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SCIENTIFIC OPINION

Genotoxicity of beauvericin

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Abstract

The European Commission (EC) asked EFSA to assess the genotoxicity of beauvericin (BEA). Relevant information, including that which has become available since the 2014 Scientific Opinion on the risks to human and animal health related to the presence of BEA and enniatins in food and feed, was reviewed. In the previous Opinion the Panel concluded that in vitro genotoxicity data were equivocal and there were no in vivo genotoxicity data available. New in vitro studies in mammalian cell lines provided no convincing evidence for induction of chromosomal damage by BEA as measured by micronucleus and chromosome aberration tests or an increase of DNA strand breaks as assessed by the Comet assay. In these studies, no concentration-dependent effects or potential for interference from associated cytotoxicity were observed. In addition, DNA double-strand breaks as measured by γ-H2AX analysis were only observed following exposure to highly cytotoxic BEA concentrations. In vivo studies (Comet and Pig-a assays, micronucleus test) with BEA were negative. In vitro gene expression studies showed no indication of a DNA damage response and (quantitative) structure activity relationship analysis was also not indicative of genotoxic potential. Some effects of BEA might play an indirect role in the formation of DNA strand breaks. These include increased reactive oxygen species, induction of cell cycle arrest and apoptosis, associated with interference in mitochondrial function and cell signalling. There was no compelling evidence of inflammatory and immunosuppressive effects. Taken together, the available data indicate that BEA is devoid of genotoxic potential.

KEYWORDS

Beauvericin, genotoxicity

CONTENTS

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

1.1.1 | Background

The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) adopted in 2014 a Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed.[1] The CONTAM Panel concluded that there might be a concern with respect to chronic exposure, but no firm conclusion could be drawn as relevant in vivo toxicity studies were not available to perform a human risk assessment. Therefore, the CONTAM Panel recommended to perform a study investigating possible health effects likely to arise from repeated exposure (i.e. 90-day study), including effects on the nervous, immune and endocrine systems, as well as screening of possible effects on reproduction and development and highlighted the need for additional in vitro and in vivo genotoxicity data.

Following a grant agreement from EFSA, a study was performed on the "In vivo toxicity and genotoxicity of beauvericin and enniatins. Combined approach to study in vivo toxicity and genotoxicity of mycotoxins beauvericin (BEA) and enniatin B (ENNB)[2]".

The outcome was discussed at the 128th,[3] 131st[4] and 132nd[5] plenary meetings of the CONTAM Panel. The CONTAM Panel concluded that it was appropriate to conduct a more in-depth analysis of the new information available, to assess the need to update the 2014 Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. It was considered that the data for a chronic toxicity assessment were probably insufficient but sufficient for a genotoxicity assessment.

It is appropriate that EFSA based on these new scientific developments re-assesses the genotoxicity of beauvericin and assesses the need to update the Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed.

1.1.2 | Terms of Reference

In accordance with Art. 29 (1) (a) of Regulation (EC) No 178/2002,[6] the Commission asks EFSA for an assessment of the genotoxicity of beauvericin based on new information available and to assess the need to update, as regards the genotoxicity of beauvericin, the 2014 Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed.

1.2 | Additional information

1.2.1 | Chemistry and analytical methods

Beauvericin (BEA, CAS: 26048-05-5) is a cyclic hexadepsipeptide that consists of alternating D-α-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methylphenylalanine moieties. The empiric formula for BEA is $C_{45}H_{57}N_3O_9$. It has a molecular weight of 784 g/mol and a melting point of 93–97°C. Its chemical structure is depicted in Figure 1 below. Determination of BEA and enniatins (ENN) is carried out mostly by liquid chromatography (LC) with ultraviolet (UV) or (multi-stage) mass spectrometry (MS, MS/MS) detection. For more detailed information about chemistry and information about analytical methods for BEA see EFSA CONTAM Panel (2014).

[1] EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), 2014. Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA Journal, 12(8), 3802. https://doi.org/10.2903/j.efsa.2014.3802

[2] Istituto Superiore di Sanità (ISS), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) and French Agency for Food, Environmental and Occupational Health & Safety (ANSES), 2018. In vivo toxicity and genotoxicity of beauvericin and enniatins. Combined approach to study in vivo toxicity and genotoxicity of mycotoxins beauvericin (BEA) and enniatin B (ENNB). EFSA Supporting Publication, EN-1406. https://doi.org/10.2903/sp.efsa.2018.EN-1406

[3] https://www.efsa.europa.eu/en/events/128th-plenary-meeting-contam-panel.

[4] https://www.efsa.europa.eu/en/events/131st-plenary-meeting-contam-panel.

[5] https://www.efsa.europa.eu/en/events/132nd-plenary-meeting-contam-panel.

[6] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. (2002). Official Journal of the European Communities, L 31, 1–24.

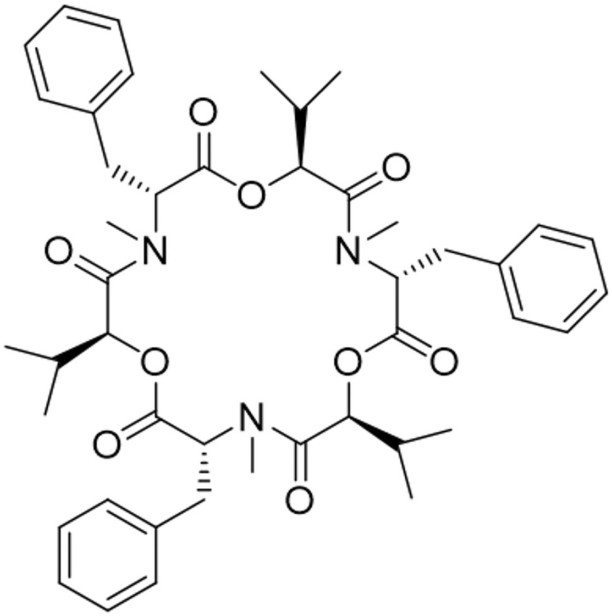

FIGURE 1 Chemical structure of beauvericin.

1.2.2 | Previous EFSA assessment

In 2014, the EFSA CONTAM Panel assessed the risks to human and animal health related to the presence of BEA and ENN in food and feed (EFSA CONTAM Panel, 2014). Given the lack of relevant toxicity data, a risk assessment was not possible for either of these structurally related compounds.

BEA possesses a wide range of biological activities, related to its ionophore properties. Both BEA and ENN are able to transport monovalent (K+) and divalent (Ca2+) cations across cell membranes, leading to intracellular disproportion of transported ions, affecting cell homeostasis. BEA and ENN are antimicrobial compounds that inhibit enzymes and induce oxidative stress. They also have cytotoxic activity towards a range of different cell types inducing apoptosis in the lower micromolar range.

The LD_{50} for acute toxicity of BEA was 100 mg/kg body weight (bw) upon oral administration to mice. There were no in vivo studies available on subchronic, chronic, reproduction and developmental toxicity, neurotoxicity or carcinogenicity of BEA. In vitro genotoxicity data were equivocal, with some studies suggesting a potential genotoxic effect. There were no in vivo genotoxicity data available.

In vitro studies indicated immunotoxicity and haematotoxicity/myelotoxicity of BEA. The CONTAM Panel concluded that there were insufficient data to establish a tolerable daily intake (TDI) or/and an acute reference dose (ARfD) for BEA.

To obtain some insights on the possible concerns for human health from BEA exposure, the CONTAM Panel calculated margins between estimated acute dietary exposure in humans and the reported LD_{50} value.

The margins ranged from about 10×10^6 to 2×10^6 for the mean dietary exposure, and from about 5×10^6 to 1×10^6 for the 95th percentile dietary exposure.

Based on the lowest observed adverse effect level (LOAEL) estimated from the therapeutic use levels of the fusafungine (an ENN analogue) drug, the Panel calculated the margins between the estimated chronic dietary exposure in humans and this LOAEL for the sum of ENN and, in the absence of toxicity data on repeated exposure, also for the structurally related BEA.

For BEA, the resulting margins of exposure ranged from about 57,000 to 1800 for the mean dietary exposure, and from about 17,000 to 1000 for the 95th percentile dietary exposure. The CONTAM Panel concluded that the large margins obtained for acute exposure to BEA do not indicate concern for human health. There might be a concern with respect to chronic exposure, but no firm conclusion could be drawn.

The CONTAM Panel noted that to perform a human risk assessment, in vivo toxicity data on BEA are needed. A study investigating possible health effects likely to arise from repeated exposure (i.e. a 90-day study), including effects on the nervous, immune and endocrine systems, as well as screening of possible effects on reproduction and development is therefore required. Additionally, in vitro and in vivo genotoxicity data are needed.

1.2.3 | Legislation

Council Regulation (EEC) No 315/933[7] stipulates that food containing a contaminant in an amount unacceptable for public health shall not be placed on the market, that contaminant levels should be kept as low as can reasonably be achieved and

[7]Council Regulation (EEC) No 315/93 of February 1993 laying down Community procedures for contaminants in food. OJ L 37, 13.2.1993, p. 1–5.

that, if necessary, the European Commission may establish maximum levels (MLs) for specific contaminants. These maximum levels are laid down in the Annex I of Commission Regulation (EU) 2023/915.[8] BEA is currently not listed in the Annex I.

2 | DATA AND METHODOLOGIES

2.1 | Collection and evaluation of data

The present assessment was developed applying a structured methodological approach, which implied developing a priori the protocol or strategy of the full risk assessment. The protocol in Annex A of this Opinion contains the method that was applied for covering all the steps of the assessment process. The CONTAM Panel used its previous risk assessment on BEA and ENN (EFSA CONTAM Panel, 2014) as a starting point for drafting the current Opinion and relevant information becoming available since then. The latter included the results from a study commissioned by EFSA (ISS, ENEA, ANSES, 2018) and studies identified in several literature searches.

2.1.1 | Data from outsourced study

Following the recommendations from the 2014 Opinion, EFSA has outsourced preparatory work that should close the data gaps on BEA and ENN, enabling to carry out a conclusive and complete risk assessment. In 2018 the final report from this project 'In vivo toxicity and genotoxicity of BEA and ENN. Combined approach to study in vivo toxicity and genotoxicity of BEA (BEA) and ENN B (ENNB)' has been published (ISS, ENEA, ANSES, 2018). The approach is composed by in vitro and short-term in vivo genotoxicity tests and a repeated-dose oral toxicity study focussing on genotoxic, immune, endocrine, nervous endpoints and a reproductive/developmental toxicity screening.

On 1 September 2023, a literature search was carried out to collect literature relevant for the assessment of the genotoxicity of BEA. This search yielded a total of 306 studies of which 20 were considered potentially relevant for the assessment (for details on the literature searches see Annex B).

On 24 January 2024, an additional literature search was carried out to collect additional relevant literature in vitro and in vivo toxicity and adverse effects in humans by BEA. This search yielded a total of 242 studies in the field of adverse effects in humans and 576 in the field of in vitro and in vivo toxicity, of which 28 and 39 studies, respectively, were considered potentially relevant for the assessment.

On 20 April 2024, the literature search on genotoxicity of BEA from 1 September 2023 was repeated (identical search terms and data bases) to include also studies published between 1 September 2023 and 20 April 2024. This search yielded 33 additional publications of which eight were considered potentially relevant for the assessment.

Details on these literature searches can be found in Annex B.

In addition to the described literature searches, a 'snowballing approach' was applied during the process of drafting the Opinion by all WG members (see Jalali & Wohlin, 2012) to obtain further relevant information published until adoption of the Opinion.

3 | ASSESSMENT

3.1 | Summary of genotoxicity in the previous Opinion

In the previous EFSA Opinion on the risks to human and animal health related to the presence of BEA and ENN in food and feed (EFSA CONTAM Panel, 2014), the CONTAM Panel concluded that in vitro genotoxicity data on BEA were equivocal, with some studies suggesting a potential genotoxic effect. A summary of the studies considered in EFSA's previous Opinion are presented in Table 1. Briefly, BEA was negative in the Ames test using Salmonella Typhimurium TA97, TA98, TA100, TA102, TA1535 strains, with and without S9 (Fotso & Smith, 2003). Some positive results were reported for induction of chromosomal damage chromosome aberrations, micronuclei (MN) and sister chromatid exchanges (SCEs) in mammalian cells in vitro (Celik et al., 2010; Klarić et al., 2008). The CONTAM Panel noted some limitations in the studies including the fact that cytotoxicity was observed particularly at the highest concentrations of BEA (EFSA CONTAM Panel, 2014).

Both positive (porcine PK15, human leukocytes) and negative (HL60 and KB-3-1 cells) results were also reported for induction of DNA breaks in studies with limitations (single concentration, unclear cytotoxicity) (Dornetshuber et al., 2009; Klarić et al., 2010).

Possible mechanisms of DNA damage induced by BEA were also investigated in the study by Dornetshuber et al. (2009). BEA has been found to weakly intercalate into double-stranded DNA and to inhibit the catalytic activity of topoisomerase I

[8]Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006 OJ L 119/103, 5.5.2023, p. 103–107.

and II. These effects, however, were observed only at high BEA concentrations. In the same study, the authors also excluded a BEA-induced increase of reactive oxygen species (ROS).

No in vivo genotoxicity data were available at the time.

3.2 | New in vitro data on genotoxicity

New data identified after 2014 EFSA Opinion include three comet assays, three MN tests and two assays to measure γ-H2AX (phosphorylation of histone H2AX on serine 139) foci in mammalian cells (Table 1).

DNA breaks, as assessed by the alkaline comet assay, were investigated in BEA-treated Caco-2 cells (1, 3, 12 μM; 24 h exposure time), Chinese hamster ovary (CHO)-K1 cells (0.1, 1, 5 μM; 24 h exposure time) and HEK293T cells (25 μM; 24 h exposure time (Mallebrera et al., 2016; Prosperini et al., 2013; Tran et al., 2020). Prosperini et al. (2013) reported an increase in the levels of DNA breaks only at the highest concentration tested (12 μM). However, the induction of apoptosis/necrosis observed even at lower BEA concentrations (38% at 3 μM) makes the results of this study inconclusive. Similarly, an increase in DNA breaks only at a single (intermediate) concentration (1 μM), with no parallel measurements of cytotoxicity, was reported by Mallebrera et al. (2016). BEA induced a concentration-dependent increase in DNA breaks in Jurkat cells at concentrations associated with cell viability in the 60%–75% range (3 and 5 μM; 24 h exposure) (Manyes et al., 2018). Finally, Tran et al. (2020) reported negative results in HEK293T cell treated with a single BEA concentration (25 μM), without providing information on cytotoxicity.

An increase in MN formation was observed at a single BEA concentration (1.25 μM) in human HepG2 cells following exposure to various concentrations of BEA for 48 h (0.312, 0.625, 1.25 and 2.5 μM) (Juan-García et al., 2019). Notably, MN formation was not concentration dependent, and the extent of cytotoxicity associated with the only concentration showing a positive effect has not been tested.

In vitro MN tests were also conducted in human lymphoblast TK6 cells and human proliferating HepaRG cells (ISS, ENEA, ANSES, 2018). TK6 cells were exposed to BEA for two time periods (for 3 h, +/− S9 and for 24 h without S9), across a wide concentration range (0.312–10 μM). The results were consistently negative both with and without S9, up to cytostatic concentrations (ranges 20%–40% and 6%–80% for 3 and 24 h treatments, respectively). In contrast, a cytokinesis-blocked MN assay in proliferating HepaRG cells exposed to BEA for 4 h in the absence of S9 (concentration range: 0.8–12.5 μM) showed a small (1.8-fold) increase in MN at a single concentration (6.3 μM associated with 30% cytostasis) which is a toxic level acceptable according to the guideline TG 487 for this test (OECD, 2023).

A multiparametric γ-H2AX and pH3 test was also performed in HepaRG cells (0.01–25 μM; 24 h exposure time) to investigate the clastogenic and/or the aneugenic potential of BEA (ISS, ENEA, ANSES, 2018). Phosphorylation of histone H2AX at serine 139 (named γH2AX) is used to visualise DNA double-strand breaks (DSBs) induced by clastogens, while phosphorylation of histone H3 at serine 10 (named pH3) identifies mitotic cells that usually increase after treatment with aneugenic compounds. An increase of γ-H2AX signal was observed with BEA only at highly cytotoxic concentrations (from 0.78 μM, 25% survival). Small increases in histone H3 phosphorylation were also induced by BEA at the same toxic concentrations (0.78 and 1.56 μM).

Induction of a γ-H2AX signal was also analysed in human lung adenocarcinoma A549 cells exposed to a large range of BEA concentrations (0.024–100 μM, 24 h exposure time) (Habauzit, Alvariño, et al., 2023). Also in this case, a significant increase in γ-H2AX levels was observed only at concentrations (25, 50, 100 μM) associated with high cytotoxicity (i.e. IC_{50}: 6.2–8.1 μM) and alterations of cell cycle progression (at 12.5 μM). The relevance of these results must be taken with caution due to the high levels of toxicity.

In summary, BEA did not induce gene mutations in *S.*Typhimurium neither with nor without metabolic activation. In vitro studies in mammalian cell lines provided no convincing evidence for induction of chromosomal damage (MN and chromosome aberration tests) by BEA. In the few observations that were positive there was no concentration-dependence and uncertain relationships with cytotoxicity. Alkaline comet assays of DNA breakage were also negative or inconclusive. In addition, DSBs formation by γ-H2AX analysis was only observed following exposure to highly cytotoxic BEA concentrations. The significance of these results is therefore uncertain. Overall, these in vitro findings do not unequivocally demonstrate genotoxicity associated with BEA exposure.

TABLE 1 Previous and new in vitro genotoxicity studies on BEA.

Reference	Test system	Cells	Concentration/treatment time	Results	Comments/additional information
Previously available studies					
Fotso & Smith (2003)	Reverse gene mutation assay (Ames test)	*S.* Typhimurium TA97, TA98, TA100, TA102, TA1535	0, 0.2, 2, 20, 500 μg/plate with and without S9 Positive controls: 2-aminofluorene, sodium azide, dexon	**Negative**	Limited concentration range
Klarić et al. (2008)	MN, Nuclear Buds (NBs) and NucleoPlasmic Bridges (NPBs)	Porcine kidney PK15 cells	0, 0.05, 0.5, 5 μg/mL (0.063, 0.63 and 6.3 μM) (24 & 48 h) Without S9 Positive controls: mitomycin C (0.03 μM), colchicine (1.2 μM)	**Positive MNs:** 24 h: 0.5 and 5 μg/mL (0.63 and 6.3 μM) 48 h: 5 mg/mL (6.3 μM) **Positive NPBs** 24 & 48 h: 5 μg/mL (6.3 μM)	No information on mitotic index but cytotoxicity reported in a different paper using the same experimental conditions: increased LDH activity, caspase 3 activation and apoptotic index observed at 48 h for the 5 μg/mL concentration (Klarić et al., 2008)
Dornetshuber et al. (2009)	Alkaline Comet assay	Human promyelocytic leukaemia HL60 cells Human epidermal carcinoma KB-3-1 cells	0, 20 μM (1 h) without S9% tail intensity Positive control: H_2O_2 (50 μM)	**Negative:** Data reported in figure only for HL60 cells No information on cytotoxicity b Nuclear features of apoptosis induced by 10 μM BEA after 24 h in KB-3-1 cells (75% of apoptotic cells)	The single concentration tested with inadequate information on cytotoxicity make these results inconclusive
	DNA intercalation (by methyl green competition assay	Salmon sperm DNA	50, 100, 150 μM	**Weakly positive:** 20% and 37% reduction at 100 and 150 μM, respectively	BEA exhibits low affinity to DNA with substantial intercalation only at high concentrations
	Suppression of Topoisomerase DNA-mediated cleavage	Plasmid relaxation assays	0, 0.01, 0.1, 1, 10, 100, 500, 1000 μM	**Weakly positive:** Only > 100 μM for Topoisomerase I and II	Inhibition observed only at high concentrations
Celik et al. (2010)	MN test	Human lymphocytes from 2 healthy donors	0, 1.25, 2.5, 5, 10 μM (48 h) Without S9 Positive control: mytomycin C (0.2 μM)	**Positive:** from 5 μM	Slight, not significant decrease in CBPI index. From this BEA cytotoxicity was recalculated to be in the range 12%–19.6%
	Chromosomal aberration			**Positive:** from 2.5 μM	Unclear toxicity
	Sister chromatid exchanges			**Positive:** all concentrations (around 2-fold)	Significant decrease in mitotic index (5 and 10 μM). Range of toxicity from 0% to 33.3%
Klarić et al. (2010)	Alkaline Comet assay Cytotoxicity by MTT	Porcine kidney PK15 cells	0, 0.1, 0.5 μM (1 and 24 h) Without S9	**Negative**:1 h **Positive:** 24 h only at 0.5 μM (all parameters)	Cytotoxicity reported only for 1 μM, 24 h in PK15 cells: 82% viability
		Human leukocytes	Tail length, tail intensity (%DNA) & tail moment No positive control	**Negative**:1 h **Positive:** 24 h only at 0.5 μM (only tail moment)	No information on cytotoxicity

(Continues)

TABLE 1 (Continued)

Reference	Test system	Cells	Concentration/treatment time	Results	Comments/additional information
New information					
Prosperini et al. (2013)	Alkaline Comet assay Cytotoxicity: MTT assay and neutral red (NR)	Colorectal carcinoma Caco-2 cells	0, 1.5, 3, 12 μM (24 h) Without S9 Tail moment Positive control: B(a)P (20 μM)	**Positive:** Only at 12 μM No parallel toxicity assay IC_{50} at 24 h: 20.6 ± 6.9 and 8.8 + 0.9 μM by MTT and NR respectively Significant increase in apoptotic/necrotic cells (38.6%) reported at 3 μM	Unclear toxicity makes the results inconclusive
Mallebrera et al. (2016)	Alkaline Comet assay Cytotoxicity: MTT assay	Chine Hamster Ovary CHO-K1	0, 0.1, 1, 5 μM (24 h) without S9% DNA in tail Positive control: B(a)P (15 μM)	**Positive:** only at 1 μM ($p < 0.05$) no clear information on cytotoxicity at the concentrations tested IC_{50} at 24 h: 10.7 μM	The lack of dose response and unclear toxicity make the results inconclusive
Manyes et al. (2018)	Alkaline Comet assay Cytotoxicity: MTT assay	Human Jurkat-T lymphocytes	0, 1.5, 3, 5 μM (24 h) without S9% DNA in tail Positive control: etoposide (5 μM)	**Positive:** 3 and 5 μM No parallel cytotoxicity assay Viability by MTT: 75% at 3 μM, unclear at 5 μM	Uncertain significance of these results because of unclear cytotoxic levels
ISS, ENEA, ANSES (2018)	MN test	Human lymphoblast cell line TK6	±S9 (3 h): 0, 0.625, 1.25, 2.5, 5, 10 μM –S9 (24 h): 0, 0.312, 0.625, 1.25, 2.5, 5 μM Positive controls: B[a]P (8 μM) and MMS (45 μM)	**Negative:** both ±S9 and short and long treatment Excessive toxicity at 10 μM (–S9, 3 h): MN not analysed	Cytostasis % (–S9, 3 h): 36.7, 20.3, 34.9, 41.2, 100% at 0.625, 1.25, 2.5, 5, 10 μM, respectively Cytostasis % (+S9, 3 h): 8.2, 0, 0, 31.9, 16.7% at 0.625, 1.25, 2.5, 5, 10 μM, respectively Cytostasis % (–S9, 24 h): 6.1, 11.6, 27.2, 79.4, 82% at 0.312, 0.625,1.25, 2.5, 5 μM
ISS, ENEA, ANSES (2018)	Cytokinesis-blocked MN test MN Test	Human hepatic undifferentiated HepaRG cells	–S9 (4 h): 0, 0.8, 1.6, 3.1, 6.3, 12.5 μM Positive control: MMS (45 μM)	**Positive:** at 6.3 μM with 30% cytostasis ($p < 0.01$) Excessive toxicity at 12.5 μM (MN not analysed)	MN increase at a single, slightly toxic concentration Cytostasis %: 14, 0, 10, 30% at 0.8, 1.6, 3.1, 6.3 μM
ISS, ENEA, ANSES (2018)	Multiparametric γ-H2AX and pH3 test Cytotoxicity: cell counting	Human hepatic undifferentiated HepaRG cells	0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5 25 μM (24 h) Positive control: MMS; Colchicine	**Positive** γ-H2AX and pH3: from 0.78 μM, a concentration with 25% survival	Uncertain significance of results obtained at highly toxic concentrations
Juan-García et al. (2019)	MN test by flow cytometry) Cytotoxicity: MTT assay	Human hepatocellular carcinoma HepG2	0, 0.312, 0.625, 1.25, 2.5 μM (48 h) without S9 Positive control: etoposide	**Positive:** only at 1.25 μM No parallel measurements of cytotoxicity in this concentration range Range cytotoxicity tested (2.5–25 μM): IC_{50} at 48 h: 7.5 μM	No concentration-dependent increase and no parallel measurements of cytotoxicity renders these results inconclusive

TABLE 1 (Continued)

Reference	Test system	Cells	Concentration/treatment time	Results	Comments/additional information
Tran et al. (2020)	Alkaline Comet assay	Human embryo kidney HEK293 cells	0, 25 μM (24 h) Positive control: H_2O_2	**Negative** No parallel measurements of cytotoxicity at the single concentration tested	Uncertain significance of this result (because of lack of information on cytotoxicity)
Habauzit, Alvariño, et al. (2023)	γ-H2AX foci Cytotoxicity: MTT assay and cell counting	Human lung adenocarcinoma A549 cells	0, 0.024, 0.049, 0.098, 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 μM Positive control: Staurosporine (0.05 μM)	**Positive:** at 25, 50, 100 μM ($p < 0.05$, $p < 0.01$, $p < 0.05$, respectively) IC_{50}: 6.2 and 8.1 μM by MTT assay and cell counting, respectively At concentrations ≥ 12.5 μM: alterations in cell cycle (decrease in G0/G1 and increase in S and G2/M	Uncertain significance of results obtained at highly toxic concentrations

Abbreviations: CBPI, cytokinesis-block proliferation index; CHO, Chinese hamster ovary; DNA, deoxyribonucleic acid; G0/G1, resting phase/first gap phase; G2/M, second gap phase/mitosis phase; H_2O_2, hydrogen peroxide; HEK-293, human embryonic kidney 293 cell line; HL60, human leukaemia cell line; HepG2, human hepatocellular carcinoma; HepaRG, human hepatic cell line; IC50, half-maximal inhibitory concentration; KB-3-1, epidermal carcinoma-derived cell line; LDH, lactate dehydrogenase; mM, micromolar; MMS, methyl methanesulfonate; MN, micronucleus; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NBs, nuclear buds; NPBs, NucleoPlasmic Bridges; NR, neutral red; PK15, porcine kidney cells lines; S, synthesis phase; S9, liver enzyme extract; TK6, lymphoblast cell line; γH2AX, phosphorylated histone at serine 139; μg, microgram; %, percentage.

3.3 | New in vivo data on genotoxicity

Table 2 describes the in vivo investigations carried out by ISS, ENEA, ANSES (2018).

In a first study, an alkaline Comet assay was performed in seven different tissues/organs and the MN test in colon in male CD-1 mice (five animals/group) exposed by oral gavage to three administrations at 0, 24 and 45 h of BEA at 50, 100 and 200 mg/kg bw per day (BEA prepared in dimethyl sulfoxide (DMSO) and resuspended in corn oil, final concentration of DMSO was 5%). These doses were established based on a preliminary dose range-finding experiment in order to determine the maximum tolerated dose (MTD) for BEA. The authors indicated that no clinical signs of toxicity were observed during the 3-day period of treatment at 200 mg/kg bw per day, and this was selected considering the high cost of the toxin and considering that this dose is 2×10^6 fold higher than the highest acute human exposure estimates for BEA (0.10 μg/kg bw per day (95th percentile)) (EFSA CONTAM Panel, 2014). In the Comet assay, BEA did not induce any increase of DNA migration in any of the organs/tissues analysed (blood, bone marrow, liver, kidney, duodenum, spleen) and BEA did not increase micronucleated, mitotic or apoptotic cell formation in colon between 50 and 200 mg/kg bw. No clinical signs of toxicity were noticed (ISS, ENEA, ANSES, 2018).

In a second study by the same investigators, male CD-1 mice were exposed by oral gavage to two administrations at 0 and 24 h of BEA at 50, 100 and 200 mg/kg bw per day (BEA prepared in DMSO and resuspended in corn oil, final concentration of DMSO was 5%) and the alkaline Comet assay was performed in liver and bone marrow and the MN tests in bone marrow 24 h after the last gavage. BEA did not induce any increase of DNA migration in the Comet assays in liver and bone marrow. BEA did not modify either the frequency of micronucleated immature erythrocytes or the immature to mature erythrocytes ratio compared with the negative control (ISS, ENEA, ANSES, 2018).

Taken together, these results suggested that BEA is devoid of in vivo genotoxicity potential after oral administration. No DNA damage was observed in first site of contact tissue (duodenum) and there is no evidence for BEA activation in the liver.

In a third study with a longer exposure duration (42 days) male and female CD1 mice (five animals/sex/group) were dosed with BEA at 0.1, 1 and 10 mg/kg bw per day, 5 days/week by gavage and the alkaline Comet assay was performed on blood, liver, kidney, duodenum and testis/ovary. Sperm from cauda epididymis were sampled for the neutral Comet assay. A Pig-a assay and a MN test were performed on leucocytes of male mice (ISS, ENEA, ANSES, 2018).

BEA did not significantly increase DNA migration in the Comet assay in blood leukocytes, liver and testis cells, but a statistically significant increase was detected for kidney and duodenum cells from only male mice and only at the intermediate dose of 1 mg/kg bw per day. At this dose level, the authors indicated that in four out of five animals the extent of DNA breakage was higher than that measured in negative control animals and that kidney was affected by BEA exposure with a decrease of absolute and relative weight and an increase of serum creatinine. However, no histopathological change was observed in the kidney. BEA did not increase DNA migration in epididymal sperm. In female mice, no statistically significant increase of DNA migration was observed in any of tissues/organs obtained from BEA-exposed mice. In the Pig-a assay, BEA did not induce significant increases in the frequencies of mutant reticulocytes (RET) or red blood cells (RBC) and did not reduce % of RET. BEA did not increase the frequency of micronucleated immature erythrocytes in blood cells of male mice after 42-days of exposure. The CONTAM Panel noted that there is limited evidence of systemic exposure (decrease in absolute and relative kidney weight, increase in creatinine and blood urea nitrogen (BUN)). Furthermore, the response in the duodenum was only seen at one dose level in one sex. This evidence suggests a lack of genotoxicity of BEA in these repeated-dose assays.

TABLE 2 In vivo genotoxicity of BEA (ISS, ENEA, ANSES, 2018).

Test organ	Animals	Concentration/treatment time	Results	Comments/additional information
First study				
Alkaline Comet assay Duodenum, colon, blood, liver, bone marrow, kidney, spleen	Male CD1 Mice	50, 100, 200 mg/kg bw, gavages at 0, 24 and 45 h	**Negative:** no significant increase of DNA migration	The electrophoresis conditions applied in this study were not optimal, then a new study was performed The top dose of 200 mg/kg bw did not induce any clinical effects on the animals and was not considered MTD as recommended by OECD TG 489 (OECD, 2016)
MN assay colon	Male CD1 Mice	50, 100, 200 mg/kg bw/day, gavages at 0, 24 and 45 h	**Negative:** no significant increase of micronucleated, mitotic or apoptotic cells frequency	
Second study				
Alkaline Comet assay liver, bone marrow	Male CD1 Mice	50, 100, 200 mg/kg bw/day, gavages at 0 and 24 h	**Negative:** no significant increase of DNA migration	
MN assay bone marrow	Male CD1 Mice	50, 100, 200 mg/kg bw/day, gavages at 0 and 24 h	**Negative:** no significant increase of MN, no impact on PCE/NCE ratio	No impact of BEA on PCE/NCE ratio indicating that the target organ might not be sufficiently exposed
Third study				
Alkaline Comet assay Blood, liver, kidney, duodenum and testis	Male CD1 Mice	0.1, 1 and 10 mg/kg bw per day, 42-daily administration by gavage	**Negative:** no significant increase of DNA migration in blood leukocytes, liver and testis cells	
			Equivocal: kidney, duodenum at 1 mg/kg bw	No dose-related increase of DNA migration in duodenum and kidney. These results could not be considered as clearly positive. Decrease of absolute and relative kidney weight and an increase of serum creatinine at 1 mg/kg bw per day
Alkaline Comet assay blood, liver, kidney, duodenum and ovary	Female CD1 Mice	0.1, 1 and 10 mg/kg bw per day, 42-daily administration by gavage	**Negative:** no significant increase of DNA migration in blood, liver, kidney, duodenum and ovary cells	
MN assay blood	Male CD1 Mice	0.1, 1 and 10 mg/kg bw per day, 42-daily administration by gavage	**Negative:** no significant increase in the frequency of micronucleated immature erythrocyte, no changes of the number of reticulocytes	The lack of changes in the frequency of reticulocytes indicated that bone marrow could not be the targeted organ of BEA and suggests that the result from this test could be considered inconclusive
Pig-a assay blood	Male CD1 Mice	0.1, 1 and 10 mg/kg bw per day, 42-daily administration by gavage	**Negative:** no significant increase of both mutant RETs and RBCs No significant reduction in % RET	The lack of changes in the frequency of RET indicated that bone marrow could not be the targeted organ of BEA and suggests that the results from this test could be considered inconclusive

Abbreviations: DNA, deoxyribonucleic Acid; MN, micronucleus; MTD, maximum tolerated dose; OECD TG, Organisation for Economic Co-operation and Development Test Guideline; PCE/NCE, polychromatic erythrocyte /normochromatic erythrocytes; RETs, reticulocytes; RBCs, rodent red blood cell.

3.4 | New in silico data on genotoxicity

In a preliminary step, Habauzit, Lemée, et al. (2023) built a large database of mycotoxins from literature. This was segregated into groups of similar compounds by clusters analysis of physicochemical/structural descriptors. One cluster was composed of BEA and ENN ($n = 14$). Five (Q)SAR models in the public domain were applied to predict in vitro mutagenicity: VEGA platform (four modules, including the ISS expert rule-base, version 1.1.5-b36), EPA T.E.S.T. (version 4.2.1), Lazar (version not specified), ADMETLab 2.0, Protox-II. BEA was predicted to be non-mutagenic by all models. The study also investigated whether putative carcinogenic effects could be outlined by (i) the analysis of modified gene expression and (ii) potential adverse outcome pathways (AOPs). Although the authors speculated about a possible link between BEA and small cell lung cancer, the CONTAM Panel considered that the evaluation based on gene expression data and AOP-helpfinder was limited and did not allow firm conclusions.

In another in silico analysis, the commercial software ACD/Percepta (ACD/Percepta Platform, version 2016) was used to predict the most common physicochemical, pharmacokinetic and toxicological properties of BEA. The system contains modules for predicting: (a) CHO/CHL mutagenicity; (b) chromosome aberrations in vitro; (c) chromosome aberrations in vivo; (d) rodent carcinogenicity. BEA was predicted as negative by the four modules. As a verification of the in silico analysis, in vitro Comet assay was applied (Tran et al., 2020, see section 3.2. on in vitro mutagenicity).

Tolosa et al. (2023) constructed a comprehensive database containing 4360 compounds (including mycotoxins and their metabolites) classified in 170 categories (clustering based on k-nearest neighbour approach from structural fingerprints). Unified groups, which included ENN and BEA were created. In addition, using the whole mycotoxins database as training set, new QSAR models for the prediction of: (a) mutagenicity (Ames test), (b) in vitro genotoxicity (MN assay) and (c) in vivo genotoxicity (MN assay) and (d) carcinogenicity (long term carcinogenicity assay in rodents) were generated in-house. The new QSAR models were applied to the compounds in the database: results of the application were reported by category, not for individual compounds. A majority of positive predictions for the four endpoints was observed for the cluster including BEA and ENN but without reporting the specific results for BEA.

Finally, the CONTAM Panel performed an analysis of structural alerts with the OECD QSAR Toolbox (version 4.7., 2024) (https://qsartoolbox.org/). No relevant structural alerts were found by the following profilers (collections of alerts): (a) genotox and non-genotox carcinogenicity by ISS; (b) DNA alerts for Ames, chromosome aberrations and MN by (On-line Alerting of Structural Integrity and Safety (OASIS); (c) in vitro mutagenicity (Ames) by ISS; (d) in vivo mutagenicity (MN) by ISS; (e) Protein binding alerts for chromosome aberrations by OASIS.

Overall, no indication of potential mutagenicity/genotoxicity was suggested by the range of (Q)SAR predictive models applied in different laboratories.

3.5 | Summary of genotoxicity

Overall, despite some equivocal positive responses, in vitro studies investigating gene mutations in bacteria, DNA breakage and chromosome damage in mammalian cells did not demonstrate genotoxicity associated with BEA exposure**.** Upon oral administration in mice, no increase in MN in bone marrow and colon was observed. Similarly, upon repeated-dose oral administration in mice, BEA did not show any evidence of in vivo genotoxicity. The CONTAM Panel noted that there was limited evidence of exposure of bone marrow. No DNA damage as measured by the Comet assay at the first site of contact tissue (duodenum) was observed and there is no evidence for BEA bioactivation in the liver. A range of (Q)SAR predictive models were applied in different laboratories and gave no indication of potential mutagenicity/genotoxicity.

Taken together, the available data did not show any genotoxic activity of BEA.

3.6 | Modes of action impacting on the interpretation of the genotoxicity studies

3.6.1 | Interaction with DNA

No reports of covalent binding of BEA to DNA were identified. Methyl green competition assays were performed to analyse whether BEA possesses the ability to intercalate into DNA. BEA was found to intercalate substantially into double-stranded DNA and to inhibit the catalytic activity of topoisomerase I and II only at a high concentration (> 100 μM) (Dornetshuber et al., 2009).

3.6.2 | Oxidative stress

Relevant to the potential for genotoxic effects as a secondary mechanism, there is substantial evidence for the generation of oxidative stress associated with mitochondrial disruption (see below) following exposure to BEA.

Lipid peroxidation as measured by malondialdehyde production was elevated by BEA in HepG2 cells at 1.25 μM within 24 h and was also elevated at 0.61 μM at 48 h. Oxidised glutathione (GSSG) was also elevated in these cells at 0.31 μM while reduced glutathione (GSH) levels were increased at 1.25 μM (Juan-García et al., 2020).

Further support for oxidative stress induced by BEA was reported in CHO-K1 cells. GSH levels (at 5 μM), glutathione peroxidase (at 1 μM), glutathione reductase (at 5 μM) and glutathione-S-transferase (at 1 μM) as antioxidant defence systems were elevated in CHO-K1. Effects were prevented by 24 h pretreatment of cells with the antioxidant N-acetyl-cysteine (at 1 mM) (Mallebrera et al., 2014).

Oxidative stress induced by BEA was also found in HepG2 cells at a concentration of 1 μM (Shi et al., 2022). BEA led to an increased production of ROS (measured using 2′-7′-dichlorodihydrofluorescein diacetate, CFH-DA, fluorescent probe), leading to the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway and Nrf2 nuclear translocation for transcriptional activation of downstream antioxidative genes. Apoptosis was induced at 1.5 μM.

Agahi et al. (2020) found that BEA downregulated the expression of B-cell lymphoma 2 protein (BCL2) and increased the ratio of GSH/GSSG, which was interpreted as evidence of oxidative stress. There was also evidence of apoptotic/necrotic changes in this cell type at 0.39 μM and above with interference in cell cycle progression observed at 0.39 and 0.78 μM, respectively (Agahi et al., 2021).

The effect of BEA treatment on oxidative stress status in brain of female mice was evaluated by measurement of the amount of ROS and GSH. These parameters did not change following BEA treatment up to the top dose of 10 mg/kg per day (ISS, ENEA, ANSES, 2018).

In salmon hepatocytes, evidence indicated that BEA induced oxidative stress (as measured by total glutathione peroxidase (GPx) enzyme activity, total H_2O_2 and total iron content). Transcriptome analysis further supported this by showing increased energy expenditure, elevated oxidative stress and iron homeostasis disturbances at sub-cytotoxic concentrations (Søderstrøm et al., 2022).

In addition to the evidence for oxidative stress, there is evidence for compensatory mechanisms that protect against elevation of ROS. In PC-12 cells, BEA not only induced oxidative stress, but also exhibited antioxidant activity (Hu et al., 2024). At 0.5 mM, BEA inhibited oxidative stress and reduced the concentrations of ROS associated with regulation of antioxidant lipids and phosphoinositide 3 kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signalling pathways.

Moreover, in the study by Mallebrera et al. (2016) in CHO-K1 cells, an increase in DNA strand breaks, as indicated by the Comet assay, was observed at 1 μM but not at 5 μM. Increased activity of superoxide dismutase at 1 μM and catalase at 5 μM were noted. This evidence indicates oxidative stress, but the authors noted that the elevation of these antioxidant activities may help to reduce the toxic effects as they represent a defence mechanism.

3.6.3 | Effects on apoptosis/cell death

As recognised in the previous EFSA CONTAM Opinion (2014) and as reviewed by Hasuda and Bracarense (2024), the toxicity of BEA is related to its ionophore properties, which initiates the disruption of monovalent potassium ions and divalent calcium cation transport across cell membranes. This was further supported by recent studies (Jestoi, 2008; Prosperini et al., 2017, Fraeyman et al., 2018, Bertero et al., 2018; Mallebrera et al., 2018). The resulting mitochondrial damage and increase in intracellular calcium may be responsible for the induction of apoptosis.

BEA induced cytotoxicity in CHO-K1 cells in a dose- and time-dependent manner, associated with inhibition of mitochondrial enzymatic activity and cell proliferation by arresting cells in G0/G1 and increasing apoptosis (Mallebrera et al., 2016).

Studies conducted in human lymphoblastic Jurkat T cells investigated the effects of BEA on mitochondrial functions. Results demonstrated BEA (1.5, 3 and 5 μM; 24 h)-induced mitochondrial damage, affecting the respiratory chain and leading to apoptosis through the caspase cascade (Alonso-Garrido et al., 2020; Escrivá et al., 2018; Escrivá et al., 2019; Manyes et al., 2018).

Several pathways involved in lung cancer pathology, as revealed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map, were analysed in A549 and NCI-H226 human lung cells treated with BEA (Habauzit, Alvariño, et al., 2023). In these cells, in addition to cell cycle inhibition and induction of apoptosis, a significant increase in γ-H2AX level was observed at cytotoxic concentrations above 12.5 μM (see Section 3.2, New in vitro data on genotoxicity). An increase in the concentration of ROS was also seen at 50 μM. In the same paper, BEA was found to increase the release of the pro-inflammatory interleukin (IL)-8.

In H4IIE cells (Wätjen et al., 2014), BEA rapidly decreased the phosphorylation of extracellular signal-regulated kinase (ERK) and strongly increased c-Jun N-terminal kinase (JNK) phosphorylation, along with selective inhibition of proto-oncogene tyrosine-protein kinase (Src) (IC_{50} = 9.8 μg/mL). These effects were associated with induction of apoptosis. In the cell line KB-3-1, cathepsin B released into the cytosol appears to be a mechanism for the activation of caspases by BEA and induction of apoptosis (Aufy et al., 2023). In A375SM human melanoma cells, BEA was found to promote caspase-dependent apoptosis through upregulation of death receptors, as well as modulation of Bcl-2 family members and suppression of ERK, JNK, p38 (mitogen-activated protein kinases), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), signal transducer and activator of transcription 3 (STAT3) and microphthalmia-associated transcription factor (MITF). These effects on cell signalling were considered by the authors to demonstrate the potential for suppression of melanoma progression (Lim et al., 2020). Also relevant to potential anti-cancer activity, Heilos et al. (2017) found a significant increase of necrotic areas, corresponding to an elevation of the numbers of apoptotic cells within grafted murine and human tumours in BALB/c and CB-17/SCID mice treated with BEA (5 mg/kg bw per day). BEA-induced mitochondrial damage, oxidative stress and apoptosis each have the potential to exert an anti-cancer effect. In addition, the modulation of the immune system may impact on carcinogenesis (Penn & Starzl, 1973; Trinchieri, 2012; Okada et al., 2021).

Aufy et al. (2023) found that BEA induced lysosomal permeabilisation, and the release of cathepsin from the epidermal carcinoma-derived cell line KB-3-1. Cathepsin B release is known to promote caspase-dependent apoptotic cell death. Escrivá et al. (2018) reported on a gene ontology analysis of the transcriptome of Jurkat cells exposed to BEA, showing overexpression of genes linked to the mitochondrial respiratory chain and oxidoreductase activity, as well as alteration of gene expression relating to apoptosis through the caspase cascade.

Further studies on gene expression changes in RGA and Caco-2 cells in relation to endocrine disruption showed that BEA reduced progestogen receptor transcriptional activity at 1 μM which was not due to pre-lethal toxicity (Fernández-Blanco et al., 2016).

3.6.4 | Effect of beauvericin on inflammation and immunotoxicity

Excessive and persistent formation of ROS from inflammatory cells is considered a hallmark of secondary genotoxicity. This, together with epigenetic changes, may lead to mutations and altered gene expression. Immunotoxicity, including chronic inflammation and oxidative stress are linked and interconnected, triggering secondary events that lead to toxic actions, including genotoxicity. Few studies investigated the immunomodulatory effects of BEA, mainly addressing inflammatory parameters as described below. A summary of the studies considered are presented in Table 3 below.

Ficheux et al. (2013) investigated the in vitro effects of BEA on human primary dendritic cells and macrophages derived from umbilical cord blood. Overall, BEA had a modest impact on the innate immune response, with the most notable effect being a reduction in endocytosis. The lowered expression of C-C chemokine receptor type 7 (CCR7), crucial for dendritic cell migration and the increased secretion of IL-10 suggest an anti-inflammatory effect.

Yoo et al. (2017) investigated BEA's anti-inflammatory effects in lipopolysaccharide (LPS)-treated RAW264.7 murine macrophage-like cells. BEA inhibited nitrous oxide (NO) production in a dose-dependent manner without causing cytotoxicity and prevented LPS-induced morphological changes. BEA significantly hindered the nuclear translocation of NF-κB subunits p65 and p50. Luciferase assays showed that BEA inhibited myeloid differentiation primary response protein (MyD88)-dependent NF-κB activation. Further analysis identified Src and spleen tyrosine kinase (Syk) as BEA's targets in upstream NF-κB signalling. These findings suggest that BEA suppresses NF-κB-dependent inflammation by targeting Src and Syk.

Shandilya et al. (2023) investigated the impact of Fusarium mycotoxins, including BEA, on the inflammatory response in a co-culture model of bovine epithelial cells (MAC-T) and macrophages (BoMac). BEA significantly decreased levels of IFN-γ, IL-8, IL-10, IL36RA and monocyte chemotactic protein 1 (MCP-1). Post-LPS challenge, IFN-γ, IL-8, IL-10, IL36RA, MIP1β and MCP-1 levels were also significantly reduced. RNA-sequencing revealed 318 differentially expressed genes, involved in key pathways including apoptosis, inflammation mediated by chemokine and cytokine signalling, and Wnt signalling. These results indicate that BEA significantly affects inflammation, decreasing both pro-inflammatory and anti-inflammatory cytokines.

Yang et al. (2022) studied BEA's effects on murine granulocyte macrophage colony stimulating factor (GM-CSF)-cultured bone marrow-derived dendritic cells (BMDCs). BEA dose-dependently activated BMDCs, increasing IL-12 and CD86 expression, and boosting IL-12p40, interferon (IFN)-β, tumour necrosis factor (TNF)-α and IL-6 production. BEA showed cytotoxicity only at > 5 μM after 16 h. BEA-treated BMDCs enhanced T cell proliferation without affecting T cells directly. BEA induced inflammatory cytokine production via a MyD88/adaptor inducing interferon-β (TRIF)-dependent pathway, potentially targeting toll-like receptor 4 (TLR4) on BMDCs. This was confirmed by NF-κB activation in TLR4/cluster of differentiation 14 (CD14)/myeloid differentiation factor 2 (MD2)-overexpressing HEK-293 cells. RNA-sequencing revealed transcriptional changes related to TLR4 signalling, metabolism, T-cell activation and other pathways. BEA demonstrated immunostimulatory effects on BMDCs via TLR4-dependent signalling. The discrepancies between these findings and the results of the other in vitro studies described above could be due to the different experimental models and conditions (monocytes/macrophages vs. dendritic cells, primary vs. cell lines, animal vs. human cells, concentrations and time).

In vivo, BEA significantly reduced weight loss, diarrhoea and mortality in mice with 2,4,6-trinitrobenezenesulfonic acid (TNBS)-induced colitis, a model of Crohn's disease (Wu et al., 2013). BEA reduced colitis signs and serum TNF-α and IFN-γ levels in a dose-dependent manner. In vitro, BEA inhibited concanavalin A (ConA)-induced T cell proliferation and LPS-induced cytokine production in macrophages by downregulating PI3K/Akt signalling. BEA suppressed T-cell activation and IFN-γ-STAT1-T-bet signalling, induced T cell apoptosis by modulating B-cell leukaemia/lymphoma 2 protein (Bcl-2), BCL2 associated agonist of cell death (Bad) and caspases.

An in vivo study conducted in CD-1 mice (ISS, ENEA, ANSES, 2018) included some immunological parameters: total serum immunoglobulin (Ig)A, IgG and IgM immunoglobulins, blood counts, spleen, thymus, mesenteric lymph nodes, anti-CD3/CD28-induced IFN-γ and IL-10 in splenocytes, spleen macrophage-induced nitric oxide (NO) production and spleen CD3+ T cells, CD4+ T cells, CD8+ T cells and CD45+ leukocytes. Taken together, the results do not demonstrate consistent effects on immune parameters and do not support the idea that BEA has an immunotoxic effect in mice under the experimental conditions and parameters investigated.

In addition to studies conducted using immune cells, two studies investigated cytokine production in non-immune cells (Habauzit, Alvariño, et al., 2023; Xu et al., 2023). Using a bovine mammary epithelial cell line, Xu et al. (2023) found that BEA downregulated TLR4 and TNF-α, and upregulated IL-6 and TGF-β. The induction of IL-6, alongside the anti-inflammatory cytokine TGF-β and the decrease in TNF-α suggests an anti-inflammatory effect. Habauzit, Alvariño, et al. (2023) demonstrated

an increase in IL-8 release in the human adenocarcinoma alveolar basal epithelial cell line A549 at 3.12 and 6.25 μM, and a decrease at higher concentrations. The IC_{50} for cell viability was 6.2 μM, thus, effects are seen at concentrations resulting in a decrease in cell viability and at higher concentrations the overt toxicity results in decreased IL-8 release compared to control.

In summary, the majority of the in vitro studies with immune cells (Ficheux et al., 2013; Shandilya et al., 2023; Yoo et al., 2017), with the exception of Yang et al. (2022), are supportive of anti-inflammatory effects of BEA, which are in line with the protective effects observed on (TNBS)-induced colitis in mice (Wu et al., 2013). In non-immune cells inconsistent results were found, with Xu et al. (2023) showing an anti-inflammatory effect and Habauzit, Alvariño, et al. (2023) demonstrating an increase in IL-8 release in A549 cells at some concentrations. The decrease in cell viability could also explain differences as cytotoxicity may trigger sterile inflammation.

TABLE 3 Studies on inflammation and immunotoxicity.

Reference	Experimental model	Dosing regimen duration	Parameters	Results
Effects in immune cells				
Ficheux et al. (2013)	Human primary dendritic cells and macrophages derived from umbilical cord blood samples	0.1–50 μM (0.32–2.4 μM for immune tests) 48 h	CD1a, CD71, HLA-DR, CD80, CD54, CD11a, CCR7 Respiratory burst activity Endocytosis TNF-α, IL-10	No effects on immature dendritic cells maturation; decrease of CCR7 expression (1.6 μM) and an increase of IL-10 secretion (1.6 and 2.4 μM) in mature dendritic cells; decrease of endocytosis (0.32–0.64 μM) ability in macrophages, with no effects in TNF-α production or respiratory burst activity
Wu et al. (2013)	CD3+ T cells isolated from female BALB/c mice lymph nodes	1.25–10 μM 30 min-72 h	ConA-induced T cell proliferation and activation	Inhibitory effects were seen at 2.5 μM (T cell proliferation at 72 h; IL-2, TNF-α, IFN-γ at 24 h; STAT1 activation at 30 min) Caspase-dependent apoptosis was observed at 1.25 mM at 24 h
Wu et al. (2013)	Supplementary material Mice peritoneal macrophages	1.25–10 μM 24 h	LPS-induced cytokine production	Inhibition of LPS-induced IL-1β, IL-12, TNF-α
Wu et al. (2013)	TNBS-induced colitis: Female BALB/c mice (aged 8–12 weeks)	1, 2 or 4 mg/kg bw, day 0 to day 7	Colon TNF-α, IFN-γ, IL-β, IL-12 Colon CD4+ T cell infiltration	Effects were seen at a dose of 2 mg/kg and onwards (colon IFN-γ, IL-1β, IL-12)
Yoo et al. (2017)	Murine macrophage-like RAW264.7 cells	0 to 7.5 μM 5 min - 18 h	NF-kB, Src, Syc activation IL-1β (mRNA) iNOS (mRNA) Production of NO	BEA (up to 7.5 μM) did not significantly affect cell viability after 9 or 12 h BEA suppresses LPS-induced NF-κB-dependent inflammation by targeting Src and Syk. NO production, inflammatory gene expression (IL-1β) and LPS-induced morphological changes in macrophages were decreased Effects were seen at concentration as low as 0.5 μM (NO production)
ISS, ENEA, ANSES (2018)	CD1 mice (5 males and 5 females)	0.1, 1, 10 mg/kg bw by gavage 5 days a week for 42 days	Total and differential WBC Spleen, thymus, mesenteric lymph nodes weight and histology Serum immunoglobulins AntiCD3/CD28-induced IFN-γ and IL-10 production in splenocytes NO production in splenic macrophages CD3+, CD4+, CD8+ and CD45+ spleen lymphocytes	Decrease in granulocytes number in females at the middle dose No changes in spleen weight and histology in both males and females. Statistically significant decrease in thymus and mesenteric lymph nodes weights in males at the middle dose. No changes in histology No effects in total IgA, IgG and IgM levels Statistically significant increase in IFN-γ and IL-10 in females at the high dose Statistically significant increase in NO in males at the high dose, and a statistically significant decrease in females at the high dose Statistically significant increase in the % and absolute number of CD3+ cells, and in the number of CD4+ cells in females at the low dose

TABLE 3 (Continued)

Reference	Experimental model	Dosing regimen duration	Parameters	Results
Yang et al. (2022)	Murine GM-CSF-cultured bone marrow-derived dendritic cells (BMDCs)	0–10 μM, 24 h	IL-12p40; IL-12p70; IL-12p40, IFN-β, TNF, IL-6 (mRNA at 6 and 12 h); T cell activation: proliferation, IFN-γ, Foxp3, IL-4 and IL-17A	Cytotoxicity at concentration >5 μM. BEA activates GM-CSF-cultured BMDCs, inducing inflammatory cytokines IL-12p40, IFN-β, TNF-α and IL-6 mRNA together with CD86 expression in a MyD88 and TRIF-dependent way. Furthermore, BEA can enhance the ability of BMDCs to induce T cell proliferation, whereas it does not have an impact on differentiation or induction on cytokine production in individual cells Effects were seen at 2.5 μM (the lowest concentration tested)
Shandilya et al. (2023)	Co-culture model of bovine mammalian epithelial cell line MAC-T and bovine macrophage cell line BoMAC	11.3 μM for 48 h and LPS for 24 h	Cytokine/chemokine multiplex analysis	The concentration used resulted in a 20% decrease in cell viability Decrease in IFN-γ, IL-8, IL-10, IL36RA and MCP-1; post-LPS challenge, decrease in IFN-γ, IL-8, IL-10, IL36RA, MIP1b and MCP-1
Effects in non-immune cells				
Habauzit, Alvariño, et al. (2023)	Human adenocarcinoma alveolar basal epithelial cells A549	0.024–100 μM, 24 h	IL-8	A statistically significant increase in IL-8 at 3.12 and 6.25 μM, with a decrease at higher concentrations due to overt cytotoxicity. The IC_{50} for cell viability was 6.2 μM.
Xu et al. (2023)	Bovine mammary epithelial cell line MAC-T cells	10 μM, 4, 24, 48 h	TLR4, IL-6, TGF-β, TNF-α (mRNA)	Cytotoxicity at 20 μM Downregulation of TLR4 ($p < 0.05$), upregulation of IL- 6 ($p < 0.001$) and TGF-β ($p = 0.01$), downregulation of TNF-α ($p < 0.001$)

Abbreviations: BALB/c, laboratory mice strain; BMDCs, bone marrow-derived dendritic cells; BoMAC, bovine macrophage cell; ConA, concanavalin A; CCR7, chemokine receptor type 7 protein; CD, cluster of differentiation; FOXP3, fork head box P3 protein; GM-CSF, granulocyte macrophage colony stimulating factor; IC50, half-maximal inhibitory concentration; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAC-T, bovine mammary epithelial cell; MCP-1, monocyte chemoattractant protein-1; MIP1β, macrophage inflammatory protein 1 beta; mRNA, messenger ribonucleic acid; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; TGF, transforming growth factor; TLR4, toll-like receptor 4; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNF, tumour necrosis factor; TRIF, TIR-domain-containing adapter-inducing interferon-β; RAW264.7, murine macrophage cell line; μM, micro molar; mM, milli molar; mg/kg: milligrams per kilogram.

3.7 | Summary of mode of action impacting on the interpretation of the genotoxicity studies

There is evidence that BEA can induce oxidative stress, interference with cell signalling and mitochondrial damage to induce cell cycle arrest and apoptosis. These effects might play an indirect role in the formation of DNA strand breaks seen at cytotoxic concentrations in vitro. In vitro and in vivo studies do not suggest inflammatory effects but rather anti-inflammatory effects.

4 | CONCLUSIONS

In vitro studies in mammalian cell lines provided no convincing evidence for induction of chromosomal damage by BEA (as assessed by MN and chromosome aberration tests) or an increase of DNA strand breakage, as assessed by the Comet assay. There was a lack of concentration-dependence of effects and potential impact of associated cytotoxicity. In addition, DSB formation by γ-H2AX analysis was only observed following exposure to highly cytotoxic BEA concentrations. Results of in vivo studies indicated that BEA is devoid of genotoxicity. Gene expression studies in vitro showed no indication of a DNA damage response, and (Q)SAR analysis also did not indicate genotoxic potential.

There is evidence that the formation of DNA strand breaks seen in vitro at cytotoxic concentrations is due to indirect effects. In particular, BEA can increase ROS and induce cell cycle arrest and apoptosis, associated with interference in cell signalling and mitochondrial function. There is no evidence of inflammatory and immunosuppressive effects of BEA.

Taken together, the available data indicate that BEA is devoid of genotoxic potential.

ABBREVIATIONS

ADMET	absorption, distribution, metabolism, excretion and toxicity
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
AOP	adverse outcome pathways
ArfD	acute reference dose
BALB/c	laboratory mice strain
BCL2	B-cell lymphoma 2 proteins
BEA	beauvericin
BMDCs	bone marrow-derived dendritic cells
BoMAC	bovine macrophage cell line
BUN	blood urea nitrogen
Ca^{2+}	divalent calcium cations
Caco-2	human colorectal adenocarcinoma cells line
CAs	chromosome Aberrations
CAS	Chemical Abstracts Service
CB-17/SCID	'severe combined immunodeficient' (SCID) mice strain
CBPI	cytokinesis-block proliferation index
CCR7	Chemokine receptor type 7 protein
CD	cluster of differentiation
CFH-DA	2′,7′-dichlorofluorescin diacetate
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
ConA	concanavalin A
CONTAM	Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA)
DMSO	Dimethyl sulfoxide
DN	double negative
DNA	deoxyribonucleic Acid
DP	double positive
DSBs	DNA double-strand breaks
EFSA	European Food Safety Authority
ENEA	Italian National Agency for New Technologies, Energy and Sustainable Economic Development.
ENN B	enniatin B
EPA T.E.S.T.	Environmental Protection Agency Toxicity Estimation Software Tool
EPA	Environmental Protection Agency
ERK	extracellular signal-regulated kinase
FOXP3	fork head box P3 protein
G0/G1	resting phase /first gap phase
GM-CSF	granulocyte macrophage colony stimulating factor
GSH	glutathione
GSH/GSSG	glutathione/oxidised glutathione ratio
GSSG	oxidised glutathione or glutathione disulfide

H2-DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
H_2O_2	hydrogen peroxide
H4IIE	rat liver hepatoma cell line
HEK-293	human embryonic kidney 293 cell line
HepaRG	human hepatic cell line
HepG2	human hepatocellular carcinoma cell line
HL60	human leukaemia cell line
IC50	half-maximal inhibitory concentration
IFN	interferon
IFN-γ-STAT1-T-bet	interferon-gamma/signal transducer and activator of transcription 1/T-box transcription factor
Ig	immunoglobulin
IL	interleukin cytokine
IL36RA	interleukin 36 receptor antagonist
iNOS	inducible nitric oxide synthase
ISS	Istituto Superiore di Sanità (Italy)
JNK	c-Jun N-terminal kinase
KB-3-1	Epidermal carcinoma-derived cell line
KEGG	Kyoto Encyclopedia of Genes and Genomes
LOAEL	lowest observed adverse effect level
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MAC-T	bovine mammary epithelial cell line
MCP-1	monocyte chemoattractant protein-1
MD2	myeloid differentiation factor 2
MIP1β	macrophage inflammatory protein 1 beta
MITF	microphthalmia-associated transcription factor
ML	maximum level
MMS	methyl methane sulfonate
MN	micronucleus
mRNA	messenger ribonucleic acid
MTD	Maximum Tolerated Dose
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
MYD88	myeloid differentiation primary response 88
NBs	nuclear buds
NCI-H226	human squamous cell lung carcinoma cell lines
NF-κB	nuclear Factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NPBs	nucleoplasmic bridges
NR	neutral red
Nrf2	nuclear factor erythroid 2-related factor 2
OASIS	On-line Alerting of Structural Integrity and Safety
OECD	Organisation for Economic Co-operation and Development
p40/p70	cytokine subunits
PC-12	rat pheochromocytoma cell lines
PCE/NCE	polychromatic erythrocyte/normochromatic erythrocytes
PI3K/AKT/mTOR	phosphoinositide 3-kinase/protein kinase b/mechanistic target of rapamycin
PK15	porcine kidney cells lines
QSAR	quantitative structure activity relationship
RAW264.7	murine macrophage cell line
RET	mutant reticulocytes
RGA	Regulatory Guidance for Assays
ROS	reactive oxygen species
S9	liver enzyme extract
SCE	sister chromatid exchange
SH-SY5Y	human derived subcloned SK-N-SH cell lines
SSB	single-strand break
STAT3	signal transducer and activator of transcription 3
TDI	tolerable daily intake
TGF	transforming growth factor
TK6	lymphoblast cell lines
TLR4	toll-like receptor 4
TNBS	2,4,6-trinitrobenzenesulfonic acid

TNF	tumour necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β.
WG	Working Group
γ-H2AX	phosphorylated histone at serine 139

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

REQUESTOR

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ANNEXES

ANNEX A

Protocol for risk assessment

Annex A contains the risk assessment protocol selected by the CONTAM Panel to draft the Opinion and is available under the Supporting Information section on the online version of the scientific output.

ANNEX B

Literature searches

Annex B contains the details on the literature searches carried out in preparation of this Opinion and is available under the Supporting Information section on the online version of the scientific output.