100 μ M of BP-3 treatment group (Fig. 1B), which indicates a causal association between BP-3 exposure and HSCR in the population study. We also examined the effect of BP-3 on the cell cycle and apoptosis after 24 h exposure using flow cytometry, but no significant differences were observed in both cell apoptosis and cell cycle (Fig. S1B and C).

3.3. The expression of RET was inhibited in BP3 contaminated cell lines

The RET proto-oncogene is considered the major disease-causing locus in HSCR, mutations of which are identified in 15–35%

sporadic cases (Brooks et al., 2005; de Groot et al., 2006). To evaluate the effect of BP-3 exposure on RET expression, we conducted RT-PCR to detect mRNA expression with BP-3 treatment from 0 to 100 μ M doses. As show in Fig. 2A, similar to HSCR stenosis tissues as reported, RET mRNA expressions were decreased in BP-3 treated 293T and SH-SY5Y cells. Meanwhile, BP-3 reduced RET protein expressions significantly in western blot experiments (Fig. 2B).

3.4. Effects of BP-3 on miR-218-RET pathway

miR-218 is predicted to target RET as well as PLAG1 by bioinformatics methods in previous research (Tang et al., 2015). To explore the potential role of miR-218 and PLAG1 in BP-3 induced effect, both gene expression levels were tested by RT-PCR in BP-3 infected 293T and SH-SY5Y cells. The results showed that miR-218 was up-regulated while PLAG1 was down regulated, which were consistent with protein levels (Fig. 2A and B). Therefore, BP-3 treatment might suppress RET and PLAG1 expression through increasing miR-218 expression. As a consequence, SLIT2, the host gene of miR-218, was assessed at mRNA level. The mRNA and protein levels of SLIT2, and its receptor ROBO1 were dose-dependently increased by BP-3 exposure (Fig. 2).

3.4.1. The validation of the miR-218-RET pathway in HSCR colon tissues

To determine whether BP-3 was involved in the pathogenesis of HSCR through miR-218-RET pathway, we detected those gene expressions in colon tissues of HSCR children and controls' at mRNA levels. As shown in Fig. 3, we confirmed the mRNA expression levels of miR-218, SLIT2 and ROBO1 in HSCR were significantly higher than those in controls ($P = 7.13 \times 10^{-8}$, $P = 5.03 \times 10^{-8}$ and 4.12×10^{-6} , respectively), while those of RET and PLAG1 were extreme lower ($P = 5.47 \times 10^{-9}$, $P = 1.02 \times 10^{-6}$).

4. Discussion

As far as we know, this is the first study to comprehensively explore the effects of BP-3 exposure (at concentrations that do not induce cytotoxic effects) on HSCR, and the possible underlying mechanism was revealed. We found maternal BP-3 exposure was associated with HSCR. BP-3 suppressed migration in 293T and SH-SY5Y cells. It also decreased RET and PLAG1 and increased miR-218, SLIT2 and ROBO1 both at the mRNA and protein levels.

HSCR is a partially penetrant oligogenic birth defect that occurs when ENS precursors fail to colonize the distal bowel during early pregnancy (Lake et al., 2013). Ganglion migration failure during 5–12 weeks of gestation is one of the leading causes of the disease. Maternal exposure to chemicals with endocrine disrupting effects can alter the development of progeny, inducing birth defects (Ngalame et al., 2013; Veiga-Lopez et al., 2013). Population study manifests that phthalate exposure of pregnant women during critical window contributes to preterm births (Ferguson et al., 2014). Nevertheless, we got barely research on mammals or primates about BP-3 toxicity in spite of its extensive exposure and potential deleterious effect (Vela-Soria et al., 2011). In our study,

Table 2
Adjusted ORs (95% CIs) for HSCR by exposure level of BP-3 in maternal urines (n = 423)^a.

Chemicals	Control/case (NO.)	Adjusted-OR ^a (95% CI) ^{BM}	p	
BP-3	low	129/37	1	
	medium	97/33	2.39 (1.10–5.21)	0.03*
	high	96/31	2.61 (1.15–5.92)	0.02*

The tables showed significances in both medium and high BP-3 exposure than low exposure in maternal urines.

*P < 0.05 compared with the low BP-3 exposure level.

^a ORs are adjusted for maternal BMI and CR level.

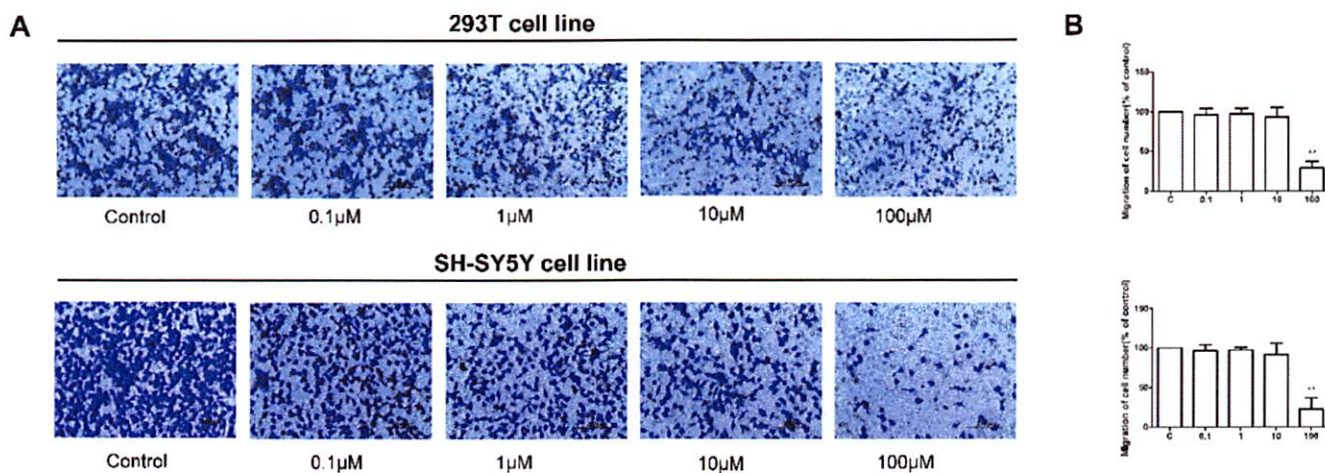


Fig. 1. Cytotoxicity measurement after BP-3 exposure. (A) The representative images of metastasis cells at the bottom of the membrane stained with crystal violet were visualized as shown in 293T and SH-SY5Y cells. (B) The quantifications of cell migration were presented as percentage migrated cell numbers, in 293T and SH-SY5Y cells respectively. ***P < 0.001, n = 5. All tests were performed for three times and presented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

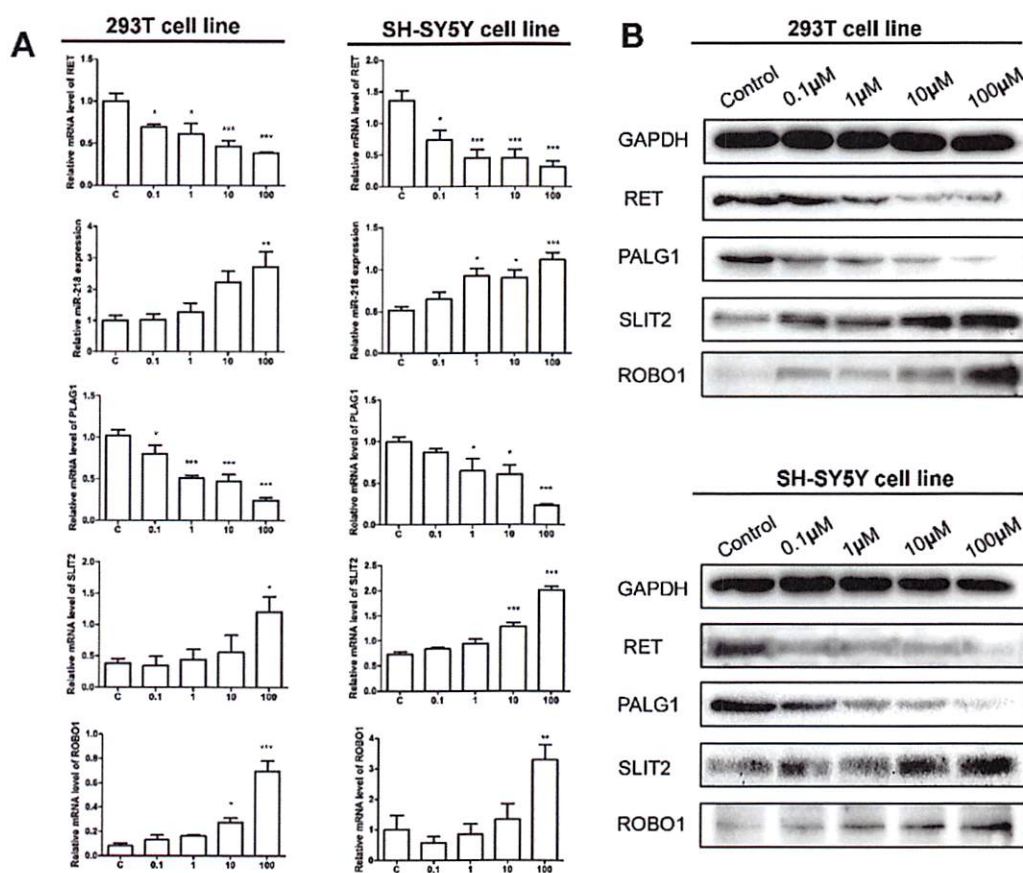


Fig. 2. mRNA and post-transcriptional expression levels of miR-218 pathway. (A) The mRNA expression level of gene of BP-3 exposure at 0, 0.1, 1, 10, 100 μM concentrations in 293T and SH-SY5Y cells. *P < 0.05, **P < 0.01, ***P < 0.001, n = 3. (B) GAPDH, RET, PLAG1, SLIT2, ROBO1 protein levels of BP-3 treatment in 293T and SH-SY5Y cells. GAPDH is used as internal control. All tests were performed for three times and presented as mean ± SE.

maternal BP-3 exposure levels were extremely consistent with disease risk of offspring's HSCR. Therefore, maternal BP-3 exposure during critic windows of fetal neural development may affect the development of offspring and induce HSCR.

It has been demonstrated that miRNAs not only regulate multiple protein coding genes, but also control all biological processes (Lin et al., 2012; Liu et al., 2012). It is universally acknowledged

that miRNAs are susceptible to environmental exposures, such as bisphenol A and nicotine. miRNA dysfunction may be a mechanism through which toxicants can exert both developmental and carcinogenic effects (Jirtle and Skinner, 2007; Izzotti et al., 2009; Avissar-Whiting et al., 2010; Hou et al., 2012). Studies showed that prenatal chemical exposure could modify the epigenome of specific genes of the offspring and social behavior in the childhood, which

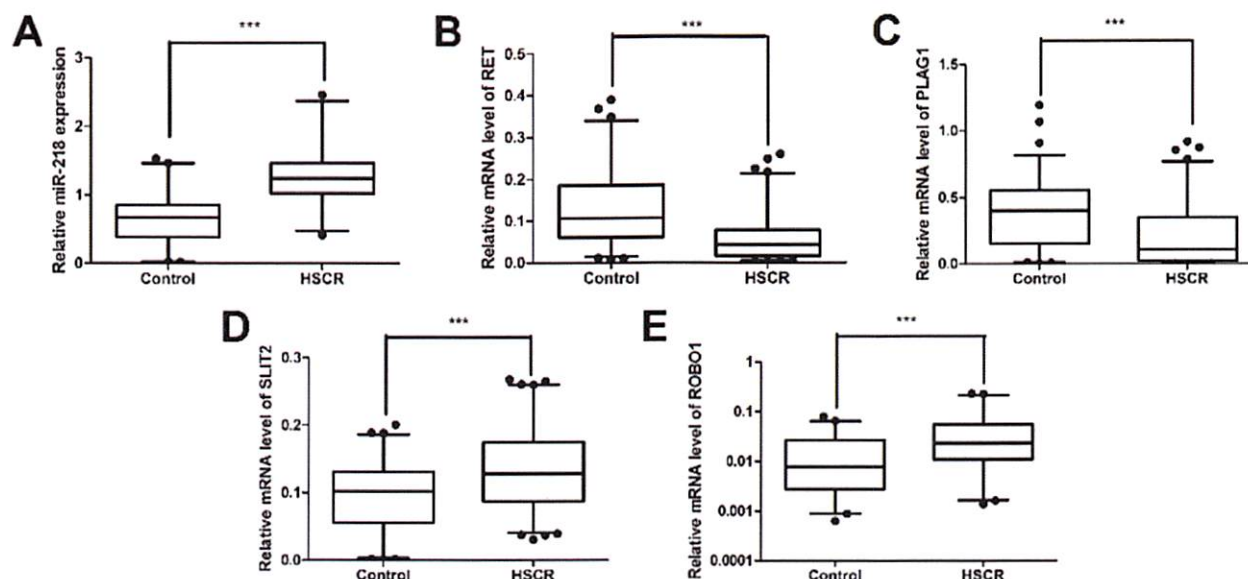


Fig. 3. Validation of population. (A)–(E) The mRNA expression levels of miR-218, RET, PLAG1, SLIT2, ROBO1, in the control and HSCR tissues. *** $P = 0.0005$, $n = 103$ controls/101 HSCR. All tests were performed for three times.

gave us clues that prenatal BP-3 exposure might alter gene expressions through epigenetic level (Furlong et al., 2014; Vilahur et al., 2014). Our former study has clarified that increased expression of miR-218 suppressed cell migration and proliferation in 293T and SH-SY5Y cells. Therefore, modified expression level of miR-218 caused by BP-3 exposure may play roles in the pathogenesis of HSCR.

Some evidences confirmed that RET was upregulated by miR-218 in acute myeloid leukemia (Diaz-Beya et al., 2013). Early researches have confirmed that miR-218 inhibits cell migration and proliferation by targeting RET and PLAG1 (Tang et al., 2015). In this study, we firstly demonstrated that BP-3 inhibited cell migration through RET and PLAG1 at the concentration of 100 μ M. To ensure the down-regulating of RET and PLAG1 were caused by the enhanced miR-218, we then examined the expression level of miR-218 in both cell lines after the exposure of BP-3. As expected, miR-218 was up-regulated. Considering the relationship between SLIT2 and miR-218 as well as ROBO1, we performed experiments to test the expression levels of both SLIT2 and ROBO1. Our study manifested BP-3 exposure led to higher expression quantities of SLIT2, and conversely lower expression quantities of ROBO1 which acted as its receptor in central nervous. Based on all the cell function experiments, we demonstrated that BP-3 contributed to the pathogenesis of HSCR via regulating genes signal transduction.

A limitation of the present study was the spot urine determination to assess exposure. However, as far as we were aware, all participants in our study had not changed their eating habits, life styles or environments for several months prior to the sample collection. Therefore, their urinary levels of BP-3 exposure are expected to be relatively stable over time. In addition, although the risk of misclassification may still exist, since this would be most likely non-differential, one would expect mainly attenuation of observed exposure–effect associations. Second, this study was the case–control design that limited our strength in making a causal conclusion. Third, although we clarify the fact that maternal BP-3 exposure brought high risk probability to offspring's HSCR, cell lines are limited in elucidating the mechanism in the present study and chemical exposure in enteric neural crest cells is needed for further investigation. What's more, other environment factors which may take part in the process of pathogenesis of HSCR are still unknown.

5. Conclusions

In conclusion, our study firstly pointed out that maternal BP-3 exposure may cause offspring's HSCR through SLIT2/ROBO1–miR-218–RET/PLAG1 pathway. These findings enhanced our understanding of BP-3 effects on embryonic development.

Conflicts of interest

The authors declare no conflict of interest with the study or preparation of the manuscript.

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Appendix A. Supplementary data


Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2015.09.019>.

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Benzophenone-3 Impairs Autophagy, Alters Epigenetic Status, and Disrupts Retinoid X Receptor Signaling in Apoptotic Neuronal Cells

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Abstract Benzophenone-3 (BP-3) is the most widely used compound among UV filters for the prevention of photodegradation. Population studies have demonstrated that it penetrates through the skin and crosses the blood-brain barrier. However, little is known about the impact of BP-3 on the nervous system and its possible adverse effects on the developing brain. We demonstrated that the neurotoxic effects of BP-3 were accompanied by the induction of apoptosis, as evidenced by apoptosis-related caspase-3 activation and apoptotic body formation as well as the inhibition of autophagy, as determined by the downregulation of autophagy-related genes, decreased autophagosome formation, and reduced LC3B-to-LC3A ratio. In this study, we showed for the first time that the BP-3-induced apoptosis of neuronal cells is mediated via the stimulation of RXR α signaling and the attenuation of RXR β /RXR γ signaling, as demonstrated using selective antagonist and specific siRNAs as well as by measuring the mRNA and protein expression levels of the receptors. This study also demonstrated that environmentally relevant concentrations of BP-3 were able to inhibit autophagy and disrupt the epigenetic status of neuronal cells, as evidenced by the inhibition of global DNA methylation as well as the reduction of histone deacetylases and histone acetyl transferases activity, which may increase the risks of neurodevelopmental abnormalities and/or neural degenerations.

Keywords Benzophenone-3 · BP-3 · Retinoid X receptors · RXR · Primary neuronal cell cultures · Autophagy

Introduction

Because of public anxiety about skin cancer caused by ultraviolet light (UV), production and consumption of sunscreen products are increasing. Nowadays, over 10,000 t of UV filters are produced annually for the global market [1]. Chemical UV filters are generally used as a mixture since none of the compounds used individually get sufficient protection against UV.

Among the filters, benzophenones (BPs) are the primary ingredients in the organic UV filter family. Benzophenone-3 (2-hydroxy-4-methoxybenzophenone, oxybenzone, 2OH-4 MeO-BP or BP-3) is the most widely used compound among BPs for the skin prevention against photodegradation [2]. Human studies have demonstrated that after topical application, BP-3 is absorbed through the skin, partially metabolized, and is excreted in the urine. BP-3 was detected in almost all (80–96%) urine samples collected from the general population in the USA [3]; pregnant women in France [4, 5]; and Danish mothers and their children, adolescents, young men, and pregnant women [6, 7]. BP-3 can be detected in the serum and urine of adult volunteers shortly after dermal application, proving that it can pass through the skin into the body [8]. It has been estimated that 10% of the applied dermal BP-3 penetrates skin into systemic circulation [9].

It is extremely disturbing that BP-3 has been detected in a large proportion of milk samples indicating that breastfed babies are exposed to BP-3 [10]. A recent study showed that maternal exposure to BP-3 is strongly associated with the onset of Hirschsprung's disease in offspring [11]. However, data on the effects of BP-3 on the nervous system are scarce. Especially little is known about the impact of BP-3 on individual

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receptors that are strongly associated with brain development such as retinoid X receptors. The only data on the apoptotic and neurotoxic effects of BP-3 on the neural cells come from SH-SY5Y neuroblastoma cells and our recently published original paper [12, 13]. BP-3 has been reported to act as endocrine disrupting chemicals (EDCs). At present, it is only known that BP-3 can weakly agonize estrogen signaling and strongly antagonize androgen-related pathways [14–16]. An association between BP-3 exposure and the estrogen-related disease endometriosis has been found [17]. Recently, retinoid X receptors have been postulated to be a target for EDCs.

The retinoid X receptor (RXR) is a type of nuclear receptor family that is encoded by three genes: *RXR α* , *RXR β* , and *RXR γ* [18]. RXRs heterodimerize with one-third of the 48 human nuclear receptor superfamily members [19]. For most of them, RXR is an obligatory partner for DNA binding and transcriptional regulation. In addition, *RXR α* is able to form homodimers and homotetramers, which is suggestive of the self-regulation of specific *RXR α* signaling pathways [20]. The diversity of RXRs suggests that they play critical roles in a wide range of cellular pathways. Recent studies have shown the prominence of RXR signaling in developing innervation and myelination in health and disease of the central nervous system [21]. Current studies in our research group have shown the involvement of RXRs in the effects of EDCs (specifically the pesticide dichlorodiphenyldichloroethylene (DDE) and nonylphenol) [22–24].

One of the most important ways of regulating gene expression is the remodeling of chromatin, including post-translational modifications of histones and DNA methylation. It has been postulated that low doses of EDCs may cause epigenetic changes, such as the incomplete methylation of specific gene regions in the young brain [25, 26]. Histone post-translational modifications include the most studied modifications—the acetylation of histones by histone acetyltransferases (HATs) and the removal of acetyl groups from histones by histone deacetylases (HDACs). These processes play important roles in cognition as well as psychiatric and neurologic diseases such as Alzheimer's disease, Huntington's disease, traumatic brain injury, post-traumatic stress disorder, stress, depression, and addiction [27].

Autophagy is a process that is mainly responsible for eliminating the cells or keeping them alive, even in conditions deprived of trophic factors. Autophagy is postulated to play a housekeeping role in removing abnormal proteins or clearing damaged organelles. The formation of autophagosomes depends on several core Atg proteins, such as the following: ULK1 complex, Beclin1:Vps34/Atg14L complex, and LC3 conjugation systems. During the process of autophagy, LC3 protein is cleaved by Atg4 to LC3A which next is modified by ubiquitin-like systems to produce LC3B. Thus, LC3A and LC3B are present in autophagosomes; both the ratio of LC3B to LC3A and the amount of LC3B only can be used to estimate

the level of autophagy. Recent studies have proposed that generally autophagy is a survival mechanism, although its dysregulation may lead to non-apoptotic cell death [28].

The present study aimed to investigate the neurotoxic and apoptotic effects of BP-3 and the impact of this chemical on the expression and function of RXRs, including *RXR α* , *RXR β* , and *RXR γ* . Neurotoxicity was estimated by measuring lactate dehydrogenase (LDH) release, which was complemented by an assessment of caspase-3 activity. These data were supported by Hoechst 33342/calcein acetoxymethyl (AM) staining, which allowed for the visualization of apoptotic nuclei and cell survival. The involvement of RXRs in the actions of BP-3 was verified using selective antagonist and agonist as well as specific siRNAs. The levels of receptor mRNAs and proteins were measured with qPCR, western blot, and enzyme-linked immunosorbent assay (ELISA), and the cellular distributions of the receptors were demonstrated using a confocal microscope. The process of autophagy was assessed by measuring the expression of autophagy-specific genes using microarray analysis and autophagosome detection, and the concentrations of autophagy-selective proteins were measured by ELISAs. Results regarding epigenetic modifications such as histone post-translational modifications and DNA methylation were complemented by an assessment of HAT and HDAC activity and the measurement of global DNA methylation.

Materials and Methods

Materials

B27 and neurobasal media were obtained from Gibco (Grand Island, NY, USA). L-glutamine, fetal bovine serum (FBS), *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitro-anilide (Ac-DEVD-*p*NA), dimethyl sulfoxide (DMSO), HEPES, CHAPS, mouse monoclonal anti-MAP2 antibody, ammonium persulfate, TEMED, TRIZMA base, Tween 20, DL-dithiothreitol, Nonidet NP-40, sodium deoxycholate, protease inhibitor (EDTA-free), bromophenol blue, 2',7'-dichlorofluorescein diacetate, RIPA buffer, the Imprint Methylated DNA Quantification Kit, the Histone Deacetylase Assay Kit, the Histone Acetyltransferase Activity Assay Kit, the Autophagy Assay Kit, protease inhibitor cocktail for mammalian tissues, and poly-ornithine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent, SDS, 30% acrylamide, 0.5 M Tris-HCl buffer, 1.5 M Tris-HCl gel buffer, and Laemmli sample buffer were from Bio-Rad Laboratories (Munich, Germany). DHA and HX 531 were from Tocris Bioscience (Minneapolis, MN, USA). 2-Mercaptoethanol was from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Immobilon-P membranes were purchased from Millipore (Bedford, MA, USA). Alexa 488-conjugated anti-goat IgG, calcein AM, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). Cy3-

conjugated anti-rabbit IgG and Cy5-conjugated anti-mouse were obtained from Jackson ImmunoResearch, Inc. (West Grove, PA, USA). The cytotoxicity detection kit and BM chemiluminescence western blotting substrate (POD) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). ELISA kits for RXR α , RXR β , RXR γ , LC3A, and LC3B were purchased from Shanghai Sunred Biological Technology Co. (Sunred, China). The culture dishes were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland). The rabbit polyclonal anti-RXR α antibody (sc-774), mouse monoclonal anti-RXR β antibody (sc-56869), mouse monoclonal anti-RXR γ antibody (sc-514134), mouse monoclonal anti- β -actin antibody (sc-47778), as well as RXR α siRNA (sc-36448), RXR β siRNA (sc-36446) and RXR γ siRNA (sc-38879) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). AllStars Negative Control siRNA AF 488, the RNeasy Mini Kit, RT² First Strand Kit, and RT² Profiler PCR Autophagy Array were obtained from Qiagen (Valencia, CA, USA). INTERFERin was obtained from PolyPlus Transfection (Illkirch, France), and the High Capacity cDNA-Reverse Transcription Kit, the TaqMan Gene Expression Master Mix, and TaqMan probes for specific genes encoding hypoxanthine phosphoribosyltransferase coding gene (*Hprt*), *Rxr α* , *Rxr β* , and *Rxr γ* were obtained from Life Technologies Applied Biosystems (Foster City, CA, USA). Quick-gDNATM MicroPrep was obtained from Zymo Research (Irvine, CA, USA).

Primary Neocortical Cell Cultures

Neocortical tissue for primary cultures was prepared from Swiss mouse embryos (Charles River, Germany) at 15–17 days of gestation and cultured as previously described [22, 29]. All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Bioethics Commission in compliance with Polish Law (21 August 1997). Animal care followed official governmental guidelines, and all efforts were made to minimize suffering as well as the number of animals used. The cells were suspended in estrogen-free neurobasal medium supplemented with B27 on poly-ornithine (0.01 mg/ml)-coated multi-well plates at a density of 2.0×10^5 cells per cm². The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ for 7 days in vitro (DIV) prior to experimentation. The number of astrocytes, as determined by the content of intermediate filament glial fibrillary acidic protein (GFAP), did not exceed 10% for all cultures [22, 30].

Treatment

Primary neuronal cell cultures were exposed to BP-3 (10–100 μ M) for 6 or 24 h. The involvement of RXR signaling

in BP-3-induced effects was verified with the high-affinity RXR antagonist HX 531 (0.1 μ M) and the RXR agonist DHA (1 μ M) as previously described [22]. Specific ligands were added to the culture media 45–60 min before BP-3. To avoid nonspecific effects in our study, agonist and antagonist of RXRs were used at concentrations that did not affect the levels of caspase-3 activity or LDH release. All the compounds were originally dissolved in DMSO and were then further diluted in culture medium to maintain DMSO concentrations below 0.1%. The control cultures were supplemented with DMSO in concentrations that were equal to those used in the experimental groups.

Identification of Apoptotic Cells

Apoptotic cells were detected via Hoechst 33342 staining at 24 h after the initial treatment as previously described [22, 29]. Neocortical cells that were cultured on glass coverslips were washed with 10 mM phosphate-buffered saline (PBS) and stained with Hoechst 33342 (0.6 mg/ml) at room temperature (RT) for 5 min. The cells containing bright blue fragmented nuclei, which was indicative of condensed chromatin, were identified as apoptotic cells. Qualitative analysis was performed using a fluorescence microscope (NIKON Eclipse 80i, NIKON Instruments Inc., Melville, New York, USA) equipped with a camera with the BCAM Viewer© Basler AG software. The level of cellular fluorescence from fluorescence microscopy images was determined using ImageJ software. To calculate the corrected total cell fluorescence (CTCF), the following equation was used: CTCF = Integrated density – (Area of selected cell \times Mean fluorescence of background).

Staining with Calcein AM

Intracellular esterase activity in the neocortical cultures was measured by calcein AM staining at 24 h after the initial treatment with BP-3 as previously described [22, 29]. To avoid the esterase activity present in the growth media, the cells were washed with PBS and incubated in 2 μ M calcein AM in PBS at RT for 10 min. The cells displaying bright green cytoplasm were identified as live cells. Fluorescence intensity was monitored at Ex/Em 494/520 nm using a fluorescence microscope (NIKON Eclipse 80i, NIKON Instruments Inc., Melville, New York, USA) equipped with a camera with the BCAM Viewer© Basler AG software. The level of cellular fluorescence from fluorescence microscopy images was determined using ImageJ software. To calculate the CTCF, the following equation was used: CTCF = Integrated density – (Area of selected cell \times Mean fluorescence of background).

Assessment of Caspase-3 Activity

Caspase-3 activity was determined according to the protocol described by Nicholson (1995) using samples treated for 6 or 24 h with BP-3 alone or in combination with the test compounds. The assessment of caspase-3 activity was performed as previously described [22, 30, 31]. Cell lysates from neocortical cultures were incubated at 37 °C using Ac-DEVD-pNA, a colorimetric substrate that is preferentially cleaved by caspase-3. The levels of *p*-nitroanilide were continuously monitored for 60 min using a Multimode Microplate Reader Infinite M200PRO (Tecan, Mannedorf, Switzerland). The data were analyzed using the Magellan software, normalized to the absorbance of vehicle-treated cells, and expressed as a percentage of control \pm SEM from three to four independent experiments. The absorbance of blanks, which acted as our enzyme-less control, was subtracted from each value.

Measurement of Lactate Dehydrogenase Activity

To quantify cell death, lactate dehydrogenase (LDH) that was released from damaged cells into the cell culture media was measured 6 or 24 h after treatment with BP-3. LDH release was measured as previously described [22, 32]. Cell-free supernatants from neocortical cultures were collected from each well and incubated at room temperature for 30 to 60 min with the appropriate reagent mixture according to the manufacturer's instructions (Cytotoxicity Detection Kit) depending on the reaction progress. The intensity of the red color that formed in the assay was measured at a wavelength of 490 nm (Infinite M200pro microplate reader, Tecan Mannedorf, Switzerland) and was proportional to both LDH activity as well as the number of damaged cells. The data were analyzed using the Magellan software, normalized to the color intensity from vehicle-treated cells (100%), and expressed as a percentage of the control value from three to four independent experiments. The absorbance of blanks, which acted as our enzyme-less control, was subtracted from each value.

Silencing of RXR α , RXR β , and RXR γ

Specific siRNAs were used to inhibit RXR α , RXR β , and RXR γ expression in neocortical cells. Each siRNA was applied separately for 6 h at 50 nM in antibiotic-free medium containing the siRNA transfection reagent INTERFERin™ as previously described [22]. After transfection, the culture media were changed, and the cells were incubated for 12 h before starting the experiment. Positive and negative siRNAs containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA were used as controls. The effectiveness of mRNA silencing was verified through the measurement of specific mRNAs using qPCR.

qPCR Analysis of mRNAs Encoding the Receptors *Rxr α* , *Rxr β* , and *Rxr γ*

Total RNA was extracted from neocortical cells that were cultured for 7 DIV (approx. 1.5×10^6 cells per sample) using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity of RNA was spectrophotometrically determined at 260 and 260/280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). Two-step real-time quantitative polymerase chain reaction (qPCR) was performed as previously described [22]. Both the reverse transcription reaction and qPCR were run on a CFX96 Real-Time System (BioRad, USA). The products of the reverse transcription reaction were amplified using TaqMan Gene Expression Master Mix containing TaqMan primer probes specific to the genes encoding *Hprt*, *Rxr α* , *Rxr β* , and *Rxr γ* . Amplification was performed in a total volume of 20 μ l containing 10 μ l of TaqMan Gene Expression Master Mix and 1.0 μ l of reverse transcription product as the PCR template. A standard qPCR procedure was utilized: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The threshold value (Ct) for each sample was set during the exponential phase, and the delta Ct method was used for data analysis. *Hprt* was used as a reference gene.

Mouse Autophagy RT² Profiler PCR Array

Total RNA was extracted from neocortical cells cultured for 7 DIV (approx. 1.5×10^6 cells per sample) using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity of RNA was spectrophotometrically determined at 260 and 260/280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). A total of 1 μ g of mRNA was reverse-transcribed to cDNA using the RT² First Strand Kit (Qiagen, Valencia, CA) and suspended in a final volume of 20 μ l as previously described [13]. Each cDNA was prepared for further use in qPCR. To analyze the signaling pathway, the RT² Profiler™ PCR Array System (Qiagen, Valencia, CA) was used according to the manufacturer's protocol. The Ct values for all wells were exported to a blank Excel spreadsheet and were analyzed with the Web-based software (www.SABiosciences.com/pcrarraydataanalysis.php).

Western Blot Analysis

The cells exposed to BP-3 for 24 h were lysed in ice-cold RIPA lysis buffer containing a protease inhibitor cocktail. The lysates were sonicated and centrifuged at 15,000 \times g for 20 min at 4 °C. The protein concentrations in the supernatants were determined using Bradford reagent (Bio-Rad Protein Assay) with bovine serum albumin (BSA) as the standard. Samples containing 40 μ g of total protein were reconstituted in the appropriate amount of

sample buffer comprised of 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM DTT, and 0.01% bromophenol blue, denatured, and separated on a 7.5% SDS-polyacrylamide gel using a Bio-Rad Mini-Protean II Electrophoresis Cell as previously described [22, 33, 34]. After electrophoretic separation, the proteins were electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA) using the Bio-Rad Mini Trans-Blot apparatus. Following the transfer, the membranes were washed, and the nonspecific binding sites were blocked with 5% dried milk and 0.2% Tween-20 in 0.02 M Tris-buffered saline (TBS) for 2 h with shaking. The membranes were incubated overnight (at 4 °C) with one of the following primary antibodies (Santa Cruz Biotechnology) diluted in TBS/Tween: anti-RXR α rabbit polyclonal antibody (diluted 1:150), anti-RXR β mouse monoclonal antibody (diluted 1:100), anti-RXR γ mouse monoclonal antibody (diluted 1:100), or anti- β -actin mouse monoclonal antibody (diluted 1:3000). The signals were developed by chemiluminescence (ECL) using BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH) and visualized using a Luminescent Image Analyzer Fuji-Las 4000 (Fuji, Japan). Immunoreactive bands were quantified using a MultiGauge V3.0 image analyzer.

Enzyme-Linked Immunosorbent Assays for RXR α , RXR β , and RXR γ

The levels of RXR α , RXR β , RXR γ , LC3A, and LC3B were determined in neocortical cells 24 h after treatment with BP-3 as previously described [22]. Specific detection of these proteins was achieved using ELISAs and the quantitative sandwich enzyme immunoassay technique. A 96-well plate was precoated with monoclonal antibodies that were specific for RXR α , RXR β , RXR γ , LC3A, and LC3B. The standards and non-denatured cell extracts were added to the wells with biotin-conjugated polyclonal antibodies specific for RXR α , RXR β , RXR γ , LC3A, and LC3B. Therefore, all native RXR α , RXR β , RXR γ , LC3A, and LC3B proteins were captured using the immobilized antibodies. The plates were washed to remove any unbound substances, and horseradish peroxidase-conjugated avidin was added to interact with the biotin bound to RXR α , RXR β , RXR γ , LC3A, and LC3B. After washing, the substrate solution was added to the wells. The enzymatic reaction yielded a blue product. The absorbance was measured at 450 nm and was proportional to the amount of RXR α , RXR β , RXR γ , LC3A, and LC3B in the sample. The protein concentration was determined in each sample using Bradford reagent—Bio-Rad Protein Assay [22, 35].

Immunofluorescence Labeling of RXR α , RXR β , and RXR γ and Confocal Microscopy

For immunofluorescence detection of RXR α , RXR β , and RXR γ , neocortical cells were cultured on glass coverslips

and subjected to immunofluorescence double-labeling as previously described [22, 36]. After 1 h of incubation in a blocking buffer (5% normal donkey serum and 0.3% Triton X-100 in 0.01 M PBS), the cells were treated for 24 h (at 4 °C) using four primary antibodies: rabbit polyclonal anti-RXR α antibody (1:50), mouse monoclonal anti-RXR β antibody (1:50), mouse monoclonal anti-RXR γ antibody (1:50), and anti-MAP2 mouse monoclonal antibody (1:100) followed by a 24-h incubation in a mixture of secondary antibodies, including Cy3-conjugated anti-rabbit IgG (1:300) and Cy5-conjugated anti-mouse IgG (1:300). The samples were subsequently washed, mounted, coverslipped, and analyzed using an LSM510 META, Axiovert 200M confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) under a Plan-Neofluor 40 \times /1.3 Oil DIC objective. A He/Ne laser and an argon laser, with two laser lines emitting at 514 and 633 nm, were used to excite the Cy3-, and Cy5-conjugated antibodies, respectively. The fluorescence signal was enhanced after combining four scans per line. A pinhole value of 1 airy unit was used to obtain flat images.

Measurement of Global DNA Methylation

Genomic DNA was extracted from neocortical tissues using the Quick-gDNATM MicroPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The quantity of DNA was spectrophotometrically determined at 260 and 260/280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). Global DNA methylation changes were measured in neocortical cells at 24 h after treatment using a specific ELISA-based kit (Imprint[®] Methylated DNA Quantification—Sigma-Aldrich; St. Louis, MO, USA) as previously described [22]. This kit contained all the reagents required to determine the relative levels of methylated DNA. The methylated DNA was detected using the capture and detection antibodies and quantified colorimetrically using an Infinite M200pro microplate reader (Tecan, Austria). The amount of methylated DNA present in the sample was proportional to the absorbance measured.

Detection of Autophagosomes

Cultured cells on 96-well plates were treated according to the manufacturer's instructions. The Autophagy Assay kit provided a simple and direct procedure for measuring autophagy in a variety of cell types using a proprietary fluorescent autophagosome marker ($\lambda_{\text{ex}} = 333/\lambda_{\text{em}} = 518$ nm). The autophagosomes were detected using an Infinite M200pro microplate reader (Tecan, Austria).

Measurement of HDAC and HAT Activity

The HDAC and HAT activities were detected using the Histone Deacetylase Assay Kit and the Histone Acetyltransferase Activity Fluorometric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Regarding HDAC kit, the measured fluorescence at $\lambda_{\text{ex}} = 365 \text{ nm}/\lambda_{\text{em}} = 460 \text{ nm}$ was proportional to the deacetylation activity. In the HAT assay, the generated product of histone acetyltransferase activity was detected fluorimetrically at $\lambda_{\text{ex}} = 535/\lambda_{\text{em}} = 587 \text{ nm}$. The kit included an active nuclear extract to be used as a positive control. The abovementioned assays provided positive and negative controls.

Data Analysis

Statistical tests were performed on raw data that were expressed as the mean arbitrary absorbance or as the fluorescence units per well containing 50,000 cells (measurements of caspase-3, LDH, autophagosomes; the fluorescence units per 1.5 million cells (qPCR, global DNA methylation, and HDAC and HAT activity); the mean optical density per 40 μg of protein (western blotting); or picograms of RXR α , RXR β , RXR γ , LC3A, and LC3B per micrograms of total protein (ELISA). Statistical analysis of cellular fluorescence related to Hoechst 33342 and calcein AM staining was performed on CTCF data using 40 counts per image. One-way analysis of variance (ANOVA) was preceded by the Levene's test of homogeneity of variances and was used to determine overall significance. Differences between the control and experimental groups were assessed using a post hoc Newman-Keuls test, and significant differences were designated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control cultures; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ versus the cultures exposed to BP-3; and \$ $p < 0.05$ and \$\$\$ $p < 0.001$ versus the siRNA-transfected control cultures. The results were expressed as the mean \pm SEM of three to four independent experiments. The number of replicates in each experiment ranged from 2 to 3, except for the measurements of caspase-3 activity and LDH release, which contained five to eight replicates. To compare the effects of BP-3 in different treatment paradigms, the results for the caspase-3, LDH, ELISA, and western blot analyses were presented as a percentage of the control.

Results

Effects of BP-3 on Caspase-3 Activity and LDH Release in Neocortical Cultures at 7 DIV

In neocortical cultures at 7 DIV, BP-3 (25–100 μM) induced an increase in caspase-3 levels to 170% of the control level at 6 h, which were further enhanced to 196% at 24-h post-

treatment (Fig. 1a). In these cells, LDH release from neocortical cells increased in a time-dependent manner to 150–180% of the control value at 6 h and to 200–290% at 24 h (Fig. 1b).

Effects of BP-3 Alone or in Combination with HX 531 on Hoechst 33342 and Calcein AM Staining in Neocortical Cultures

In the present study, a 24-h exposure to BP-3 (25 μM) was necessary to develop an apoptotic morphology in cell nuclei. Apoptotic cells were detected by Hoechst 33342 staining as formation of bright blue fragmented nuclei containing condensed chromatin (Fig. 2). Furthermore, treatment with BP-3 reduced the density of calcein AM-stained living cells at 7 DIV, as indicated by the decreased number of cells exhibiting light-colored cytoplasm. Co-treatment with RXR antagonist-HX 531 (0.1 μM) inhibited the BP-3-induced effects. Quantitative analysis of relevant fluorescence signals showed that at 7 DIV, 25 μM BP-3 caused an increase in formation of condensed chromatin by 386% of the control level and reduced number of live cells by 56%. Treatment with HX 531 (0.1 μM) inhibited the effect of BP-3 in respect to the apoptotic fragmentation of cell nuclei by 231% and enhanced cell viability by 35% (Fig. 2).

Effects of BP-3 Alone or in Combination with DHA and HX 531 on BP-3-Induced Caspase-3 Activity and LDH Release in Neocortical Cultures

Neocortical cultures exposed to BP-3 (25 μM) for 24-h caused a greater than 50% increase in caspase-3 activity in the neuronal cells. Co-treatment with the selective RXR agonist DHA (1 μM) did not change the effect of BP-3 on caspase-3 activity. The RXR antagonist HX 531 (0.1 μM) inhibited the BP-3 (25 μM)-induced caspase-3 activity by 40% (Fig. 3a).

The selective RXR agonist DHA (1 μM) did not affect BP-3-induced LDH release. However, the high-affinity RXR antagonist HX 531 (0.1 μM) diminished BP-3-induced LDH release by 25% (Fig. 3b).

Effect of BP-3 on the mRNA Levels of *Rxr α* , *Rxr β* , and *Rxr γ*

According to our data, treatment with BP-3 (25 μM) affected the mRNA levels of *Rxr α* , *Rxr β* , and *Rxr γ* . A 3-h exposure of the neocortical cultures to BP-3 caused a 25% decrease in *Rxr β* and a 55% decrease in *Rxr γ* but caused a 100% increase in *Rxr α* mRNA compared with the control (Fig. 4a). The pattern of mRNA expression was changed after prolonged exposure to BP-3. After 6 h of treatment, BP-3 decreased the mRNA expression level of *Rxr α* (26%) but did not change the expression level of *Rxr β* in neocortical cells (Fig. 4b). After 24 h of exposure, BP-3 did not change the mRNA expression levels of any *Rxrs* (Fig. 4c). These data were normalized to *Hprt*.

Fig. 1 Time-course effects of BP-3 (10, 25, 50, 75, and 100 μ M) on caspase-3 activity (a) and LDH release (b) in primary cultures of mouse neocortical cells at 7 DIV. The cells were treated with BP-3 for 6 and 24 h. The results are presented as a percentage of the control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control cultures

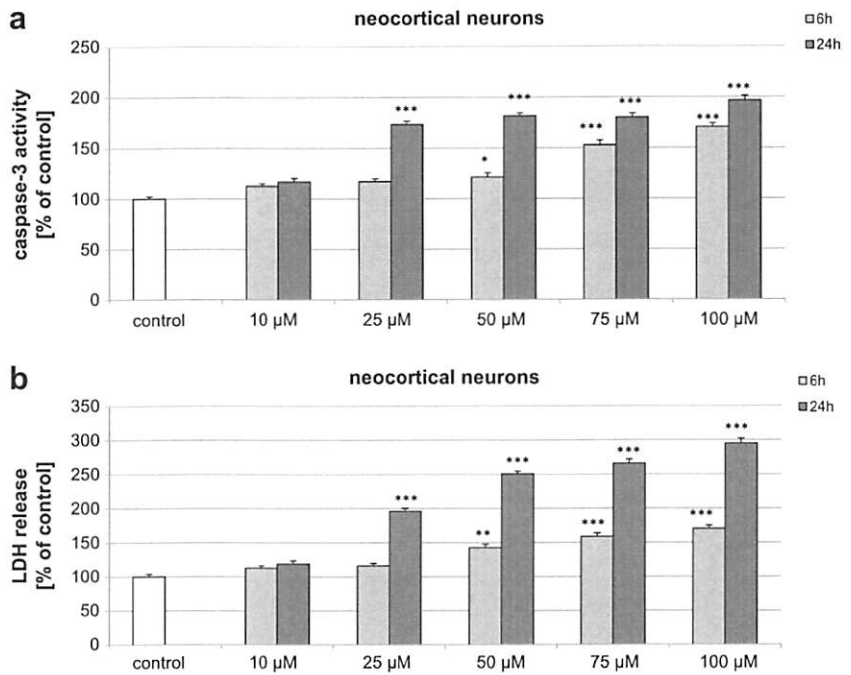
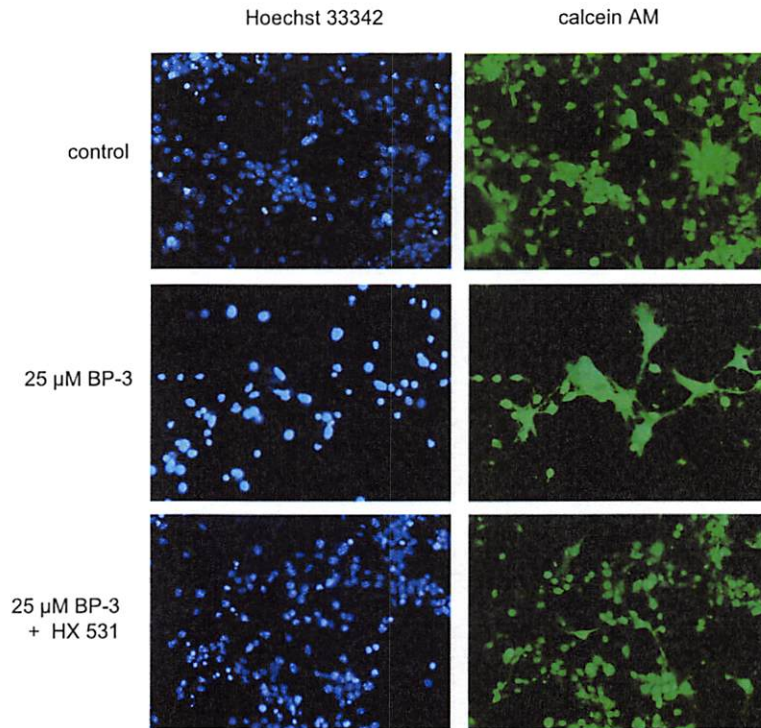


Fig. 2 Influence of BP-3 (25 μ M) and HX 531 (0.1 μ M) on Hoechst 33342 (first column) and calcein AM (second column) staining in mouse neocortical cultures at 7 DIV, examined 24 h post-treatment. Cells with bright fragmented nuclei with condensed chromatin were identified as cells undergoing apoptosis, whereas cells with light-colored cytoplasm were identified as live cells. Statistical analysis of relevant fluorescence signals was performed on CTCF data using 40 counts per image. * $p < 0.05$ and *** $p < 0.001$ versus control cultures



Corrected total cell fluorescence (CTCF)	control	25 μ M BP-3	25 μ M BP-3 + HX 531
Hoechst 33342	0.153 (100 %)	0.591 (386 %)***	0.238 (155 %)*
calcein AM	49.328 (100 %)	21.609 (44 %)***	38.802 (79%)*

Fig. 3 Impact of the RXR agonist and antagonist on BP-3-induced caspase-3 activity (a) and LDH release (b) in neocortical cultures at 7 DIV. The primary neocortical cultures were treated with BP-3 (25 μ M) for 24 h. The results were normalized to the absorbance of vehicle-treated cells and are expressed as a percentage of control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. *** p < 0.001 versus control cultures; ## p < 0.01 and ### p < 0.001 versus the cultures exposed to BP-3

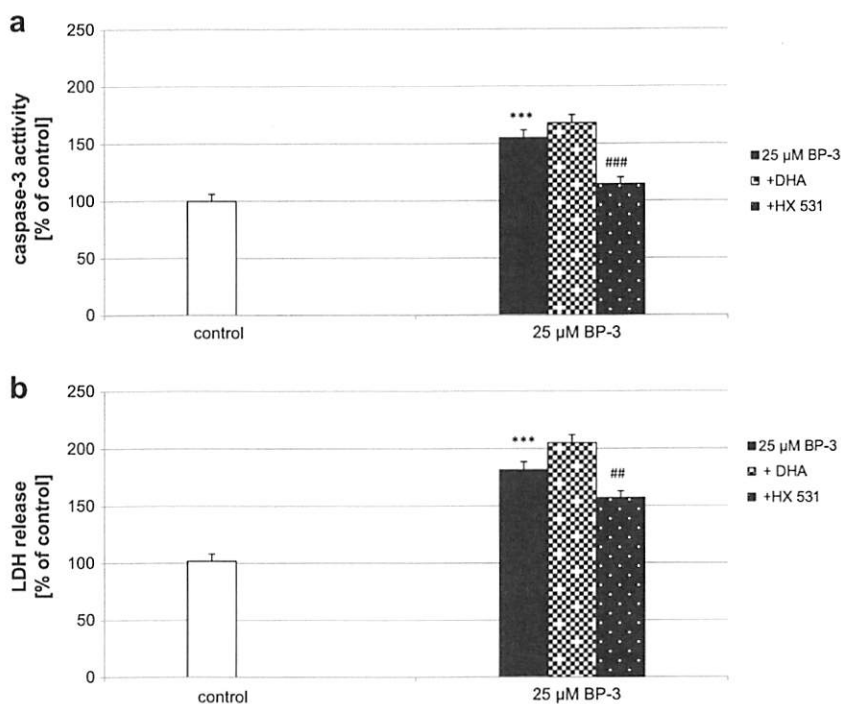


Fig. 4 Effect of BP-3 (25 μ M) on the mRNA expression levels of *Rxra*, *Rxr β* , and *Rxr γ* in neocortical cultures at 7 DIV. Each bar represents the mean \pm SEM of three independent experiments. The number of replicates for each experiment ranged from 2 to 3. ** p < 0.01 and *** p < 0.001 versus control cultures

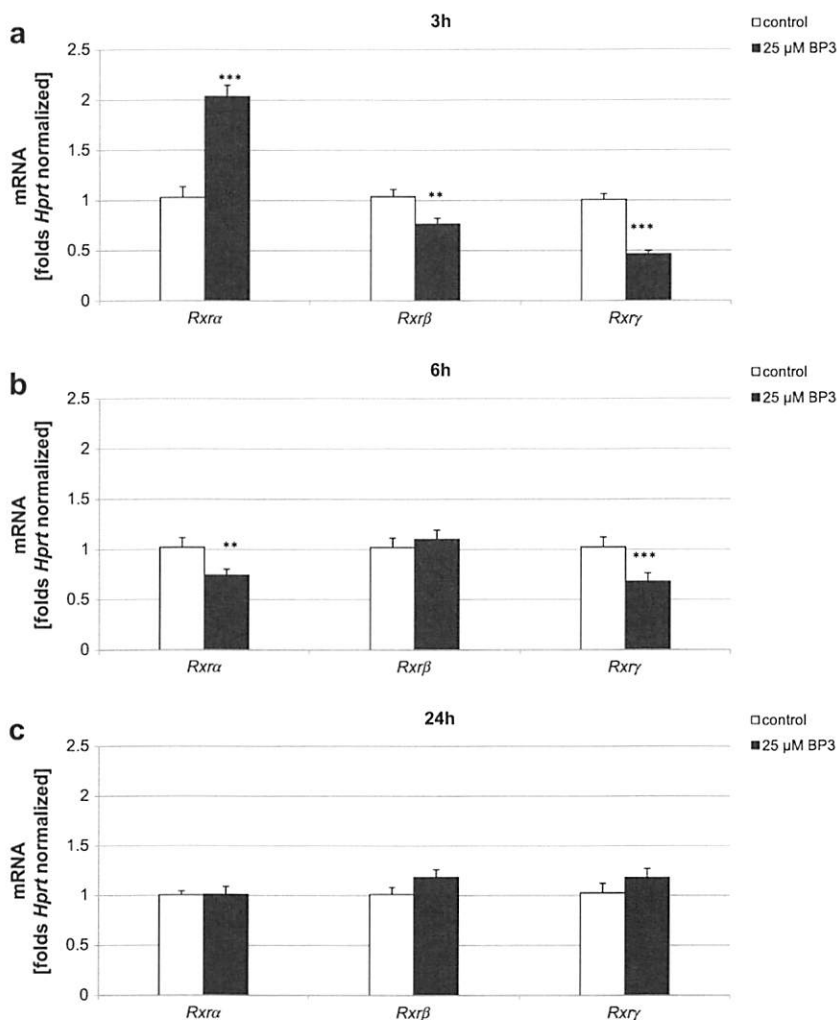
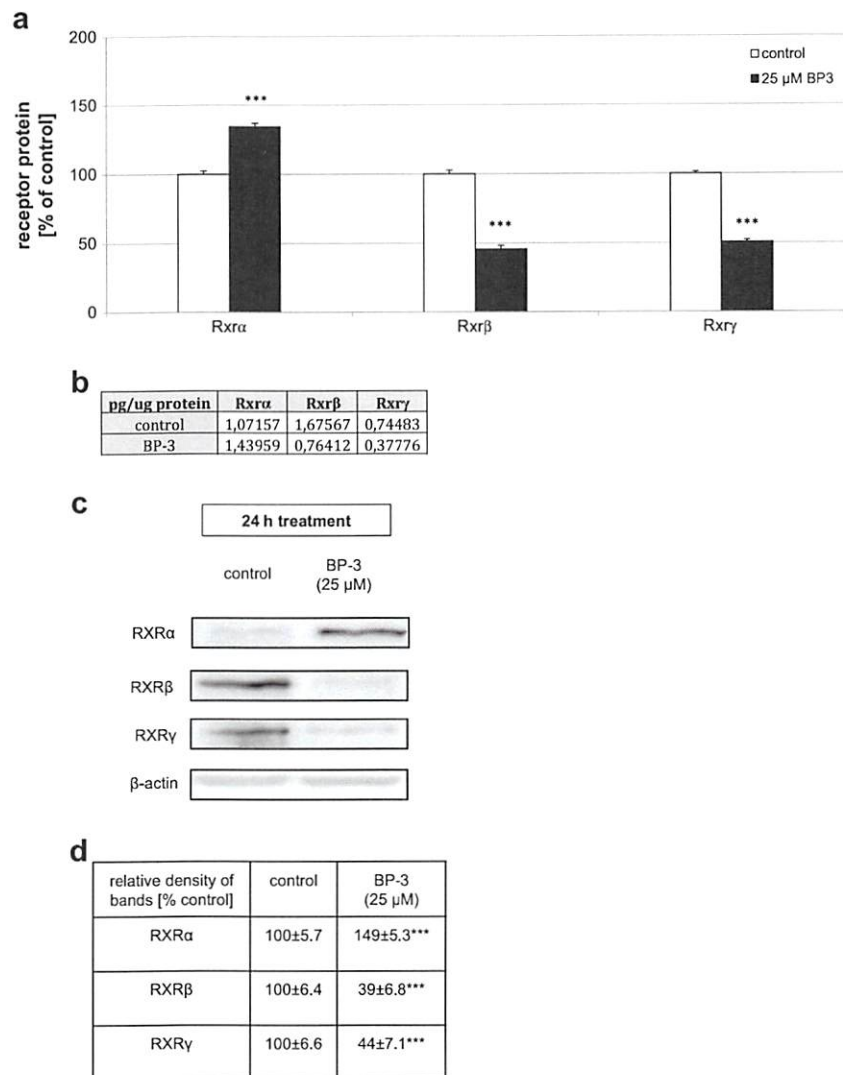


Fig. 5 Effects of BP-3 on the protein levels of RXR α , RXR β , and RXR γ in mouse neocortical cultures at 7 DIV. The neocortical cells were cultured for 7 DIV and then treated for 24 h with BP-3 (25 μ M). The concentrations of the receptors were measured using specific ELISAs and are presented as a percentage of the control (a) and as picogram of specific protein, i.e., RXR α , RXR β , and RXR γ , per microgram of total protein (b). For the western blot analyses, protein samples were denatured, electrophoretically separated, transferred to PVDF membranes, and subjected to immunolabeling (c). The relative protein levels of RXR α , RXR β , and RXR γ were presented as a percentage of the control (d). Each bar or value represents the mean of three independent experiments \pm SEM. The number of replicates in each experiment ranged from 2 to 3. *** $p < 0.001$ versus control cultures



Effects of BP-3 on the Protein Expression Levels of RXR α , RXR β , and RXR γ in Mouse Neocortical Cells

A 24-h exposure to BP-3 was necessary to detect changes in the protein levels of the receptors. In the cultures treated with BP-3, RXR α expression enhanced and reached 1.43 pg/ μ g of total protein (134% of the control). In cultures exposed to BP-3 (25 μ M) for 24 h, the concentration of RXR β was 0.76 pg/ μ g of total protein, and it was 56% less than that in control cultures. In the cultures exposed to BP-3, the level of RXR γ reached 0.38 pg/ μ g, which was 49% less than controls (Fig. 5a, b).

Western blot analysis determined the relative protein expression levels of RXR α , RXR β , and RXR γ in mouse neocortical cells at 7 DIV. Exposure to BP-3 (25 μ M) for 24 h decreased the relative RXR β and RXR γ protein levels by 61 and 56%, respectively. Treatment with BP-3 (25 μ M) increased the relative RXR α protein level by 49% (Fig. 5c, d).

Effect of BP-3 on the Distribution of RXR α , RXR β , RXR γ , and MAP2 Staining in Neocortical Cells

Immunofluorescence labeling was performed in parallel with the measurements of receptor protein levels. Confocal microscopy revealed that RXR α , RXR β , and RXR γ were localized to neocortical cells at 7 DIV. A 24-h exposure to BP-3 (25 μ M) increased RXR α staining but reduced RXR β - and RXR γ -specific immunofluorescence. MAP2 staining confirmed the neural localization of the receptors and revealed the BP-3-induced inhibition of neurite outgrowth (Fig. 6).

Influence of BP-3 on Caspase-3 Activity and LDH Release in Neocortical Cells Transfected with RXR α , RXR β , and RXR γ siRNAs

A 24-h exposure to BP-3 (25 μ M) only slightly reduced caspase-3 activity and LDH release in the RXR β and RXR γ siRNA-transfected cells, suggesting that the transfected cells

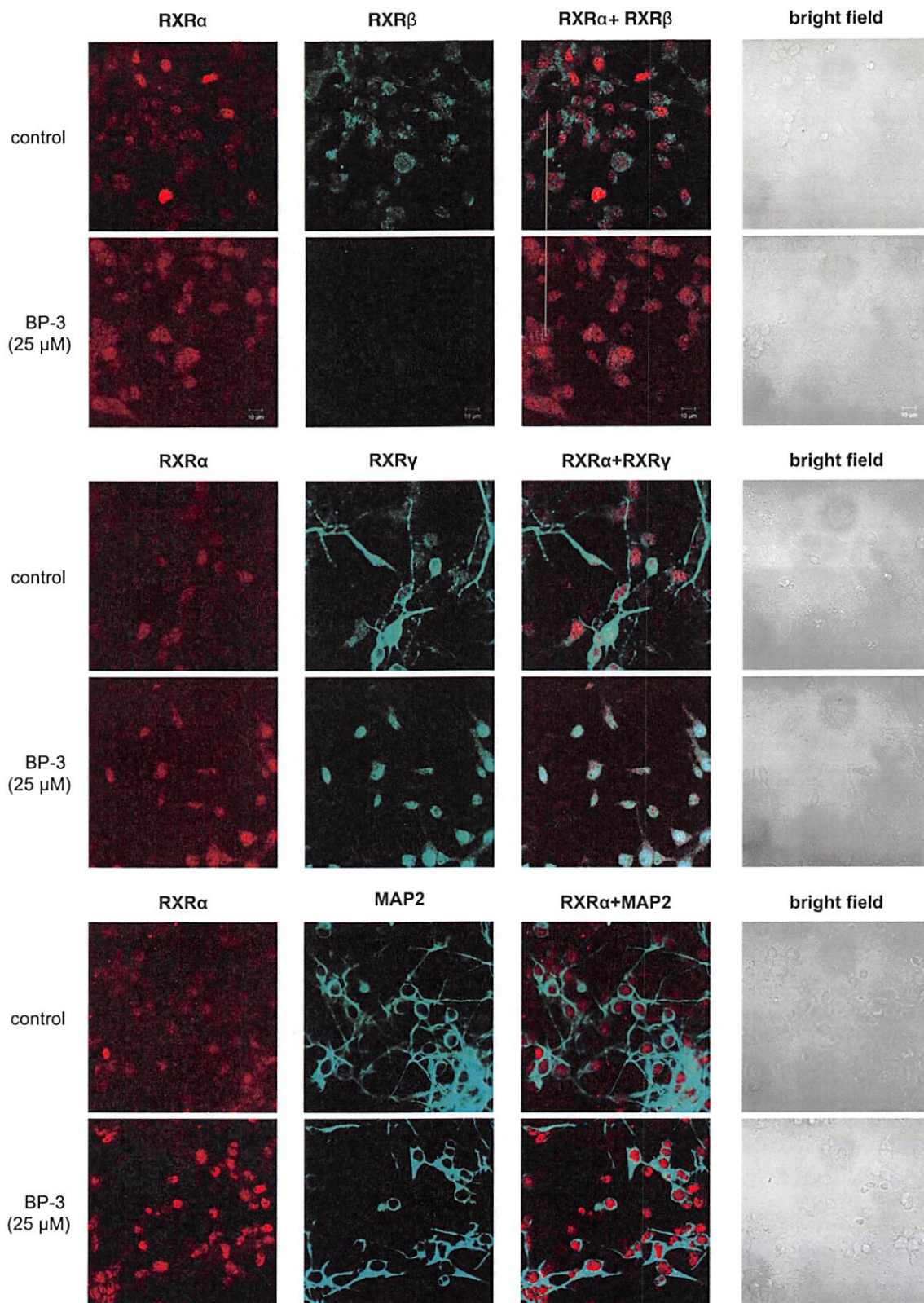
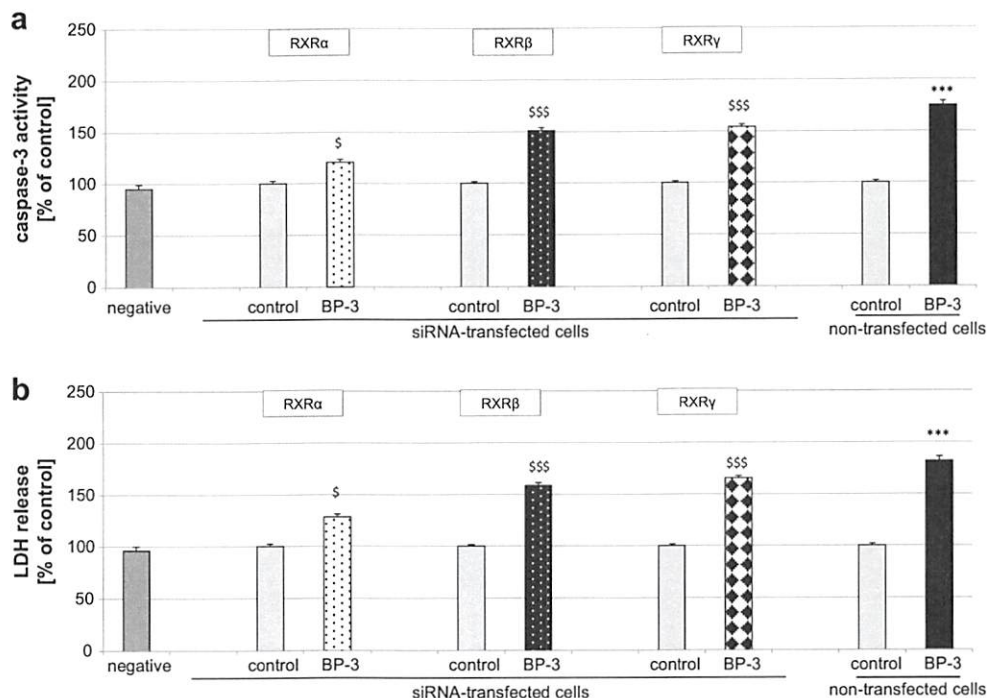


Fig. 6 Influence of BP-3 on the cellular distributions of RXR α (red), RXR β (blue), RXR γ (blue), and MAP2 (blue) in mouse neocortical cultures at 7 DIV. The overlay of RXR α /RXR β , RXR α /RXR γ , and RXR α /MAP2 (red plus blue) staining with the bright field images are

shown. The primary neocortical cultures were treated with BP-3 (25 μM) for 24 h. Analyzed using an LSM510 META, Axiovert 200M confocal laser scanning microscope under a Plan-Neofluor 40×/1.3 Oil DIC objective

Fig. 7 Effect of BP-3 (25 μ M) on caspase-3 activity (a) and LDH release (b) in RXR α , RXR β , and RXR γ siRNA-transfected neocortical cells. Each bar represents the mean \pm SEM of three to four independent experiments. The number of replicates in each experiment ranged from 5 to 8. *** p < 0.001 versus the non-transfected control cultures; $^{\$}$ p < 0.05 and $^{\$ \$ \$}$ p < 0.001 versus the siRNA-transfected control cultures



were slightly less vulnerable to BP-3 than the non-transfected cells. In comparison to the non-transfected cells, the effects of BP-3 were reduced by 20% with respect to caspase-3 levels and by 30% with respect to LDH release in the siRNA-transfected cells (Fig. 7a, b).

A 24-h exposure of RXR α siRNA-transfected cells to 25 μ M BP-3 reduced caspase-3 activity and LDH release to 55 and 50% of the control values, respectively (Fig. 7a, b). These cells were much less vulnerable to BP-3 than the non-siRNA-treated wild-type cells.

The effectiveness of mRNA silencing was verified by qPCR. In this study, siRNA treatment decreased the *Rxr α* mRNA level by 83% (equal to 0.17-fold), the *Rxr β* mRNA level by 64% (equal to 0.36-fold), and the *Rxr γ* mRNA level by 68% (equal to 0.32-fold) compared to the non-transfected wild-type cells.

Influence of BP-3 on Global DNA Methylation in Neocortical Cultures

A 24-h exposure of neocortical cells to BP-3 (25 μ M) caused changes in the level of global DNA methylation. The treatment with BP-3 reduced the methylation level by 55% of the control value (Fig. 8).

Effects of BP-3 on HDAC and HAT Activity in Neocortical Cultures

A 24-h exposure of neocortical cultures to BP-3 reduced the levels of HDAC and HAT activities. Treatment with BP-3 decreased the activities of HDAC and HAT by 32 and 17% of the control value, respectively (Fig. 9a, b).

Fig. 8 Influence of BP-3 on global DNA methylation in neocortical cultures at 7 DIV. Primary neocortical cultures were treated with BP-3 (25 μ M) for 24 h. Total DNA was extracted from cells followed by ELISA. Each bar represents the mean of three independent experiments \pm SEM. The number of replicates in each experiment ranged from 2 to 3. *** p < 0.001 versus control cultures

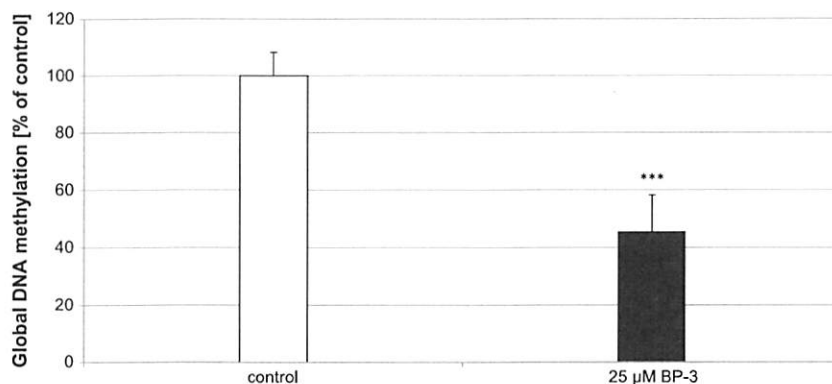
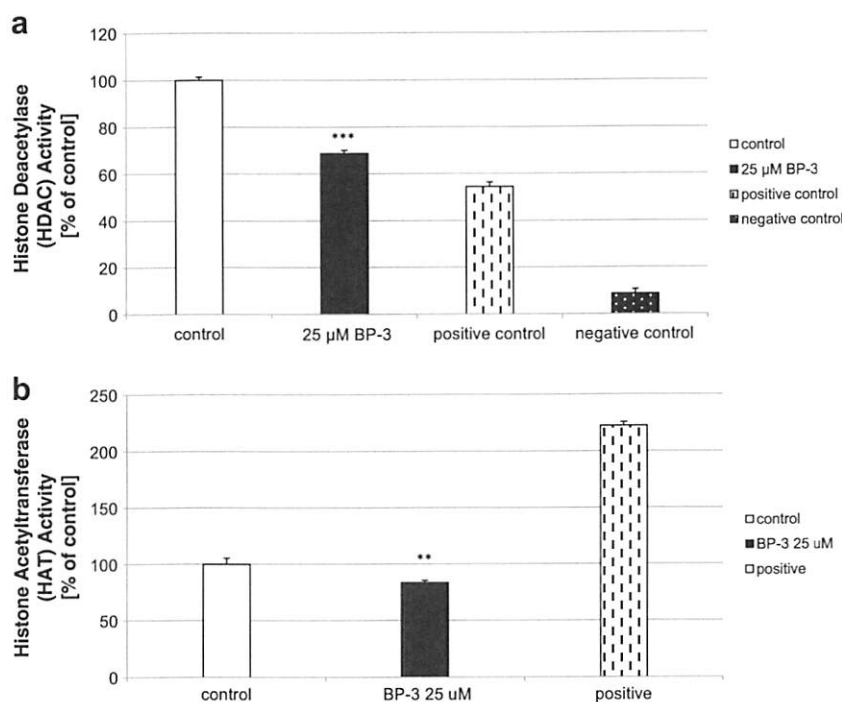


Fig. 9 Effects of BP-3 (25 μ M) on HDAC (a) and HAT (b) activity in the primary cultures of mouse neocortical cells at 7 DIV. The cells were treated with BP-3 for 24 h. The results are presented as a percentage of the control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. ** $p < 0.01$ and *** $p < 0.001$ versus control cultures



Effects of BP-3 on the Expression Profiles of Genes Involved in Autophagy Using a Mouse Autophagy RT² Profiler PCR Array

To validate that BP-3 impairs autophagy in neuronal cells, we analyzed a total number of 84 key genes that are involved in this process. Among them, 71 genes were differentially expressed in response to BP-3 treatment: 60 were downregulated (green color) and 11 were upregulated (red color) in the BP-3-treated samples. The downregulated genes were *Akt1*, *Ambr1*, *App*, *Atg10*, *Atg16l1*, *Atg16l2*, *Atg3*, *Atg4b*, *Atg4d*, *Atg7*, *Atg9a*, *Atg9b*, *Bcl2*, *Bid*, *Cdkn1b*, *Cdkn2a*, *Cln3*, *Ctsb*, *Ctsd*, *Ctss*, *Cxcr4*, *Dram1*, *Eif2ak3*, *Eif4g1*, *Esr1*, *Gaa*, *Gabarapl1*, *Gabarapl2*, *Hdac1*, *Hdac6*, *Hgs*, *Hsp90aa1*, *Hspa8*, *Htt*, *Igf1*, *Irgm1*, *Lamp1*, *Map11c3a*, *Map11c3b*, *Mapk14*, *Mapk8*, *Mtor*, *Npc1*, *Pik3c3*, *Pik3r4*, *Prkaal*, *Pten*, *Rab24*, *Rb1*, *Rgs19*, *Rps6kb1*, *Snca*, *Sqstm1*, *Tgfb1*, *Tgm2*, *Tmem74*, *Ulk1*, *Ulk2*, *Uvrag*, and *Wip1*. The upregulated genes were *Bad*, *Bak1*, *Bax*, *Bcl2l1*, *Bnip3*, *Casp3*, *Casp8*, *Dapkl*, *Fas*, *Nfkb1*, and *Trp53* (Fig. 10).

Effects of BP-3 on Autophagosome Detection

A 24-h exposure of neocortical cultures to BP-3 reduced the level of autophagosomes in mouse neuronal cell cultures. Treatment with BP-3 decreased autophagosome level by 29% compared to the control value (Fig. 11).

Effects of BP-3 on the Protein Expression Levels of LC3A and LC3B in Mouse Neocortical Cells

In cultures exposed to BP-3 (25 μ M) for 24 h, the concentration of LC3A was 1.23 pg/ μ g of total protein, which was 126% higher than that in control cultures. In the cultures exposed to BP-3, the level of LC3B reached 0.21 pg/ μ g, which was reduced by 75% compared to controls (Fig. 12a, b).

Discussion

The primary aim of the present study was to evaluate the neurotoxic effects of BP-3 with an emphasis on apoptosis, autophagy, the epigenetic status of neuronal cells, and the molecular mechanisms involving RXRs. The results of the study demonstrated that BP-3 caused neurotoxicity, as evidenced by the concentration-dependent activation of caspase-3 and LDH release in mouse neocortical cells. In the present study, neocortical cells responded to 25–100 μ M BP-3. This was in line with our previous study in which 25 μ M BP-3 was determined to be the lowest effective concentration at 24 h of exposure [13]. The used concentration is environmentally relevant since BP-3 has been found in human adipose tissue at concentrations up to 5 mg/kg (\sim 22 μ M) [37]. The ability of BP-3 to cross the blood-brain barrier has been shown; after it was applied by gavage, the *Er α* and *Er β* mRNA expression levels were changed in the rat pituitary gland [38]. Moreover, a BP-3 analogue (BP-4) changed the expression levels of many genes

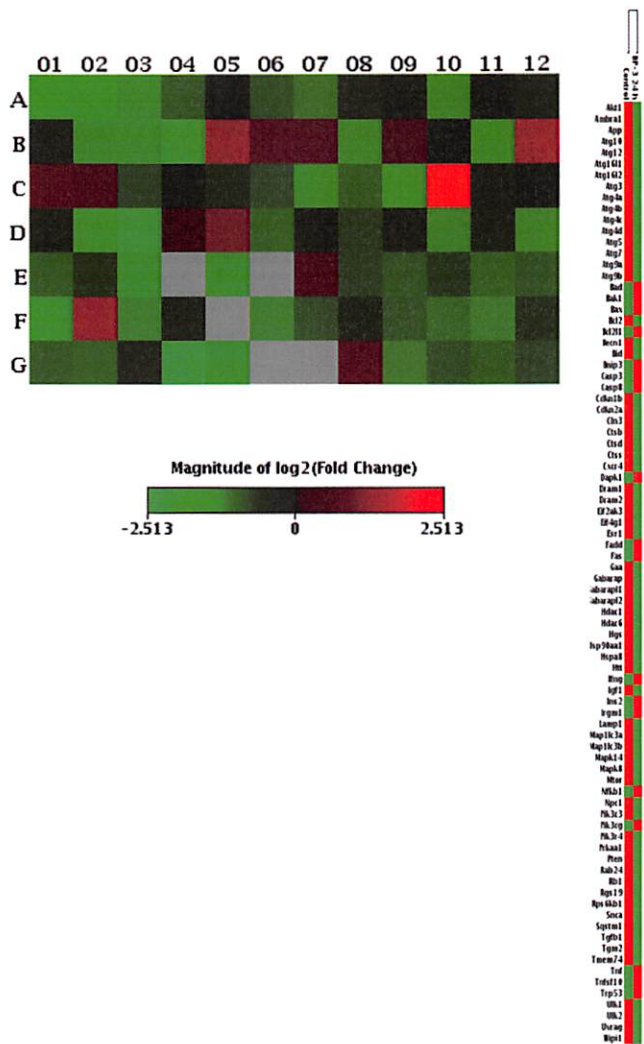


Fig. 10 Gene expression patterns of autophagy showing the 71 genes that were significantly differentially expressed between the control and BP-3-treated groups. Among these genes, 60 genes were downregulated (green color) and 11 genes were upregulated (red color) in the BP-3-treated samples compared to the control

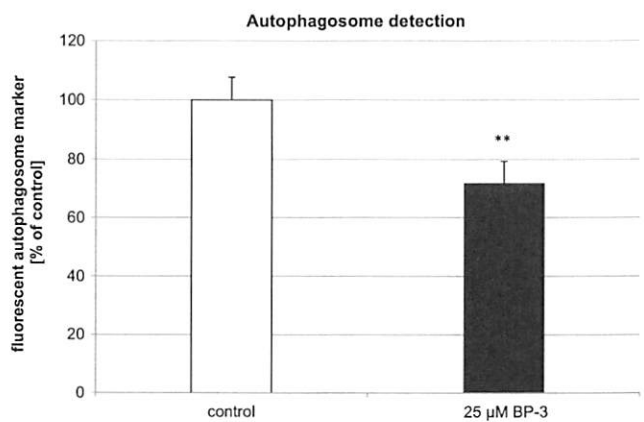


Fig. 11 The effect of 25 μM BP-3 on autophagosome levels at 24 h. The data are expressed as the mean ± SEM of four independent experiments, consisting of eight replicates per treatment group. ***p* < 0.001 versus the control

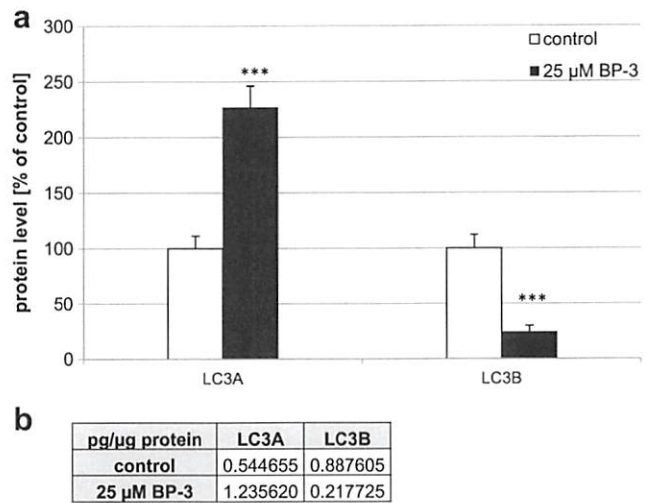


Fig. 12 Effects of BP-3 on the protein levels of LC3A and LC3B in mouse neocortical cultures at 7 DIV. The neocortical cells were cultured for 7 DIV and then treated for 24 h with BP-3 (25 μM). The concentrations of the receptors were measured using ELISA and are presented as a percentage of the control (a) and as picogram of the specific protein (LC3A or LC3B) per microgram of total protein (b). ****p* < 0.001 versus control cultures

in the *Danio rerio* brain when it was added to water [39]. In the present study, the neurotoxic effects of BP-3 involved enhanced LDH release and impaired cell survival (calcein AM staining), which were accompanied by the induction of apoptosis as estimated by caspase-3 activation and increased apoptotic body formation (Hoechst 33342 staining).

In this study, both the neurotoxic and apoptotic effects of BP-3 were inhibited by HX 531, which is a potent RXR antagonist. These data suggest that RXR receptors are involved in BP-3-induced effects in neuronal cells. Previously, we demonstrated an important role of RXRα- and RXRβ-intracellular signaling in the propagation of DDE- and nonylphenol-induced apoptosis during the early stages of neural development [22–24]. Recently, the involvement of RXRs in apoptotic signaling in retina pigment epithelial cells and gastrointestinal cancer cells has been shown [40, 41]. The role of RXRs in neuronal survival and neurotoxicity is complex and depends on RXR heterodimerization partners. Interestingly, A/B domain region of RXR receptors was found to cause growth inhibition or retinoid-induced apoptosis [42]. Elevated levels of RXRα gene and protein expression have been found in individuals suffering from dementia [43], and knockout of RXRγ impairs the working memory in mice [44]. In the present study, BP-3 altered the mRNA expression levels of *Rxrα*, *Rxrβ*, and *Rxrγ* in a time-dependent manner. Increased expression of *Rxrα* mRNA and reduced expression levels of *Rxrβ* and *Rxrγ* mRNA were observed at 3 h of exposure, which mirrored the alterations in the estimated protein levels of the receptors at 24 h of exposure. These profiles were also concurred with the immunofluorescent labeling of RXRα, RXRβ, and RXRγ following BP-3 treatment. Based on these data, we hypothesize that the BP-3-

induced apoptosis of neuronal cells is mediated by the attenuation of RXR β /RXR γ and the stimulation of RXR α signaling pathways. To test this hypothesis, we used gene-specific siRNAs. Compared to non-transfected wild-type cells, silencing of RXR α caused a substantial reduction of BP-3-induced caspase-3 activity and LDH release; however, silencing of RXR β and RXR γ did not affect the BP-3-induced effects. Therefore, we suggest that the BP-3-induced apoptosis of neuronal cells is mediated by the stimulation of RXR α signaling and the attenuation of RXR β /RXR γ signaling, which is in line with the BP-3-induced alterations in RXR expression levels.

Previously, we demonstrated that stimulation of ER β /GPR30 and impairment of ER α /PPAR γ signaling were involved in propagation of BP-3-induced apoptosis [13]. Taking into account our previous and present data, one may assume that in neuronal cells BP-3 stimulates RXR α /ER β /GPR30 and inhibits RXR β /RXR γ /ER α /PPAR γ intracellular pathways. It has been documented that ER β may downregulate ER α /PPAR γ and disrupt RXR α /PPAR γ signaling [45]. RXR α is an obligatory heterodimer partner of PPAR γ as well as RXR β /RXR γ [46]. We suggest that in our study, BP-3 by upregulation of ER β impaired RXR β /RXR γ /ER α /PPAR γ signaling. We also postulate that BP-3 was able to destroy RXR β and RXR γ heterodimers but not RXR α homodimers, possibly due to stronger covalent bonding in homodimers than in heterodimers.

In addition to the demonstration that the BP-3-induced apoptosis involves the activation of RXR α signaling and the impairment of RXR β /RXR γ signaling, we showed that BP-3 inhibited global DNA methylation as well as reduced HDAC and HAT activities in mouse embryonic neuronal cells. Aberrant DNA methylation and other epigenetic modifications have been found to be associated with development and normal cellular homeostasis as well as growing number of human diseases. Low doses of a pesticide DDT have been postulated to cause hypomethylation of specific gene regions in the young brain and impaired hippocampal neurogenesis [25]. Recently, we showed evidence of the involvement of global DNA hypomethylation in DDT-induced depressive-like effects and the DDE-induced apoptosis of primary neuronal cells [22, 47]. Exposures to xenobiotics such as tributyltin (TBT) and triphenyltin (TPT) have been shown to alter HDAC and HAT activity [48]. The global DNA hypomethylation as well as diminished HDAC and HAT activity suggest of chromosomal instability thus can cause inappropriate gene expression pattern. We postulate that global DNA hypomethylation and diminished HDAC activity are responsible for the BP-3-induced increase in the RXR α expression level, whereas diminished HAT activity corresponds to reduced expression of RXR β /RXR γ in response to BP-3 treatment in our study.

Based upon our data, we suggest that altered epigenetic status caused by BP-3 treatment may not only be involved in apoptosis and the disruption of RXR signaling but may also affect autophagy. Autophagy is neuroprotective and is responsible for degrading damaged organelles and misfolded

proteins. Dysregulation of autophagy has been linked to neural degenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and encephalopathy. In the present study, the attenuation of the autophagic process was confirmed by the downregulation of genes involved in autophagy as detected by the microarray analysis, decreased autophagosome formation, and the reduced ratio of LC3B to LC3A. We hypothesize that BP-3 induced the downregulation of genes related to autophagy through decreased HAT activity in mouse neurons.

Conclusions

In summary, we showed for the first time that the BP-3-induced apoptosis of neuronal cells is mediated via the stimulation of RXR α signaling and the attenuation of RXR β /RXR γ signaling, as demonstrated by the use of selective antagonist and specific siRNAs as well as by measuring the mRNA and protein expression levels (qPCR, ELISA, western blot, and immunofluorescent labeling) of the receptors. This study also demonstrated that the use of BP-3 at environmentally relevant concentrations was able to inhibit autophagy and disrupt the epigenetic status of neuronal cells, which may increase the risk of neurodevelopmental abnormalities and/or neural degeneration.

Abbreviations

AM	acetoxymethyl
ANOVA	analysis of variance
BP-3	benzophenone-3
BPA	bisphenol A
BPs	benzophenones
DDE	dichlorodiphenyldichloroethylene
DIV	days in vitro
DMSO	dimethyl sulfoxide
EDCs	endocrine disrupting chemicals
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HAT	histone acetyltransferase
HDAC	histone deacetylase
Hprt	hypoxanthine-guanine phosphoribosyltransferase
LDH	lactate dehydrogenase
PBS	phosphate-buffered saline
qPCR	quantitative polymerase chain reaction
RT	room temperature
RXR	retinoid X receptor
TBT	tributyltin
TPT	triphenyltin
UV	ultraviolet light

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants performed by any of the authors.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

All members of the research team received approval from the local ethical committee on animal testing. Animal care followed official governmental guidelines, and all efforts were made to minimize suffering and the number of animals used.

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Oxybenzone HEL Monograph - 7 of 7

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To: IEM Committee <IEM.Committee@mauicounty.us>; County Clerk <County.Clerk@mauicounty.us>;

Cc: cadowns@haereticus-lab.org <cadowns@haereticus-lab.org>;

📎 5 attachments (5 MB)

66 Wnuk Mol Neurobiol [2017] BP3 apoptosis in mouse neuronal cells.pdf; 67 Zhang Chlorination of oxybenzone- Kinetics, transformation, disinfection byproducts formation, and genotoxicity changes.docx; 68 Li Journal of Hazardous Materials [2016] BP3 transformation chlorine.pdf; 69 Sherwood Altered UV absorbance and cytotoxicity of chlorinated sunscreen agents.docx; 70 Duirk Reaction of benzophenone UV filters in the presence of aqueous chlorine- kinetics and chloroform formation.docx;

Aloha Chair and Maui County Council,

Mahalo Nui Loa for inviting us to provide testimony before your committee. It was an honor and privilege.

We are submitting the attached studies to you, as requested by the Infrastructure and Environmental Management committee after our presentations on Oxybenzone and Octinoxate's effects on marine life and human health. After reviewing the attached studies, we are confident that you will all feel comfortable with your vote to support the legislation to ban the sale of SPF Sunscreen products containing Oxybenzone and/or Octinoxate.

Mahalo,

Craig Downs – Executive Director – Haereticus Environmental Laboratory

Joe DiNardo – Retired Personal Care Industry Toxicologist & Formulator

Notes:

- Because of the size of the files there will be several Emails sent per topic; all will be numbered appropriately.
- The first Email on the topic will contain the main article (Dermatology Paper – Oxybenzone Review, Oxybenzone HEL Monograph or Octinoxate HEL Monograph) and the references used to support the main article will be included.
- Chemical names used in the attached research papers may vary – please feel free to ask us to clarify any concerns you may have associated with terminology:
 - 1) Oxybenzone = Benzophenone-3 (BP-3) and metabolites maybe noted as Benzophenone-1 and 4-Methylbenzophenone
 - 2) Octinoxate = Ethylhexyl Methoxycinnamate (EHMC) = Octyl Methoxycinnamate (OMC)

Apoptosis Induced by the UV Filter Benzophenone-3 in Mouse Neuronal Cells Is Mediated via Attenuation of $Er\alpha$ / $Ppar\gamma$ and Stimulation of $Er\beta$ / $Gpr30$ Signaling

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Abstract Although benzophenone-3 (BP-3) has frequently been reported to play a role in endocrine disruption, there is insufficient data regarding the impact of BP-3 on the nervous system, including its possible adverse effects on the developing brain. Our study demonstrated that BP-3 caused neurotoxicity and activated apoptosis via an intrinsic pathway involving the loss of mitochondrial membrane potential and the activation of caspases-9 and -3 and kinases p38/MAPK and Gsk3 β . These biochemical alterations were accompanied by ROS production, increased apoptotic body formation and impaired cell survival, and by an upregulation of the genes involved in apoptosis. The BP-3-induced effects were tissue-specific and age-dependent with the most pronounced effects observed in neocortical cells at 7 days in vitro. BP-3 changed the messenger RNA (mRNA) expression levels of *Er α* , *Er β* , *Gpr30*, and *Ppar γ* in a time-dependent manner. At 3 h of exposure, BP-3 downregulated estrogen receptor mRNAs but upregulated *Ppar γ* mRNA. After prolonged exposures, BP-3 downregulated the receptor mRNAs except for *Er β* mRNA that was upregulated. The BP-3-induced patterns of mRNA expression measured at 6 and 24 h of exposure reflected alterations in the protein levels of the receptors and paralleled their immunofluorescent labeling. *Er α* and *Ppar γ* agonists diminished, but *Er β* and *Gpr30* agonists stimulated the BP-3-induced apoptotic and neurotoxic effects. Receptor

antagonists caused the opposite effects, except for ICI 182,780. This is in line with a substantial reduction in the effects of BP-3 in cells with siRNA-silenced *Er β* /*Gpr30* and the maintenance of BP-3 effects in *Er α* - and *Ppar γ* siRNA-transfected cells. We showed for the first time that BP-3-affected mRNA and protein expression levels of *Er α* , *Er β* , *Gpr30*, and *Ppar γ* , paralleled BP-3-induced apoptosis and neurotoxicity. Therefore, we suggest that BP-3-evoked apoptosis of neuronal cells is mediated via attenuation of *Er α* /*Ppar γ* and stimulation of *Er β* /*Gpr30* signaling.

Keywords Benzophenone-3 · BP-3 · Estrogen receptors · Peroxisome proliferator-activated receptor gamma · Primary neuronal cell cultures

Introduction

The toxicity of ultraviolet light (UV) filters used in sunscreens and cosmetics to attenuate the negative effects of harmful UV radiation on skin and hair has become a concern. More than approximately 10,000 tons of UV filters are produced annually for the global market [1]. The variety of such compounds and the percentage of different filters added to industrial products are expanding with the increasing demand to prevent UV radiation-induced cell damage. Organic chemicals that absorb UVA (400–315 nm) or UVB (315–280 nm) radiation are added at concentrations of up to 10% to sunscreen products for skin protection. Due to public anxiety regarding skin damage caused by UV light, the use of UV screens is increasing, even though the benefit with respect to the prevention of melanoma remains controversial. In Europe, 28 chemical substances that function as UV filters are approved for use in industry, but the European Commission has reported many of them as hormonally active substances or endocrine-

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disrupting chemicals (EDCs). Chemical filters are almost always used in the mixtures but none of them are used individually in cosmetic in acceptable concentrations and does not provide adequate protection. These compounds are approved as harmless when used in small doses, but it should be noted that daily use involves at least several different cosmetics.

Benzophenone-3 (2-hydroxy-4-methoxybenzophenone, oxybenzone, 2OH-4 MeO-BP or BP-3) is a commonly used sunscreen agent, absorbing UVB and UVA radiation. The industrial use of BP-3 has increased over the past decade [2]. BP-3 is utilized as a flavor ingredient; a fragrance enhancer; a perfume fixative, an additive for plastics, coatings, and adhesive formulations; an ultraviolet curing agent in sunglasses; and as an agent to prevent UV light from damaging scents and colors in perfumes and soaps. It can be added to plastic packaging as a UV blocker, which allows manufacturers to package their products in clear glass or plastic rather than in dark packaging. Additionally, BP-3 has been approved by the US Food and Drug Administration as an indirect food additive [3]. Population studies have demonstrated that it penetrates through the skin and is excreted in the urine. BP-3 was found in >95% of urine samples collected from the US general population and in 100% of an investigated group of Danish children at concentrations ranging from 0.4 to 21,700 ng/ml [4–8]. In some cases, as much as 10% of the applied dermal dose was absorbed into systemic circulation [9]. BP-3 was found in 83% of blood samples from investigated adults [10].

The majority of reports have concentrated on the negative effects of BP-3 on the reproductive system. Epidemiological studies have provided evidence of a strong correlation between BP-3 exposure and increased ratios of diagnosed endometriosis [11]. However, the most disturbing finding is the fact that breastfed babies are exposed to BP-3, as this UV filter was detected in human milk samples [12]. In addition to mother milk, other ways of exposures to BP-3 are possible. BP-3 was found to cross blood-brain barrier after being applied in gavages [13]. It has been demonstrated that bisphenol A (BPA), which chemical structure is very similar to BP-3, easily crosses the blood–placental barrier [14, 15]. Therefore, fetal brain could be exposed to a much higher concentration of BP-3 than the concentration that was detected in human milk fat by Schlumpf et al. [12]. The high concentrations of BP-3 that have been detected in mother's urine may reduce birth weight in girls and increase birth weight and head circumference in boys [6, 16]. It has been postulated that the BP-3 concentration in urine may reach 16.7 µg/l and it may reflect less than 1% of the concentration of BP-3 to which healthy volunteers were exposed [17, 18]. Furthermore, a strong association between maternal exposure to BP-3 and the onset of Hirschsprung's disease in offspring has been shown [19]. In addition to the above-mentioned studies, there is insufficient data on the impact of BP-3 on the nervous system, including its possible adverse effects on the developing brain. The only

relevant report showed apoptotic and neurotoxic effects of BP-3 on SH-SY5Y neuroblastoma cells [20].

BP-3 has frequently been reported to play a role in endocrine disruption [21–24]. This disruption can occur by altering normal hormone levels, inhibiting or stimulating the production and metabolism of hormones, or changing the way hormones travel through the body, thus affecting the functions that these hormones control. EDCs have been postulated to exert their actions through nuclear hormone receptors, including estrogen receptors and peroxisome proliferator-activated receptor gamma (PPAR γ), either by activating or by suppressing them. Although BP-3 has been recognized as a ligand for nuclear estrogen receptors (ERs), there is no data regarding its interactions with newly identified membrane ER, G protein-coupled receptor 30 (GPR30), and the nuclear receptor PPAR γ , which is known to interact with ERs. Identifying chemicals that display hormonal activity is now a major research focus because they could disrupt brain development and cause abnormalities observed during ontogeny or at the onset of neurodegenerative diseases.

Primarily, ERs mediate the actions of endogenous estrogen hormones, which influence a wide variety of developmental and functional aspects in the mammalian central nervous system (CNS). This includes the survival of neurons, the growth and plasticity of neurites, their synaptic connections and transmission, and motor skills and higher cognitive functions [25]. Experimental and epidemiologic studies have provided information that estrogens protect against Alzheimer's disease (AD) in postmenopausal women and positively affect the symptoms of Parkinson's disease and tardive dyskinesia [26]. The idea of a neuroprotective function of estrogen is supported by observations that females are less vulnerable to acute insults associated with cerebral ischemia, neurotrauma, and hypoxia and that estradiol or estrogen-like compounds protect against stroke [27, 28]. Surprisingly, EDCs interference with nuclear receptors implicated in metabolism has remained scarcely studied. Peroxisome proliferator-activated receptors are ligand-activated transcription factors that regulate genes important in cell differentiation and various metabolic processes, especially lipid and glucose homeostasis [29]. These receptors are important for neuronal development and brain function. Agonists of PPAR γ have been used to reduce neurodegenerative changes in mouse models of neurodegenerative diseases [30] and have also shown benefits in experimental models of stroke and ischemia [31]. Concomitantly, studies of PPAR γ activation showed effects against oxidative stress, mitochondrial dysfunction, and apoptosis in several cell models that resemble AD, Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and spinal cord injuries (SCI) [32]. A signal cross-talk between nuclear ERs and the PPAR γ has also been shown [33]. However, there is no data on the interactions of BP-3 with these receptors in mammalian neurons.

Recently, we showed that the pesticide dichlorodiphenyltrichloroethane (DDT) impaired Gpr30-mediated intracellular signaling in mouse neurons [34]. We also demonstrated an involvement of the retinoid X receptor (Rxr), which is an obligatory heterodimer partner of Ppar γ , in the neurotoxic and apoptotic actions of nonylphenol and dichlorodiphenyldichloroethylene (DDE) [35, 36]. The present study aimed to investigate the neurotoxic and apoptotic effects of BP-3 and the impact of this chemical on the expression and functions of ERs, including classical Er α /Er β , the membrane Gpr30 and Ppar γ . Neurotoxicity was estimated by measuring lactate dehydrogenase (LDH) release, which was complemented by an assessment of caspase-3 and -8 activities and a loss of mitochondrial membrane potential and ROS production. These data were supported by Hoechst 33342/calcein acetoxyethyl (AM) staining, which allowed for apoptotic nuclei and cell survival to be visualized, respectively, and by measurements of the expression of genes involved in apoptosis, as detected by a microarray analysis. Specific inhibitors of caspase-8, caspase-9, glycogen synthase kinase-3 β (Gsk3 β), and p38/MAPK were employed to indicate which of these enzymes participated in BP-3-induced caspase-3-dependent apoptosis. The involvement of classical and membrane ERs and Ppar γ in BP-3 actions was verified using selective antagonists and agonists. The levels of specific messenger RNAs (mRNAs) and proteins were measured with quantitative polymerase chain reaction (qPCR), western blot, and ELISA, and the cellular distributions of receptors were demonstrated using a confocal microscope.

Materials and Methods

Materials

B27 and neurobasal media were obtained from Gibco (Grand Island, NY, USA). L-glutamine; fetal bovine serum (FBS); N-acetyl-Asp-Glu-Val-Asp *p*-nitro-anilide (Ac-DEVD-*p*NA); dimethyl sulfoxide (DMSO); HEPES; CHAPS; mouse monoclonal anti-MAP-2 antibody; ammonium persulfate; TEMED; TRIZMA base; Tween 20; DL-dithiothreitol; Nonidet NP-40; sodium deoxycholate; protease inhibitor (EDTA-free); bromophenol blue; 2',7'-dichlorofluorescein diacetate; Gsk3 β ; p38/MAPK; caspase-9 and caspase-8 inhibitors (SB 216763, SB 203580, Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone trifluoroacetate salt hydrate, Z-Leu-Glu(OMe)-Thr-Asp(OMe) fluoromethyl ketone); RIPA buffer; protease inhibitor cocktail for mammalian tissues; and polyornithine were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent, SDS, 30% acrylamide, 0.5 M Tris-HCl buffer, 1.5 M Tris-HCl gel buffer, and Laemmli sample buffer were from Bio-Rad Laboratories (Munich, Germany). 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 2,3-bis(4-Hydroxyphenyl)-propionitrile (DPN), G1, ICI

182780, GW1929, methylpiperidino-pyrazole (MPP), 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), G15, and GW9662 were from Tocris Bioscience (Minneapolis, MN, USA). 2-mercaptoethanol was from Carl Roth GmbH+ Co. KG, (Karlsruhe, Germany). Immobilon-P membranes were purchased from Millipore (Bedford, MA, USA). Alexa 488-conjugated anti-goat IgG, calcein AM, and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). Cy3-conjugated anti-rabbit IgG and Cy5-conjugated anti-mouse were obtained from Jackson ImmunoResearch, Inc. (West Grove, PA, USA). The cytotoxicity detection kit and BM Chemiluminescence Western Blotting Substrate (POD) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). ELISA assay kits for Er α , Er β , Gpr30 and Ppar γ were purchased from Shanghai Sunred Biological Technology Co. (Sunred, China). The culture dishes were obtained from TPP Techno Plastic Products AG (Trasadingen, Switzerland). Rabbit polyclonal anti-Er α antibody (sc-7207), goat polyclonal anti-Er β antibody (sc-6822), rabbit polyclonal anti-Er β antibody (sc-8974), rabbit polyclonal anti-Gpr30 antibody (sc-134576), mouse monoclonal anti-Ppar γ antibody (sc-7273), mouse monoclonal anti- β -Actin antibody (sc-47778), Er α siRNA (sc-29306), Er β siRNA (sc-35326), Gpr30 siRNA (sc-60744), and Ppar γ siRNA (sc-29456) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). AllStars Negative Control siRNA AF 488, RNeasy Mini Kit and RT² Profiler PCR Apoptosis Array were obtained from QIAGEN (Valencia, CA, USA). INTERFERin was obtained from PolyPlus Transfection (Illkirch, France), and the high-capacity complementary DNA (cDNA)-Reverse Transcription Kit, the TaqMan Gene Expression Master Mix and TaqMan probes corresponding to specific genes encoding *Hprt*, *Era*, *Er β* , *Gpr30*, and *Ppar γ* were obtained from Life Technologies Applied Biosystems (Foster City, CA, USA). JC-1 was obtained from Biotium, Inc. (Hayward, CA, USA).

Primary Neocortical and Hippocampal Neuronal Cell Cultures

Neocortical and hippocampal tissues for primary cultures were prepared from Swiss mouse embryos (Charles River, Germany) at 15–17 days of gestation and cultured as previously described [37]. All procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Bioethics Commission in compliance with Polish Law (21 August 1997). Animal care followed official governmental guidelines, and all efforts were made to minimize suffering and the number of animals used. The cells were suspended in estrogen-free neurobasal medium with a B27 supplement on poly-ornithine (0.01 mg/ml)-coated multi-well plates at a density of 2.0×10^5 cells/cm². The cultures were maintained at

37 °C in a humidified atmosphere containing 5% CO₂ for 7 days in vitro (DIV) prior to experimentation. The number of astrocytes, as determined by the content of intermediate filament glial fibrillary acidic protein (GFAP), did not exceed 10% for all cultures [38].

Treatment

Primary neuronal cell cultures were exposed to BP-3 (1–100 µM) for 6 or 24 h. To assess whether the effects of BP-3 were tissue-dependent, we examined these effects in neocortical and hippocampal cultures. The involvement of ER signaling in BP-3-induced effects was verified with the high-affinity estrogen receptor antagonist ICI 182,780 (1 µM), also known to act as a membrane estrogen receptor Gpr30 agonist [39], the Er α antagonist methyl-piperidino-pyrazole (MPP; 1 µM), the Er α agonist 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 1 µM), the Er β antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5,-a]pyrimidin-3-yl]phenol (PHTPP; 1 µM), the Er β agonist 2,3-bis(4-Hydroxyphenyl)propionitrile (DPN; 1 µM), the Gpr30 antagonist G-15 (10 µM), and the Gpr30 agonist G-1 (1 µM). BP-3-induced Ppar γ activation was examined using the receptor agonist GW1929 (1 µM) and antagonist GW9662 (1 µM). For apoptotic signaling, we used a cell permeable Gsk3 β inhibitor SB 216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione; 1 µM) and a p38/MAPK inhibitor SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; 1 µM) and caspase-8 and caspase-9 inhibitors: Z-Leu-Glu(O-Me)-Thr-Asp(O-Me)-fluoromethyl ketone (Z-LETD-FMK; 40 µM) and Z-Leu-Glu(O-Me)-His-Asp(O-Me)-fluoromethyl ketone trifluoroacetate salt hydrate (Z-LEHD-FMK; 40 µM), respectively. GW1929, GW9662, ICI 182780, MPP, PPT, DPN, and PHTPP were added to the culture media 45–60 min before BP-3 was added. The other agents were introduced simultaneously with BP-3. To avoid non-specific effects in our study, specific receptor ligands and SB 216763, SB 203580, and the caspase inhibitors were used at concentrations that did not affect the control levels of caspase-3 activity or LDH release. All the compounds were originally dissolved in DMSO and then further diluted in culture medium to maintain the DMSO concentration below 0.1%. The control cultures were treated with DMSO in concentrations equal to those used in the experimental groups.

Identification of Apoptotic Cells

Apoptotic cells were detected via Hoechst 33342 staining at 24 h after the initial treatment, as previously described [37]. Neocortical cells cultured on glass coverslips were washed with 10-mM phosphate-buffered saline (PBS) and exposed to Hoechst 33342 (0.6 mg/ml) staining at room temperature (RT) for 5 min. The cells containing bright blue fragmented

nuclei, indicating condensed chromatin, were identified as apoptotic cells. Qualitative analysis was performed using a fluorescence microscope (NIKON Eclipse 80i, NIKON Instruments Inc., Melville, NY, USA) equipped with a camera with BCAM Viewer© Basler AG software.

Staining with Calcein AM

The intracellular esterase activity in the neocortical cultures was measured based on calcein AM staining at 24 h after the initial treatment with BP-3 [37]. To avoid the esterase activity present in the growth media, the cells were washed with PBS and incubated in 2 µM calcein AM in PBS at RT for 10 min. The cells displaying bright green cytoplasm were identified as living cells. Fluorescence intensity was monitored at Ex/Em 494/520 nm using a fluorescence microscope (NIKON Eclipse 80i, NIKON Instruments Inc., Melville, New York, USA) equipped with a camera with BCAM Viewer© Basler AG software.

Assessment of Caspase-3 Activity

Caspase-3 activity was determined according to Nicholson, using samples treated for 6 or 24 h with BP-3 alone or in combination with the test compounds [40]. The assessment of caspase-3 activity was performed as previously described [38]. Cell lysates from neocortical and hippocampal cultures were incubated at 37 °C using a colorimetric substrate preferentially cleaved by caspase-3, called Ac-DEVD-*p*NA (N-acetyl-aspartyl-glu-val-aspartyl-*p*-nitro-anilide). The levels of *p*-nitroanilide were continuously monitored for 60 min using a Multimode Microplate Reader Infinite M200PRO (Tecan, Mannedorf, Switzerland). The data were analyzed using Magellan software, normalized to the absorbency of vehicle-treated cells and expressed as a percentage of control \pm SEM from three to four independent experiments. The absorbance of blanks, acting as no-enzyme controls, was subtracted from each value.

Measurement of Lactate Dehydrogenase Activity

To quantify cell death, lactate dehydrogenase (LDH) release from damaged cells into the cell culture media was measured 6 or 24 h after treatment with BP-3. LDH release was measured as previously described [41]. Cell-free supernatants from neocortical and hippocampal cultures were collected from each well and incubated at room temperature for 30 to 60 min with the appropriate reagent mixture according to the manufacturer's instructions (Cytotoxicity Detection Kit) depending on the reaction progress. The intensity of the red color that formed in the assay, measured at a wavelength of 490 nm (Infinite M200pro microplate reader, Tecan Mannedorf, Switzerland), was proportional to both LDH activity and the number of damaged cells. The data were analyzed using

Magellan software, normalized to the color intensity from vehicle-treated cells (100%) and expressed as a percentage of the control value from three to four independent experiments. The absorbance of blanks, acting as no-enzyme controls, was subtracted from each value.

Assessment of Loss of the Mitochondrial Membrane Potential

The loss of the mitochondrial membrane potential was measured using a JC-1 Assay Kit, which utilizes the cationic dye 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbo-cyanine iodide. In healthy cells, this dye aggregates and stains mitochondria bright red, whereas in apoptotic cells, the mitochondrial membrane potential collapses, and the dye remains in the cytoplasm in a green fluorescent monomeric form. The loss of mitochondrial membrane potential, a hallmark of apoptosis, was assessed in neocortical cultures 24 h after treatment with BP-3 (25 μ M). The cells were incubated in the JC-1 solution for 25 min, and the red (550/600 nm) and green (485/535 nm) fluorescence intensities were measured using an Infinite M1000 microplate reader (Tecan, Austria). The data were analyzed using Tecan i-control software, normalized to the fluorescence intensity of vehicle-treated cells, and expressed as the red to green fluorescence ratio \pm SEM of three to four independent experiments. The fluorescence intensity of blanks, acting as no-enzyme controls, was subtracted from each value.

ROS Formation

To determine the ability of BP-3 to induce ROS production in the neocortical neurons, 5 μ M H2DCFDA was applied as previously described [42, 43]. After diffusion into the cell, H2DCFDA is deacetylated by cellular esterases into a non-fluorescent compound that is subsequently oxidized by ROS into 2,7'-dichlorofluorescein (DCF). The cells were incubated in serum- and phenol red-free neurobasal medium containing H2DCFDA for 40 min before BP-3. After this time, the culture medium was replaced with fresh medium to remove extracellular residual DCF. The DCF fluorescence was measured after 3–24 h of BP-3 treatment and detected using an Infinite M1000 microplate reader (Tecan, Austria). The data were analyzed using Tecan i-control software and normalized to the fluorescence intensity in vehicle-treated cells (% of control). The means \pm SEM from eight separate samples were calculated from four independent experiments.

Silencing of *Er α* , *Er β* , *Gpr30*, and *Ppar γ*

Specific siRNAs were used to inhibit *Er α* , *Er β* , *Gpr30*, and *Ppar γ* expression in neocortical cells. Each siRNA was applied separately for 6 h at 50 nM in antibiotic-free medium

containing the siRNA transfection reagent INTERFERin™. After transfection, the culture media were changed, and the cells were incubated for 12 h before starting the experiment. Positive and negative siRNAs containing a scrambled sequence that did not lead to the specific degradation of any known cellular mRNA were used as controls. The effectiveness of mRNA silencing was verified through the measurement of specific mRNAs using qPCR.

qPCR Analysis of mRNAs Specific to Genes Encoding the Receptors *Er α* , *Er β* , *Gpr30*, and *Ppar γ*

Total RNA was extracted from neocortical cells cultured for 7 DIV (approx. 1.5×10^6 cells/sample) using an RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The quantity of RNA was spectrophotometrically determined at 260 nm and 260/280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). Two-step real-time PCR was performed. Both the reverse transcription reaction and quantitative polymerase chain reaction (qPCR) were run on a CFX96 Real-Time System (Bio-Rad, USA). The products of the reverse transcription reaction were amplified using TaqMan Gene Expression Master Mix containing TaqMan primer probes specific to the genes encoding *Hprt*, *Er α* , *Er β* , *Gpr30*, and *Ppar γ* . Amplification was performed in a total volume of 20 μ l of a mixture containing 10 μ l TaqMan Gene Expression Master Mix and 1.0 μ l reverse transcription product as the PCR template. A standard qPCR procedure was performed: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The threshold value (C_t) for each sample was set during the exponential phase, and the delta delta C_t method was used for data analysis. *Hprt* (hypoxanthine phosphoribosyltransferase coding gene) was used as a reference gene.

Mouse Apoptosis RT² Profiler PCR Array

Total RNA was extracted from neocortical cells cultured for 7 DIV (approx. 1.5×10^6 cells/sample) using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The quantity of RNA was spectrophotometrically determined at 260 and 260/280 nm (ND/1000 UV/Vis; Thermo Fisher NanoDrop, USA). A total of 1 μ g mRNA was reverse transcribed to cDNA using an RT² First Strand Kit (QIAGEN, Valencia, CA) and suspended in a final solution of 20 μ l. Each cDNA was prepared for further use in qPCR. To analyze the signaling pathway, the RT² Profiler™ PCR Array System (QIAGEN, Valencia, CA) was used according to the manufacturer's protocol. The C_t values for all wells were exported to a blank Excel spreadsheet and were used with the Web-based software (www.SABiosciences.com/pcrarraydataanalysis.php).

Western Blot Analysis

The cells exposed for 24 h to BP-3 were lysed in ice-cold RIPA lysis buffer containing a protease inhibitor cocktail. The lysates were sonicated and centrifuged at 15,000×g for 20 min at 4 °C. The protein concentrations in the supernatants were determined using Bradford reagent (Bio-Rad Protein Assay) with bovine serum albumin (BSA) as the standard. Samples containing 40 µg of total protein were reconstituted in the appropriate amount of sample buffer comprising 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM DTT, and 0.01% bromophenol blue, denatured and separated on 7.5% SDS-polyacrylamide gel using a Bio-Rad Mini-Protean II Electrophoresis Cell, as previously described [44]. After electrophoretic separation, the proteins were electrotransferred to PVDF membranes (Millipore, Bedford, MA, USA) using the Bio-Rad Mini Trans-Blot apparatus. Following the transfer, the membranes were washed, and non-specific binding sites were blocked with 5% dried milk and 0.2% Tween-20 in 0.02 M Tris-buffered saline (TBS) for 2 h while shaking. The membranes were incubated overnight (at 4 °C) with one of the following primary antibodies (Santa Cruz Biotechnology): anti-Er α rabbit polyclonal antibody (diluted 1:200), anti-Er β rabbit polyclonal antibody (diluted 1:200), anti-Gpr30 rabbit polyclonal antibody (diluted 1:150), anti-Ppary mouse polyclonal antibody (diluted 1:150) or anti- β -actin mouse monoclonal antibody (diluted 1:3000) diluted in TBS/Tween. The signals were developed by chemiluminescence (ECL) using BM Chemiluminescence Blotting Substrate (Roche Diagnostics GmbH) and visualized using a Luminescent Image Analyzer Fuji-Las 4000 (Fuji, Japan). Immunoreactive bands were quantified using a MultiGauge V3.0 image analyzer.

Enzyme-Linked Immunosorbent Assays for Er α , Er β , Gpr30, and Ppary

The levels of Er α , Er β , Gpr30, and Ppary were determined in neocortical cells 24 h after treatment with BP-3. Specific detection of these proteins was obtained using enzyme-linked immunosorbent assays (ELISAs) and the quantitative sandwich enzyme immunoassay technique. A 96-well plate was pre-coated with monoclonal antibodies specific to Er α , Er β , Gpr30, or Ppary. The standards and non-denatured cell extracts were added to the wells with biotin-conjugated polyclonal antibodies specific for Er α , Er β , Gpr30, or Ppary. Therefore, all native Er α , Er β , Gpr30, or Ppary proteins were captured using the immobilized antibodies. The plates were washed to remove any unbound substances, and horseradish peroxidase-conjugated avidin was added to interact with the biotin bound to Er α , Er β , Gpr30, or Ppary. After washing, the substrate solution was added to the wells. The enzyme reaction yielded a blue product. The absorbance was measured at 450 nm and was

proportional to the amount of Er α , Er β , Gpr30, or Ppary. The protein concentration was determined in each sample using Bradford reagent (Bio-Rad Protein Assay).

Immunofluorescent Labeling of Er α , Er β , Gpr30, and Ppary and Confocal Microscopy

For immunofluorescence detection of Er α , Er β , Gpr30, and Ppary, neocortical cells were grown on glass coverslips and subjected to immunofluorescence double-labeling, as previously described [45]. After 1 h of incubation in a blocking buffer (5% normal donkey serum and 0.3% Triton X-100 in 0.01 M PBS), the cells were treated for 24 h (at 4 °C) using five primary antibodies: rabbit polyclonal anti-Er α antibody (1:50), goat polyclonal anti-Er β antibody (1:50), rabbit polyclonal anti-Gpr30 antibody (1:50), mouse monoclonal anti-Ppary antibody (1:50), and anti-MAP2 mouse monoclonal (1:100), followed by a 24-h incubation in a mixture of secondary antibodies, including Alexa Fluor 488-conjugated anti-rabbit IgG (1:300), Cy3-conjugated anti-rabbit IgG (1:300), and Cy5-conjugated anti-mouse IgG (1:300). The samples were subsequently washed, mounted, coverslipped and analyzed using an LSM510 META, Axiovert 200 M confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) under a Plan-Neofluar 40×/1.3 Oil DIC objective. An He/Ne laser and an argon laser, with two laser lines emitting at 488, 514, and 633 nm, were used to excite the Alexa Fluor 488-, Cy3- and Cy5-conjugated antibodies, respectively. The fluorescence signal was enhanced after summing four scans per line. A pinhole value of 1 airy unit was used to obtain flat images.

Data Analysis

Statistical tests were performed on raw data expressed as the mean arbitrary absorbance or fluorescence units per well containing 50,000 cells (measurements of caspase-3, LDH), the fluorescence units per 1.5 million cells (qPCR), the mean optical density per 40 µg of protein (Western blotting) or picogram of Er α , Er β , Gpr30, and Ppary per microgram of total protein (ELISAs). One-way analysis of variance (ANOVA) was preceded by the Levene test of homogeneity of variances and used to determine overall significance. Differences between control and experimental groups were assessed using a post hoc Newman-Keuls test, and significant differences were designated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (versus control cultures), # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ (versus the cultures exposed to BP-3) and \$ $p < 0.05$ and \$\$\$ $p < 0.001$ (versus the siRNA-transfected control cultures). The results were expressed as the mean \pm SEM of three to four independent experiments. The number of replicates in each experiment ranged from 2 to 3, except for measurements of caspase-3 activity and LDH release, with replicates ranging

from 5 to 8. To compare the effects of BP-3 in various brain tissues and different treatment paradigms, the results corresponding to caspase-3, LDH, and western blot analysis were presented as a percentage of the control.

Results

Effects of BP-3 on Caspase-3 and Caspase-8 Activities and LDH Release and Mitochondrial Membrane Potential in 7 DIV Neocortical Cultures

In 7 DIV neocortical cultures, BP-3 (25–100 μ M)-induced caspase-3 increased to 160% of the control level at 6 h and was further enhanced to 190% at 24 h post-treatment (Fig. 1a). In these cells, LDH release values increased in neocortical cells in a time-dependent manner to 150–180% of the control value at 6 h and to 210–280% at 24 h (Fig. 1b). BP-3 at concentrations of 1–50 μ M was not effective at activating caspase-8, but BP-3 at concentrations of 75–100 μ M increased enzyme activity to 125–150% of the control value. (Fig. 1c). In 7 DIV cultures, treatment with 25 μ M BP-3 decreased the mitochondrial membrane potential by 31% (Fig. 1d).

Effects of BP-3 on Caspase-3 Activity and LDH Release in 7 DIV Hippocampal Cultures

In hippocampal cultures exposed to 1–100 μ M BP-3, the activity of caspase-3 increased to 150% at 6 h and to 160% at 24 h (Fig. 2a). LDH release increased with the duration of BP-3 treatment and was elevated to 150 and 208% of the vehicle control at 6 and 24 h, respectively (Fig. 2b). BP-3-induced caspase-3 activity and LDH release in hippocampal cells were lower than those in neocortical cells.

Effects of BP-3 on Caspase-3 Activity and LDH Release in 2 and 12 DIV Primary Cultures of Mouse Neocortical Cells

To assess whether the effects of BP-3 depended on the age of the neuronal tissue, we studied them in 2 and 12 DIV neocortical cultures. In 2 DIV cultures, 25 μ M BP-3 activated caspase-3 to 120% of the control value (Fig. 3a). BP-3-induced LDH release was enhanced to 131–138% of the control level at 6 h, after which it decreased to 119–126% at 24 h (Fig. 3b).

In 12 DIV cultures treated with 25–50 μ M BP-3, the activity of caspase-3 rose to 131 and 148% of the control level at 6 and 24 h, respectively (Fig. 3c). In these cells, the LDH release was elevated to 121–145% of the control value (Fig. 3d). The effects of BP-3 in 2 and 12 DIV neocortical cultures were lower compared to the effects in 7 DIV neocortical cultures.

Effects of BP-3 on ROS Production in 7 DIV Primary Cultures of Mouse Neocortical Cells

Following exposure to 25 μ M BP-3, ROS production increased in the neurons in a time-dependent manner. Compared with the controls, ROS production reached values of 153% at 3 h, 196% at 6 h, and 214% at 24 h (Fig. 4).

Impact of the Caspase-9, Caspase-8, Gsk3 β , and p38/MAPK Inhibitors on BP-3-Induced Caspase-3 Activity and LDH Release

In the presence of the caspase-9 inhibitor Z-LEHD-FMK (40 μ M), the activity of caspase-3 was diminished by 78% at 24 h of exposure (Fig. 5a). A 24-h treatment with Gsk3 β inhibitor SB 216763 (1 μ M) and p38/MAPK inhibitor SB 203580 (1 μ M) decreased BP-3-stimulated caspase-3 activity by 55 and 31%, respectively (Fig. 5a). However, exposure to the caspase-8 inhibitor Z-LETD-FMK (40 μ M) did not significantly affect the BP-3-activated caspase-3 in mouse neocortical cells at 7 DIV.

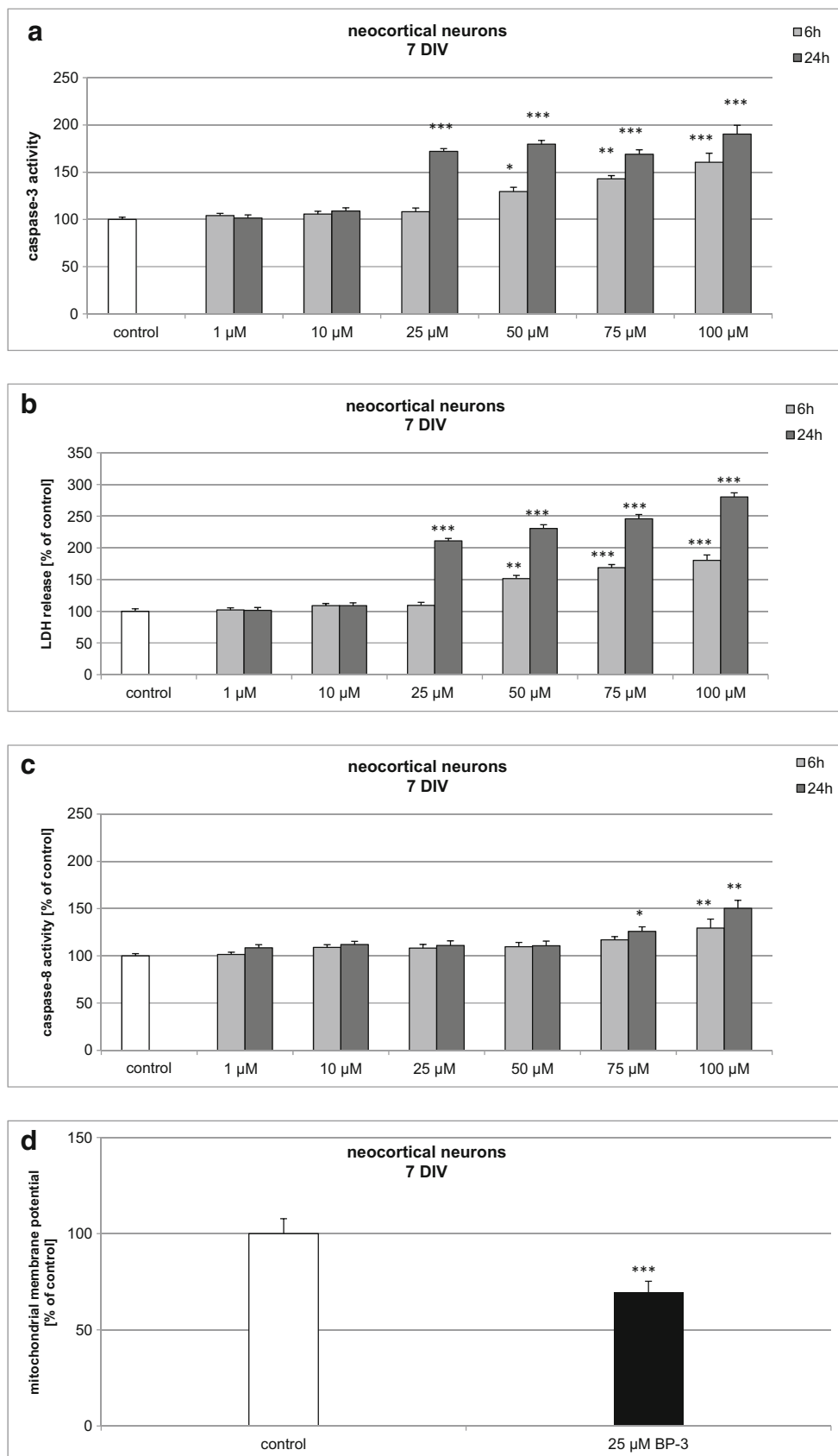
In neocortical cultures, caspase-9, Gsk3 β , and p38/MAPK inhibitors decreased BP-3-stimulated LDH release by 67, 57, and 46%, respectively (Fig. 5b). Co-treatment with caspase-8 inhibitor Z-LETD-FMK (40 μ M) did not significantly influence the BP-3-induced effect in 7 DIV neocortical cultures.

Effects of BP-3 on the Expression Profiles of Genes Involved in Apoptosis Using a Mouse Apoptosis RT² Profiler PCR Array.

To validate that BP-3 induced apoptosis in neuronal cells, we analyzed a total number of 84 key genes involved in programmed cell death. Among them, 46 genes were differentially expressed in response to BP-3: 45 were upregulated (red color) and 1 was downregulated (green color) in the BP-3-treated samples. The upregulated genes were *Anxa5*, *Apa1*, *Api5*, *Atf5*, *Bad*, *Bag1*, *Bag3*, *Bcl10*, *Bcl2l11*, *Birc2*, *Birc3*, *Birc5*, *Bnip2*, *Bnip3*, *Bnip3l*, *Card10*, *Casp1*, *Casp2*, *Casp3*, *Casp4*, *Casp9*, *Cflar*, *Dad1*, *Dffa*, *Dffb*, *Diablo*, *Fadd*, *Fas*, *Fasl*, *Gadd45a*, *Igfl1r*, *Ltr*, *Mapk1*, *Mcl1*, *Naip2*, *Nfkb1*, *Polb*, *Prdx2*, *Ripk1*, *Tnfrsf10b*, *Tnfsf12*, *Traf2*, *Traf3*, *Trp53bp2*, *Trp73*, and *Xiap*. The only downregulated gene was *Card10* (Fig. 6).

Effect of BP-3 on mRNA Levels of *Erc α* , *Er β* , *Gpr30*, and *Ppar γ*

Treatment with BP-3 (25 μ M) evoked changes in the mRNA levels of *Erc α* , *Er β* , *Gpr30*, and *Ppar γ* . A 3-h exposure of the neocortical cultures to BP-3 caused a 45% decrease in *Erc α* , a 23% decrease in *Er β* , and a 60% decrease in *Gpr30* but caused a 100% increase in *Ppar γ* mRNA compared with the control



◀ **Fig. 1** Time-course effects of BP-3 (1, 10, 25, 50, 75, and 100 μM) on caspase-3 activity (a), LDH release (b), caspase-8 activity (c), and mitochondrial membrane potential (d) in primary cultures of mouse neocortical cells at 7 DIV. The cells were treated with BP-3 for 6 and 24 h. The results are presented as a percentage of the control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control cultures

(Fig. 7a). The pattern of mRNA expression was changed after prolonged exposure to BP-3, especially with respect to *Er β* and *Ppar γ* . At 6 and 24 h of the experiment, BP-3 stimulated the mRNA expression of *Er β* (39–58%) but inhibited the expression of *Ppar γ* (32–39%) in neocortical cells (Fig. 7b, c). These data were normalized to *Hprt* as a control.

Effects of BP-3 on the Protein Expression Levels of *Er α* , *Er β* , *Gpr30*, and *Ppar γ* in Mouse Neocortical Cells

A 24-h exposure to BP-3 was necessary to detect changes in the protein levels of the receptors. In the control cultures, *Er α* and *Ppar γ* reached 2.90 and 9.51 pg per microgram of total protein, respectively. A 24-h exposure to BP-3 (25 μM) decreased *Er α* and *Ppar γ* levels by 21–27% of the control values (Fig. 8a, b).

In cultures exposed to BP-3 (25 μM) for 24 h, the concentration of *Er β* was 0.83 pg per micrograms of total protein,

and it was 45% larger than in control cultures (Fig. 8a, b). In the cultures exposed to BP-3, the level of *Gpr30* reached 0.05 pg and it was reduced by 32% compared to the control.

The western blot analysis demonstrated the constitutive protein expression of *Er α* , *Er β* , *Gpr30*, and *Ppar γ* in mouse neocortical cells at 7 DIV (Fig. 8c). Exposure to BP-3 (25 μM) decreased the relative *Er α* , *Gpr30*, and *Ppar γ* protein levels by 28, 19, and 64%, respectively, at 24 h post-treatment. Treatment with BP-3 (25 μM) increased the relative *Er β* protein level by 66% (Fig. 8c, d).

Effects of BP-3 Alone or in Combination with Receptor Agonists/Antagonists on BP-3-Induced Caspase-3 Activity and LDH Release in Neocortical Cultures

A 24-h exposure of neocortical cultures to BP-3 (25 μM) caused almost a 50% increase in caspase-3 activity in the neuronal cells. Co-treatment with the selective *Er β* and *Gpr30* agonists, DPN (1 μM) and G1 (10 μM), enhanced the action of BP-3 on caspase-3 activity by 21 and 56%, respectively. The selective *Er α* agonist PPT and *Ppar γ* agonist GW 1929 (both 1 μM) significantly diminished the effect of 25 μM BP-3 in the mouse neuronal cell cultures (Fig. 9a).

The selective *Er α* agonist PPT (1 μM) and *Ppar γ* agonist GW 1929 (1 μM) effectively inhibited BP-3-induced LDH

Fig. 2 Time-course effects of BP-3 (1, 10, 25, 50, 75, and 100 μM) on caspase-3 activity (a) and LDH release (b) in primary cultures of mouse hippocampal cells at 7 DIV. The cells were treated with BP-3 for 6 and 24 h. The results are presented as a percentage of the control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control cultures

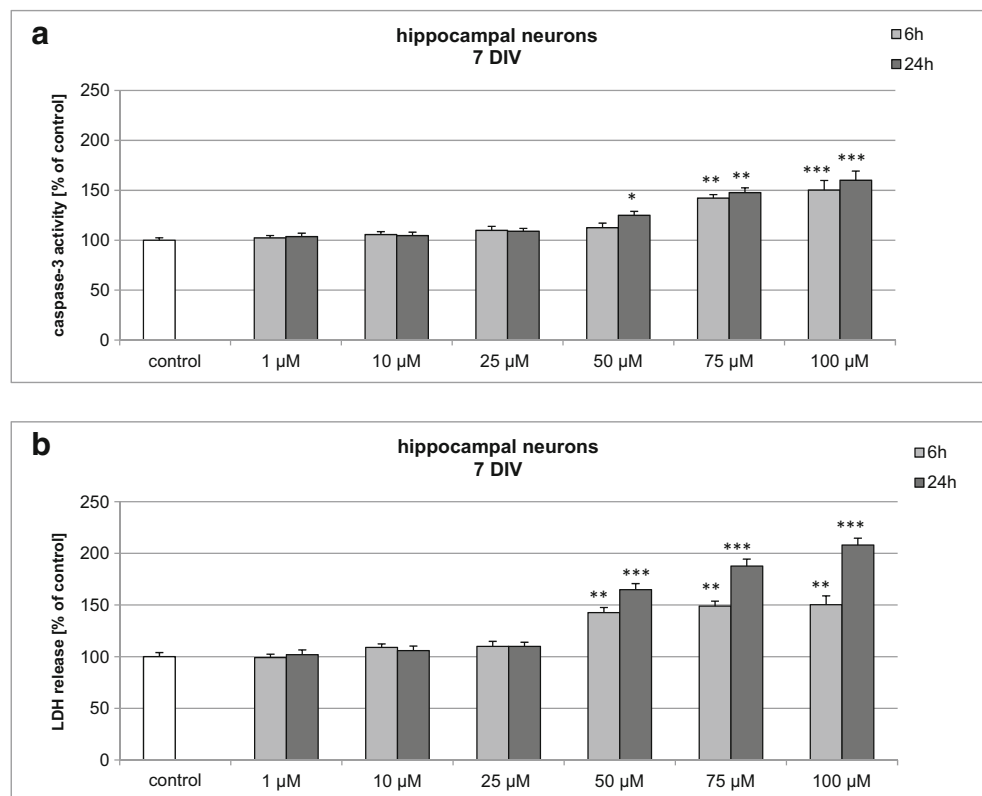
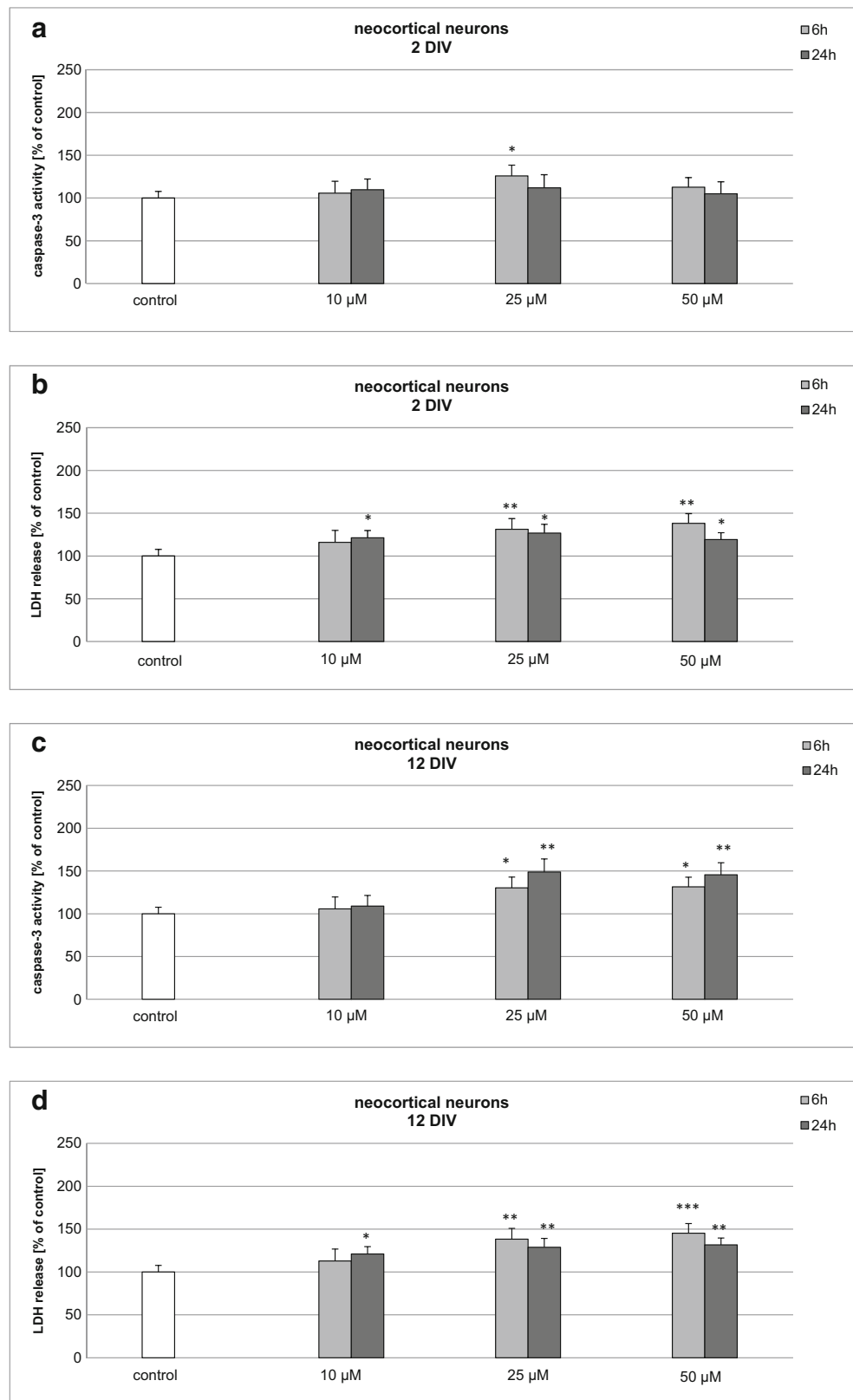


Fig. 3 Time-course effects of BP-3 (10, 25, 50 μM) on caspase-3 activity (**a, c**) and LDH release (**b, d**) in primary cultures of mouse neocortical cells at 2 and 12 DIV. The cells were treated with BP-3 for 6 and 24 h. The results are presented as a percentage of the control. Each *bar* represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control cultures



release. In addition, high-affinity estrogen receptor antagonist ICI 182780 (1 μM) and selective Er β or Gpr30 agonists intensified BP-3-induced LDH release (Fig. 9b).

The Er β antagonist PHTPP (1 μM) and the Gpr30 antagonist G15 (10 μM) inhibited the BP-3 (25 μM)-induced caspase-3 activity by 52 and 46%, respectively, whereas the

Fig. 4 The effect of 25 μM BP-3 on ROS production after 3, 6, and 24 h. The data are expressed as the means \pm SEM of four independent experiments, each of which consisted of eight replicates per treatment group. *** $p < 0.001$ versus the control

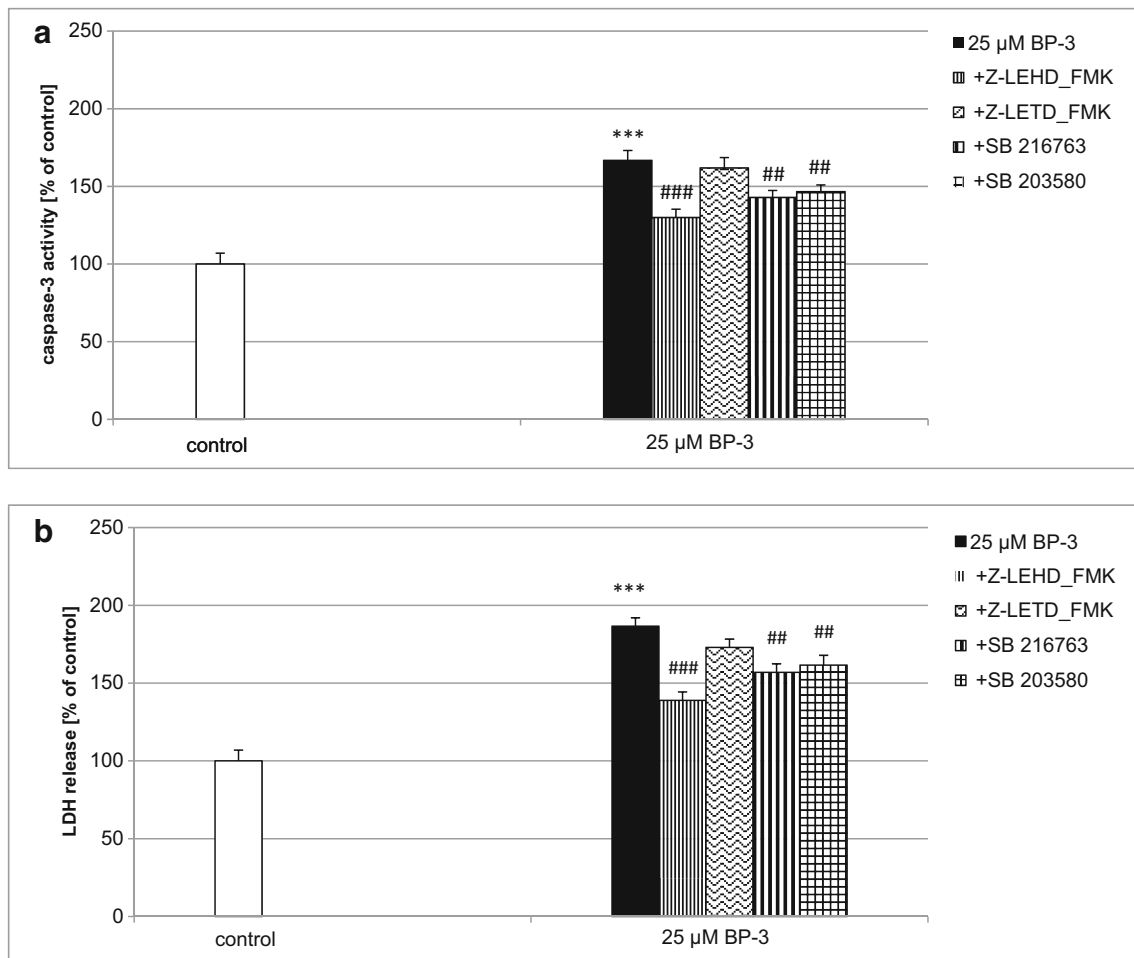
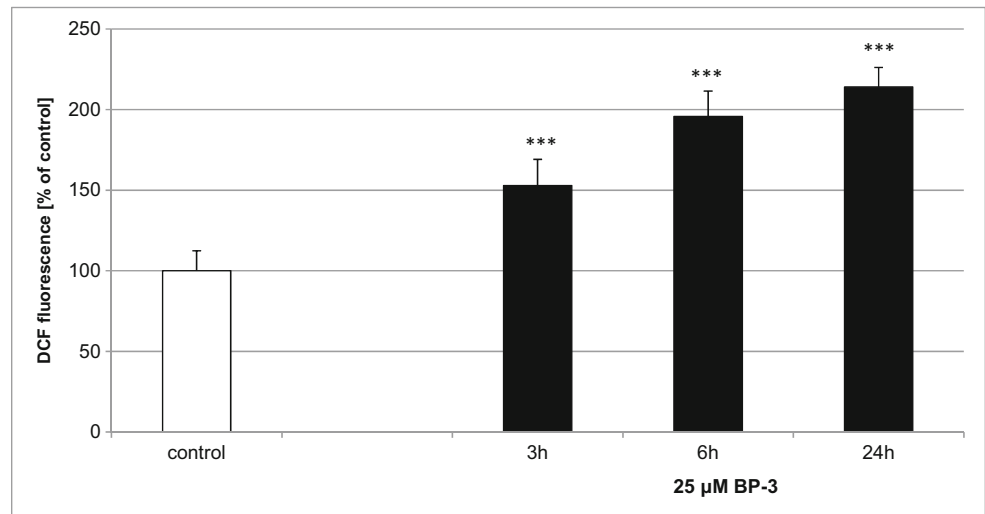
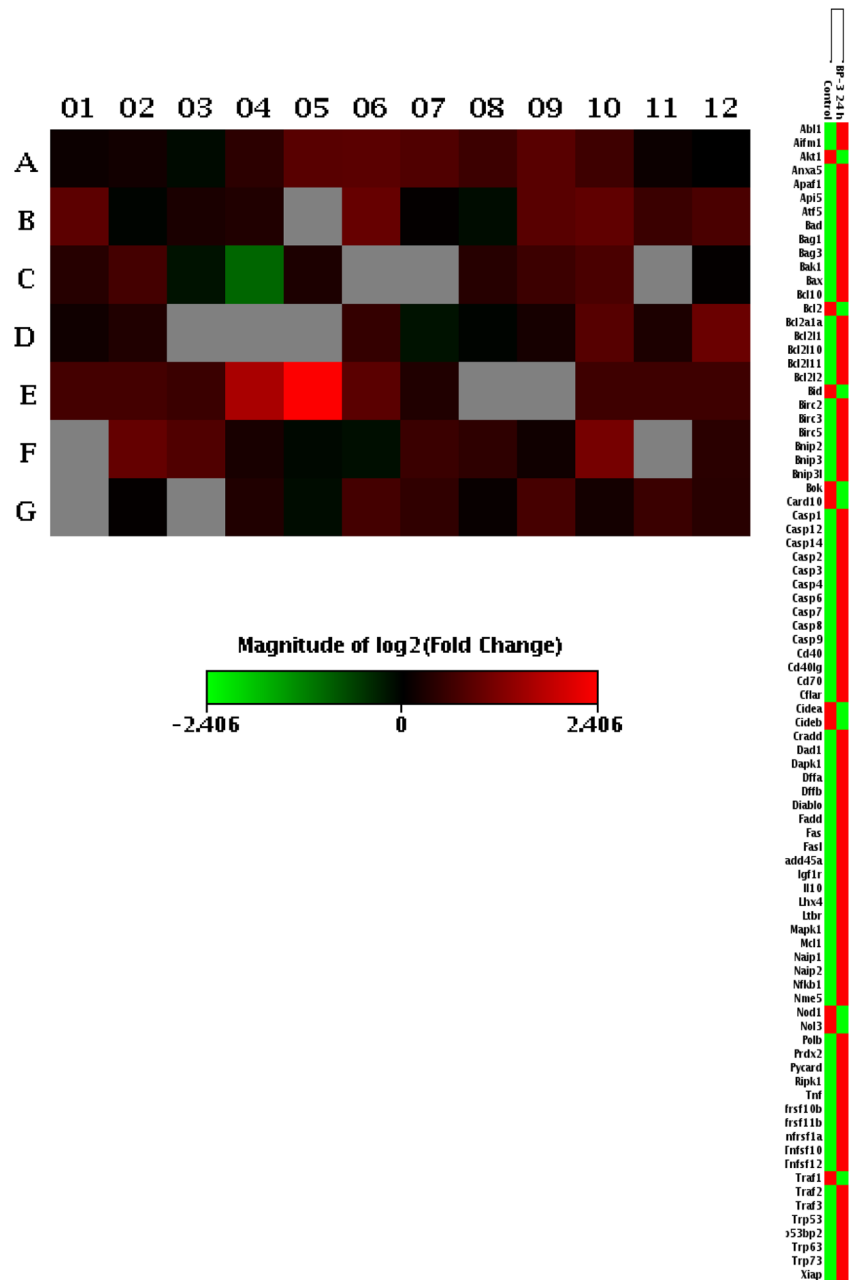


Fig. 5 Impact of the caspase-9, caspase-8, Gsk3 β , and p38/MAPK inhibitors on BP-3-induced caspase-3 activity and LDH release in 7 DIV neocortical cultures. Primary neocortical cultures were treated with BP-3 (25 μM) for 24 h. The caspase-9 inhibitor Z-LEHD-FMK (40 μM), caspase-8 inhibitor Z-LETD-FMK (40 μM), Gsk3 β inhibitor SB 216763 (1 μM) and p38/MAPK inhibitor SB 203580 (1 μM) were

added into the culture media simultaneously with BP-3. The results were normalized to the absorbency in vehicle-treated cells and expressed as a percentage of the control. Each *bar* represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. *** $p < 0.001$ versus control cultures; ## $p < 0.01$, and ### $p < 0.001$ versus the cultures exposed to BP-3

Fig. 6 Gene expression patterns of apoptosis in the control and BP-3 groups showing the 46 genes that were differentially expressed with statistical significance between control and BP-3 group. Among these genes, 45 genes were upregulated (*red color*), and 1 gene was downregulated (*green panel*) in the BP-3-treated samples



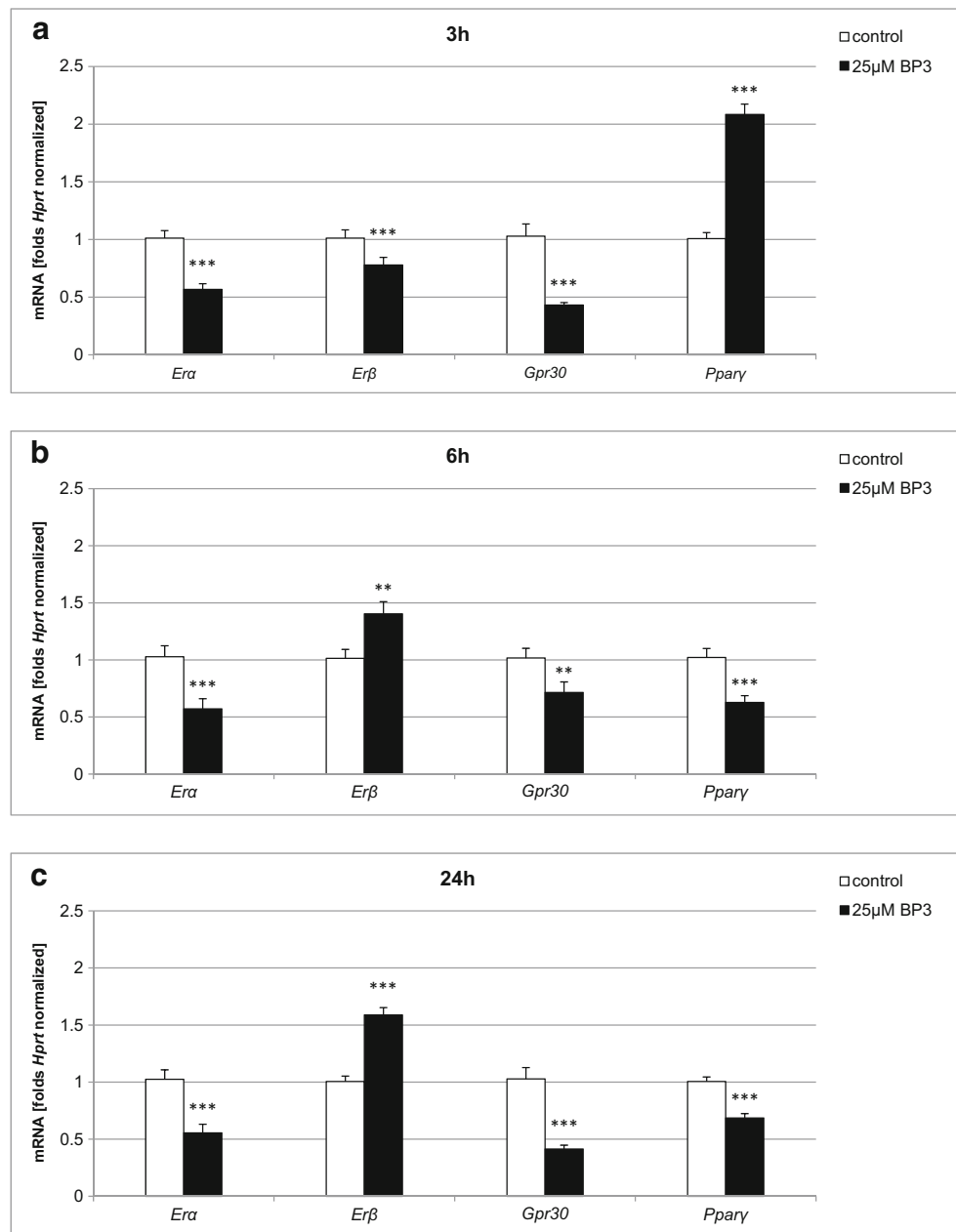
selective $\text{Er}\alpha$ antagonist MPP (1 μM) and the $\text{Ppar}\gamma$ antagonist GW 9662 did not change this effect. Treatment of the neocortical cultures with the high-affinity estrogen receptor antagonist ICI 182780 (1 μM), which was recently found to possess properties of a $\text{Gpr}30$ agonist, potentiated the BP-3-stimulated caspase-3 activity by 40% (Fig. 9c).

As demonstrated in Fig. 9d, 1 μM PHTPP or G15 reduced LDH release by 48 and 53% in the cultures subjected to BP-3 (25 μM) for 24 h. In this study, exposure to MPP or GW 9662 did not affect BP-3-induced LDH release in the mouse neocortical cultures. In addition, the high-affinity estrogen receptor antagonist ICI 182780 (1 μM) intensified BP-3-induced LDH release (Fig. 9d).

Effects of BP-3 Alone or in Combination with MPP, PHTPP, G15, and GW 9662 on Hoechst 33342 and Calcein AM Staining in Neocortical Cultures

In the present study, a 24-h exposure to BP-3 was necessary to develop an apoptotic morphology of cell nuclei. A continuous 24-h exposure of neocortical cultures to BP-3 (25 μM) induced apoptosis in mouse neuronal cells, as evidenced by Hoechst 33342 staining. The formation of bright blue fragmented nuclei containing condensed chromatin was labeled with Hoechst 33342 (Fig. 10). Treatment with BP-3 reduced the density of calcein AM-stained living cells at 7 DIV, as indicated by the decreased number of cells

Fig. 7 Effect of BP-3 (25 μ M) on the mRNA expression levels of *Erx*, *Er β* , *Gpr30*, and *Ppar γ* in neocortical cultures at 7 DIV. The extraction of total RNA at 3, 6, and 24 h post-treatment from the neocortical cells was followed by reverse transcription and quantitative polymerase chain reaction (qPCR). The products of the reverse transcription reaction were amplified using TaqMan probes and primers corresponding to the specific genes. *Hprt* was used as a reference gene. Each bar represents the mean \pm SEM of three independent experiments. The number of replicates for each experiment ranged from 2 to 3, $**p < 0.01$, and $***p < 0.001$ versus control cultures



exhibiting light-colored cytoplasm. Co-treatment with MPP (1 μ M) and GW 9662 (1 μ M) did not significantly affect Hoechst 33342 and calcein AM staining. However, the addition of PHTPP (1 μ M) and G15 (10 μ M) inhibited the BP-3-induced effects.

Influence of BP-3 on Caspase-3 Activity and LDH Release in Neocortical Cells Transfected with *Erx*, *Er β* , *Gpr30*, and *Ppar γ* siRNAs

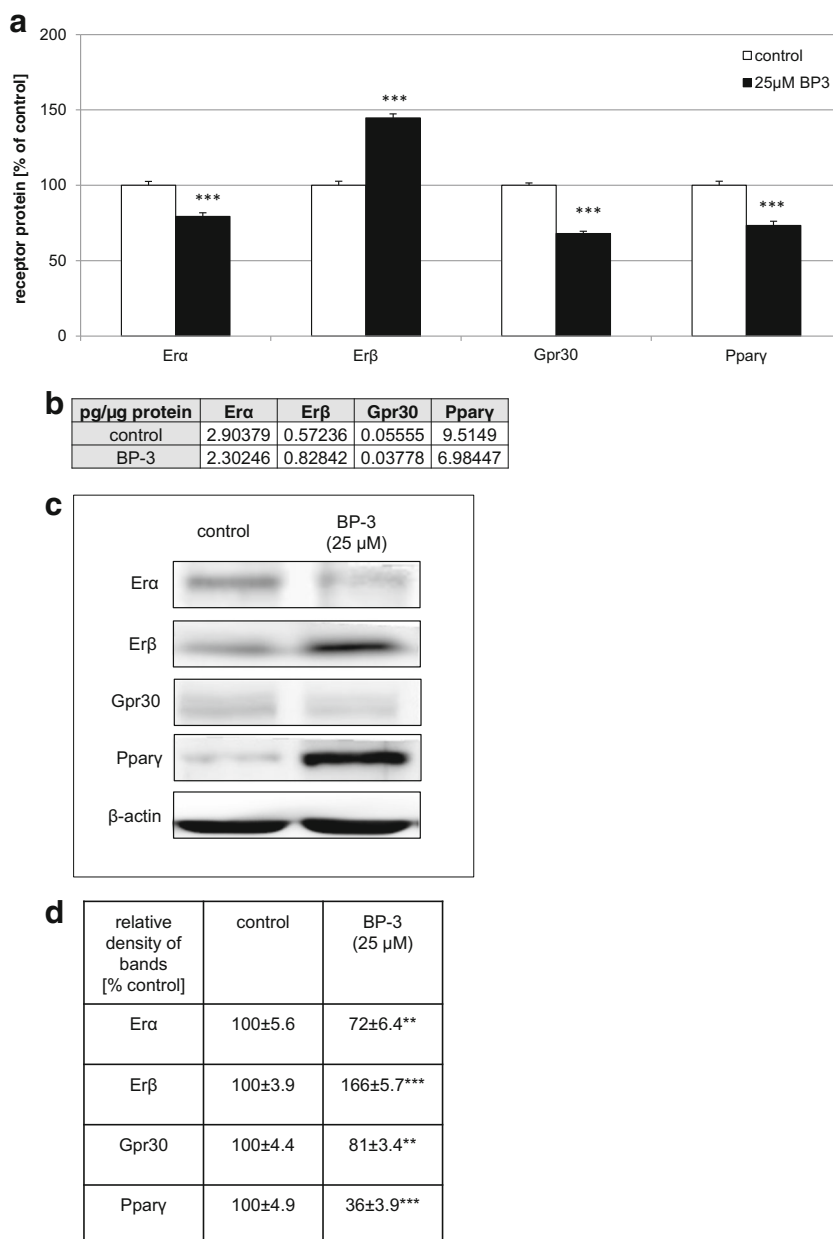
A 24-h exposure to BP-3 (25 μ M) only slightly enhanced caspase-3 activity and LDH release in the *Er β* and *Gpr30* siRNA-transfected cells, suggesting that the transfected cells

were less vulnerable to BP-3 than the non-transfected cells. In comparison to non-transfected cells, in the siRNA-transfected cells, the effects of BP-3 were reduced by 55% with respect to caspase-3 and by 65% with respect to LDH (Fig. 11a, b).

A 24-h exposure of *Erx* and *Ppar γ* siRNA-transfected cells to 25 μ M BP-3 increased caspase-3 activity and LDH release by 85 and 75% over the control, respectively (Fig. 11a, b). These cells were vulnerable to BP-3 in the same way as the non-siRNA-treated wild cells.

The effectiveness of mRNA silencing was verified by measuring the specific mRNAs using qPCR. In this study, mRNA silencing decreased the *Erx* mRNA concentration by 62% (equal to 0.38-fold), the *Er β* mRNA concentration by 68%

Fig. 8 Effects of BP-3 on the protein levels of Era α , Erb β , Gpr30, and Ppary in mouse neocortical cultures at 7 DIV. The neocortical cells were cultured for 7 DIV and then treated for 24 h with BP-3 (25 μ M). The concentrations of the receptors were measured using specific ELISAs and are presented as a percentage of the control (a) and as pg of Era α , Erb β , Gpr30, and Ppary per microgram of total protein (b). For the western blot analyses protein samples were denatured, electrophoretically separated, transferred to PVDF membranes, and subjected to immunolabeling (c). The signals were developed by chemiluminescence (ECL) and visualized using a Luminescent Image Analyzer Fuji-Las 4000 (Fuji, Japan). Immunoreactive bands were quantified using an image analyzer (ScienceLab, MultiGauge V3.0), and the relative protein levels of Era α , Erb β , Gpr30, and Ppary were presented as a percentage of the control (d). Each bar or value represents the mean of three independent experiments \pm SEM. The number of replicates in each experiment ranged from 2 to 3. $**p < 0.01$, and $***p < 0.001$ versus control cultures

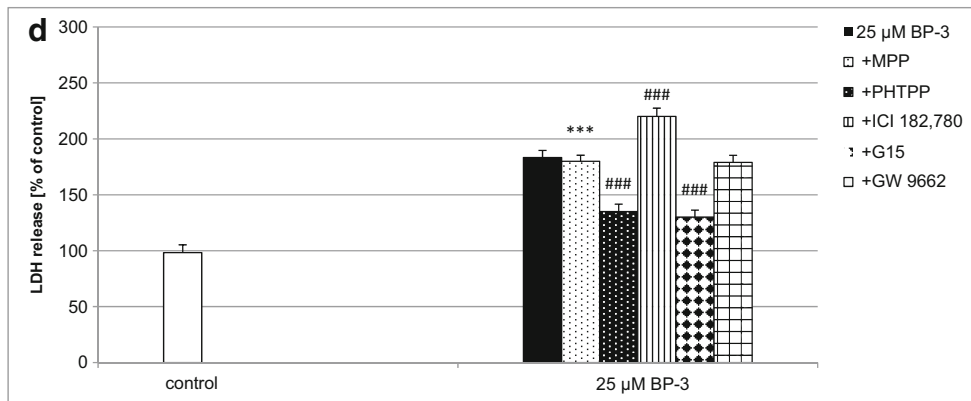
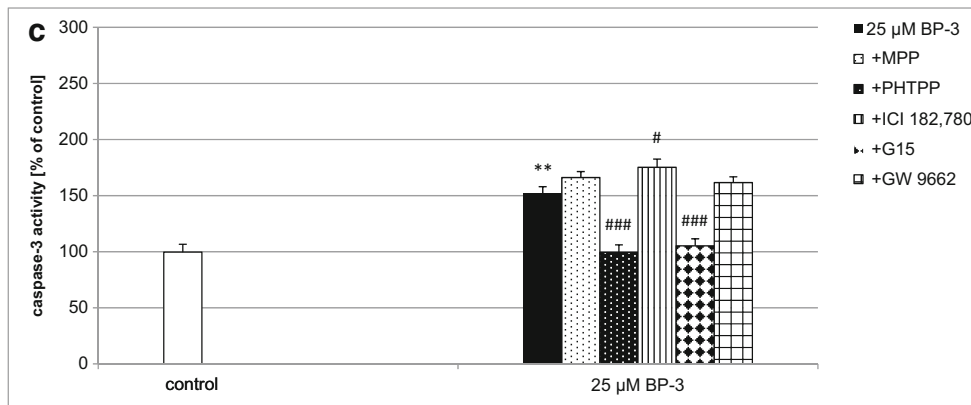
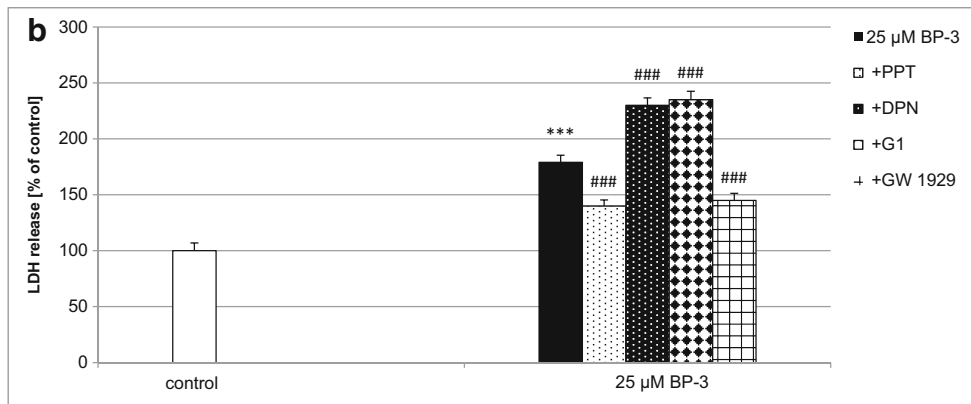
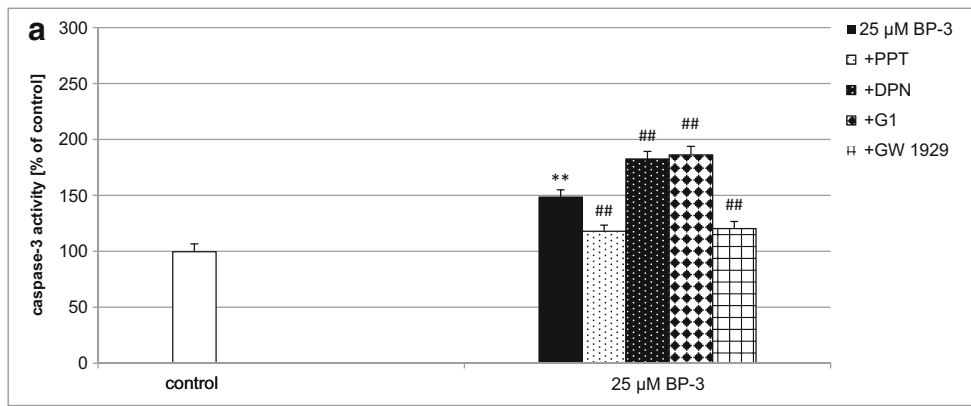


(equal to 0.32-fold), the Gpr30 mRNA concentration by 59% (equal to 0.41-fold), and the Ppary mRNA concentration by 69% (equal to 0.31-fold) compared to the non-transfected wild-type cells.

Effect of BP-3 on the Distribution of Era α , Erb β , Gpr30, Ppary, and MAP2 Staining in Neocortical Cells.

Immunofluorescence labeling and confocal microscopy revealed that Era α , Erb β , Gpr30, and Ppary were localized to neocortical cells at 7 DIV. A 24-h exposure to BP-3

Fig. 9 Impact of Era α , Erb β , Gpr30, and Ppary agonists and antagonists on BP-3-induced caspase-3 activity (a, c) and LDH release (b, d) in 7 DIV neocortical cultures. The primary neocortical cultures were treated with BP-3 (25 μ M) for 24 h. The Era α , Erb β , and Ppary agonists (PPT, DPN, GW 1929) and antagonists (MPP, PHTPP, ICI 182780, GW9662; 1 μ M) were added to the culture media approximately 45–60 min before BP-3 was added. The membrane ER agonist G1 (1 μ M) and antagonist G15 (10 μ M) were added to the culture media simultaneously with BP-3. The results were normalized to the absorbency in vehicle-treated cells and are expressed as a percentage of control. Each bar represents the mean of three to four independent experiments \pm SEM. The number of replicates in each experiment ranged from 5 to 8. $**p < 0.01$, $***p < 0.001$ versus control cultures, $#p < 0.05$, $##p < 0.01$ and $###p < 0.001$ versus the cultures exposed to BP-3



(25 μ M) increased Er β staining but reduced Er α -, Gpr30, and Ppar γ -specific immunofluorescence. MAP2 staining confirmed the neural localization of receptors and revealed the BP-3-induced inhibition of neurite outgrowth (Fig. 12).

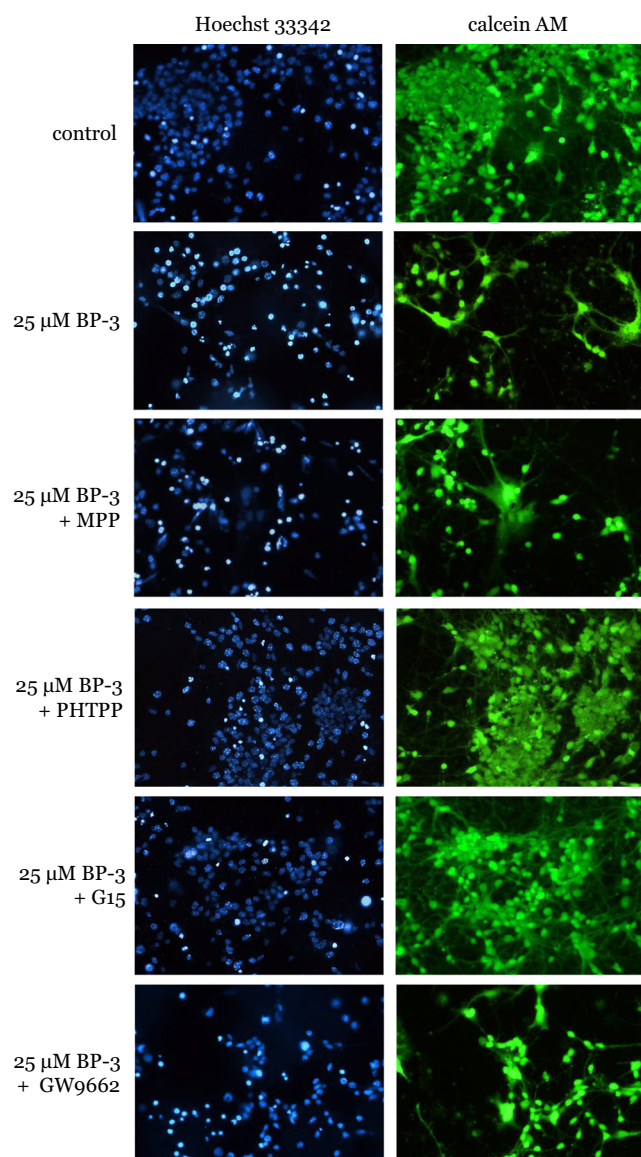


Fig. 10 Influence of BP-3 (25 μ M) and MPP (1 μ M), PHTPP (1 μ M), G15 (10 μ M), GW 9662 (1 μ M) on Hoechst 33,342 (first column) and calcein AM (second column) staining in mouse neocortical cultures at 7 DIV, examined 24 h post-treatment. The Er α , Er β , and Ppar γ antagonists (MPP, PHTPP, GW9662) were added to the culture media approximately 45–60 min before BP-3 was added. The membrane ER antagonist G15 (10 μ M) was added to the culture media simultaneously with BP-3. The cells were cultured on glass coverslips, washed with 10 mM PBS, and exposed to Hoechst 33342 (0.6 μ g/ml) at RT for 5 min. The cells were subsequently rewashed and incubated with 2 μ M calcein AM at RT for 10 min. Cells with bright fragmented nuclei showing condensed chromatin were identified as undergoing apoptosis, whereas cells with light-colored cytoplasm were identified as living cells

Discussion

The primary aim of the present study was to evaluate the apoptotic and neurotoxic effects of BP-3 on neuronal cells with a particular emphasis on the molecular mechanisms of its actions, including estrogen receptors and Ppar γ . We demonstrated that BP-3 evoked concentration-dependent activation of caspase-3 and LDH release in neocortical and hippocampal tissues. In the paradigms examined in the present study, neocortical tissue responded to 25–100 μ M BP-3, whereas hippocampal cells exhibited weaker vulnerability. The extended vulnerability of neocortical cells to BP-3 could be due to low expression of ERs in this tissue which limits ER-related neuroprotection [46]. Indeed, in comparison to the hippocampal cells [42], in neocortical cells Er α protein is present in much lower concentration as evidenced in the present study. Recently, it has been observed that BP-3 used in similar concentrations (0.1–100 μ M) caused toxic effects in neuroblastoma cells [20]. The BP-3 concentrations used in our study are environmentally relevant because BP-3 has been found in human adipose tissue at concentrations up to nearly 5 mg/kg, which is equal to approximately 22 μ M [47]. In the cited article of Wang et al. [47], provided data on accumulation of BP-3 in adipose tissue were related to the concentrations of the compound in males and females of Caucasian and African-American origins and expressed as mean values. However, the authors underlined approximately threefold lower levels of accumulated BP-3 in African-Americans both males and females. Therefore, the actual levels of accumulated BP-3 in Caucasians could be much higher than the values of 30.3 and 62.2 ng/g. BP-3 is able to cross blood–brain barrier since it has altered Er α and Er β mRNA expression levels in rat pituitary gland after being applied in gavages [13]. Moreover, BP-4 which is BP-3 analogue when added to water, displayed multiple effects on gene expression in the zebrafish brain [48].

In the present study, the biochemical alterations were accompanied by increased apoptotic body formation and impaired cell survival, evidenced by Hoechst 33342 and calcein AM staining and by the upregulation of genes involved in apoptosis detected by microarray analysis. The BP-3-induced effects were age-dependent with the most pronounced enzyme activities observed at 7, but not at 2 and 12 DIV. Based on our results, BP-3 induced ROS formation, suggesting that ROS initiated the intrinsic apoptotic pathway in neuronal cells. Indeed, we indicated that BP-3 caused a substantial loss of mitochondrial membrane potential, and inhibitors of intrinsic-related caspase-9 and kinase Gsk3 β reduced the effects induced by BP-3. The cytotoxic effects of BP-3, which involved activation of caspase-3, LDH release, decreased mitochondrial membrane potential, and ROS formation, were previously observed in the HaCaT cell line [49]. Moreover, a reduction of ROS scavengers, i.e., GSH, in response to BP-3

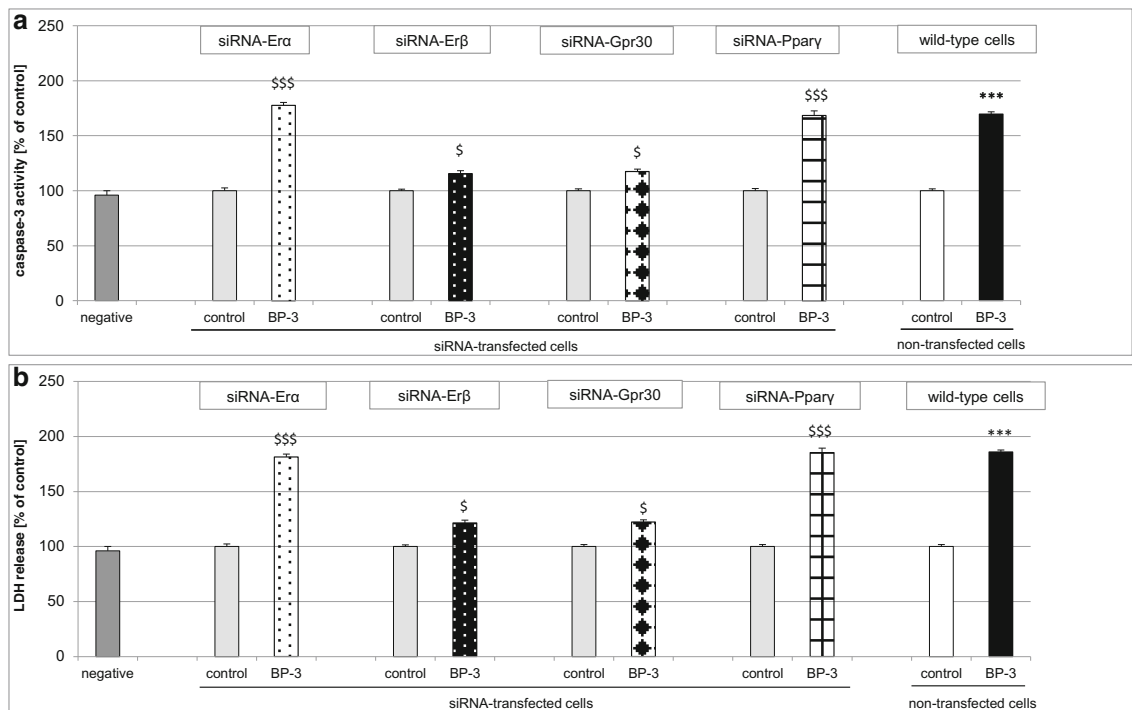


Fig. 11 Effect of BP-3 (25 μ M) on caspase-3 activity (**a**) and LDH release (**b**) in Er α , Er β , Gpr30, and Ppar γ siRNA-transfected neocortical cells. The primary neocortical cultures were transfected with 50 nM Er α , Er β , Gpr30, and Ppar γ siRNAs in INTERFERINTM-containing medium without antibiotics for 6 h. The results were normalized to the absorbance in vehicle-treated cells and are expressed

as a percentage of the control, either in siRNA-transfected or non-transfected cells. Each bar represents the mean \pm SEM of three to four independent experiments. The number of replicates in each experiment ranged from 5 to 8. *** p < 0.001 versus the non-transfected control cultures, $^{\$}p$ < 0.05, $^{$$$}p$ < 0.001 versus the siRNA-transfected control cultures

was observed in *Tetrahymena thermophila* [50]. In our study, the apoptotic effects of BP-3 were inhibited by an inhibitor of p38/MAPK but not by an inhibitor of extrinsic-related caspase-8, thus confirming the prevalence of an intrinsic pathway in BP-3-induced apoptosis in neural cells. Furthermore, BP-3 only partially engaged extrinsic-related caspase-8 in neuronal apoptosis when used in higher concentrations. We postulate that BP-3 by stimulating p38/MAPK may inhibit Er and Ppar γ functions in neuronal cells.

Until now, UV filters, including BP-3, have been shown to have endocrine-disrupting capacities and act via sex steroid receptors, i.e., classical estrogen and androgen receptors. However, there is no data regarding the mechanisms of action of BP-3 in the mammalian nervous system that involve classical and newly recognized membrane estrogen receptors and Ppar γ . Classical estrogen receptors (Er α , Er β) and Ppar γ are nuclear receptors acting as ligand-activated transcription factors. The estrogen receptors when located on the plasma membranes may rapidly activate intracellular pathways such as ERK 1/2 and PI3K kinases [51, 52]. As for Ppar γ , there are no reports on membrane localization of the receptor. In the present study, BP-3 altered the mRNA expression levels of Er α , Er β , Gpr30, and Ppar γ in a time-dependent manner. We demonstrated that 3–24-h exposures caused

decreases in Er α and Gpr30 mRNAs. Moreover, 6–24-h exposures to BP-3 caused a significant increase in Er β mRNA but decreased Ppar γ mRNA. Recently, BP-3 was found to have a direct impact on the ER-related insect endocrine pathway, activating the ecdysone receptor gene *EcR* [53]. BP-3 also transcriptionally activated human Er α and Er β in transfected human embryonic kidney cells HEK293 [54]. Furthermore, BP-3 was found to cause a strong activation of *hER β* in reporter gene assays in HELN cells [55], which is similar to the substantial upregulation of Er β mRNA observed in our study. We showed that BP-3-induced patterns of mRNA expression detected at 6 and 24 h reflected alterations in the protein levels of the receptors, as indicated by the use of specific ELISAs and western blot analyses. These profiles were also in parallel with the immunofluorescent labeling of Er α , Er β , Gpr30, and Ppar γ in response to BP-3. Basing on these data, we hypothesize that the BP-3-evoked apoptosis of neuronal cells is mediated via the attenuation of Er α /Gpr30/Ppar γ and the stimulation of Er β signaling pathways, though we did not differentiate the effects between nuclear and membrane receptors.

The proposed hypothesis has been partially confirmed by the use of selective receptor ligands and specific siRNAs. According to our data, Er α and Ppar γ agonists diminished,

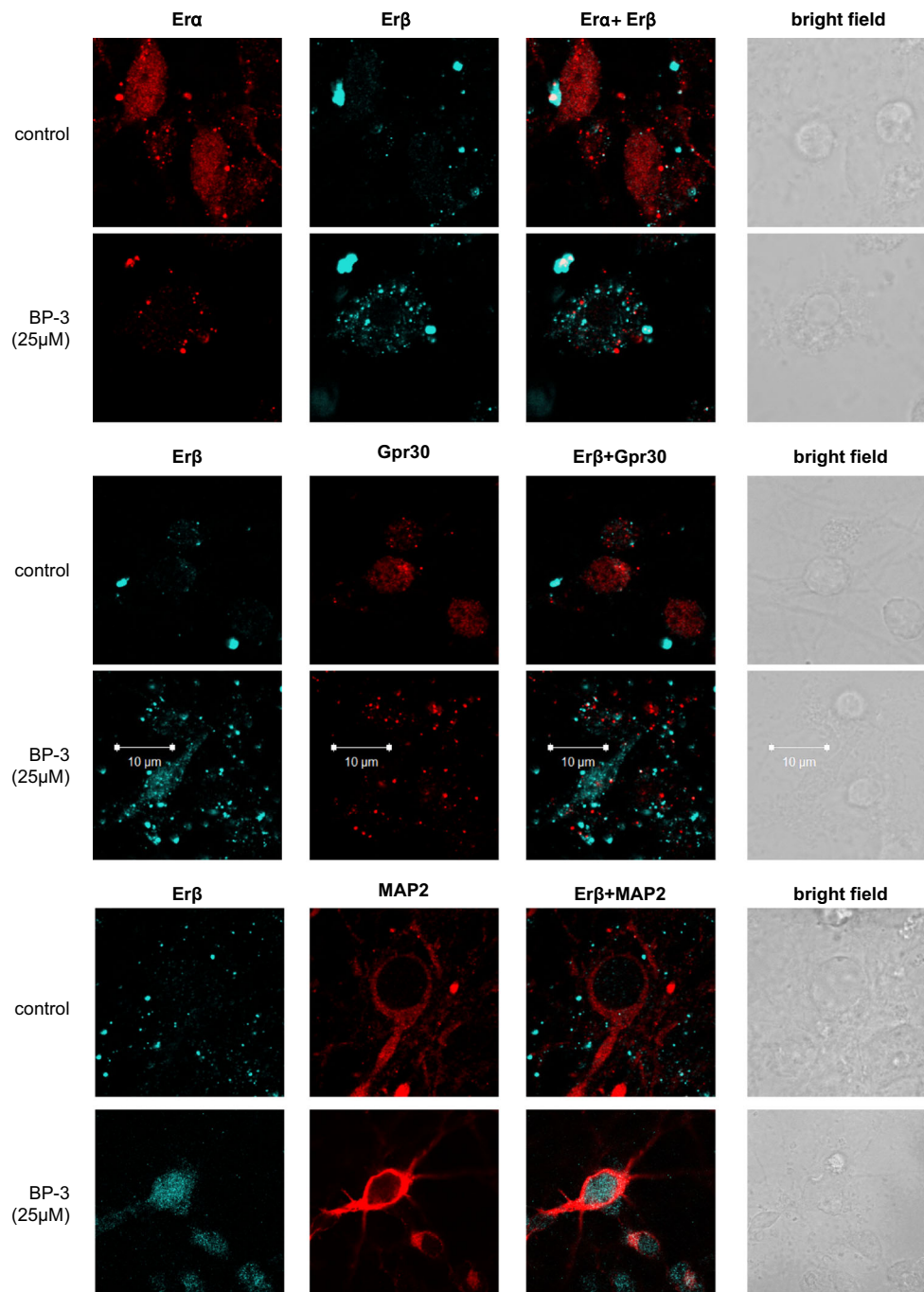


Fig. 12 Influence of BP-3 on the cellular distributions of Er α (red), Er β (blue), Gpr30 (red), Ppary (blue), and MAP2 (red) in mouse neocortical cultures at 7 DIV. The overlay of Er α /Er β , Er β /Gpr30, Er α /Ppary, Er β /Map2, and Ppary/MAP2 (red plus blue) and the bright field images are also shown. The primary neocortical cultures were treated with BP-3

(25 μ M) for 24 h. The cells were cultured on glass coverslips and subjected to immunofluorescent double-labeling. The samples were analyzed using an LSM510 META Axiovert 200 M confocal laser scanning spectral microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) with a Plan-Apochromat 63 \times /1.4 Oil DIC objective

but Er β and Gpr30 agonists stimulated the BP-3-induced apoptotic and neurotoxic effects. In comparison to receptor agonists, receptor antagonists caused opposite effects, except for ICI 182,780, which is known to act not only as an Er α /Er β antagonist but also as a Gpr30 agonist. These results were complemented by double Hoechst/calcein AM staining,

according to which the receptor antagonists PHTPP and G15 improved viability and attenuated BP-3-induced apoptosis, whereas MPP and GW9662 did not change these parameters. This is in line with a substantial reduction of the effects of BP-3 in the cells with siRNA-silenced Er β /Gpr30 and the maintenance of the effects of BP-3 in Er α - and Ppary- siRNA-

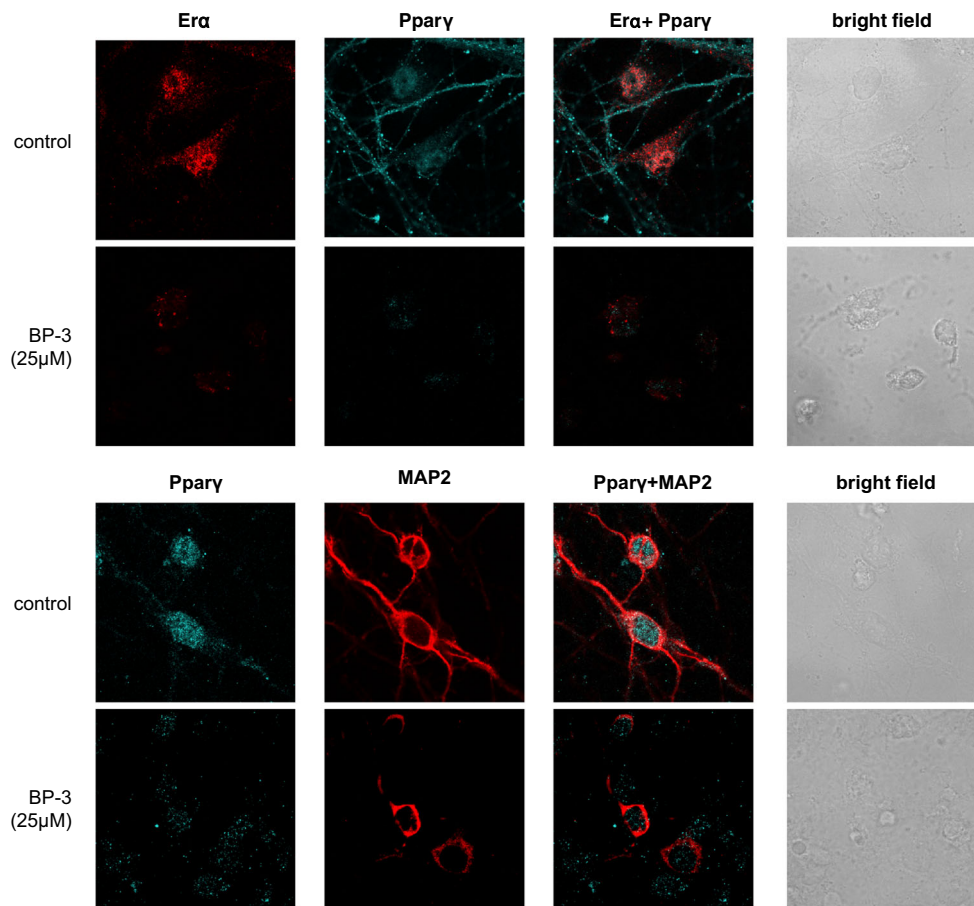


Fig. 12 (continued)

transfected cells. Therefore, we suggest that BP-3-evoked apoptosis of neuronal cells is mediated via an attenuation of $Er\alpha$ / $Ppary$ and stimulation of $Er\beta$ / $Gpr30$ signaling. Recently, it has been shown that activation of $Ppary$ inhibited caspase-3 activity and protected murine cortical neurons against ischemia [56, 57]. Reduced expression of $Ppary$ accompanied neurotoxicity of TBBPA [58] which is in line with the effect of BP-3 in our study. We hypothesize that the BP-3-evoked downregulation of $Ppary$ is at least partially due to the upregulation of $Er\beta$. A negative cross-talk between $Er\beta$ and $Ppary$ has previously been shown in adipose tissue of $\beta ERKO$ mice [59]. It is also possible that the reduced expression of $Er\alpha$ observed in our study was linked to the cross-talk of $Ppary$ with its heterodimeric partner, the nuclear receptor $Rxr\alpha$, through the mechanism previously detected in the vitellogenin A2 promoter [60]. We demonstrated that the BP-3-affected mRNA and protein expression levels of $Er\alpha$, $Er\beta$, and $Ppary$ measured by qPCR, ELISAs, western blot, and immunofluorescent labeling of neuronal cells are parallel to BP-3-induced apoptosis and neurotoxicity. Although BP-3 reduced $Gpr30$ mRNA and protein expression, it accelerated the function of $Gpr30$ in neuronal cells, thus pointing to the involvement of $Gpr30$ in BP-3 neurotoxicity.

Conclusions

In summary, our study demonstrated that BP-3 caused neurotoxicity and activated apoptosis via an intrinsic pathway involving the loss of mitochondrial membrane potential and the activation of caspases-9 and -3 and kinases p38/MAPK and $Gsk3\beta$. We showed for the first time that BP-3-evoked apoptosis of neuronal cells is mediated via the attenuation of $Er\alpha$ / $Ppary$ and the stimulation of $Er\beta$ / $Gpr30$ signaling, as demonstrated using selective ligands and specific siRNAs and by measurements of mRNA and protein expression (qPCR, ELISA, western blot, immunofluorescent labeling) of the receptors.

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AM, acetoxymethyl; ANOVA, analysis of variance; BP-3, benzophenone-3; BSA, bovine serum albumin; CNS, central nervous system; DDE, dichlorodiphenyldichloroethylene; DDT, dichlorodiphenyltrichloroethane; DIV, days in vitro; DMSO, dimethyl sulfoxide; EDCs, endocrine-disrupting chemicals; ELISA, enzyme-linked immunosorbent assay; ERs, estrogen receptors; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; $Gpr30$, G protein-coupled receptor 30; HD, Huntington's disease; Hprt, hypoxanthine-guanine

phosphoribosyltransferase; LDH, lactate dehydrogenase; mRNA, messenger RNA; PBS, phosphate-buffered saline; Ppar, peroxisome proliferator-activated receptors; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RT, room temperature; Rxr, retinoid X receptor; SCI, spinal cord injuries; SEM, standard error of the mean; TBS, tris-buffered saline; UV, ultra violet light

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Compliance with Ethical Standards

Disclosure of Potential Conflicts of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. All members of the research team received approval from the local ethical committee on animal testing. Animal care followed official governmental guidelines, and all efforts were made to minimize suffering and the number of animals used.

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Chlorination of oxybenzone: Kinetics, transformation, disinfection byproducts formation, and genotoxicity changes.

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Author information

Abstract

UV filters are a kind of emerging contaminant, and their transformation behavior in water treatment processes has aroused great concern. In particular, toxic products might be produced during reaction with disinfectants during the disinfection process. As one of the most widely used UV filters, oxybenzone has received significant attention, because its transformation and toxicity changes during chlorine oxidation are a concern. In our study, the reaction between oxybenzone and chlorine followed pseudo-first-order and second-order kinetics. Three transformation products were detected by LC-MS/MS, and the stability of products followed the order of tri-chloro-methoxyphenyl > di-chlorinated oxybenzone > mono-chlorinated oxybenzone. Disinfection byproducts (DBPs) including chloroform, trichloroacetic acid, dichloroacetic acid and chloral hydrate were quickly formed, and increased at a slower rate until their concentrations remained constant. The maximum DBP/oxybenzone molar yields for the four compounds were 12.02%, 6.28%, 0.90% and 0.23%, respectively. SOS/umu genotoxicity test indicated that genotoxicity was highly elevated after chlorination, and genotoxicity showed a significantly positive correlation with the response of tri-chloro-methoxyphenyl. Our results indicated that more genotoxic transformation products were produced in spite of the elimination of oxybenzone, posing potential threats to drinking water safety. This study shed light on the formation of DBPs and toxicity changes during the chlorination process of oxybenzone.



Transformation of benzophenone-type UV filters by chlorine: Kinetics, products identification and toxicity assessments



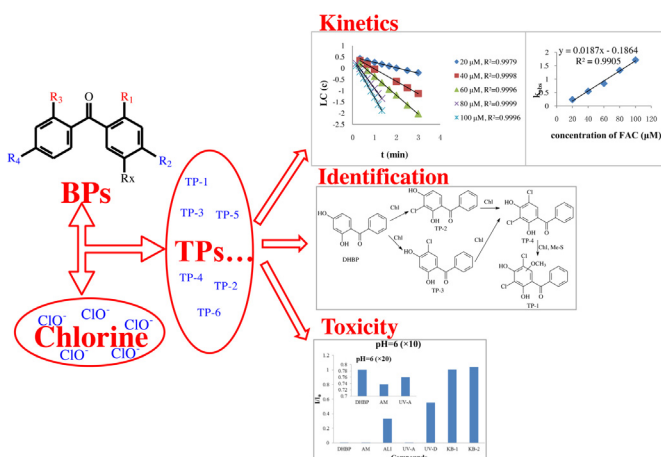
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HIGHLIGHTS

- Chlorination kinetics of three benzophenone-type UV filters (BPs) was studied.
- Chlorination of BPs followed second-order reaction.
- The transformation products (TPs) of six BPs were identified.
- Several transformation pathways were proposed.
- Mostly enhanced toxicity of TPs after chlorination was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

The present study focused on the kinetics, transformation pathways and toxicity of several benzophenone-type ultraviolet filters (BPs) during the water chlorination disinfection process. The transformation kinetics of the studied three BPs was found to be second-order reaction, which was dependent on the concentration of BPs and chlorine. The second-order rate constants increased from 86.7 to 975 M⁻¹ s⁻¹ for oxybenzone, 49.6–261.7 M⁻¹ s⁻¹ for 4-hydroxybenzophenone and 51.7–540 M⁻¹ s⁻¹ for 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid with the increasing pH value from 6 to 8 of the chlorination disinfection condition. Then the transformation products (TPs) of these BPs were identified by HPLC-QToF analysis. Several transformation pathways, including electrophilic substitution, methoxyl substitution, ketone groups oxidation, hydrolysis, decarboxylation and ring cleavage reaction, were speculated to participate in the chlorination transformation process. Finally, according to the toxicity experiment on luminescent bacteria, *Photobacterium phosphoreum*, enhanced toxicity was observed for almost all the TPs of the studied BPs except for 2,2'-dihydroxy-4,4'-dimethoxybenzophenone; it suggested the formation of TPs with more toxic than the parent compounds during the chlorination process. The present study provided a foundation to understand the transformation of BPs during chlorination disinfection process, and was of great significance to the drinking water safety.

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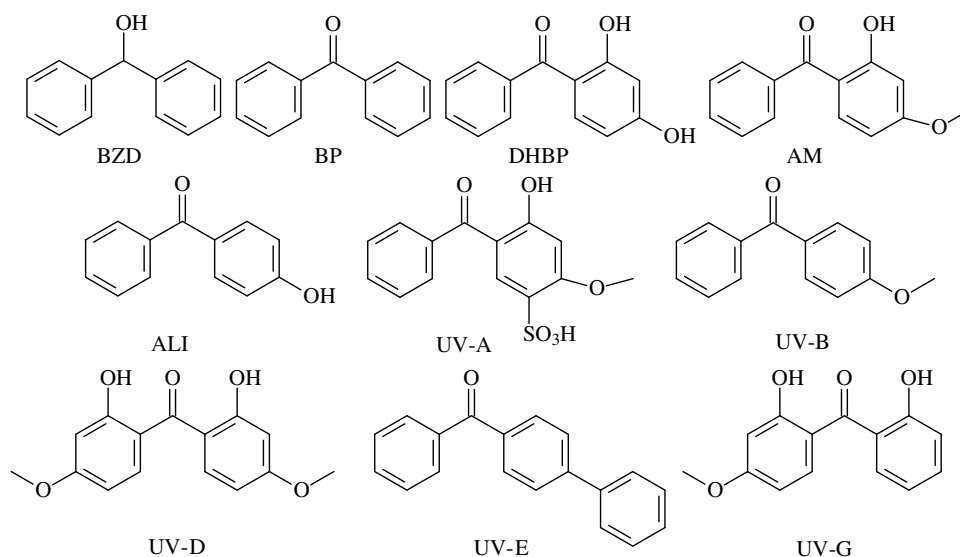


Fig. 1. Structures of the studied BPs.

1. Introduction

Benzophenone-type UV filters (BPs) can absorb or reflect UV radiation, which are broadly used in personal care products (PCPs) and synthetic polymer products [1–6]. The content of some BPs may account for as much as 10% of the product weight [6,7]. Oxybenzone (AM), 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid (UV-A), 2,2'-dihydroxy-4,4'-dimethoxybenzophenone (UV-D), 2,2'-dihydroxy-4-methoxybenzophenone (UV-G) and 2,4-dihydroxybenzophenone (DHBP) are widely used as additives in sunscreen products [3,8–10]; benzophenone (BP), 4-methoxybenzophenone (UV-B) and 4-benzoylbiphenyl (UV-E) are used as inhibitors to prevent the degradation of polymers and pigments, such as plastics, packaging materials, glassworks, textiles and so on [4,5]. Benzhydrol (BZD), DHBP, UV-G and 4-hydroxybenzophenone (ALI) were transformation products of BPs [11]. The extensive usage and mis-disposition of these products led to the increasing BPs residues in the environment [3,6,7,10,12–14].

Because of their physical and chemical properties, i.e. stability and lipophilicity, BPs may bio-accumulate in the environment [15,16] and cause long-term effect on environment and humans. It was documented that some BPs exhibited potential endocrine disrupting activity [17,18], multiple hormonal activities [19] and even genotoxicity and reproductive toxicity [20,21]. Especially, BP is classified as “chemicals suspected of endocrine disrupting effects” [22]. Witorsch et al. summarized the estrogenicity of benzophenone derivatives [23].

Chlorination by free aqueous chlorine (FAC) is one of the most common methods for drinking water disinfection [24]. However, although the parent compounds were transformed during the chlorination process, many intermediates or transformation products (TPs) may simultaneously form unexpectedly in some cases [25–27]. BPs are ubiquitous in the water system, i.e. pool water, waste water, river, lake and so on, and the residue concentration may be up to $550 \mu\text{g m}^{-3}$ [28]. Especially, AM in waste water ranged from 0.7 to $7.8 \mu\text{g L}^{-1}$ and 0.01–0.7 $\mu\text{g/L}$ before and after wastewater treatment plants [16], and 56–68 ng L^{-1} in river Glatt [12]. Thereby, the residue BPs may undergo transformation during disinfection process, including chlorination, oxidation, hydrolysis, decarboxylation and condensation [29,30]. TPs thus accompany chlorination, which may pose hazards, even greater than those of the parent compounds, on the environment and humans. Hence,

it is of great significance to monitor the transformation rates, TPs and their toxicity for the safety of the drinking water. However, up to date, very few researches concentrated on this topic of BPs from all these three aspects [27,31–34]. Negreira et al. [27] studied the kinetics and TPs of AM in the presence of aqueous chlorine. The half-lives of AM under different experimental conditions were obtained and several chlorine substituted TPs were identified. However, no toxicity assessment was conducted. Liu et al. [34] studied the acute toxicity of the BPs after the chlorination disinfection process with the conclusion of increased toxicity of UV-A.

The present study carried out systematic experiments on the kinetics, TPs and toxicity of BPs by aqueous chlorine disinfection. The objectives were: (1) to calculate the chlorination rates of the BPs under different chlorination conditions; (2) to identify the TPs and propose the chlorination transformation pathways; (3) to evaluate the total toxicity of TPs. It would provide a good understanding of the behavior of BPs during chlorination disinfection procedure, and provide references for the water safety.

2. Materials and methods

2.1. Chemical reagents and apparatus

NaCl, NaH_2PO_4 , Na_2HPO_4 , acetic acid (HAc), sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$), sodium hypochlorite solution (NaClO, 30% approximately) and BP were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). They were all analytical grade. Methanol was acquired from Fisher Scientific (high performance liquid chromatographic (HPLC) grade, New Jersey, USA). The other BPs, DHBP, AM, ALI, UV-A, UV-D, UV-G, BZD, UV-B and UV-E, were purchased from Alfa Aesar (purity $\geq 98\%$, Tianjin, China). The structures of the studied BPs are depicted in Fig. 1.

Stock solutions of each BPs were prepared in methanol at the concentration of 1 mg mL^{-1} separately and were kept at 4°C before usage. All the purified water used in the experiments was produced by a Heal Force NW system (Hong Kong, China).

All the glassware and plastic containers were washed by pure water and methanol and dried before usage. The blank tests were conducted in every experiment to evaluate the interference from containers and no interference was found.

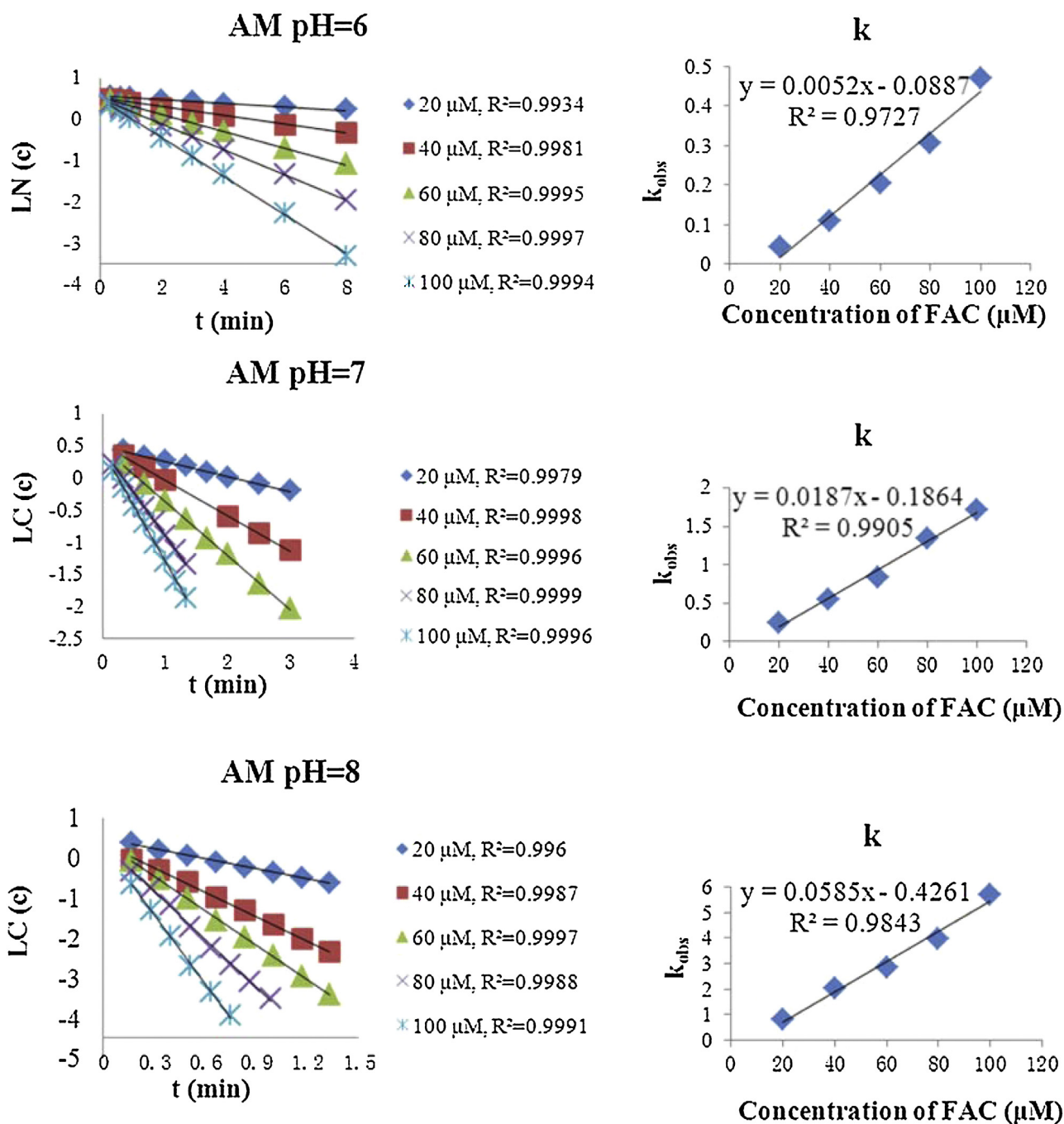


Fig. 2. Chlorination kinetics of AM, ALI and UV-A at different pHs. The concentration of free aqueous chlorine ranged from 20 to 100 $\mu\text{mol L}^{-1}$ and the initial concentration of each BPs was 2 $\mu\text{mol L}^{-1}$.

2.2. Kinetic experiments

The state of HClO varies with pH value of the solution, thus it could affect the disinfection efficiency greatly [25]. To keep the pH of the chlorination solution stable, all the kinetic experiments were conducted in the 10 mmol L^{-1} phosphate buffer solution (PBS).

2.2.1. Preparation of the PBS

NaH_2PO_4 and Na_2HPO_4 were dissolved in pure water to attain 10 mmol L^{-1} aqueous solutions, respectively. They were mixed at a certain proportion to get the desired pH values of 6, 7 and 8, monitored by a pH meter (Mettler Toledo, Shanghai, China), to represent the pH ranges of real water samples, such as tap, swimming pool

and wastewater [27]. The freshly prepared PBS was used in three days.

2.2.2. Determination of FAC

The NaClO solution was titrated by standard $\text{Na}_2\text{S}_2\text{O}_3$ solution to obtain the exact concentration of FAC ([FAC], the concentration of effective chlorine). To ensure the accuracy of the experiment, the NaClO solution was kept in dark under 4 °C and titrated every week.

2.2.3. Kinetic experiments

A certain volume of NaClO solution was added to PBS (10 mL) in a glass bottle to get specified [FAC]. Immediately, the stock solution of each BPs was added to get the concentration of 2 $\mu\text{mol L}^{-1}$,

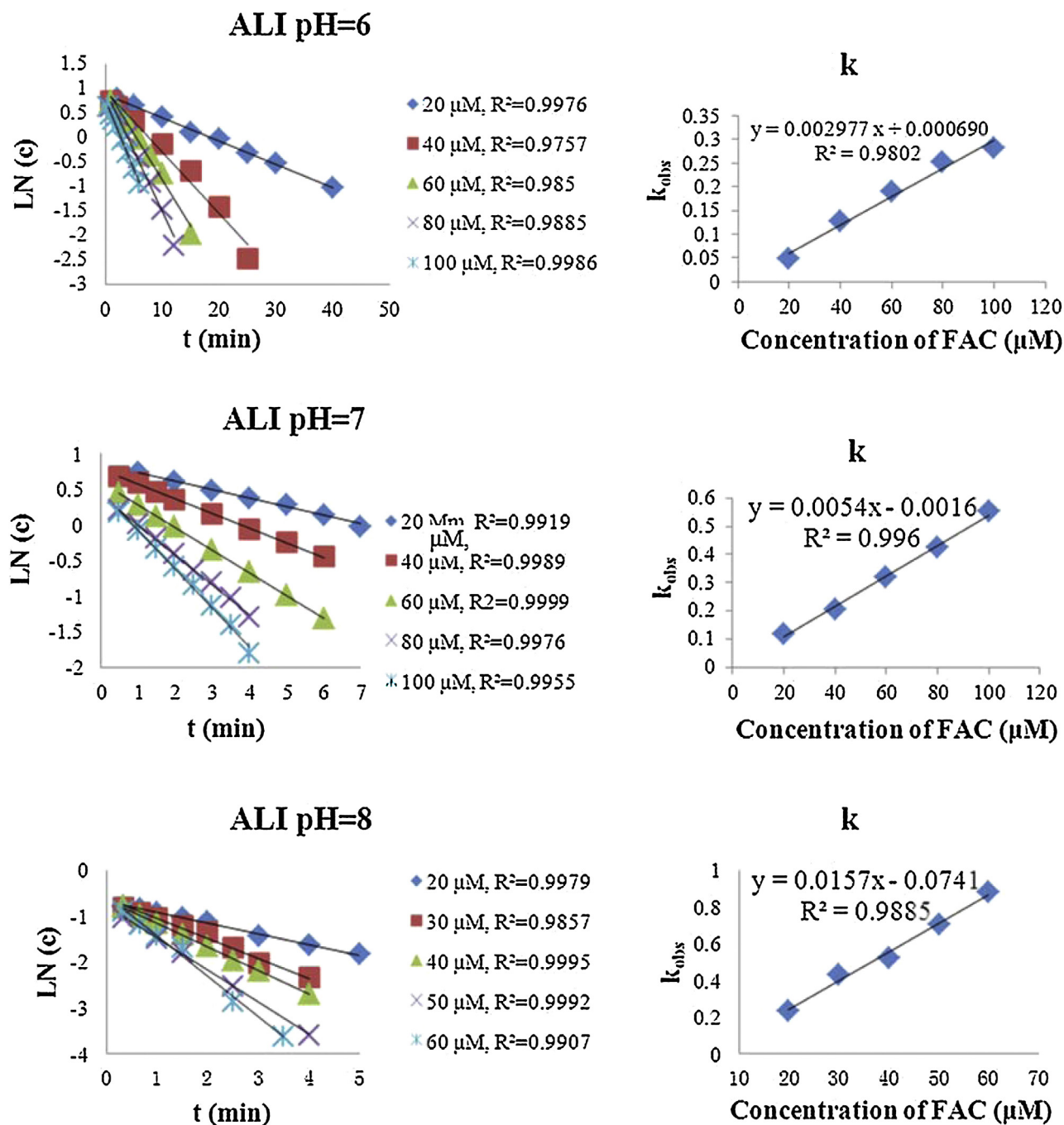


Fig. 2. (Continued).

respectively. At different time intervals, 1 mL solution was collected and transferred to a HPLC sample vial containing 10 μL $\text{Na}_2\text{S}_2\text{O}_3$ (0.5 mol L^{-1}), which was the reducing agent to terminate the reaction [27], immediately followed by shaking to quench the chlorination and analysis by a HPLC system. The kinetic experiments were conducted three times in parallel.

2.2.4. HPLC analysis

A SHIMADZU UFLC system equipped with a LC-20AD pump, a SIL-20A autosampler and a SPD-M20A UV detector (Kyoto, Japan) was applied for the determination. A Dionex C18 column ($2.1 \times 150 \text{ mm}$, $2.2 \mu\text{m}$, USA) was used for separation. The mobile phase was the mixture of 90% methanol-10% aqueous (1% HAC), the flow rate was 0.1 mL min^{-1} and $5 \mu\text{L}$ was injected into the HPLC for

analysis. The wavelengths for AM, ALI and UV-A were 290, 292 and 286 nm, respectively.

2.3. Identification of TPs

To detect as many TPs as possible, during the chlorination, initial concentration of each analyte was set at $20 \mu\text{g mL}^{-1}$ (about $100 \mu\text{mol L}^{-1}$), and the [FAC] was set at $200 \mu\text{mol L}^{-1}$. To keep the pH value of the chlorination system stable, 20 mM PBS was applied for this part.

NaClO solution was added to 20 mL PBS, followed by adding 400 μL of each BP's stock solutions individually, to reach the ratio of [FAC] to each BP' concentration at 2:1. The system was quickly mixed by slight shaking and left still in dark for 1 h. Afterwards,

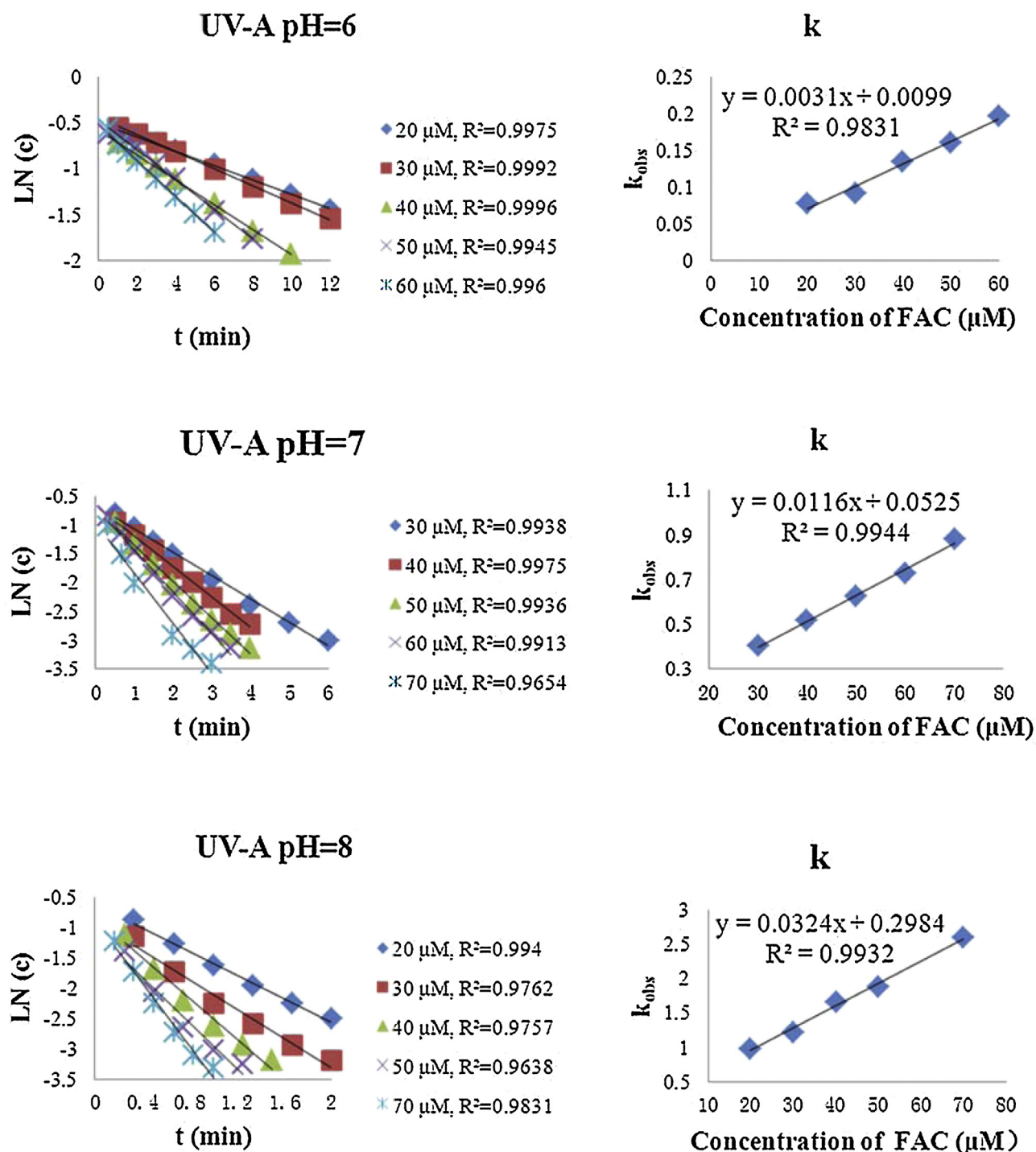


Fig. 2. (Continued).

$\text{Na}_2\text{S}_2\text{O}_3$ solution (0.5 mol L^{-1} , $100 \mu\text{L}$) was added to stop the chlorination reaction. Then, solid phase extraction (SPE) was applied to purify and enrich the TPs (200 mg C18 , 3 mL methanol and 3 mL water for activation, 20 mL reaction solutions for sample loading and $300 \mu\text{L}$ methanol- H_2O ($19:1, \text{ v:v}$) for elution). The eluents were injected into HPLC system for analysis.

A Dionex HPLC-DAD system (Ultimate 3000HPLC, USA) with a Dionex C18 column ($2.2 \mu\text{m}$, $2.1 \times 150 \text{ mm}$) was applied for the preliminary experiment to confirm the chlorination conditions. The mobile phase was the mixture of methanol and 1% HAC solution by

gradient elution, as listed in Supplementary Information 1 (S.I. 1). The injection volume was $5 \mu\text{L}$.

A HPLC-QToF system (Waters, xevo, G2, Massachusetts, USA) was used for identification. The chromatographic condition was listed in S.I. 1. The MS conditions were as below: negative mode; ion source temperature: 100°C ; desolvation temperature: 350°C ; cone gas: 50 L h^{-1} ; desolvation gas: 800 L h^{-1} ; capillary voltage: 2.6 kV ; sampling cone: 40 V ; m/z : $50\text{--}500$.

2.4. Toxicity experiments

Two marine bacteria, i.e. *P. phosphoreum* [34,35] and *vibrio fisheri* [36–38], were commonly used photobacteria in the assessment of the acute toxicity of water quality. Herein, the total toxicity assessments of the TPs after chlorination were performed according to the Chinese standard (GB/T 15441–1995) [35], which is a national standard method based on the *P. phosphoreum*. The freeze-dried bacteria were obtained from the Institution of Soil Science, Chinese Academy of Science (Nanjing, China).

2.4.1. Sample preparation

The chlorination was carried out in 10 mmol L⁻¹ PBS (pH = 6, 7 and 8). Initial concentration of the BPs was 4 μmol L⁻¹ and the [FAC] was about 200 μmol L⁻¹. The reaction time was set at 72 h without quenching procedure. Two blank tests were conducted. Methanol instead of the analyte was used to investigate the influence of FAC on luminescence of the bacteria, named as KB-1; methanol instead of the analyte without the addition of FAC was used to investigate the influence of solvent (methanol), named as KB-2. The control group was NaCl solution at the concentration of 30 g L⁻¹. Before toxicity assessment, the chlorination solutions were diluted 10 or 20 times by NaCl solution (30 g L⁻¹) to keep the similar osmotic pressure to the control group. Stock solution of each BPs was diluted to 4 μmol L⁻¹ by PBS and then diluted 10 times by NaCl solution (30 g L⁻¹) for toxicity test of the parent compounds.

2.4.2. Revival of the bioluminescent bacteria

The ampulla bottle containing 1 g *P. phosphoreum* was rapidly added with 1 mL pre-cooled NaCl solution (20 g L⁻¹) once opened. The bacteria solution was adequately mixed and kept in dark for 30 min to get a stable activation level.

2.4.3. Toxicity assessments

Two milliliter portion of each solution, including the diluted chlorination solution and diluted blank solution as the test group, and NaCl solution (30 g L⁻¹) as the control group, were separately added to the cell. Then 10 μL solution of the reactivated *P. phosphoreum* was added to each cell in order, followed by slight shaking to mix well. After 15-min incubation under ambient temperature, the cell was put in a Hitachi F-4600 fluorescence spectrophotometer (Japan) in luminescent mode for determination. The toxicity test for each sample was carried out in triplicate.

2.4.4. Data analysis

The total toxicity of the TPs was reflected by the parameter of inhibition rate calculated by the following Eq. (1):

$$\text{Inhibition} = 1 - \frac{I}{I_0} \quad (1)$$

I is luminescence intensity of the chlorination solution after 15-min incubation; I_0 is luminescence intensity of 30 g L⁻¹ NaCl solution after 15-min incubation.

3. Results and discussion

3.1. Chlorination kinetic study of BPs

In our preliminary experiments, it was found that BPs without phenolic hydroxyl group, such as BZD, BP, UV-B and UV-E herein, could not be transformed in the presence of FAC (data not shown); the BPs with two phenolic hydroxyl groups, e.g. DHBP, UV-D and UV-G, were chlorinated so fast that it was difficult to quench the reaction at exact time. Thus, chlorination kinetics were only performed for AM, ALI and UV-A.

All the kinetic experiments were performed under the condition of large excess of chlorine ($[FAC]_0 \geq 10 \times [analyte]_0$). Under this condition, the reaction would subject to the following Eq. (2) if the chlorination reaction is a second-order reaction:

$$\frac{d[analyte]_t}{dt} = -k \times [FAC]_t \times [analyte]_t \quad (2)$$

$[FAC]_t$ is the concentration of chlorine at the time of t ; $[analyte]_t$ is the concentration of the BPs at the time of t ; k is the second-order rate constant. If $[FAC]_0 \geq 10 \times [analyte]_0$ ($[FAC]_0$ is the initial [FAC]; $[analyte]_0$ is the initial concentration of BPs), the $[FAC]$ could be regarded as a constant, i.e. $[FAC]_t = [FAC]_0$; thus, $k \times [FAC]_t$ could be regarded as a constant. Hence, Eq. (2) could be described as follows:

$$\frac{d[analyte]_t}{dt} = -k_{obs} \times [analyte]_t \quad (3)$$

k_{obs} is the observed pseudo-first-order rate constant and Eq. (3) could be also written as below:

$$\text{Ln}[analyte]_t = -k_{obs} \times t + \text{Ln}[analyte]_0 \quad (4)$$

According to Eq. (4), k_{obs} could be calculated by the linearity slope plotted between $\text{Ln}[analyte]_t$ versus t , and k could be determined by $k_{obs} = k \times [FAC]_0$.

In the kinetic experiment, initial concentration of the BPs was 2 μmol L⁻¹ and the minimum [FAC] was 20 μmol L⁻¹. Under this condition, the chlorination exhibited pseudo-first order kinetics depending on the concentration of analytes, and it could be demonstrated by the linear plot of $\text{Ln}[analyte]_t$ versus t , as shown in Fig. 2. It can be seen that as [FAC] rose from 20 μmol L⁻¹ to 100 μmol L⁻¹, k_{obs} increased linearly for all the studied UV filters at each investigated pH.

According to $k_{obs} = k \times [FAC]_0$, the second-order rate constant k , as shown in Eq. (2), could be obtained by plotting k_{obs} versus [FAC], as shown in Fig. 2. With the increasing pH values of the buffer solution from 6 to 8, k increased for all the studied BPs. This phenomenon agreed well with some other chlorination reactions between aqueous chlorine and phenols or compounds containing phenolic hydroxyl group [39–41].

The good linear correlation coefficients (≥ 0.9727) for all the studied BPs at each pH value indicated that the aqueous chlorine reaction followed second-order kinetics based on the concentration of chlorine and the analytes [36,39]. With the increasing pH of PBS from 6 to 8, the second-order rate constants increased from 86.7 to 975 M⁻¹ s⁻¹ for AM, 49.6–261.7 M⁻¹ s⁻¹ for ALI, and 51.7–540 M⁻¹ s⁻¹ for UV-A, which were similar to the previous work [31]. These trends may be highly related to the different states of the BPs under different pH conditions [25]. With the increasing pH from 6 to 8, more phenolate state of BPs generated, which facilitated the chlorination and resulted in higher reaction rate constants.

3.2. Identification of the TPs

In the preliminary experiment, the concentration ratios of FAC to BPs were investigated at 2:1, 5:1 and 10:1. With the increasing [FAC], the amount of TPs decreased for each analyte, and no new TPs signal appeared. Thus 2:1 of the concentration ratio was set to investigate the TPs formation at pH 6, 7 and 8. Under different pH conditions, the TPs profiles were similar despite that some peak areas of the TPs varied and the detectable TPs under pH 8 were slightly more than those at pH 6 and 7, as shown in S.I. 2. As discussed above, the different states of BPs under different pH conditions may account for this observation. Under pH 8, BPs dissociated more which was in favor of the transformation of BPs, and thus generated more TPs [25]. Therefore, 2:1 of the concentra-

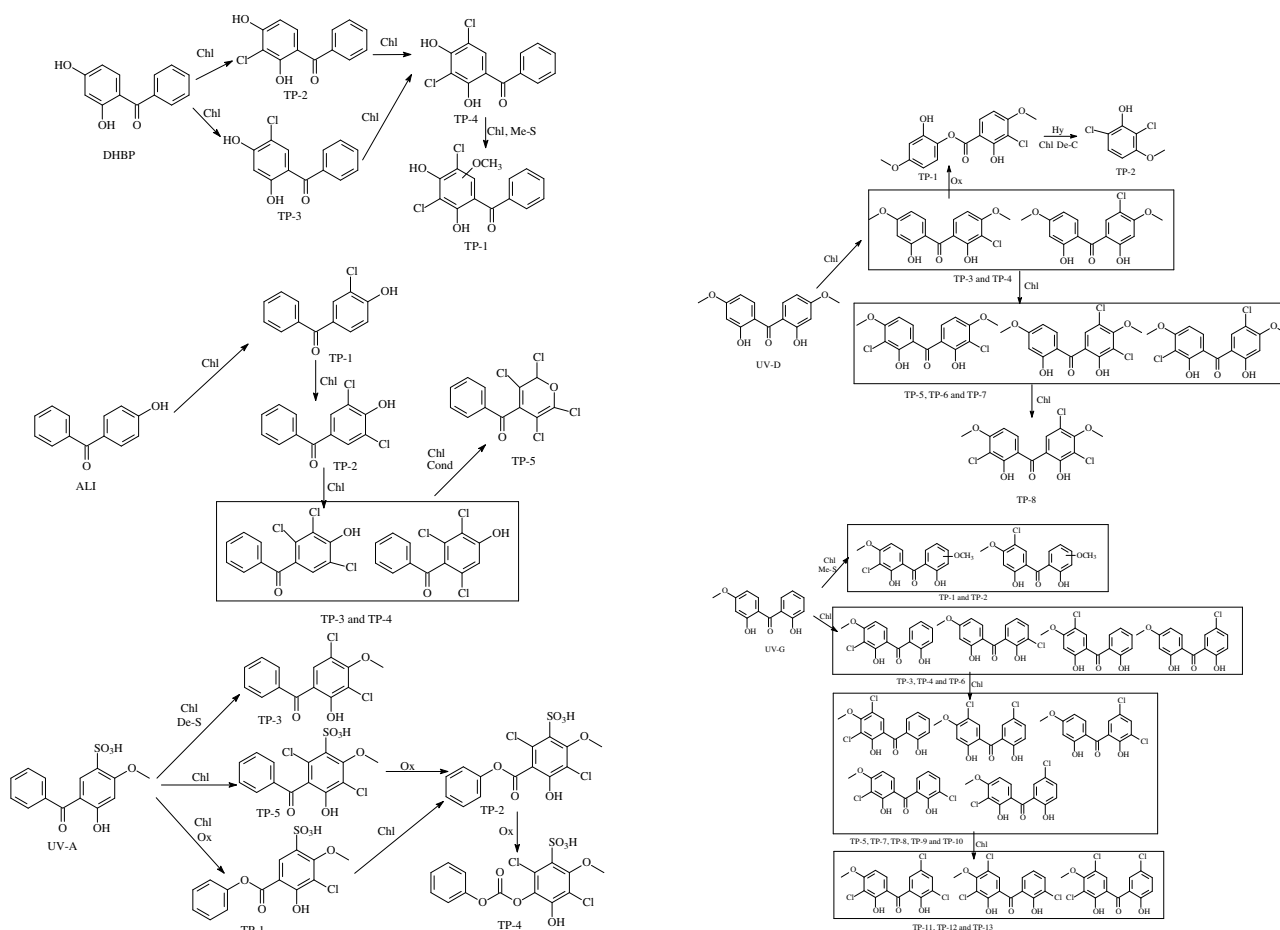


Fig. 3. Proposed transformation pathways of BPs during chlorination.

tion ratio of FAC versus BPs at pH 8 were used in the identification experiments.

Table 1 summarizes the retention time, experimental and theoretical exact mass of $[M-H]^-$ and proposed empirical formula of the TPs and the relative mass errors. Most of the TPs were the products of hydrogen replacement by one or more chlorine molecules in the aromatic ring for all BPs (The structures and corresponding MS spectra of TPs are listed in S.I. 3), which was generally observed for phenolic chemicals [25,32,42].

The position of the substitution could not be exactly confirmed by HPLC-QToF experiment. However, based on the structures of the parent BPs as shown in Fig. 1, all of the studied transformable BPs contained phenolic hydroxyl groups. Considering the activity of phenolic hydroxyl groups in electrophilic substitution reactions, the most likely substitution positions could be the *ortho*- and *para*-carbons to the phenolic hydroxyl group [27,33,43]. For all BPs except ALI, mono-, di- or tri-substituted TPs were individually identified based on the electrophilic substitution. However, for ALI, two tri-substituted TPs formed, despite that only two active carbons were available to be substituted (Fig. 3). It was possible that some other reactions were involved, and the substitution position could not be confirmed. For AM, only one TP was detected and identified.

For UV-A and UV-D, besides chlorine substitution, one or two oxygen molecules were inserted according to the exact mass besides the electrophilic substitution. This may result from oxidation of the ketone group and formation of esters TPs [29,30]. For UV-D, the TP-2 may result from the integrated reaction of substitution, oxidation, hydrolysis and decarboxylation [29]. For ALI, the TP-5 may result from the combined reaction of substitution

and benzene ring-opening reaction [29]. For TP-1 and TP-2 of UV-G, besides chlorine substitution, a methoxyl group substitution was speculated to occur, but the position could not be confirmed. Additionally, the same reaction occurred in the chlorination of DHBP (TP-1). These substituted methoxyl group might result from methanol in the buffer solution [44,45].

3.3. Proposed transformation pathway

On account of the identified TPs, tentative reaction pathways for BPs were proposed in Fig. 3. Firstly, the BPs were converted into mono-, di- and tri-chlorinated substitution TPs by electrophilic substitution reaction for all BPs, which was very common in the chlorination of compounds containing phenolic hydroxyl groups [25,27,29,30,39]. Furthermore, the substituted TPs might undergo an oxidation reaction on the ketone group and transform into esters (TP-1 and TP-2 of UV-A, and TP-1 of UV-D), and the esters could be further oxidized to form di-carbonate TPs (TP-4 of UV-A) [29,30]. Immediately, the esters underwent hydrolysis and decarboxylation reaction, generating TPs of chlorinated phenols (TP-2 of UV-D). Meanwhile, ring cleavage reaction was also possible during the chlorination process (TP-6 of ALI). Moreover, methoxyl group substitution may be possible in the presence of methanol during the chlorination process (TP-1 of DHBP and TP-1, TP-2 of UV-G) [44,45].

3.4. Toxicity study

As demonstrated in Section 3.1, BPs containing phenolic hydroxyl group could be transformed in the presence of FAC and these TPs were stable. Since the toxicity of the TPs was unclear, tox-

Table 1
LC-Q-TOF scan data of the TPs and reaction type.

Compound	t(min)	[M-H] ⁺ experimental(amu)	Formula	[M-H] ⁺ exact mass ^a (amu)	Mass error(amu)	Relative mass error (e ⁶)	Reactiontype ^b	
DHBP	TP1	4.621	310.9893	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0015	4.9	Chl, Me-S
	TP2	6.658	247.0183	C ₁₃ H ₉ O ₃ Cl	247.0162	0.0021	8.5	Chl
	TP3	7.614	247.0186	C ₁₃ H ₉ O ₃ Cl	247.0162	0.0024	9.7	Chl
	TP4	9.095	280.9799	C ₁₃ H ₈ O ₃ Cl ₂	280.9772	0.0027	9.5	Chl
AM	TP1	10.102	294.9930	C ₁₄ H ₉ O ₃ Cl ₂	294.9929	0.0001	0.4	Chl
ALI	TP1	3.662	231.0241	C ₁₃ H ₉ O ₂ Cl	231.0213	0.0028	12.2	Chl
	TP2	4.395	264.9857	C ₁₃ H ₈ O ₂ Cl ₂	264.9823	0.0034	12.8	Chl
	TP3	5.317	298.9460	C ₁₃ H ₇ O ₂ Cl ₃	298.9433	0.0027	8.9	Chl
	TP4	5.542	298.9463	C ₁₃ H ₇ O ₂ Cl ₃	298.9433	0.0030	9.9	Chl
	TP5	6.918	320.9082	C ₁₂ H ₆ O ₂ Cl ₄	320.9044	0.0038	11.9	Chl, Ox,Hy, Cle
UV-A	TP1	5.693	356.9834	C ₁₄ H ₁₁ O ₇ SCl	356.9836	0.0002	0.6	Chl, Ox,
	TP2	8.573	390.9449	C ₁₄ H ₁₀ O ₇ SCl ₂	390.9446	0.0003	0.7	Chl, Ox,
	TP3	10.887	294.9933	C ₁₄ H ₁₀ O ₃ Cl ₂	294.9929	0.0004	1.4	Chl, De-S
	TP4	11.532	406.9404	C ₁₄ H ₁₀ O ₈ SCl ₂	406.9395	0.0009	2.2	Chl, Ox
	TP5	15.099	374.9496	C ₁₄ H ₁₀ O ₆ SCl ₂	374.9497	0.0001	0.3	Chl
UV-D	TP1	4.569	323.0324	C ₁₅ H ₁₃ O ₆ Cl	323.0322	0.0002	0.5	Chl, Ox
	TP2	9.095	190.9668	C ₇ H ₆ O ₂ Cl ₂	190.9667	0.0001	0.7	Chl,Hy, De-C
	TP3	12.228	307.0385	C ₁₅ H ₁₃ O ₅ Cl	307.0373	0.0012	3.8	Chl
	TP4	14.768	307.0381	C ₁₅ H ₁₃ O ₅ Cl	307.0373	0.0008	2.5	Chl
	TP5	16.074	340.9993	C ₁₅ H ₁₂ O ₅ Cl ₂	340.9984	0.0009	2.8	Chl
	TP6	16.996	340.9991	C ₁₅ H ₁₂ O ₅ Cl ₂	340.9984	0.0007	2.2	Chl
	TP7	19.259	340.9989	C ₁₅ H ₁₂ O ₅ Cl ₂	340.9984	0.0005	1.6	Chl
	TP8	21.73	374.9598	C ₁₅ H ₁₁ O ₅ Cl ₃	374.9594	0.0004	1.1	Chl
	TP1	6.187	307.0392	C ₁₅ H ₁₃ O ₅ Cl	307.0373	0.0019	6.1	Chl, Me-S
	TP2	7.805	307.0385	C ₁₅ H ₁₃ O ₅ Cl	307.0373	0.0012	3.8	Chl, Me-S
UV-G	TP3	8.45	277.0282	C ₁₄ H ₁₁ O ₄ Cl	277.0268	0.0014	5.2	Chl
	TP4	9.163	277.0284	C ₁₄ H ₁₁ O ₄ Cl	277.0268	0.0016	5.9	Chl
	TP5	9.737	310.9894	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0016	5.2	Chl
	TP6	11.009	277.0283	C ₁₄ H ₁₁ O ₄ Cl	277.0268	0.0015	5.5	Chl
	TP7	11.409	310.9897	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0019	6.1	Chl
	TP8	11.965	310.9896	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0018	5.8	Chl
	TP9	12.228	310.9897	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0019	6.1	Chl
	TP10	12.924	310.9897	C ₁₄ H ₁₀ O ₄ Cl ₂	310.9878	0.0019	6.1	Chl
	TP11	13.409	344.9507	C ₁₄ H ₉ O ₄ Cl ₃	344.9488	0.0019	5.5	Chl
	TP12	14.924	344.9507	C ₁₄ H ₉ O ₄ Cl ₃	344.9488	0.0019	5.5	Chl
	TP13	15.29	344.9510	C ₁₄ H ₉ O ₄ Cl ₃	344.9488	0.0022	6.3	Chl

^a mass calculated for ³⁵Cl.^b Chl, electrophilic substitution; Ox, oxidation; Hy,hydrolysis; De-C, decarboxylation; De-S, desulfonation;Me-S,methoxy group substitution; Cle, ring cleavage reaction.**Table 2**
Comparison of the inhibition rates between the TPs and parent compounds of BPs under different pH conditions.

	DHBP	AM	ALI	UV-A	UV-D	UV-G	KB-1	KB-2
pH = 6 ($\times 10^3$) ^a	0.9988	0.9981	0.6677	0.9979	0.4477	<0.1 ^b	<0.1	<0.1
RSD (n = 3, %)	0.20	0.07	1.27	0.19	10.32	–	–	–
pH = 7 ($\times 10$)	0.7041	0.6870	0.3280	<0.1	<0.1	<0.1	<0.1	<0.1
RSD (n = 3, %)	5.19	6.05	3.29	–	–	–	–	–
pH = 8 ($\times 40$)	0.8432	0.8951	0.9564	0.9306	0.7571	0.2439 ^c	0.9292	<0.1
RSD (n = 3, %)	1.17	0.30	0.50	0.43	1.53	31.36	0.76	–
parent compounds ^d	<0.1	0.2890	0.1507	<0.1	0.5895	<0.1	<0.1	–
RSD (n = 3, %)	–	15.48	29.97	–	2.73	–	–	–

^a ($\times 10$),The chlorination solutions were diluted 10 times by NaCl solution (30 g L⁻¹).^b When the inhibition rates were less than 0.1, the test results were inaccurate, so the data and related RSDs were not shown.^c The solution was diluted 10 times by NaCl solution (30 g L⁻¹).^d The parent BPs were diluted by PBS at the pH of 6 and then diluted 10 times by NaCl solution (30 g L⁻¹).

icity evaluation of the TPs could be of great importance for water safety. In this experiment, a marine bacteria, *P. phosphoreum*, was used for the toxicity assessment [34,35]. Table 2 illustrated the inhibition rate (15 min), which reflected the total toxicity of the solutions after chlorination under different pHs. The greater the inhibition rate was, the more toxicity the solution possessed. In the case of UV-G under pH=8 and UV-D under pH=6, the inhibition rates were relatively small and the toxicity results were not that accurate (RSD $\geq 10\%$). These results could only be used to reflect the trends of the toxicity. Under pH 6 and pH 7, the inhibition rates of KB-1 and KB-2 were weak (<0.1), the inhibition was mainly caused by the TPs. Compared to the parent BPs, the inhibition rate was greatly increased after chlorination except UV-D and UV-G, which indicated that some more toxic TPs of BPs

generated during the chlorination process. The toxicity-decreased phenomenon was only observed for UV-D. For UV-G, because of the weak inhibition, the toxicity change could not be determined by the present method. Furthermore, the solutions of DHBP, AM and UV-A under pH 6 almost inhibited the bacteria completely, which were largely higher than under pH 7 at the same conditions; this might result from the different composition of the TPs mixtures. Under pH 8, the KB-1 solution showed strong inhibition effect while KB-2 showed weak inhibition, which indicated that the inhibition effect was mainly caused by the addition of NaClO solution and possibly depended on the state of the NaClO. In this case, the real toxicity of the TPs could not be determined.

4. Conclusion

In this study, the kinetics, transformation pathways and total toxicity of several benzophenone-type UV filters (BPs) were studied. The second-order rate constants increased from 86.7 to 975 M⁻¹ s⁻¹ for oxybenzone, 49.6–261.7 M⁻¹ s⁻¹ for 4-hydroxybenzophenone, 51.7–540 M⁻¹ s⁻¹ for 2-hydroxy-4-methoxybenzophenone-5-sulfonic acid with the increasing pH values from 6 to 8. The transformation products (TPs) were identified, and the transformation pathways were proposed as chlorine substitution, oxidation, hydrolysis, benzene rings cleavage reaction and methoxyl substitution. Furthermore, the total toxicity evaluation of the TPs was assessed based on the bioluminescent bacteria experiment. The results of the toxicity experiment indicated that some more toxic TPs formed during chlorination process, and the toxicity of the TPs depended on the pH value of the solutions. These results indicated that extensive usage of BPs-containing products may cause environmental and human health's issues, and those products should be wisely used. For high BPs-containing water, the disinfection process should be optimized to degrade these compounds to a large degree; considering the possible more toxicity of TPs, the toxicity of the disinfected water should be monitored. Anyway, in the toxicity assessments, we only obtained the total toxicity information of TPs, and the individual toxicity of TP was still unknown. Some more toxicity assessments for individual TP, especially for those having more toxicity, need further study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhazmat.2016.02.059>.

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Altered UV absorbance and cytotoxicity of chlorinated sunscreen agents.

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Abstract

Sunscreens are widely utilized due to the adverse effects of ultraviolet (UV) radiation on human health. The safety of their active ingredients as well as that of any modified versions generated during use is thus of concern. Chlorine is used as a chemical disinfectant in swimming pools. Its reactivity suggests sunscreen components might be chlorinated, altering their absorptive and/or cytotoxic properties. To test this hypothesis, the UV-filters oxybenzone, dioxybenzone, and sulisobenzene were reacted with chlorinating agents and their UV spectra analyzed. In all cases, a decrease in UV absorbance was observed. Given that chlorinated compounds can be cytotoxic, the effect of modified UV-filters on cell viability was examined. Chlorinated oxybenzone and dioxybenzone caused significantly more cell death than unchlorinated controls. In contrast, chlorination of sulisobenzene actually reduced cytotoxicity of the parent compound. Exposing a commercially available sunscreen product to chlorine also resulted in decreased UV absorbance, loss of UV protection, and enhanced cytotoxicity. These observations show chlorination of sunscreen active ingredients can dramatically decrease UV absorption and generate derivatives with altered biological properties.

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Reaction of benzophenone UV filters in the presence of aqueous chlorine: kinetics and chloroform formation.

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Abstract

The transformation of two benzophenone UV filters (Oxybenzone and Dioxybenzone) was examined over the pH range 6-11 in the presence of excess aqueous chlorine. Under these conditions, both UV filters were rapidly transformed by aqueous chlorine just above circumneutral pH while transformation rates were significantly lower near the extremes of the pH range investigated. Observed first-order rate coefficients ($k(\text{obs})$) were obtained at each pH for aqueous chlorine concentrations ranging from 10 to 75 μM . The $k(\text{obs})$ were used to determine the apparent second-order rate coefficient ($k(\text{app})$) at each pH investigated as well as determine the reaction order of aqueous chlorine with each UV filter. The reaction of aqueous chlorine with either UV filter was found to be an overall second-order reaction, first-order with respect to each reactant. Assuming elemental stoichiometry described the reaction between aqueous chlorine and each UV filter, models were developed to determine intrinsic rate coefficients ($k(\text{int})$) from the $k(\text{app})$ as a function of pH for both UV filters. The rate coefficients for the reaction of HOCl with 3-methoxyphenol moieties of oxybenzone (OXY) and dioxybenzone (DiOXY) were $k(1,\text{OxY}) = 306 \pm 81 \text{ M}^{-1}\text{s}^{-1}$ and $k(1,\text{DiOxY}) = 154 \pm 76 \text{ M}^{-1}\text{s}^{-1}$, respectively. The $k(\text{int})$ for the reaction of aqueous chlorine with the 3-methoxyphenolate forms were orders of magnitude greater than the un-ionized species, $k(2,\text{OxY}) = 1.03(\pm 0.52) \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k(2_1,\text{DiOxY}) = 4.14(\pm 0.68) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. Also, $k(\text{int})$ for the reaction of aqueous chlorine with the DiOXY ortho-substituted phenolate moiety was $k(2_2,\text{DiOxY}) = 2.17(\pm 0.30) \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. Finally, chloroform formation potential for OXY and DiOXY was assessed over the pH range 6-10. While chloroform formation decreased as pH increased for OXY, chloroform formation increased as pH increased from 6 to 10 for DiOXY. Ultimate molar yields of chloroform per mole of UV filter were pH dependent; however, chloroform to UV filter molar yields at pH 8 were 0.221 CHCl_3/OXY and 0.212 $\text{CHCl}_3/\text{DiOXY}$.