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A critical assessment of the estrogenic potency of benzyl salicylate

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ABSTRACT

Benzyl salicylate (BS) is a natural ingredient of essential oils and a widely used fragrance chemical. A number of *in vitro* screening studies have evaluated the estrogenic potential of BS with ambiguous results. Lack of dose-response information for the positive control 17β-estradiol (E2) in most studies makes an assessment of the relative potency and efficacy challenging. Notwithstanding this difficulty, BS has been added as the only fragrance ingredient to the list of the first 14 substances to be screened as potential endocrine disruptors by the European Scientific Committee for Consumer Safety (SCCS) and it is included in the Community rolling action plan (CoRAP) of the European REACH regulation to be assessed for the same property. Here we review all literature evidence and present new data to quantify the *in vitro* potency and efficacy of BS vs. E2 with full dose response analysis in both an estrogen response element (ERE) depending reporter gene assay and in the MCF7 cell proliferation (E-screen) assay. In both assays, very similar results for BS were found. BS is a partial agonist exhibiting 35–47 % maximal efficacy and it is active only close to the cytotoxic concentration. The extrapolated concentration to achieve 50 % efficacy is 21′000′000 higher as compared to E2 in the reporter gene assay. A ca. 36′000′000 higher concentration of BS as compared to E2 is required to reach equivalent partial cell proliferation stimulation in the MCF7 proliferation assay. This potency is significantly below the agonistic activity of known chemicals which cause estrogenic effects in *in vivo* assays. Importantly, in this study the weak agonistic activity is for the first time directly related to the activity of E2 in a full quantitative comparison in human cell lines which may help ongoing evaluations of BS by regulatory bodies.

1. Introduction

Benzyl salicylate (BS; benzyl 2-hydroxybenzoate) is a natural ingredient of essential oils such as Jasmin and Ylang Ylang. It is manufactured at high tonnage as a widely used synthetic fragrance chemical. A number of screening studies have evaluated *in vitro* the estrogenic potential of BS in different assays [1–7]. These various studies came to different results which may be partly attributed to the different test systems and the inconsistent use of positive controls. Especially the lack of dose-response information for the positive control 17β-estradiol (E2) in the studies with human cells [1,2] makes an assessment of the relative potency and efficacy challenging. Notwithstanding this difficulty, BS has been added, based on these *in vitro* results, as the only fragrance ingredient to the list of the first 14 substances to be screened with priority as potential endocrine disruptors by the European Scientific Committee for Consumer Safety (SCCS). In addition, BS was added to the CoRAP (Community Rolling Action Plan) of the European REACH regulation to be assessed for the same property.

On the other hand, there is no evidence from OECD test guideline (TG) studies which had to be conducted under the REACH regulation, that BS has any effects on estrogen-sensitive endpoints *in vivo*. BS was tested up to a dose of 2500 ppm in the diet (158–324 mg/kg bw/d depending on the phase of the study) in an OECD TG 421 reproductive/developmental toxicity screening study in rats [8]. This test dose represents the maximal tolerable dose. The no observed adverse effect level (NOAEL) was 2500 ppm for all endpoints evaluated in the TG 421

Abbreviations: ATCC, American Type Culture Collection; BPA, Bisphenol A; BS, Benzyl salicylate; CAT, chloramphenicol acetyl transferase gene; CoRAP, Community Rolling Action Plan; DMSO, Dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle Medium; E2, 17β-estradiol; ERE, estrogen response element; ER, estrogen receptor; FBS, foetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-*N*-ethanesulfonic acid; HRPT, Human Relevant Potency Threshold; MoA, Mode of action; 4−OHT, 4-hydroxy-tamoxifen; OECD, Organisation of Economic Co-operation and Development; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; SCCS, European Scientific Committee for Consumer Safety: SRB, sulforhodamine B; TG, test guideline; YES, Yeast Estrogen screen; NOAEL, no observed adverse effect level.

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study, since BS had no effects in the F_0 generation on male and female mating and fertility, male copulation and female conception indices, estrous cycle lengths, mean number of days between pairing and coitus, gestation lengths, and the process of parturition. There were no test substance-related effects on mean number of pups born, pup survival, live litter size, mean sex ratio, anogenital distance, areolae/nipple anlagen (males only), thyroid hormone levels (total T4) and thyroid weights in the F_1 generation. In addition, there were no clinical observations or necropsy findings in the F_1 generation that could be attributed to F_0 maternal administration of BS. Thus this study provided no evidence of effects on estrogen-sensitive endpoints *in vivo*. A higher concentration of 6000 ppm in the diet in a 90 day repeated dose study (OECD TG 408) led to reduced food consumption and reduced body weight [8]. However, in this 90 day study [8], no effects of BS on absolute uterus weight was recorded up to a dose of 6000 ppm in the diet (429 mg/kg bw/d).

The only reported *in vivo* evidence for an estrogenic effect of BS comes from a study from Zhang et al. [7] using the uterotrophic assay in both mice and rats. In rats, an increase of uterine weight was observed for BS at all doses tested (1.23–100 mg/kg bw/d). However there was no clear dose-response with an increase at all doses to 115 % – 135 % of the control values, while E2 led to a dose dependent increase to up to 375 % at the highest concentration (0.4 mg/kg bw/d). Similarly, in mice E2 led to a dose-dependent increase of uterine weight of 117 % – 478 % of the control values, while the effect of BS was at 114 %–119 % of control values in most tested doses (11, 33, and 300 mg/kg bw/d) except for a higher effect (138 %) at a single dose of 100 mg/kg bw/d, but without apparent dose-response. This low efficacy compared to the positive control and an unclear dose-response relationship across a wide range of doses in this study makes data interpretation difficult. Given the lack of effects at 158–324 mg/kg bw/d on reproductive parameters in the OECD TG 421 study and the lack of effects on the uterus weight up to high doses in a 90 day study questions the significance of the effects of BS observed by Zhang et al.

Here we review all literature evidence and present new data to quantify the potency and efficacy of BS vs. E2 with full dose response analysis in both an estrogen response element (ERE) depending reporter gene *in vitro* assay and in the MCF7 cell proliferation (E-screen) *in vitro* assay.

2. Materials and methods

2.1. Test chemicals

BS of commercial fragrance grade (>99 % purity based on GC-analysis) from Givaudan International SA was used. Estradiol (E2) (E2758, 99 % purity), Bisphenol A (BPA) (239658, 99.8 % purity) and (Z)-4-Hydroxytamoxifen (H7904, 99.75 % purity) were sourced from Sigma-Aldrich.

2.2. T47D-KBluc reporter gene assay

The T47D-KBluc cell line was initially developed by the US Environmental Protection Agency [9] and was purchased from ATCC (ATCC CRL-2865). It has a very similar setup as the VM7Luc4E2 assay described in OECD TG 455 [10] The T47D-KBluc cells contain the endogenously expressed estrogen receptors ERα and ERβ as well as an estrogen response element (ERE) linked to a luciferase gene. Cells were grown at 37 °C and 5% CO_2 in RPMI-1640 containing phenol red (Gibco; A1049101) which was supplemented with 9% heat inactivated fetal bovine serum (FBS; ThermoFisher Scientific, 10270), Penicillin/Streptomycin (final concentration 100 U/mL; ThermoFisher Scientific, 15140−122) and 0.75 μL/mL Insulin (10 mg/mL sol. in HEPES ~0.2 U/mL; Sigma-Aldrich; I0516) (growth medium). The cells were seeded at 20′000 cells per well in 96-well plates in growth medium. After 24 h, the medium was changed to pre-test medium (same as growth medium, but without antibiotics and with charcoal-stripped FBS (9%; ThermoFisher Scientific, 12676−029) in order to avoid background activation of the receptor). 48 h after seeding, the medium was changed to test medium (same as pre-test medium, but with only 5% charcoal-stripped FBS) containing the test chemicals dissolved and diluted in DMSO (final concentration of 1% DMSO). Twelve dilutions of the test chemicals were tested in triplicate and in three independent experiments. After 24 h incubation in presence of the test compounds, cell viability was measured with the PrestoBlue® assay (Invitrogen, A13262). The medium was aspirated and 100 μL of PrestoBlue® reagent diluted 10-fold in DPBS (ThermoFisher Scientific, 14200−083) was added to each well. Plates were incubated for 30 min at 37 °C and 5 % CO_2 and the fluorescence was determined at 560 excitation and 590 nm emission. The supernatant was then removed, and the same cells were subsequently lysed with Passive Lysis Buffer (Promega, E1941) and luciferase activity was determined (Biotek, synergy H1 luminometer with automatic injection of 50 μL of the luciferase substrate to each well and integration of the luciferase activity for 2 s). E2 was tested as positive control in each assay plate in a full dose response analysis (2.4×10^{-13} – 5×10^{-7} M) to determine potency and 100 % efficacy. The T47D-KBluc reporter gene assay was subjected to an internal validation with performance standards from OECD TG 455, and results of this internal validation are shown in the Supporting information, (Table S2 and Figure S4).

2.3. MCF7 cell proliferation assay

MCF7 cells (ECACC 86012803) were obtained from Sigma-Aldrich and the MCF7 BUS cell line used for the E-Screen [11] from the Laboratory of A. Soto. MCF7 ECACC 86012803 cells were grown at 37 °C and 5 % CO_2 in DMEM medium with phenol red (ThermoFisher Scientific, 22320−022) supplemented with 9 % heat inactivated FBS and 10 μg/mL Gentamicin (Sigma-Aldrich, G1272). The MCF7 BUS cells were grown at 37 °C and 5 % CO_2 in DMEM medium with phenol red (ThermoFisher Scientific, 11965−092) supplemented with 5 % heat inactivated FBS and Penicillin/Streptomycin.

Sub-confluent cells (both MCF7 cell lines) were harvested and seeded in assay medium (DMEM without phenol red (ThermoFisher Scientific, 11880−028), supplemented with 9 % charcoal stripped FBS and Gentamicin (10 μg/mL) at a density of 5000 cells/well in 96-well plates. 24 h after cell seeding, the medium was replaced with assay medium containing eleven different dilutions of the test chemicals which were diluted in DMSO (final DMSO concentration of 0.5 %). Cells were incubated for 6 days and the cell yield was determined with the PrestoBlue® cell viability assay as described above. Cell numbers from each treatment (six replicates) were normalized to the average of solvent control cultures to correct for differences in the initial seeding density and to calculate fold-induction of cell proliferation vs. solvent control. Two independent experiments were done with the ECACC 86012803 cells and two independent experiments with the MCF7 BUS cells.

For deriving EC50 values and equipotent concentrations, dose-response curves were fitted with GraphPad Prism 6, using the “log (concentration) vs. normalized response - variable slope algorithm”, after normalizing the data to 100 % efficacy for E2.

3. Results

3.1. Literature review on yeast and human cell line reporter gene assays with BS

All identified studies which screened BS for estrogenic activity in reporter gene assays are summarized in Table 1. In a first study by Miller et al. 2001 [12], using the yeast YES assay with the recombinant human estrogen receptor α, a submaximal induction with 15 % efficacy relative to E2 was observed. This submaximal effect occurred at 5×10^{-5} M, while the positive control E2 had an EC50 of 2×10^{-10} M. The EC50

Table 1
Effects of BS in published reports using reporter gene assays.

Study	Assay details	Effect of BS	Positive control	Effect of positive control	Activity of BS vs. E2
Miller et al., [12]	YES assay	Submaximal induction, ca. 15 % efficacy vs. E2 at 5 × 10^{-5} M	E2	EC50 2 × 10^{-10} M	BS 600′000-fold weaker than E2; E2 EC50 36-fold higher than in human cell assays
Kunz et al. [4,5]	YES assay with estrogen α receptor	Submaximal induction, ca. 12 % efficacy vs. E2 at 10^{-3} M; EC50 rel. to this efficacy 1.66 × 10^{-4} M	E2	EC50 2.6 × 10^{-10} M	BS reported to be 860′000-fold weaker than E2 based on EC50
Charles and Darbre [1]	Estrogen-response-element (ERE) upstream of CAT gene in MCF7 cells containing endogenous ER	Submaximal induction, ca. 60 % efficacy vs. E2 at 2 × 10^{-4} M EC50 rel. to 60% efficacy 10^{-4} M; no effect at 5 × 10^{-5} M	E2	Only tested at 10^{-8} M, i.e. 2000-fold > EC50 of E2	No direct comparison to E2 possible
Tox21 [14]	BG1 ER-luc cell assay (luciferase gene under control of ERα receptor)	6 separate tests on 2 separate samples: sample 259418 inconclusive agonist in 3 experiments with 31 % efficacy; sample 256928 active agonist in 3 experiments with 36 % efficacy	E2	100 % efficacy at minimal test conc. of 1.18 × 10^{-9} M, i.e. 200-fold > EC50 of E2	No direct comparison to E2 possible
Tox21 [14]	ER-α -BLA HEK293 cell-based assay with β-lactamase gene under control of ERα receptor	2 × 9 separate tests on 2 separate samples: All 18 experiments concluded that BS is inactive (average efficacy < 3%)	E2	100 % efficacy at minimal test conc. of 9.8 × 10^{-10} M, i.e. 200-fold > EC50 of E2	BS not active
Present study	T47D-KBluc cell-based assay with luciferase gene under control of ER receptor	3 independent experiments in triplicate each; maximal efficacy 47 %; extrapolated EC50 of 7.1 × 10^{-5} M	E2	EC50 3.3 × 10^{-12} M	BS partial agonist; potency 21′000′000- fold below E2

value of E2 is ca. 36-times higher as compared to typical EC50 values of E2 in human cell line assays [10] and the lower potency of E2 in the yeast-based YES assay was also observed by other authors [13]. Kunz et al. 2006 reported a maximal efficacy of 12 % for BS in the YES assay and an EC50 of 1.7 × 10^{-4} M [4,5]. The EC50 of the positive control E2 was 2.6 × 10^{-10} M which is similar to the one reported by Miller et al.

The first study using human cells was reported by Charles and Darbre 2009 [1] with a modified MCF7 cell line containing an endogenously expressed estrogen receptor and a stably integrated Estrogen-response-element (ERE) upstream of a chloramphenicol acetyl transferase gene (CAT). Submaximal induction of the reporter gene with ca. 60 % maximal efficacy compared to E2 was found at 2 × 10^{-4} M. Relative to this 60 % efficacy, an EC50 of around 10^{-4} M can be estimated based on the graphical data in the publication and no effect was observed at 5 × 10^{-5} M. E2 as positive control was tested, but only at a single dose of 10^{-8} M, i.e. at a saturation dose which is more than three orders of magnitude above the typical EC50 of E2 [10]. Thus no potency comparison to E2 can be made from this study.

In the large Tox21 screening studies [14] two different batches of BS were tested on the BG1 ER-luc cell line assay (referred to as VM7Luc4E2 in OECD TG 455) with a luciferase gene under control of the ERα receptor. For the two samples, 31 % and 36 % maximal efficacy was found vs. E2 at the highest test concentration of 9.2 × 10^{-5} M (Figure S2 in Supporting information). No direct comparison to E2 is possible, as the Tox21 screening tested E2 at a minimal concentration of 1.18 × 10^{-9} M which is 200-fold above the EC50 of 5.6 × 10^{-12} M for the VM7Luc ER TA assay reported in OECD TG 455 [10]. In parallel, the ER-alpha-BLA HEK293 cell-based assay with the β-lactamase gene under control of the human ERα-receptor was used in Tox21. No activity was found for BS in 18 repeated experiments conducted with the two BS batches up to a maximal test concentration of 7.6 × 10^{-5} M. Based on these contradictory data, BS is rated as an inconclusive agonist by Tox21 [14].

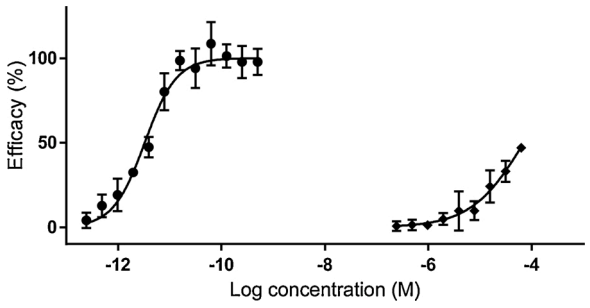

Fig. 1. Potency and efficacy of BS in comparison to E2 in the T47D-KBluc reporter gene assay. E2 closed circles; BS, closed diamonds. Note the logarithmic scale on the x-axis. Average and standard deviation of three independent experiments, each conducted in triplicate.

In summary, these literature studies indicate that BS is at most a partial agonist, with maximal efficacy observed in different studies between 12 % and 60 %. The most active dose is around 1–2 × 10^{-4} M. None of the studies in human cells measured the full dose-response of E2 and therefore potency comparisons cannot be made within the studies and only in reference to literature data.

3.2. Full dose response analysis of BS in the T47D-KBluc reporter cell line assay

To fill the data gap from this literature analysis and to get an accurate estimate of the potency and efficacy of BS in comparison to E2, we conducted three independent experiments with the T47D-KBluc reporter gene assay. Fig. 1 shows the results for all tested concentrations in the non-cytotoxic range as analyzed with GraphPad Prism. BS is cytotoxic at ≥1.25 × 10^{-4} M with a reduction of cell viability by >80 % (Supporting information, Figure S1). At 6.25 × 10^{-5} M a maximal efficacy of 47 % for luciferase induction compared to E2 is observed. Extrapolated, this gives an estimated EC50 of 7.1 × 10^{-5} M. A full sigmoidal dose-response curve over the tested range was obrserved with E2 and the derived EC50 is 3.3 × 10^{-12} M, which is similar to the value of 5.6 × 10^{-12} M in the VM7Luc ER TA assay reported in OECD TG 455 [10]. Thus, the potency of BS in this test is around 21′000′000-fold lower as compared to E2, and it is only a partial agonist close to cytotoxic concentrations, confirming literature reports in respect to efficacy.

The dependence of the observed luciferase induction on the estrogen receptor was additionally investigated in experiments conducted with a simultaneous exposure of the cells to the ER antagonist 4-hydroxytamoxifen (4−OHT; 1 μM) [15]. The effects of both E2 and BS were suppressed by 4−OHT, indicating that the induction of the luciferase by BS is indeed also ER-dependent (Fig. 2).

3.3. Literature review of MCF7 cell proliferation assays with BS

Three studies tested the effect of BS on proliferation of estrogen-dependent MCF7 cells (E-Screen) (Table 2). Jimenez-Diaz et al. [3]

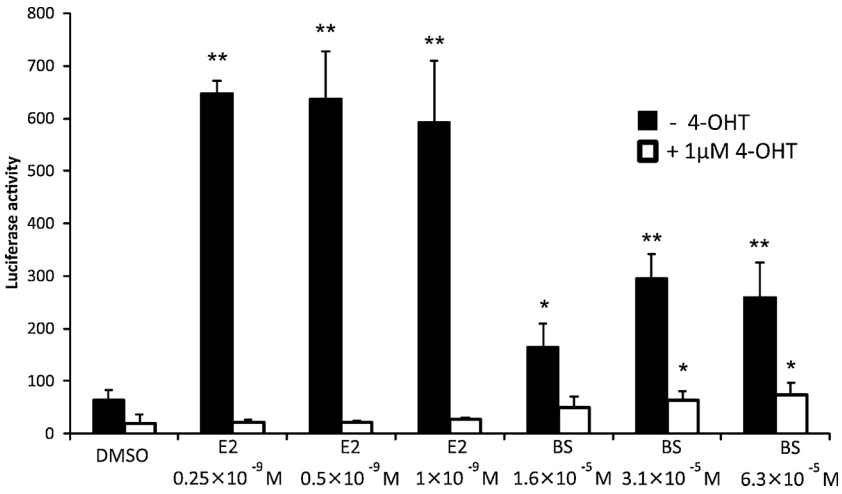

Fig. 2. E2- or BS-induced luciferase signal in presence of the estrogen receptor antagonist 4-hydroxy-tamoxifen (4−OHT). T47D-KBluc were stimulated with E2 or BS in presence (open bars) or absence (closed bars) of 1 μM 4−OHT. Note: Doses of E2 used in this experiment are 50 – 100 fold above the concentration required for maximal induction. Average of three independent experiments conducted in triplicate. Each treatment was compared to the corresponding DMSO control with a *T*-test. Statistical differences are indicated with * ($p < 0.05$) and ** ($p < 0.01$).

found no BS-induced cell proliferation up to a concentration of 1×10^{-5} Hashimoto et al. [2] reported ca. 240 % cell proliferation as compared to the negative control in presence of BS at 1×10^{-5} M, a decreased proliferation compared to the solvent control at 1×10^{-4} M and no increase of proliferation at lower concentrations (1×10^{-6} M to 1×10^{-9} M). No positive control was included in the study. Therefore the data could not be further evaluated, neither for potency nor efficacy. Charles and Darbre [1] studied growth stimulation of MCF7 cells over 7 days, and found that E2 led to 5.23 cell divisions, while BS induced 3.2 divisions at 8×10^{-5} M as compared to 1.8 divisions in the non-exposed control treatment. This yields a final increase of cell yield compared to the control treatment for E2 of 10-fold and 2.7-fold for BS. No effect was observed for BS at 5×10^{-6} M, similar to the results of Jimenez-Diaz et al., but contradicting the results of Hashimoto et al. As for the experiments with the reporter cell line assay in the same publication, E2 was only tested at a single dose of 10^{-8} M, i.e. more than three orders of magnitude above the typical EC50 of E2 [10] and thus no potency comparison can be made from this study [1].

In summary, two studies found no effects at 10^{-5} M on the proliferation of MCF7 cells, one study noted effects at this concentration while contradicting results were found at 10^{-4} M. None of these studies allows for a direct comparison with E2 as the only study testing E2 in a dose-response manner [3] found no BS-induced cell proliferation.

3.4. Full dose response analysis of BS in the MCF7 cell proliferation assay

In this study, BS was tested in direct comparison to E2 and Bisphenol A (BPA) in the MCF7 cell proliferation assay with the MCF7 BUS cell line and in addition in two repetitions with the MCF7 ECACC 86012803 cell line. It had been reported that the MCF7 BUS cell line showed the highest proliferative response to E2 as compared to other MCF7 lines [16,17]. BS was active as a very weak partial agonist and, similar to the T47D-KBluc reporter gene assay, again close to cytotoxic levels only. The maximal BS-induced cell proliferation was, on the average of four independent experiments, around 2.7-fold higher compared to the non-exposed control cells, while E2 reached, on the average, a maximal induction of 7.2-fold which is in the range observed in the literature [17]. The maximal cell proliferation in the MCF7 BUS cell line was similar to the proliferation of the commercial MCF7 ECACC 86012803 cell line. A representative experiment with the MCF7 BUS cell line is shown in Fig. 3. All four independent experiments are summarized in Figure S3 and Table S1 in the Supporting information. To directly compare the potency of BS to E2, we assessed the equipotent dose of E2 needed to reach the maximal proliferation reached by BS (Table S1). On average 3.6×10^{-12} M E2 led to an equipotent effect after 6 days compared to the maximal proliferation induced by BS at 1×10^{-4} M (average of the four independent experiments). Thus the average difference between the two chemicals in potency assessed is 36′000′000 fold. These results partly confirm the literature reports indicating a very weak proliferation effect at 10^{-4} M, but not at 10^{-5} M.

BPA was tested as additional control. BPA is a full agonist giving similar maximal cell yield compared to E2, with an EC50 of ca. 3.4×10^{-7} M, i.e. of a potency ca. 30′000 fold below E2 in agreement with the data of Andersen et al. [18]. Cell proliferation of the MCF7 cell lines was completely inhibited for E2 and BS in presence of the E2 antagonist 4−OHT (Fig. 3).

Table 2
Effects of BS reported in the MCF7 cell proliferation assay (E-screen).

Study	Assay details	Effect of BS	Positive control	Effect of positive control	Activity of BS vs. E2
Jimenez-Diaz et al., [3]	6 d MCF7 proliferation assay, SRB[a] staining, colorimetric	No cell proliferation up to 1×10^{-5} M	E2	Data not shown (E2 tested at tested at 10^{-13} – 10^{-9} M)	No estrogenic activity up to 1×10^{-5} M
Hashimoto et al., [2]	6 d MCF7 proliferation assay, SRB staining, colorimetric	Cell proliferation (ca. 240 % of neg. control) at 1×10^{-5} M; no proliferation at 1×10^{-4} M (slight cytotoxicity) and at 1×10^{-6} - 1×10^{-9} M	None	Not tested	Data only given as graph, only one concentration per order of magnitude, no positive control
Charles and Darbre [1]	7 d MCF7 proliferation assay, cell nuclei count	1.8 doublings in control, 5.23 doublings with E2 at 10^{-8} M, 3.2 doublings with 8×10^{-5} M BS; (i.e. BS: 270 % cells of control;) LOAEC ca. 2×10^{-5} M	E2	Only tested at 10^{-8} M, i.e. almost 2000-fold above the EC50 of E2 (E2: 1080 % cells of control)	No direct comparison to E2 possible
Present study	6 d MCF7 proliferation assay, Cell yield by PrestoBlue®	4 independent experiments each in six replicates. Maximal efficacy ca. 28 % at 1×10^{-4} M	E2	EC50 ca. 1.2×10^{-11} M	BS partial agonist; potency for equal proliferation ca. 36′000′000- fold below E2

[a] SRB, sulforhodamine B.

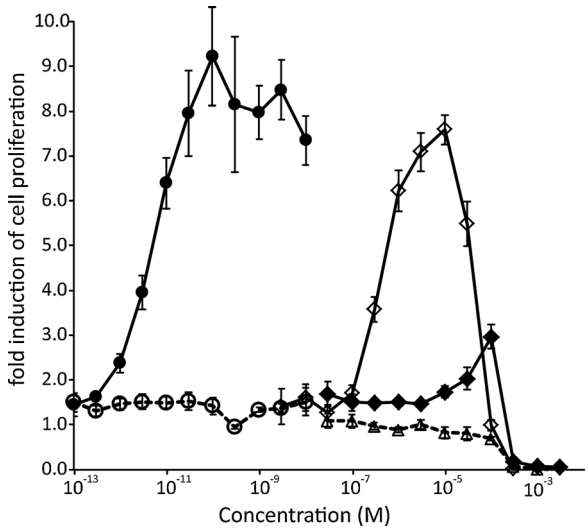

Fig. 3. Potency and efficacy of BS in comparison to E2 and BPA in the MCF7 BUS cell proliferation assay. E2, closed circles; BPA, open diamonds; BS, closed diamonds. Data are shown as fold-induction of cell yield as compared to control cells treated with solvent only. In parallel, cells were treated with E2 and 1 μM 4−OHT (open circles) or BS and 1 μM 4−OHT (open triangles). Cells were seeded at an initial density of 5000 cells per well and incubated in presence of test chemicals for 6 days. Shown is fold-induction of cell proliferation assessed with the PrestoBlue® cell viability assay as compared to untreated cells. Results from one representative experiment in the MCF7 BUS cell line conducted in six replicates for each concentration and each chemical is shown. Results of three additional repetitions are shown in the Supporting information (Figure S3).

4. Discussion

This study compares the *in vitro* potency of BS directly to E2 on the estrogen receptor in human cell lines expressing endogenously ERα and ERβ. BS is a very weak partial agonist close to cytotoxic levels in the T47D-KBluc reporter gene assay and the MCF7 cell proliferation assay. The potency is over seven orders of magnitude lower as compared to E2 in both assays. This very low potency and the partial agonism only make it rather unlikely, that BS exerts any estrogen-receptor dependent effect *in vivo*.

Lately Borgert et al. [19] proposed a Human Relevant Potency Threshold (HRPT) for chemicals acting on the estrogen receptor. This approach is based on a comparison of *in vitro* potency with *in vivo* effects in either uterotrophic assays or from human clinical evidence. This analysis is partly based on different dietary phytoestrogens which have intermediate potency *in vitro*, and different potencies in animal tests and in humans. Some of the phytoestrogens with higher activities have some proven beneficial effects based on a supposed estrogen MoA *in vivo* when taken at high-dose as a standardized food supplement [19]. Borgert et al. proposed that this HRPT threshold is 10^4 – fold below the potency of E2. Our data indicate that the potency of BS is > 1000-fold below this putative HRPT (Fig. 4).

Chemicals which are positive in the *in vivo* uterotrophic assay typically have a clearly higher potency as compared to the potency of BS *in vitro*. BPA has an EC50 of 5.33×10^{-7} M according to OECD TG 455 and we found a similar EC50 in the comparable T47D-KBluc assay (3.3×10^{-7} M, Table S2). BPA is a full agonist and has a >200-fold higher potency *in vitro* than BS, whereas the typical dose for effects observed in the uterotropic assay is 10–100 mg/kg/d [20]. The estrogenic soy isoflavone genistein widely present in the human diet has a 2000-fold higher potency in the T47D-KBluc assay as compared to BS and is typically positive in the uterotrophic assay at 10 mg/kg/d [20]. These two examples indicate that an *in vivo* effect would not be expected below an unrealistic dose of 2–20 g/kg/d. This also indicates that the very weak but statistically significant effect observed in the rat study by Zhang et al. [7] at 3.7–100 mg/kg bw/d is unlikely to be caused by the estrogenic mode-of action of BS due to the low potency of BS. Furthermore, BS is an ester which is rapidly hydrolyzed by carboxyl esterases in microsomes isolated from different tissues, with highest activities in microsomes isolated from the intestine and the liver [21]. Thus actual exposure to the parent molecule in an *in vivo* situation will be significantly reduced by first-pass metabolism in the intestine and the liver as compared to the *in vitro* situation, which will further decrease potency of BS *in vivo*. This has also been observed for phthalate esters which have an EC50 of 2–6 × 10^{-6} M in reporter gene assays. Although they are >10-times more potent than BS *in vitro*, phthalate esters are still negative in uterotrophic assays [20]. These quantitative comparisons further indicate that any reproducible ER-mediated effects *in vivo* even by testing BS at high concentrations in animal studies are not to be expected, which is confirmed by negative findings from the guideline studies conducted under the REACH regulation [8]. BS is mainly used at low concentrations in topical products containing fragrances, thus consumer exposure is by repeated dermal exposure. Possible effects upon repeated application had been studies in the two guideline studies (OECD TG 421 and 408) and no indications for effects on estrogen-sensitive endpoints had been found *in vivo* also by repeated application [8].

The published studies found inconsistent *in vitro* results, especially when using the MCF7 cell proliferation assay. One reason could be that the study by Hashimoto [2] used only one test concentration per order of magnitude or that a lower maximal test concentration was used [3]. Most importantly, the positive control E2 was often tested at a concentration far above the EC50, which makes potency comparisons to BS in these studies difficult, a data gap which we can fill with the data presented in this report.

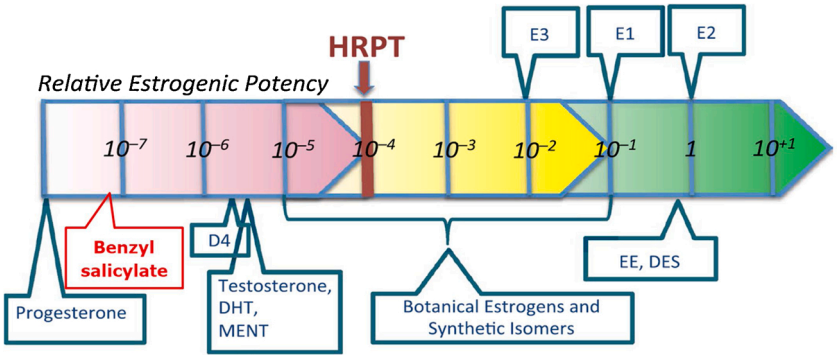

Fig. 4. Potency of BS vs. the human relevant potency threshold (HRPT) estimated by Borgert et al. (Figure modified from [19]). Potency of BS is over three orders of magnitude below the HRPT and also orders of magnitude below the typical botanical estrogens widely found in human nutrition.

Author statement

Andreas Natsch and Heike Laue reviewed the literature, designed the study and wrote the manuscript. Lu Hostettler and Tina Haupt performed and evaluated the experiments.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. All authors are employees of Givaudan, a company manufacturing and using benzyl salicylate. The authors receive no benefits except their salary for conducting this research.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.05.001.

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