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REVIEW ARTICLE

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Comprehensive review of ensulizole toxicology data and human exposure assessment for personal care products

Kimberly G. Norman[a], Lewis E. Kaufman[b], Peter Griem[c], Linda Loretz[a], Alexandra Kowcz[a], Samuel M. Cohen[d], Anthony R. Scialli[e], Alan R. Boobis[f], David Jacobson-Kram[g], Rita Schoeny[h], Thomas J. Rosol[i], Gary M. Williams[j], Norbert E. Kaminski[k], F. Peter Guengerich[l] and J. F. Nash[m]

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ABSTRACT

A comprehensive review of existing toxicity and human exposure data for the ultraviolet filter ensulizole (2-phenylbenzimidazole-5-sulfonic acid) as currently used in over-the-counter sunscreen formulations was conducted. Authorized maximum ensulizole usage levels in consumer end-use products worldwide range from 3% to 8%, with the maximum usage level limited to 4% in the United States, Canada, and Australia. Postmarketing clinical safety studies of ensulizole have reported only occasional local skin effects, none of which were associated with systemic toxicity. Ensulizole has been investigated *in vitro*, in animal toxicity studies, and in human studies for its pharmacokinetics, pharmacodynamics, and potential toxicological properties. Experimentally determined values of 4% for oral absorption in rats and of 0.26% for dermal absorption in humans were used for risk calculation purposes. There was no evidence of ensulizole bioaccumulation from rat *in vivo* studies, consistent with its high water solubility and low octanol/water partition coefficient. Ensulizole is not classifiable as an irritant, although local skin irritation with no systemic effects was noted in a 3-month repeated-dose dermal toxicity study in rabbits. Ensulizole is non-(photo)sensitizing, non-phototoxic, and has demonstrated low toxicity in acute (oral, dermal, and intraperitoneal) and subchronic repeated-dose studies in mammalian species. Subchronic 3-month no-observed-adverse-effect levels (NOAELs) were identified at 100 mg/kg/day (dermal rabbit) and 1000 mg/kg/day (oral rat OECD 408 study), the highest doses tested, respectively. Ensulizole is considered non-genotoxic, based on negative *in vitro* studies. No *in vivo* genotoxicity or long-term carcinogenicity studies were identified. Carcinogenicity risk is not expected based on the negative genotoxicity data, empirical evidence from repeated-dose toxicity and developmental toxicity studies, and the absence of effects on the androgen, estrogen, thyroid, immune, developmental, or reproductive systems. Based on the selected rat subchronic NOAEL of 1000 mg/kg/day and conservative assumptions for estimating the systemic exposure dose (SED) from the application of sunscreen products, margins of safety (defined as NOAEL/SED) >100 were obtained for ensulizole. Therefore, the available data show that ensulizole does not pose risks to human health when used in sunscreen products at concentrations up to 4%, the permitted maximum usage level in the United States, Canada, and Australia.

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Ensulizole; 27503-81-7; toxicology; exposure assessment; sunscreen; UV filter; personal care products; cosmetics; risk assessment

Abbreviations: AR: androgen receptor; ASEAN: Association of Southeast Asian Nations; ATSDR: Agency for Toxic Substances and Disease Registry; AUC: area under the curve; CatSper: cationic channel of sperm; CERAPP: Collaborative Estrogen Receptor Activity Prediction Project; CHLF: Chinese hamster lung fibroblasts; CHO: Chinese hamster ovary; CoMPARA: Collaborative Modeling Project for Androgen Receptor Activity; C_{max}: maximum (peak) concentration; DMSO: dimethyl sulfoxide; DNCB: 2,4-dinitrochlorobenzene; EATS: estrogen, androgen, thyroid, and steroidogenesis; ECHA: European Chemicals Agency; EPA: U.S. Environmental Protection Agency; ER: estrogen receptor; FAO: Food and Agriculture Organization; FDA: U.S. Food and Drug Administration; GCP: good clinical practice; GD: gestation day; GLP: good laboratory practice; GPMT: guinea pig maximization test; hERα: human recombinant ER, α-subtype; HPRT: hypoxanthine-guanine phosophoribosyltransferase; IARC: International Agency for Research on Cancer; IC_{50}: half maximal inhibitory concentration; ICDRG: International Contact Dermatitis Research Group; IV: intravenous; JECFA: Joint FAO/WHO Expert Committee on Food Additives; LD_{50}: median lethal dose; LOAEL: lowest-observed-adverse-effect level; MERCOSUR: Mercado Común del Sur

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(The Southern Common Market); MMTV: mouse mammary tumor virus; MoE: margin of exposure; MoS: margin of safety; MUsT: maximal usage trial; NOAEL: no-observed-adverse-effect level; NTP: U.S. National Toxicology Program; OECD: Organization for Economic Cooperation and Development; OEHHA: California Office of Environmental Health Hazard Assessment; OTC: over-the-counter; PACD: photoallergic contact dermatitis; PBSA: 2-phenylbenzimidazole-5-sulfonic acid (ensulizole); PK: pharmacokinetics; PoDsys: point of departure for systemic effects; QSAR: quantitative structure–activity relationship; SCCNFP: Scientific Committee on Cosmetic Products and Non-Food Products; SCCP: Scientific Committee on Consumer Products; SCCS: Scientific Committee on Consumer Safety; SED: systemic exposure dose; SIDS: screening information dataset; STTA: stably transfected transcriptional activation; TEA: triethanolamine; TG: test guideline; U.S.: United States; UV: ultraviolet; Vd: volume of distribution; WHO: World Health Organization

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Introduction

Ensulizole (2-phenylbenzimidazole-5-sulfonic acid [PBSA]; CAS no. 27503-81-7; Table 1) is a water-soluble, photostable sunscreen active ingredient that absorbs efficiently at short ultraviolet (UV) wavelengths (i.e. UVB; Figure 1). It is used in conjunction with other UV filters in sunscreen products due to its minimal protection against UVA wavelengths (Nash 2006). Moreover, it is more commonly found in daily use facial products (e.g. moisturizers) and rarely in recreational sunscreen products, which are often water resistant.

Ensulizole has a long history of safe use in sunscreen products in the United States (U.S.) and globally. In the U.S., ensulizole is a Category I (generally recognized as safe and effective) UV filter under the U.S. Food and Drug Administration (FDA) 1999 final monograph, but it is currently under review by the FDA under over-the-counter (OTC) reform legislation (USFDA 2019). Ensulizole is authorized for use in consumer end-use products at concentrations (w/v) ranging from 3% in Japan to 8% in the European Union, China, the Southern Common Market (MERCOSUR), and the Association of Southeast Asian Nations (ASEAN). In the U.S., Canada, and Australia, OTC or therapeutic usage level has been limited to a maximum of 4% (USFDA 2023). These permitted levels are based on review of ensulizole safety, efficacy, and postmarket surveillance data by numerous regulatory bodies and safety experts around the world. To date, none of the global regulatory bodies that have approved ensulizole for use in sunscreen products have reported any health or safety concerns related to its use.

The purpose of the present assessment is to (1) develop a comprehensive summary of the pharmacokinetic (PK) and toxicological data that exist for ensulizole; (2) estimate the systemic exposure dose (SED) to ensulizole from the topical application of sunscreen products; and (3) provide an estimate of the derived margin of safety (MoS) (defined as no-observed-adverse-effect levels [NOAEL]/SED) for ensulizole when used in sunscreen products at concentrations up to 4%. This assessment incorporates data generated since the various regulatory reviews were published, and specifically addresses regulatory questions regarding carcinogenicity and developmental and reproductive toxicology raised in an ongoing review that has been undertaken by the FDA.

Materials and methods

A literature search was performed to identify clinical and nonclinical studies that evaluated ensulizole disposition and toxicity. Studies were primarily identified through the following expert organizations and state, federal, and international regulatory electronic databases to identify substance-specific information and data: the European Chemicals Agency (ECHA) database, PubChem, the U.S. Environmental Protection Agency (EPA) CompTox Dashboard, the EPA Integrated Risk Information System (IRIS), the U.S. National Toxicology Program (NTP), ChemIDPlus, the Organization for Economic

Table 1. Ensulizole identifiers and physicochemical properties.

Identifier/Property	Attribute
INCI/INN/USAN	Ensulizole
FDA UNII	9YQ9DI1W42
IUPAC	2-Phenyl-3*H*-benzimidazole-5-sulfonic acid
CAS registry	27503-81-7
EINECS (EC)	248-502-0
Synonyms	2-Phenylbenzimidazole-5-sulfonic acid Benzimidazole, 2-phenyl, 5-sulfonic acid
Trade names	Neo Heliopan Hydro, Eusolex 232, Novantisol
Molecular formula	$C_{13}H_{10}N_2O_3S$
Molecular weight	274.3 g/mol
Physical description	Colorless to yellowish powder
Melting point	>300 °C
Boiling point	>300 °C
Density	1.62 g/cm^3
Solubility	Water (109 mg/L at 25 °C), miscible in ethanol
Partition coefficient (Log K_{ow})	−1.42 (at 25 °C)
Vapor pressure	Expected to be low based on a melting point >300 °C
Self-ignition temperature	>400 °C

CAS: Chemical Abstracts Service; EC: European Commission; EINECS: European Inventory of Existing Commercial Chemical Substances; INCI: International Nomenclature Cosmetic Ingredient; INN: International Nonproprietary Name; IUPAC: International Union of Pure and Applied Chemistry; UNII: Unique Ingredient Identifier; USAN: United States Adopted Name.
Sources: SCCP 2006; Drugbank 2023; ECHA 2023; NCBI 2023; USEPA 2023.

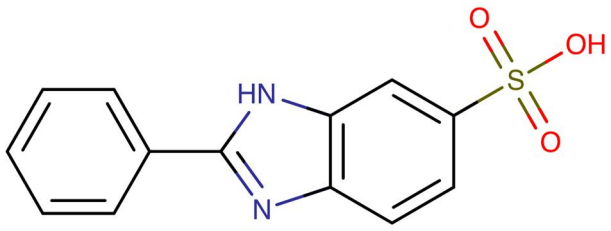

Figure 1. Chemical structure of ensulizole (2-phenylbenzimidazole-5-sulfonic acid).

Co-operation and Development (OECD) eCHEM Portal, OECD Screening Information DataSet (SIDS), the International Program on Chemical Safety (IPCS) INCHEM catalog, the FDA databases, GESTIS Substance database, the Agency for Toxic Substances and Disease Registry (ATSDR), the International Agency for Research on Cancer (IARC) publications, the California Office of Environmental Health Hazard Assessment (OEHHA) chemical database, the database that provides chemical evaluations from the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), and opinions from the European Commission scientific committees, especially the Scientific Committee on Consumer Safety (SCCS) and its predecessors, the Scientific Committee on Consumer Products (SCCP) and the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP).

The information and data obtained from these databases were supplemented with additional published literature retrieved from the PubMed database from its inception date, using the following search construct:

> (ensulizole OR 2-phenylbenzimidazole-5-sulfonic acid OR 27503-81-7) AND (human OR clinical OR nonclinical OR animal) AND (toxic* OR abnormal OR adverse OR develop* OR repro* OR endo* OR carc* OR geno* OR metabol* OR pharmaco* OR immuno*)

This search generated papers from which titles and abstracts were screened to identify potentially relevant articles. Articles that examined ensulizole and its physicochemical properties, PK, pharmacodynamics, and/or toxicity were included for full-article review; those focused on efficacy/photoprotection, environmental, or invertebrate systems were largely excluded. Relevant unpublished reports of toxicity studies performed by a manufacturer were included. Additional literature was identified and manually obtained through references cited by published papers, reviews, and regulatory documents acquired from the searches.

Many of the studies described herein were compliant with Good Laboratory Practice (GLP) regulations and/or a particular test guideline provided by the OECD, the European Union, the EPA, and/or the FDA. This information and the specific method or OECD testing guideline (TG) number are acknowledged when applicable for studies described in this ensulizole profile.

Results

Pharmacokinetics and toxicokinetics

The data from one human PK study, two *in vivo* animal toxicokinetic studies, and one OECD TG 428 *in vitro* percutaneous absorption study suggest low oral (<5%) and dermal (<0.5%) absorption of ensulizole. There was no evidence for bioaccumulation in any of the organs investigated in the available studies. For risk assessment purposes, an oral absorption of 4% and a dermal penetration value of 0.26% were used. It is not known whether clinical maximal usage trial (MUsT) studies have been conducted with ensulizole. None were found using the methodology described.

The human skin penetration of ^{14}C-ensulizole from a cosmetic gel formulation containing 8% ensulizole was determined *in vivo* using a standardized tape-stripping method (non-GLP but compliant with Good Clinical Practice [GCP]) (SCCP 2006; ECHA 2023). One gram of a cosmetic gel containing 80 mg of ^{14}C-ensulizole (1.76 MBq/g gel) was applied to an area of 333 cm^2 on the upper forearm of each of six healthy male volunteers. The skin was protected with a non-occlusive cover. After 6 h, the remaining gel on the skin was

removed by skin washings with cotton wool plugs soaked in ether. Subsequently, the whole area of treated skin was stripped 10 times with adhesive films. Blood, urine, and feces samples were collected over a 120-h period. Plasma samples, urine, feces, methanol extracts of the cotton wool plugs and adhesive films were analyzed for total radioactivity using liquid scintillation counting. The plasma radioactivity values were always lower than two times the background value. In most of the human volunteers, no noticeable plasma levels of radioactivity were observed; therefore, specific penetration rates could not be established. Total excretion of radioactivity ranged from 0.11% to 0.26% of the applied dose (SCCP 2006; ECHA 2023). Based on this skin penetration study in human volunteers, the dermal penetration of ensulizole, applied in the form of a cosmetic gel formulation, was determined to be 0.26% and this value was used in further calculations.

A GLP-compliant toxicokinetic study of ^{14}C-labeled ensulizole sodium salt was conducted in pregnant Wistar rats according to a method equivalent to EPA Office of Pesticide Programs Guideline 85-1 (Metabolism and Pharmacokinetics) (SCCP 2006; ECHA 2023). The pregnant rats were administered either a single intravenous (IV) injection (1 mg/kg) or an oral dose (1000 mg/kg) of ^{14}C-labeled ensulizole sodium salt on gestation day (GD) 18. After a single IV injection, the peak plasma concentrations (C_{max}) of ^{14}C were reached after 5 min (C_{max} 4.12 μg/mL, volume of distribution [V_d] = 0.25 L), and the ^{14}C radioactivity was eliminated from plasma with an apparent half-life of 0.4 h. After oral administration, the C_{max} of ^{14}C concentrations were reached after 15 min, indicating rapid absorption from the gastrointestinal tract. Liver and kidneys in pregnant rats exhibited the highest concentrations of ^{14}C radioactivity after both IV and oral administration. The lowest concentrations were measured in the fetuses, showing that the placenta was a barrier to exposure. Approximately 66% of the IV-injected radioactivity was excreted via the kidneys. After oral administration, 2.5% of the dose was found in urine within 48 h. As no residual radioactivity was present in the rats after both modes of administration, it was concluded that the dose had been excreted completely within 48 h. Based on comparison of the plasma area under the curve (AUC) values and the excretion data after IV and oral administration, it was concluded that 3%–4% of the dose had been absorbed from the gastrointestinal tract following oral administration. The study suggests that there was no bioaccumulation potential in any of the studied organs after IV and oral administration of ensulizole in rats (SCCP 2006; ECHA 2023).

In a toxicokinetic screening study (non-GLP), male Wistar rats were administered a single dose of approximately 0.2 mg of ^{14}C-labeled ensulizole sodium salt either orally or by IV. The animals were placed in metabolism cages and exhaled air, urine, and feces were collected over a period of 72 h, after which residual radioactivity in the carcasses was determined. Approximately 96% of orally dosed ensulizole radioactivity was excreted by 72 h via urine and feces. Negligible amounts were excreted in the expired air following either route of exposure (SCCP 2006).

An OECD TG 428 *in vitro* percutaneous absorption study was conducted using 4% ensulizole in two cosmetic formulations (oil-in-water emulsion and water-in-oil emulsion) on female pig skin (SCCP 2006). The two sunscreen formulations were applied separately at a dose of 150–200 μg/cm^2 or 0.5 mg/cm^2 to the stratum corneum of the skin disks. The formulations remained on the skin surface for 1.5 or 24 h under non-occluded conditions. Ensulizole content was determined by high performance liquid chromatography. The recoveries for the water-in-oil and oil-in-water formulations were 96.6% (range 89.9%–100.4%) and 100.9% (range 90.8%–101.6%), respectively. At 24 h, the total absorbed amounts of ensulizole from epidermis, dermis, and receptor fluid were 6.6 and 10.9 μg/cm^2 for the same respective formulations. Substance present in the receptor fluid alone was below the analytical limit of detection (SCCP 2006).

Recent studies have explored predictive modeling to describe the expected absorption of dermally applied ensulizole. Among these is an integrated mechanistic dermal absorption model designed to simulate steady-state plasma concentrations of seven UV filters, including ensulizole, following facial application. This model was trained using *in vitro* permeation test data integrated with previously published *in vitro* permeation test and clinical PK data via a Bayesian Markov chain Monte Carlo method and then validated using real-world *in vivo* datasets (Hamadeh et al. 2024). Predictions of plasma concentrations of ensulizole following applied doses of 1 or 2 mg/cm^2, once or twice a day, to the face, neck, and hands were <0.5 ng/ml, the FDA threshold (Wang and Ganley 2019). Consistent with earlier work, these data showed limited, if not negligible, skin penetration of this sunscreen active material.

Toxicology

Acute toxicity

According to the data presented below for oral, dermal, and intraperitoneal rodent acute toxicity studies and the EPA hazard determination guidelines (see Supplemental Table 1), ensulizole has low acute mammalian toxicity via all tested routes of exposure.

In acute oral toxicity studies (GLP status and method not specified), median lethal dose (LD_{50}) values for ensulizole sodium salt were >1600 to >66,000 mg/kg and >5000 to >6600 mg/kg in rats and mice, respectively (Hoffman 1971; SCCP 2006; ECHA 2023).

In two acute dermal toxicity studies in Wistar rats (non-GLP) conducted according to a method equivalent or similar to OECD TG 402, 30% (w/v) aqueous solutions of ensulizole sodium salt and ensulizole triethanolamine (TEA) salt (equivalent to 3000 mg/kg) were applied occlusively to the animals' skin for 24 h, followed by an observation period of 14 days. The dermal LD_{50} values in both studies were >3000 mg/kg (SCCP 2006; ECHA 2023).

In two acute intraperitoneal toxicity studies in Wistar rats (non-GLP, method not specified), the LD_{50} values were 1046 and 1513 mg/kg for ensulizole sodium salt and ensulizole TEA salt, respectively (SCCP 2006; ECHA 2023).

Repeated-dose toxicity

The data presented below for two available subchronic toxicity studies indicate that ensulizole has low repeated-dose toxicity according to the EPA hazard determination guidelines (see Supplemental Table 1). From these studies, a 3-month oral systemic NOAEL in rats of 1000 mg/kg/day and a 91-day dermal systemic NOAEL in rabbits of 100 mg/kg/day (the highest doses tested in both studies) were identified. The 3-month oral rat systemic NOAEL of 1000 mg/kg/day was selected as the point of departure for risk assessment purposes. No studies were identified that investigated the subacute (<1 month) or chronic (>3 months) toxicity of ensulizole.

An OECD TG 408 (non-GLP) subchronic oral toxicity study was conducted to determine the repeated-dose toxicity of ensulizole. Wistar SPF rats were administered ensulizole in 5% aqueous Tylose (nonionic, water-soluble cellulose, w/v) by oral gavage daily at doses of 0, 100, 330, or 1000 mg/kg/day for 3 months. Endpoints evaluated for all animals included mortality, clinical signs of toxicity, body weight, food intake, water consumption, hematology, clinical chemistry, urinalysis, organ weights (thymus, thyroid, heart, lung, liver, spleen, kidneys, adrenals, testes, ovaries), and macroscopic and microscopic pathology. During the study, one control group male and single females in the 330 and 1000 mg/kg/day groups died. However, these mortalities were not considered test article-related. There were no test material-related effects, including clinical pathology, organ weight, macroscopic necropsy, or histopathological changes noted at any dose level. It was concluded that the treatment with ensulizole was well tolerated at all tested dose levels. The 3-month oral NOAEL for ensulizole was 1000 mg/kg/day, the highest dose tested (Bomhard and Schilde 1978; SCCP 2006; ECHA 2023).

A GLP-compliant subchronic toxicity study was conducted to determine the dermal toxicity of ensulizole in rabbits. New Zealand white rabbits received topical applications of formulations containing 0%, 1%, and 5% ensulizole (w/v) at doses of 2 mL/kg/day (equivalent to 0, 20, and 100 mg/kg/day), 5 days/week for 13 weeks (91 days) under occluded conditions (Weisenburger 1991). The formulation without active substance was considered as the vehicle control group. All treatment and control group animals exhibited a range of dermal irritation symptoms including erythema, edema, desquamation, and red raised areas at the site of application, which were scaled as slight, moderate, or marked (severe). Signs of dermal irritation occurred early in the study and continued or worsened throughout the study for all groups. Macroscopic examination of the application sites showed treatment-related low incidences of moderate erythema, edema, and/or scabbed areas. No treatment-related biologically meaningful changes or differences were observed for body weights, organ weights, or hematologic endpoints. Microscopic examination of the application sites showed treatment-related changes in high-dose group females and low-dose group males and females. These changes included acanthosis, multifocal ulceration, and/or necrosis of the epidermis and chronic active inflammatory changes. Both sexes in the control group, some high-dose group males, and one female each in the low- and high-dose groups exhibited a chronic inflammatory response in the dermis. There were no microscopic changes in the other tissues examined, confirming lack of systemic toxicity. Under the study conditions, the 91-day local toxicity lowest-observed-adverse-effect level (LOAEL) and the 91-day systemic toxicity NOAEL for ensulizole were determined to be 20 and 100 mg/kg/day (highest dose tested), respectively (Weisenburger 1991).

Irritation, sensitization, and photosafety

Skin irritation. Data from two rabbit OECD TG 404-compliant skin irritation studies and three other non-GLP studies in rabbits show that ensulizole is not acutely irritating to skin under the conditions tested.

In an OECD TG 404-compliant dermal irritation study, 0.5 mL of undiluted and diluted (16% aqueous, w/v) solutions of ensulizole TEA salt were applied to the skin of six New Zealand white rabbits (three/sex) for 4 h under occlusive conditions. The animals were observed for skin alterations, clinical signs, and body weight changes for a period of 8 days. Skin grading was conducted according to the Draize scale, with mean scores (24/48/72 h after application) ranging from 0 (none) to 4 (severe) for erythema and edema (Draize et al. 1944). The undiluted test material caused slight irritation, with one rabbit showing erythema on Day 4 and slight scaling on Days 6 and 7. A second rabbit showed slight scaling on Days 5 and 6. The mean erythema and edema scores after application of undiluted material were 0.17 and 0, respectively. Erythema was fully reversible within 5 days. Treatment with a 16% aqueous solution of ensulizole TEA salt (w/v) did not result in any skin reactions. Under the study conditions, ensulizole TEA salt was not considered to be irritating to skin (SCCP 2006; ECHA 2023).

In an OECD TG 404-compliant dermal irritation study, 0.5 mL of a 10% (w/v) aqueous solution of ensulizole sodium salt was applied to the skin of three female Mol:Russian rabbits for 4, 10, and 24 h under occlusion. No erythema or edema reactions were observed in any of the animals up to 72 h post-application after exposure durations of 4, 10, or 24 h (all erythema and edema scores were 0). Under the study conditions, ensulizole sodium salt was not considered to be irritating to skin (Jacobson 1991; SCCP 2006; ECHA 2023).

In two dermal irritation studies (non-GLP), 0.5 mL of a 30% (w/v) aqueous solution of ensulizole sodium salt and ensulizole TEA salt were applied to the skin of six male New Zealand white rabbits (three each for intact and abraded skin) for 24 h under occlusive conditions. No abnormalities were recorded over a 7-day observation period in either study. Under the study conditions, ensulizole sodium salt and ensulizole TEA salt were not considered to be irritating to skin (SCCP 2006).

In a non-GLP dermal irritation study conducted according to a method similar to OECD TG 404, 1 mL of a 10% aqueous solution (w/v) of ensulizole sodium salt was applied to the skin of six New Zealand white rabbits (three each for intact and scarified skin) for 24 h under occlusive conditions. No

skin reactions were observed on either intact or scarified skin. Under the study conditions, ensulizole sodium salt was not considered to be irritating to skin (Hoffman 1971; SCCP 2006).

In another dermal irritation study, 1 mL of a 10% aqueous solution (w/v) of ensulizole sodium salt was applied dermally to six rabbits (three each for intact and scarified skin) for 24 h under occlusion and the application was repeated daily for 5 days. No skin reactions were observed on either intact or scarified skin. Under the study conditions, ensulizole sodium salt was not considered to be irritating to skin (Hoffman 1971; SCCP 2006).

Eye irritation. The results obtained from four *in vivo* nonclinical eye irritation studies show that ensulizole is not irritating to eyes.

In an OECD TG 405-compliant *in vivo* eye irritation study, 0.1 mL Eusolex 232 TS liquid containing ensulizole and TiO_2 (30% aqueous solution, w/v) was instilled into the conjunctival sac of the left eye of each of three rabbits. The right eyes remained untreated and served as controls. The animals were investigated for eye irritation 1 h after treatment and then daily for a period of 8 days. Effects on the cornea, iris, and conjunctivae were evaluated according to the Draize scale. Slight reddening of the conjunctiva was observed in one animal only on Day 1. No other effects were observed during the 8-day observation period. Based on these study results, ensulizole was not considered to be an ocular irritant (SCCP 2006; ECHA 2023).

In an *in vivo* ocular irritation study (non-GLP), 0.1 mL ensulizole TEA salt (30% aqueous solution, w/v) was instilled into the conjunctival sac of the left eye of each of three New Zealand white rabbits. The right eyes remained untreated and served as controls. The animals were observed up to 7 days. Effects on the cornea, iris, and conjunctivae were evaluated according to the Draize scale. No abnormalities were recorded over a 7-day observation period. Based on these study results, ensulizole TEA salt was not considered to be an ocular irritant (SCCP 2006).

In an *in vivo* ocular irritation study (non-GLP), 0.1 mL ensulizole sodium salt (10% aqueous solution, w/v) was instilled into the conjunctival sac of the right eye of each of 12 albino rabbits. The left eyes remained untreated and served as controls. After 1 min, the right eyes were rinsed in six animals, but not in the other six. No reaction was observed at the single 24-h observation period. Based on these study results, ensulizole sodium salt was not considered to be an ocular irritant (Hoffman 1971; SCCP 2006).

In an *in vivo* ocular irritation study (non-GLP), 0.1 mL ensulizole sodium salt (30% aqueous solution, w/v) was instilled into the conjunctival sac of the left eye of each of three New Zealand white rabbits (SCCP 2006). The right eyes remained untreated and served as controls. The animals were observed up to 7 days. Effects on the cornea, iris, and conjunctivae were evaluated according to the Draize scale. No abnormalities were recorded over a 7-day observation period. Based on these study results, ensulizole sodium salt was not considered to be an ocular irritant (SCCP 2006).

Skin sensitization. The results of five *in vivo* guinea pig sensitization studies suggest that ensulizole has no skin sensitization potential.

In an OECD TG 406-compliant guinea pig maximization test (GPMT), intradermal injections of 0.1 mL Freund's complete adjuvant in water, 1% w/v ensulizole (w/v) in arachis oil, and Freund's adjuvant with 1% w/v ensulizole in arachis oil (1:1, w/v) were administered in the induction phase to groups of Dunkin Hartley guinea pigs into three separate sites. After 1 week, the same area was treated with a single topical application of 0.2–0.3 mL 50% w/w ensulizole in arachis oil under occlusive conditions for 48 h. On Day 21, the shorn right flank of each animal was treated with 0.1–0.2 mL 25% and 10% w/w ensulizole in arachis oil under occlusion for 24 h in the challenge phase. Cutaneous reactions were evaluated at 24 and 48 h after removal of the dressing. There was no concurrent positive control, but historical controls with 2,4-dinitrochlorobenzene (DNCB) were available. There were no adverse reactions reported at ensulizole and vehicle control sites of the test or control animals at the 24- and 48-h observations. It was concluded that ensulizole did not induce skin sensitization in guinea pigs under the conditions of the study (Tuffnell 1992a; SCCP 2006; ECHA 2023).

In an OECD TG 406 GPMT study, intradermal injections of 0.1 mL Freund's adjuvant, 12.5% ensulizole (w/v; in Eusolex 232–TS liquid containing 40% ensulizole and TiO_2, no further details are available), and Freund's adjuvant with 12.5% ensulizole (w/v, in Eusolex 232–TS) into three separate sites were administered to guinea pigs (strain not available) in the induction phase. After 1 week, the same area was treated with a single topical application of 40% ensulizole (w/v, no details about vehicle and volume are available). After 1 week, in the challenge phase, the animals were treated topically with 25% ensulizole (w/v, no details about vehicle and volume are available). Cutaneous reactions were evaluated at 24 and 48 h after removal of the dressing. There was no concurrent positive control, but historical control data with DNCB were available. There were no adverse reactions reported at ensulizole and vehicle control sites of the test or control animals at the 24- and 48-h observations. It was concluded that ensulizole did not induce skin sensitization in guinea pigs under the conditions of the study (SCCP 2006).

In another OECD TG 406 GPMT study, intradermal injections of 0.1 mL Freund's complete adjuvant in water, 25% w/v ensulizole sodium salt in water, and Freund's adjuvant with 25% w/v ensulizole sodium salt in water (1:1, all w/v) into three separate sites were administered to groups of Dunkin Hartley guinea pigs during the induction phase. After 1 week, the same area used previously for intradermal injections was treated with a single topical application of 0.2–0.3 mL undiluted ensulizole sodium salt under occlusive conditions for 48 h. On Day 21, the shorn right flank of each animal was treated with 0.1–0.2 mL undiluted ensulizole sodium salt and 75% v/v ensulizole sodium salt in water (w/v) at separate sites under occlusive conditions for 24 h during the challenge phase. Cutaneous reactions were evaluated at 24 and 48 h after removal of the dressing. No reactions were observed. There was no concurrent positive control, but historical

controls with DNCB were available. There were no adverse reactions reported at ensulizole sodium salt and vehicle control sites of the test or control animals at the 24- and 48-h observations. It was concluded that ensulizole sodium salt did not induce skin sensitization in guinea pigs under the conditions of the study (Tuffnell 1992b; SCCP 2006; ECHA 2023).

In a skin sensitization study (non-GLP), Pirbright white guinea pigs (5/sex/dose) were topically treated on the left flank with 5% or 30% solutions of ensulizole sodium salt (w/v, presumably water was used as the vehicle but not stated) five times weekly for 2 weeks. Water was used as the vehicle control. After 7 days, 0.5% and 3% solutions of ensulizole sodium salt (w/v) were applied to the animals in the challenge phase, respectively. No reaction was observed at the site of application. All animals tested with the positive control (2% DNCB, w/v) produced an erythematous reaction 3 h after application of the elicitation dose. It was concluded that ensulizole sodium salt did not induce skin sensitization in guinea pigs under the conditions of the study (Frohberg 1974a; SCCP 2006).

In another non-GLP skin sensitization study, Pirbright white guinea pigs (5/sex/dose) were topically treated on the left flank with 5% or 30% solutions of ensulizole TEA salt (w/v, presumably water was used as the vehicle but not stated) five times weekly for 2 weeks. Water was used as the vehicle control. After 7 days, 0.5% and 3% solutions of ensulizole TEA salt (w/v) were applied to the animals in the challenge phase, respectively. No reactions were observed at the site of application. All animals tested with the positive control (2% DNCB) produced an erythematous reaction 3 h after application of the elicitation dose. It was concluded that ensulizole TEA salt did not induce skin sensitization in guinea pigs under the conditions of the study (Frohberg 1974b; SCCP 2006).

Photosafety. The results of two *in vitro* and two *in vivo* phototoxicity studies and one *in vivo* dermal photosensitization study suggest that ensulizole does not have dermal photoirritation or photosensitization potential.

In an *in vitro* 3T3 Neutral Red Uptake phototoxicity test conducted according to OECD TG 432, 391–50,000 mg/L (5%, w/v) of ensulizole diluted in Earle's buffered salt solution was evaluated both in the presence and absence of irradiation (no details were available on UV dose). Cytotoxicity was observed in both the irradiated and non-irradiated samples. The negative control (sodium lauryl sulfate) was confirmed to be non-phototoxic, whereas the positive control (chlorpromazine) was phototoxic in the 3T3 fibroblasts. Ensulizole was assessed to be non-phototoxic in 3T3 fibroblasts (photoirritation factor: 1.4) (SCCP 2006).

Scalp treatment formulations containing 4% ensulizole were tested in an *in vitro* phototoxicity test (non-GLP) both in the presence and in the absence of irradiation (1.7 mW/cm^2 for 50 min) using the BALB/c mouse fibroblast cell line, 3T3, at concentrations of 0.000272% to 0.004%. The negative control was non-phototoxic, whereas the positive control was phototoxic in the test system. Ensulizole was assessed to be non-phototoxic in 3T3 fibroblasts (photoirritation factor: 0) (Kirk 2007).

A GLP-compliant dermal phototoxicity study was conducted to evaluate ensulizole at 50% (w/v) and 100% (undiluted) using 10 female test and 5 female control Pirbright white (Bor:DHPW [SPF]) guinea pigs according to the Vinson and Borselli method (Vinson and Borselli 1966; Buchholz 1991b). After application of the ensulizole solutions to separate sites on the shaved backs of the test animals, the skin was irradiated with UVB at 0.10 J/cm^2 for 50 sec and UVA at 10.26 J/cm^2 for 26 min; control animal skin was not irradiated. Skin reactions were evaluated and compared in the two groups 24 and 48 h after the end of irradiation. The areas treated with the positive control (8-methosypsoralen) showed a phototoxic reaction only in the irradiated animals. There were no skin reactions observed in ensulizole-treated areas (both nonirradiated and irradiated) and untreated areas (negative control, both nonirradiated and irradiated) of the animals. Under the study conditions, ensulizole was assessed to be non-phototoxic (Buchholz 1991b; SCCP 2006).

Non-GLP testing was conducted in Naval Medical Research Institute mice (5/sex/group) to determine potential phototoxic properties of ensulizole (5% aqueous solution) (SCCP 2006). After application of 5% ensulizole solution (w/v) to animal backs, the skin of test animals was irradiated with light from a quartz lamp (Q600; no details available on UV dose). Skin reactions were evaluated and compared 48 and 72 h after the end of irradiation. No skin effects were observed in non-irradiated animals. In both irradiated groups (test and controls), slight to moderate erythema of the same intensity and frequency was seen 48 and 72 h post-irradiation. Under the study conditions, ensulizole was assessed to be non-phototoxic (SCCP 2006). However, the study is considered of lower reliability due to the lack of information on the UV dose and its non-GLP status.

A GLP-compliant dermal photosensitization study was conducted to evaluate ensulizole using 10 test and 5 control Pirbright white (Bor:DHPW [SPF]) guinea pigs according to the Vinson and Borselli method (Vinson and Borselli 1966; Buchholz 1991a). In the induction phase, the test animals were administered 10 daily applications of ensulizole (100%) over two periods of 5 consecutive days. Each dose application was followed 30 min later by irradiation (UV A + B at 10.44 J/cm^2 for 100 min). In the challenge phase 2 weeks later, four different concentrations of ensulizole (25%, 50%, 75%, and 100% w/v) were applied to each of the test animals at two adjacent sites per concentration; one site was protected from light and the other was irradiated (UVA at 10.06 J/cm^2 for 101 min). Skin reactions were evaluated 24 and 48 h after the end of irradiation. The test animals showed no dermal irritation throughout the 2-week induction period. During the same period, all positive control (hexachlorophene)-treated animals showed moderate erythema after the sixth exposure and edema after the seventh exposure. In the challenge phase, positive control animals revealed a photosensitizing reaction. In the test group, no photoallergenic skin reactions were observed either on the irradiated or on the protected side, indicating that ensulizole was not a photosensitizer under the conditions of this study (Buchholz 1991a; SCCP 2006; ECHA 2023).

Genotoxicity and carcinogenicity

Genotoxicity. Five *in vitro* genotoxicity studies were reviewed, which demonstrated that ensulizole did not cause gene mutations or chromosomal aberrations.

A GLP-compliant OECD TG 471 bacterial reverse mutation assay (i.e. Ames test) was conducted to determine the mutagenic potential of ensulizole (Herbold 1992; SCCP 2006; ECHA 2023). *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98, and TA100 were exposed to ensulizole dissolved in dimethyl sulfoxide (DMSO) at concentrations of 0, 300, 1000, 3000, 10,000, and 75,000 μg/plate in the presence and absence of metabolic activation (i.e. S9 liver homogenate fraction from Aroclor 1254-induced rats). Ensulizole did not increase the number of revertants in any test strain in a dose-related and biologically relevant manner compared to the negative control, with or without metabolic activation. The results obtained for negative and positive controls confirmed the validity of the study. Under the study conditions, ensulizole was not mutagenic in the Ames test with or without metabolic activation (Herbold 1992; SCCP 2006; ECHA 2023).

Another GLP-compliant study was conducted according to a method similar to OECD TG 471 (Ames test) to determine the mutagenic potential of ensulizole. *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 and *Escherichia coli* strains WP2 and WP2 uvrA were exposed to ensulizole dissolved in DMSO at concentrations of 0, 50, 250, 1250, 2500, 5000, and 10,000 μg/plate in the presence and absence of metabolic activation (S9 liver homogenate fraction from Aroclor 1254-pretreated rats). Cytotoxicity was not observed. Ensulizole did not show mutagenic activity, either in the absence or in the presence of the metabolic activation system. The results obtained for negative and positive controls confirmed the validity of the study. Under the study conditions, ensulizole was not mutagenic with or without metabolic activation (Herbold 1992; SCCP 2006; ECHA 2023).

An OECD TG 473 study was conducted to determine the genotoxic potential of ensulizole using an *in vitro* chromosomal aberration test in human peripheral blood lymphocytes. In one experiment for metaphase analysis, the human lymphocyte cultures were treated with 0.33, 3.3, and 20 mM ensulizole in medium for 24 h without metabolic activation and 3.5 h of treatment with metabolic activation (S9 liver homogenate fraction from Aroclor 1254-pretreated rats). In another experiment for metaphase analysis, the human lymphocyte cultures were treated with 1.0, 3.3, and 10 mM ensulizole for 4 h without metabolic activation and 3.5 h with metabolic activation. The results obtained for negative and positive (mitomycin C and cyclophosphamide) controls confirmed the validity of the study. Ensulizole did not induce an increase in the number of structural or numerical chromosome aberrations with or without metabolic activation when compared to controls (King 2002; SCCP 2006; ECHA 2023).

An OECD TG 473 study was conducted to determine the genotoxic potential of ensulizole using an *in vitro* chromosomal aberration test in Chinese hamster ovary (CHO) cells. The cell cultures were treated with 2.8, 3.8, and 5 μL/mL ensulizole in ethanol with and without metabolic activation (liver S9 homogenate fraction from Aroclor-induced rat) for 8 or 12 h. The results obtained for negative and positive controls confirmed the validity of the study. Ensulizole did not induce a statistically significant increase in chromosome aberrations with or without metabolic activation when compared to controls (Putman and Morris 1991).

An OECD TG 476 study was conducted to determine the *in vitro* mammalian mutagenicity of ensulizole using the hypoxanthine-guanine phosphoribosyltransferase (HPRT) test in Chinese hamster lung fibroblasts (CHLF V79). The cell cultures were treated with 87.5, 175, 350, 700, 1400, and 2800 μg/mL ensulizole in a preliminary dose range-finding study and in two main experiments with and without metabolic activation (liver S9 homogenate fraction from phenobarbital/β-naphthoflavone-treated rats). In the first experiment, the treatment period was 4 h with and without metabolic activation. The second experiment was performed with a treatment time of 4 h with and 24 h without metabolic activation. Cytotoxicity was not observed up to the highest tested dose. No biologically relevant or reproducible dose-dependent increase of the mutation frequency was observed in either of the main experiments. The results obtained for negative and positive (ethylmethanesulfonate and 7,12-dimethylbenzanthracene) controls confirmed the validity of the study. Under the study conditions, ensulizole was not mutagenic with or without metabolic activation (Wollny 2013; ECHA 2023).

Photomutagenicity. Three photomutagenicity or photoclastogenicity studies showed that ensulizole was not photogenotoxic under the conditions tested.

A study was conducted to assess the photomutagenicity of ensulizole in a GLP-compliant modified Ames test. *S. typhimurium* (TA102 and TA1537) and *E. coli* WP2 and WP2pkM101 were exposed to 25–2500 μg/plate of ensulizole. The liquid cultures were irradiated with 10–1800 mJ/cm^2 UVA and 0.34–61.2 mJ/cm^2 UVB. Solvent and dark controls, as well as appropriate positive controls for each strain, were included. Under the study conditions, ensulizole was not photomutagenic in *S. typhimurium* (TA1537 and TA102) and *E. coli* (WP2 and WP2pkM101) (SCCP 2006).

A GLP-compliant *in vitro* yeast gene conversion study was conducted to assess the photomutagenicity potential of ensulizole. *Saccharomyces cerevisiae* (D7 strain) cells were exposed to 50–1000 μg/mL ensulizole and irradiation doses of 168–3360 mJ/cm^2 UVA and 2.5–50 mJ/cm^2 UVB. Appropriate positive and negative controls were run concurrently and showed the expected effects. Under the study conditions, ensulizole was not photomutagenic for *S. cerevisiae* (SCCP 2006).

A GLP-compliant study was conducted to assess the photoclastogenicity of ensulizole in an *in vitro* chromosomal aberration assay in CHO cells. The CHO cells were exposed to 250–1000 μg/mL ensulizole and irradiation doses of 200–800 mJ/cm^2 UVA and 6.8–27.2 mJ/cm^2 UVB. An appropriate positive control was tested concurrently. Under the study conditions, ensulizole was not photoclastogenic in CHO cells (SCCP 2006).

Beyond these photomutagenicity studies, there have been reports in the literature that UVB-photoexcited ensulizole can generate reactive oxygen species and free radicals through Type I and Type II mechanisms (Inbaraj et al. 2002), which mechanistically explains the oxidation of guanines *in vitro* in isolated DNA at concentrations of 10 μM (Stevenson and Davies 1999). Follow-up *in vitro* research was conducted to determine whether ensulizole (up to 4 mM) could enhance the formation of DNA damage upon UVA or UVB exposure (Bastien et al. 2010). As expected, this study showed that ensulizole protected against formation of cyclobutane pyrimidine dimers *in vitro*. However, the substance photosensitized the formation of oxidized guanines and DNA strand breaks after UVA and UVB irradiation, although it did not induce single-strand breaks, double-strand breaks, or the formation of measurable oxidized pyrimidines (Bastien et al. 2010). Later research has shown that complexation of ensulizole with cyclodextrin inhibits the formation of free radicals generated by ensulizole exposed to simulated sunlight, suppressing its photosensitizing potential (Scalia et al. 2004).

Developmental and reproductive toxicity

Although the database is limited to the two studies described below, the available information suggests that ensulizole does not elicit developmental toxicity. Also, no effects of ensulizole on reproductive organs were observed in available oral or dermal repeated-dose toxicity studies. However, specific reproductive toxicity studies, including data on fertility, are not available.

In a GLP-compliant developmental toxicity study conducted according to EEC Directive 79-831 (Annex V, Part B), pregnant female Wistar rats (25 animals/group) were orally administered ensulizole sodium salt by gavage at dose levels of 0 or 1000 mg/kg/day on GD 6–15. A control group of animals was dosed with the vehicle alone (water). Water consumption in the treatment group was increased during the exposure period and continued until GD 18 (no other details available); this effect was considered to be treatment-related but not adverse. There were no other signs of toxicity in dams. Fetuses did not show any malformations. The nature and frequency of skeletal variations were comparable in test and control groups. There was no evidence of any other embryo or fetotoxic effects. Under the study conditions, the NOAEL was 1000 mg/kg/day for maternal toxicity and for embryotoxicity/fetotoxicity (SCCP 2006; ECHA 2023).

In a GLP-compliant developmental toxicity screening study, pregnant female Charles River mice (12 animals/group) were orally administered ensulizole by gavage at doses of 0, 40, 120, and 400 mg/kg/day on GD 8–12. The control animals were dosed with the vehicle (water). All dams were allowed to deliver. Surviving dams and pups were euthanized on lactation day 3. No treatment-related effects were observed on maternal clinical signs, mortality, behavior, and body weights, pup survival, and pup body weights. There was no evidence of maternal or developmental toxicity at any dose level. Under the study conditions, the NOAEL for maternal and developmental toxicity was 400 mg/kg/day (highest dose tested) (Johnson 1990).

Estrogen, androgen, thyroid, and steroidogenesis-related activity

Table 2 summarizes the relevant studies available for the assessment of potential estrogen, androgen, thyroid, and steroidogenesis (EATS)-mediated adversity and/or EATS-mediated endocrine activity of ensulizole, organized according to the OECD conceptual framework levels (OECD 2018). Quantitative structure–activity relationship (QSAR) modeling suggests no androgen or estrogen receptor binding and no agonist or antagonist activity. *In vitro* testing supports a lack of activity at the level of the estrogen and androgen receptors (AR). There were no estrogenic effects in uterotrophic assays, no androgen agonist/antagonist effects or 5α-reductase inhibition in a Hershberger assay, and no toxicity to the reproductive organs or system in the previously described repeated-dose subchronic (oral and dermal) and developmental toxicity studies in rats and mice. Overall, the data show a lack of EATS-mediated adversity and/or EATS-mediated endocrine activity for ensulizole. Results of the *in silico*, *in vitro*, and uterotrophic investigations are detailed below.

Ensulizole was evaluated using the EPA Collaborative Modeling Project for Androgen Receptor Activity (CoMPARA) consensus QSAR models to predict potential AR activity and the EPA Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) Potency Level consensus QSAR models to predict potential estrogen receptor (ER) activity (USEPA 2023). Ensulizole was predicted to be inactive for AR and ER binding, agonist, or antagonist activity.

In a battery of unpublished studies (see details in Table 2) sponsored by the NTP, including six *in vitro* endocrine-related assays conducted by CeeTox, Inc., and uterotrophic and Hershberger assays conducted by Integrated Laboratory Systems, Inc. (ILS), there were no indications of estrogenic or androgenic activity or inhibition of aromatase activity (CeeTox 2013c, 2013a, 2013f, 2013b, 2013d). Decreased testosterone and estradiol levels of up to 30% were noted in the H295R steroidogenesis assay at individual ensulizole concentrations ranging from 0.001 to 10 μM, but these lacked consistency with concentration and across replicates (CeeTox 2013e; NTP 2024). There were no estrogenic (uterotrophic) effects noted in the uterotrophic assay at ensulizole doses up to 1000 mg/kg/day (ILS 2012b; NTP 2024). There were no androgenic or anti-androgenic effects noted in the Hershberger assay at ensulizole doses up to 1000 mg/kg/day (ILS 2012a; NTP 2024).

An *in vitro* screening study was conducted to determine the potential AR binding of ensulizole sodium salt. The assay utilized a recombinant protein incorporating both the hinge region and ligand binding domain of the rat AR (which is identical to that of the human AR) fused to thioredoxin as receptor and labeled methyltrienolone [17α-methyl-^{3}H] as ligand. Ensulizole sodium salt in DMSO was tested at concentrations ranging from 1000 to 1,000,000 nM in two independent experiments. Positive controls were dihydrotestosterone (high AR affinity) and androstenedione (low AR affinity). The quantity of radiolabeled competitive ligand methyltrienolone bound in the presence of ensulizole sodium salt was

Table 2. Assessment of ensulizole-related estrogen, androgen, thyroid, developmental, and reproductive data according to OECD conceptual framework.

Assay/data	Results	References
Level 1—Existing data and non-test information		
EPA CoMPARA consensus QSAR models	Inactive in AR binding, agonist, or antagonist activity	Mansouri et al. (2020)
EPA CERAPP Potency Level consensus QSAR models	Inactive in ER binding, agonist, or antagonist activity	Mansouri et al. (2016)
Level 2—In vitro (mammalian and non-mammalian) assays: select endocrine mechanism(s)/pathways(s)		
ER binding assay (rat uterine cytosol) (OPPTS 890.1250, GLP-compliant)	Ensulizole was classified as "non-interacting" with the ER at soluble concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M.	CeeTox (2013c); NTP (2024)*
AR binding assay (rat prostate cytosol) (OPPTS 890.1150, GLP-compliant)	Ensulizole was classified as a "non-binder" (mean specific binding >75%) of the AR at soluble concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M.	CeeTox (2013a); NTP (2024)*
Human recombinant aromatase assay (OPPTS 890.1200, GLP-compliant)	Ensulizole was classified as a non-inhibitor (mean aromatase activity of 102% ± 1% SD) at the highest soluble concentration of $10^{-3.5}$ M.	CeeTox (2013f); NTP (2024)*
AR transactivation activity assay (MDA-kb2 cells) (GLP-compliant)	Ensulizole did not show agonism or antagonism of AR-mediated transactivation in the test system.	CeeTox (2013b); NTP (2024)*
ER transcriptional activation assay (human cell line [HeLa-9903]) (OECD 455, OPPTS 890.1300, GLP-compliant)	Ensulizole was not an agonist of hERα in the test system.	CeeTox (2013d); NTP (2024)*
H295R steroidogenesis assay (OPPTS 890.1550, GLP-compliant)	Decreases (∼ 30%) in mean testosterone and estradiol levels (inconsistent and only occasionally statistically significant) were noted at several ensulizole concentrations between 0.001 and 10 μM.	CeeTox (2013e); NTP (2024)*
In vitro screening study to determine the potential AR binding of ensulizole sodium salt using rat recombinant fusion protein to thioredoxin as receptor and labeled methyltrienolone [17α-methyl-3H] as ligand	No affinity for the AR receptor was noted.	Freyberger (2002a); SCCP (2006)
In vitro screening study to determine the potential AR agonistic and antagonistic effects of ensulizole using STTA assays	Ensulizole was not an AR agonist or antagonist in the AR-Ecoscreen™ and 22Rv1/MMTV AR transactivation assays.	Lee et al. (2018)
In vitro screening study to determine the potential ER binding of ensulizole sodium salt using the hERα as receptor and radiolabeled estradiol [2,4,6,7,16,17-3 H(N)] as ligand	Ensulizole showed no ER affinity.	Freyberger (2002b); SCCP (2006)
In vitro screening study to determine the potential ER agonistic and antagonistic effects of the chemicals using STTA assays	No ER agonist or antagonist effects were noted in the STTA assay using the ERa-HeLa-9903 cell line or in the BG1Luc ER transactivation assay.	Lee et al. (2016)
In vitro study to determine if chemical UV filters may mimic the physiological action of progesterone on cationic channel of sperm (CatSper) and affect Ca^{2+} signaling in human sperm cells	Ensulizole did not mimic the physiological action of progesterone on the CatSper.	Rehfeld et al. (2016)
In vitro study to determine the ability of chemical UV filters to affect acrosome reaction, penetration, hyperactivation, and viability in human sperm cells	Ensulizole did not induce acrosome reaction, increase penetration in viscous medium, induce a change in the proportion of hyperactivated cells, or decrease sperm viability.	Rehfeld et al. (2018)
Level 3—In vivo (mammalian) assays: select endocrine mechanism(s)/pathway(s)		
In vivo uterotrophic assay (juvenile female Wistar rats)	No estrogenic effects were noted at up to 200 mg/kg/day ensulizole sodium salt (subcutaneous administration).	Krötlinger (2002); SCCP (2006); ECHA (2023)
In vivo uterotrophic assay (ovariectomized female Sprague Dawley rats) (OPPTS 890.1600, GLP-compliant)	No estrogenic (uterotrophic) effects were noted at 320 or 1000 mg/kg/day ensulizole (oral administration).	ILS (2012b); NTP (2024)*
In vivo Hershberger assay (castrated male Sprague Dawley rats) (OPPTS 890.1400, GLP-compliant)	No androgen agonist/antagonist activity or 5α-reductase inhibition were noted at 320 or 1000 mg/kg/day ensulizole (oral administration).	ILS (2012a); NTP (2024)*
Level 4—In vivo (mammalian) assays: adverse effects on endocrine relevant endpoints		
3-month repeated-dose oral toxicity study in Wistar SPF rats (OECD TG 408)	The NOAEL was 1000 mg/kg/day, the highest dose tested (no thyroid or reproductive organ-related effects).	Bomhard and Schilde (1978); SCCP (2006); ECHA (2023)
91-day repeated-dose dermal toxicity study in New Zealand white rabbits	The NOAEL was 100 mg/kg/day, the highest dose tested (no thyroid or reproductive organ-related effects).	Weisenburger (1991)

(*continued*)

Table 2. Continued.

Assay/data	Results	References
Developmental toxicity study in female Wistar rats (EEC Directive 79-831, Annex V, Part B)	The NOAEL was 1000 mg/kg/day for maternal toxicity and embryotoxicity/teratogenicity (limit test).	SCCP (2006); ECHA (2023)
Developmental toxicity screening study in female Charles River mice	The NOAEL for maternal and developmental toxicity was 400 mg/kg/day, the highest dose tested.	Johnson (1990)
Level 5—In vivo (mammalian) assays: more comprehensive data over more extensive parts of an organism's lifecycle		
No studies available		

AR: androgen receptor; CERAPP: Collaborative Estrogen Receptor Activity Prediction Project; CoMPARA: Collaborative Modeling Project for Androgen Receptor Activity; EPA: U.S. Environmental Protection Agency; ER: estrogen receptor; GLP: good laboratory practice; hERα, human recombinant estrogen receptor, alpha-subtype; NOAEL: no-observed-adverse-effect level; OECD: Organization for Economic Co-operation and Development; OPPTS: Office of Prevention, Pesticides, and Toxic Substances (U.S. EPA); QSAR: quantitative structure–activity relationship; SD: standard deviation; STTA: stably transfected transcriptional activation; TG: test guideline; UV: ultraviolet.

*Unpublished study report (sponsored by the U.S. National Toxicology Program).

comparable to that of the control. Ensulizole sodium salt showed no AR affinity and therefore half maximal inhibitory concentration (IC_{50}) values could not be calculated. The positive controls verified the efficacy of the test system by yielding IC_{50} values of 4.6–4.7 nM for dihydrotestosterone and 2.0–2.4 μM for androstenedione (Freyberger 2002a; SCCP 2006).

An *in vitro* screening study was conducted to determine the potential AR agonistic and antagonistic effects of ensulizole using stably transfected transcriptional activation (STTA) assays. Ensulizole in DMSO was tested at concentrations ranging from 10^{-1} to 10^{-7} mg/mL in the AREcoScreen™ and 22Rv1/mouse mammary tumor virus (MMTV) cell lines in two independent experiments. Dihydrotestosterone, mestanolone (AR agonist positive), and di (2-ethylhexyl) phthalate (AR agonist negative) were reference substances for the agonist assay, and hydroxyflutamide and bisphenol A were reference substances for the antagonist assay. All reference control substance tests passed the acceptability criteria of the study. Ensulizole showed no evidence of AR agonistic or antagonistic activity in the AR-Ecoscreen™ and 22Rv1/MMTV AR TA assays (Lee et al. 2018).

An *in vitro* screening study was conducted to determine the potential ER binding of ensulizole sodium salt using the human recombinant ER, α-subtype (hERα) as receptor and radiolabeled estradiol [2,4,6,7,16,17-^{3}H(N)] as ligand. Ensulizole sodium salt in DMSO was tested at concentrations ranging from 1000 to 1,000,000 nM in two independent experiments. Positive controls were 17β-estradiol (high ER affinity) and genistein (low ER affinity). Within the experimental error, the quantity of radiolabeled competitive ligand estradiol bound in the presence of ensulizole sodium salt was comparable to that of the control. Ensulizole sodium salt showed no ER affinity; therefore, IC_{50} values could not be calculated. The positive controls verified the efficacy of the test system by yielding IC_{50} values of 1.7–2.1 nM for 17β-estradiol and 175 nM for genistein (Freyberger 2002b; SCCP 2006).

An *in vitro* screening study was conducted to determine the potential ER agonistic and antagonistic effects of ensulizole using STTA assays. Ensulizole in DMSO was tested at concentrations ranging from 10^{-1} to 10^{-7} mg/mL in hERα-HeLa-9903 and 1 to 9.77×10^{-4} mg/mL in BG1Luc-4E2 cell lines in two independent experiments. Reference substances for the agonist assays were 17β-estradiol, 17α-estradiol (ER agonist positive), and corticosterone (ER agonist negative), whereas bisphenol A (ER agonist positive) and corticosterone (ER agonist negative) were reference substances for the antagonist assays. All reference control substance tests passed the acceptability criteria of the study. Ensulizole showed no evidence of ER agonist or antagonist effects in the STTA assay using the hERα-HeLa-9903 cell line or in the BG1Luc ER TA assay (Lee et al. 2016).

An *in vitro* study was conducted to determine whether chemical UV filters mimic the physiologic action of progesterone on the cationic channel of sperm (CatSper) and affect Ca^{2+} signaling in human sperm cells, by measuring the intracellular free Ca^{2+} concentration using a fluorometric assay (Rehfeld et al. 2016). Sperm cells were loaded with the fluorescent Ca^{2+} indicator Fluo-4 (10 μM) for 45 min at 37 °C. The sperm pellet was resuspended in human tubular fluid medium to 5×10^6 sperm/mL and aliquots of 50 μL were loaded into the wells of a multi-well plate. Fluorescence was recorded before and 232 sec after injection of 25 μL of the chemical UV filters (10 μM), negative control (buffer with vehicle), and positive control (progesterone, 5 μM) to duplicate wells. To compare the results from different experiments, the relative maximally induced Ca^{2+} signal from a given experiment was calculated by dividing the maximal Ca^{2+} signal with that of the paired positive control. For ensulizole, the mean relative maximal Ca^{2+} signal was −3.08% ($n = 3$). Therefore, ensulizole was not found to mimic the physiologic action of progesterone on the CatSper (Rehfeld et al. 2016).

As a follow-up study to their 2016 experiment, an *in vitro* study was conducted by Rehfeld and collaborators to determine the ability of chemical UV filters to affect acrosome reaction, penetration, hyperactivation, and viability in human sperm cells. The capacity of the UV filters to induce acrosome reaction and increase sperm penetration was associated with the ability of the UV filters to induce Ca^{2+} influx. Under the study conditions, ensulizole did not induce acrosome reaction, increase penetration in viscous medium, induce a change in the proportion of hyperactivated cells, or decrease sperm viability (Rehfeld et al. 2018).

A GLP-compliant *in vivo* uterotrophic screening study was conducted according to the OECD draft protocol B (OECD 2000) in juvenile female Wistar rats to assess the estrogenic properties of ensulizole sodium salt. The animals were subcutaneously administered ensulizole sodium salt in corn oil at

dose levels of 0, 50, and 200 mg/kg/day for 3 days. Two additional groups of juvenile female rats were treated once daily for 3 consecutive days with 0.3 and 1 μg/kg 17α-ethinylestradiol (positive control). A corn oil vehicle group and an untreated animal group were included as negative controls. There was no difference between the control and treatment group animals regarding general behavior, mortality, body weight, feed intake, uterus weight, or gross pathology. Enlargement of the uterus and increased uterine weights were noted in positive control animals. The results obtained for negative and positive controls confirmed the validity of the study. Under the study conditions, no estrogenic effects were detected in the uterotrophic assay on juvenile female rats at subcutaneous doses of up to 200 mg/kg/day ensulizole sodium salt (Krötlinger 2002; SCCP 2006; ECHA 2023).

A GLP-compliant *in vivo* uterotrophic study was conducted in ovariectomized female Sprague Dawley rats to determine the estrogenic properties of ensulizole. Ovariectomized adult female rats were orally administered ensulizole in corn oil at dose levels of 0, 320, and 1000 mg/kg/day for 3 days. The results obtained for negative and positive controls confirmed the validity of the study. Administration of ensulizole at neither dose level affected body weights, body weight gains, or uterine weights (wet and blotted) compared to the vehicle control. Under the study conditions, no estrogenic effects were detected in the ovariectomized rat uterotrophic assay up to the 1000 mg/kg/day oral limit dose of ensulizole (ILS 2012b).

Carcinogenicity

As described in Cohen et al. (2025), there are four fundamental modes of action for human carcinogens: (1) mutagenicity, (2) immunosuppression, (3) increased estrogenic activity, and (4) cytotoxicity and increased regenerative cell proliferation. Short-term animal studies and various *in vitro* and *in silico* investigations can adequately screen for these modes of action. Although no carcinogenicity studies are available for ensulizole, no carcinogenic effects are anticipated from the use of ensulizole as a dermal sunscreen ingredient, as it is not cytotoxic or genotoxic *in vitro*, even at doses that were 15× higher than the OECD maximum recommended dose of 5 mg/plate (OECD 2020). Ensulizole also demonstrated low toxicity in repeated-dose (Bomhard and Schilde 1978; Weisenburger 1991) and developmental (Johnson 1990; SCCP 2006; ECHA 2023) toxicity testing. There were no signs of induction of preneoplastic hyperplasia in any tissues in these studies and no indication of effects on the endocrine or immune systems. Moreover, Cohen et al. (2025) found no evidence of a biologically relevant carcinogenic mode of action for ensulizole, and the estimated systemic exposure in humans fell well below concentrations that have any reported biological activity.

Neurotoxicity and immunotoxicity

No studies were identified in which the potential neurotoxicity or immunotoxicity of ensulizole was assessed. However, no indications of any such effects were observed in the repeated-dose toxicity studies described above, including no effects on immune or hematopoietic tissues or hematologic evaluations.

Postmarketing safety data

The results of several prospective and retrospective postmarketing safety evaluations of sunscreens containing ensulizole are presented. There were no adverse effects noted beyond local effects, none of which were related to systemic toxicity. The postmarketing data are supportive of the view that sunscreen products containing ensulizole are safe when used as directed.

Schauder and Ippen summarized the published and unpublished 15-year experience with sunscreen allergy and photoallergy (1981–1996) encompassing 402 patients with suspected clinical photosensitivity who were patch- and photopatch-tested with commercial sunscreens and facial cosmetics containing different UV filters (Schauder and Ippen 1997). Eighty patients (20%) demonstrated allergic and/or photoallergic contact dermatitis (PACD) to one or more UV filter(s). In 47 patients with photodermatoses or photoaggravated dermatoses and in 33 subjects with normal photosensitivity, 91 allergic and 84 photoallergic reactions to UV filters were observed. Sunscreens containing ensulizole (1% in water; 10% in petrolatum, w/v) were associated with one allergic and seven photoallergic reactions collectively, which were considered "rare" incidences by the authors.

Two Swedish dermatology clinics included seven sunscreen UV filters in the standard photopatch protocols from 1990 to 1997 in which 355 patients with suspected photosensitivity were tested. In 28 patients (7.9%), a total of 42 allergic reactions were found. Most (80%) of the reactions were of photocontact origin. More than 21 irritant and/or phototoxic reactions of doubtful relevance were noted in 14 patients (16 on irradiated and 5 on non-irradiated test sites). Two cases of photocontact allergy to ensulizole were reported (Berne and Ros 1998).

Two cases of facial dermatitis in users of ensulizole-containing facial moisturizers were identified in a clinical investigation at University Hospitals Cleveland Medical Center (Cleveland, Ohio, USA). Only a weak reaction was noted on patch testing, but strong reactions were noted 24 h after UVA exposure. In neither case was there a positive reaction to any sunscreen present in the North American photopatch series, suggesting a lack of any cross-reaction patterns (Nedorost 2005).

A retrospective multicenter study (2004–2006) was conducted in Italy to evaluate the results of photopatch testing in 1082 patients with allergens proposed for use in Italy, and other substances suggested by each patient's personal history (Pigatto et al. 2008). The application of proposed allergens was made in duplicate to the skin on the upper back of subjects for 2 days. After 2 days, any post-removal reactions

were recorded. Then, one of the treated parts was covered by opaque material and the other was irradiated with 5 J/cm^2 UVA. Further readings were made post-irradiation according to the International Contact Dermatitis Research Group (ICDRG) protocol. To determine relevance, patients with positive reactions were reevaluated based on their clinical history, presence of the allergen, and the existence of an appropriate temporal relationship between UV exposure and the appearance of dermatitis. Almost one-quarter (21.6%, $n = 234$) of the patients tested positive to at least one test substance of the standard photopatch testing or to added substances with a total of 290 reactions. Two hundred four (204) of the reactions were typically photoallergic, 68 reactions were allergic, and, within this group, 10 were photoaugmented reactions and 18 reactions were phototoxic. No photoallergic reactions were reported in any subject treated with ensulizole (10% in petrolatum, w/v) (Pigatto et al. 2008).

A retrospective study (2003–2007) was conducted evaluating the results of photopatch tests performed with an extended series of photoallergens to identify the main photoallergens used in Portugal. Eighty-three patients (58 females/25 males, mean age 54.8 years) were tested with a photoallergen series including ensulizole, irradiated at Day 2 with 5 J/cm^2 UVA. Thirty-six of 83 patients (43.3%) had at least one positive reaction, with 21 (25.3%) reacting in the photoallergen series. Only one positive reaction was reported for 10% ensulizole in petrolatum (w/v) (Cardoso et al. 2009).

In a randomized double-blind study, an assessment of the irritation potential of sunscreens used in photopatch testing was conducted in 94 healthy volunteers according to the European consensus methodology (Kerr et al. 2009). Patches containing 2%, 5%, or 10% ensulizole in petrolatum (w/v) were applied to the left and right non-paravertebral skin of the upper back of healthy volunteers. After 2 days, the patches were removed, and a set on one side of the back was covered with a UV-opaque material, while the other was irradiated with 5 J/cm^2 UVA. Blinded readings of both sites were made pre-irradiation, immediately post-irradiation, and at Days 1, 2, and 3 post-irradiation. All reactions were graded using both the ICDRG scale and an erythema scale for grading photopatch test reactions. Of the 94 subjects recruited, 80 were analyzed after withdrawals and exclusions. No irritation or photoirritation reactions were produced in subjects by 2%, 5%, or 10% ensulizole in petrolatum (w/v) (Kerr et al. 2009).

A prospective, multicenter photopatch study was conducted with 1031 patients with suspected PACD in 30 centers across 12 European countries (Kerr et al. 2012). The frequency of PACD with commonly used organic UV filters and topical nonsteroidal anti-inflammatory drugs, including newer agents in Europe, was evaluated. Photopatch testing was conducted according to the European consensus methodology. The test agents were applied to the skin of the back and removed after 24 or 48 h, depending on the center. One set was irradiated with 5 J/cm^2 UVA, while the other set was covered with a UV-impermeable material. Readings of the test sites were made at five different time points: pre-irradiation, immediately after, and 24, 48, and 72 h post-irradiation. All reactions were graded using the ICDRG system. Three hundred and forty-six PACD reactions in 200 (19.4%) subjects occurred. There were no reported PACD reactions related to 10% ensulizole in petrolatum (Kerr et al. 2012).

After the use of two different sunscreens, a generalized, extremely pruritic eruption was reported in a 79-year-old male. Photopatch testing was performed with TRUE Test, the Spanish Photobiology Group sunscreen series, and the patient's own products. The sunscreen products were applied to the skin of the upper back with Finn Chambers. One panel was removed 48 h later and irradiated with UVA (5 J/cm^2). The photopatch test was evaluated 2 days after irradiation. A positive reaction was observed 4 days after application of 10% ensulizole in petrolatum (w/v, 2 days after irradiation) on both the irradiated and non-irradiated sides. No reaction was seen with the patient's own products, although ensulizole was present in one of the products according to the product label (Barrientos et al. 2019).

Risk characterization

To characterize the risk associated with the use of an ensulizole-containing sunscreen product, an MoS approach was used. This well-established risk estimate, also known as a margin of exposure (MoE), was calculated as the ratio of the point of departure for systemic effects (PoD_{sys}), often an NOAEL obtained from animal toxicology studies, to the estimated human SED as shown in Eq. (1). Although an MoS is not a probabilistic statement of risk, the concern regarding the exposure evaluated decreases as the value of the MoS increases. Accordingly, MoS values greater than 100 are generally interpreted to be acceptable and protective for non-genotoxic and non-carcinogenic effects, whereas values lower than 100 suggest that the chemical exposure risk may not be acceptable (SCCS 2023).

$$\mathrm{MoS} = \frac{\mathrm{PoDsys}}{\mathrm{SED}} \tag{1}$$

Estimation of systemic exposure dose

The expected SED to ensulizole through use of an ensulizole-containing sunscreen product was calculated using Eq. (2). Terms in the equation are: the daily sunscreen application rate (A), unit conversion factor (UC), ensulizole concentration in the sunscreen (C), dermal absorption of ensulizole (D), and a typical individual's body weight (BW). Conservative assumptions were applied in choosing these terms (see Table 3).

$$\mathrm{SED(mg/kg/day)} = A\left(\frac{\mathrm{g}}{\mathrm{day}}\right) \times \mathrm{UC}\left(1000\frac{\mathrm{mg}}{\mathrm{g}}\right) \times C\left(\frac{\%}{100}\right) \times \left(\frac{D(\%)}{\mathrm{BW}}\right) \tag{2}$$

Ensulizole SEDs were calculated for three separate scenarios: (1) 97.2 g/day maximum amount based on a MUsT, (2) 28 g/day based on the recommended sunscreen application per day by the American Academy of Dermatology, and (3)

Table 3. Assumptions for ensulizole systemic exposure dose calculations.

Term	Assumed value	Rationale
A (sunscreen applied)	97.2 g/day	Maximum amount based on FDA guidance for industry (i.e. assumed body surface area of 16,200 cm^2, sunscreen applied to 75% of body area, applied amount of 2 mg/cm^2, and 4 applications/day)
	28 g/day	Recommended amount of sunscreen applied per day to cover human adult body (American Academy of Dermatology, 2025)
	3.46 g/day	The value used for safety evaluations of facial application products (1.73 g/application × 2 applications/day)
UC (unit conversion factor)	1000 mg/g	
C (ensulizole concentration within product)	0.04	4% is the maximum concentration of ensulizole allowed in product formulations in the United States (USFDA 2019)
D (dermal absorption of ensulizole)	0.0026	0.26% of the applied dermal dose of ensulizole is used for the bioavailability
BW (body weight)	60 kg	Typical body weight of an adult human female
	70 kg	Typical body weight of an adult human male

FDA: U.S. Food and Drug Administration.

3.46 g/day based on estimates for facial application of a sunscreen product.

Scenario 1. Assuming that a 60-kg individual applies 97.2 g of a 4% ensulizole-containing sunscreen product per day with 0.26% dermal absorption of ensulizole, the SED of ensulizole was calculated below to be 0.17 mg/kg/day:

$$\text{SED} = \left(97.2\frac{\text{g}}{\text{day}}\right) \times \left(1000\frac{\text{mg}}{\text{g}}\right) \times 0.04 \times \left(\frac{0.0026}{60\ \text{kg}}\right) = 0.17\ \text{mg/kg/day}$$

Scenario 2. Assuming that a 60-kg individual applies 28 g of a 4% ensulizole-containing sunscreen product per day with 0.26% dermal absorption of ensulizole, the SED of ensulizole was calculated below to be 0.049 mg/kg/day:

$$\text{SED} = \left(28\frac{\text{g}}{\text{day}}\right) \times \left(1000\frac{\text{mg}}{\text{g}}\right) \times 0.04 \times \left(\frac{0.0026}{60\ \text{kg}}\right) = 0.049\ \text{mg/kg/day}$$

Scenario 3. Assuming that a 60-kg individual applies 3.46 g (facial application only) of a 4% ensulizole-containing sunscreen product per day with 0.26% dermal absorption of ensulizole, the SED of ensulizole was calculated below to be 0.006 mg/kg/day:

$$\text{SED} = \left(3.46\frac{\text{g}}{\text{day}}\right) \times \left(1000\frac{\text{mg}}{\text{g}}\right) \times 0.04 \times \left(\frac{0.0026}{60\ \text{kg}}\right) = 0.006\ \text{mg/kg/day}$$

Ensulizole-specific MoS calculations

The NOAEL of 1000 mg/kg/day for general toxicity in the OECD TG 408 90-day rat study (Bomhard and Schilde 1978; SCCP 2006; ECHA 2023) was selected as the point of departure for risk assessment purposes. The other available subchronic toxicity study (rabbit dermal NOAEL of 100 mg/kg/day, highest dose tested) was limited in its dosing range due to local skin irritation and no LOAEL was identified in the study (Weisenburger 1991), which was not the case in the rat study (oral dosing; OECD TG 408). These subchronic (3-month) repeated-dose toxicity studies were more robust with a broad range of endpoints evaluated when compared to the available embryo-fetal toxicity studies, which were largely limited in scope to developmental endpoints (though no developmental effects were observed and NOAELs were consistent with that in the OECD TG 408 90-day oral toxicity study). Based on data obtained from a toxicokinetic study in rats (SCCP 2006; ECHA 2023) and in line with the approach taken by the SCCP (2006), an oral bioavailability of 4% was used in the MoS calculations. Using the estimated systemic doses of 0.17, 0.049, or 0.006 mg/kg/day for the three exposure scenarios, the following MoS values of 235, 816, and 6667, respectively, were calculated for ensulizole based on general toxicity in the current safety evaluation.

Scenario 1.

$$\text{MoS} = \frac{1000\ \text{mg/kg/day (NOAEL)} \times 0.04(\text{oral bioavailability})}{0.17\ \text{mg/kg/day(SED)}} = 235$$

Scenario 2.

$$\text{MoS} = \frac{1000\ \text{mg/kg/day (NOAEL)} \times 0.04(\text{oral bioavailability})}{0.049\ \text{mg/kg/day(SED)}} = 816$$

Scenario 3.

$$\text{MoS} = \frac{1000\ \text{mg/kg/day (NOAEL)} \times 0.04(\text{oral bioavailability})}{0.006\ \text{mg/kg/day(SED)}} = 6667$$

Ensulizole safety conclusion

In conclusion, ensulizole can be considered safe when used as a sunscreen UV filter at concentrations up to 4%, the maximum permitted usage level in the United States, Canada, and Australia. Furthermore, it is not anticipated that ensulizole will pose any risk of carcinogenicity to humans based on the negative genotoxicity data, low toxicity in repeated-dose

Table 4. Summary of ensulizole toxicological endpoints and risk characterization.

Property	Key ensulizole-related results
Pharmacokinetics and toxicokinetics	An estimated human dermal bioavailability of 0.26% was obtained from an *in vivo* GCP-compliant tape-stripping study. Oral absorption was 4% in a GLP-compliant toxicokinetic study in pregnant rats. No maximal usage trials have been conducted with ensulizole.
Acute toxicity	Low acute mammalian toxicity: • Mouse oral LD_{50} value >5000 mg/kg (GLP status and method not specified) • Rat oral LD_{50} value >1600 mg/kg (GLP status and method not specified) • Rat dermal LD_{50} values >3000 mg/kg (two non-GLP studies similar to OECD TG 402) • Rat intraperitoneal LD_{50} values of 1046 and 1513 mg/kg (non-GLP studies of unspecified method)
Repeated-dose toxicity	Low repeated-dose toxicity: • NOAEL of 1000 mg/kg/day (highest dose tested) in a non-GLP 3-month rat subchronic oral toxicity study (OECD TG 408) • NOAEL of 100 mg/kg/day (highest dose tested) for systemic toxicity in a GLP-compliant 91-day rabbit subchronic dermal toxicity study (only local dermal irritation effects with no concomitant microscopic changes were noted at all dose concentrations)
Irritation, sensitization, and photosafety	Ensulizole is a nonirritant, non-sensitizer, non-phototoxic, and a non-photosensitizer.
Genotoxicity and carcinogenicity	Negative genotoxicity/photomutagenicity results were found in bacterial reverse mutation assays, an *in vitro* chromosomal aberration test in human peripheral blood lymphocytes, *in vitro* chromosomal aberration tests in Chinese hamster ovary cells, an *in vitro* HPRT gene mutation assay in Chinese hamster lung fibroblasts, and an *in vitro* study in *Saccharomyces cerevisiae* yeast. Although no long-term carcinogenicity study on ensulizole has been performed, no carcinogenicity is expected based on the negative genotoxicity data and the empirical evidence from repeated-dose and developmental toxicity studies with ensulizole.
Hormonal, developmental, and reproductive toxicity	Ensulizole was predicted to be inactive for AR and ER binding, agonist, and antagonist activity based on EPA QSAR modeling, was negative for estrogen, androgen, thyroid, and steroidogenesis activity in *in vitro* assays, demonstrated no estrogenic effects in uterotrophic assays or androgen agonist/antagonist activity in a Hershberger assay, and showed no embryotoxicity or teratogenicity in developmental toxicity studies or effects on the reproductive organs in repeated-dose toxicity studies.
Immunotoxicity and neurotoxicity	No immunotoxicity or neurotoxicity studies were available for ensulizole, but there were no indications for any such effects from repeat-dose studies.
Risk characterization	No remarkable clinical effects beyond occasional local reactions have been reported for the use of ensulizole in sunscreen products. The NOAEL of 1000 mg/kg/day for general toxicity from an OECD TG 408 rat subchronic toxicity study was used for the current safety evaluation along with three distinct exposure scenarios: high (FDA's high end use; four full-body applications), medium (American Academy of Dermatology's recommended application amount for sunscreen), and low (non-recreational use; facial product). Using conservatively estimated systemic doses of 0.17, 0.049, or 0.006 mg/kg/day, MoS values of 235, 816, and 6667, respectively, were obtained for ensulizole.

AR: androgen receptor; EPA: U.S. Environmental Protection Agency; ER: estrogen receptor; GCP: good clinical practice; GLP: good laboratory practice; HPRT: hypoxanthine-guanine phosphoribosyltransferase; LD_{50}: median lethal dose (the dose at which 50% of the animals died); NOAEL: no-observed-adverse-effect level; MoS: margin of safety; OECD: Organization for Economic Co-operation and Development; QSAR: quantitative structure–activity relationship; TG: test guideline.

and developmental toxicity studies (including no histopathologic changes in evaluated organs), and lack of effects on the androgen or estrogen systems.

Discussion

Based on the absence of reported remarkable clinical effects following commercial usage of ensulizole in sunscreen products, this substance has a very good safety profile. Postmarketing clinical safety studies of ensulizole have reported only occasional local effects, none of which were associated with systemic toxicity. Global regulatory authorities have supported this conclusion by registering ensulizole for use at maximum levels ranging from 3% to 8% in consumer end-use products, with the United States, Canada, and Australia limiting its OTC or therapeutic usage level to a maximum of 4%. Substantial human dermal safety and PK, nonclinical, and postmarketing data were available to assess ensulizole's safety as a sunscreen active ingredient in OTC sunscreen formulations. Table 4 provides an overall summary of the available data.

In experimental human studies, no dermal irritation, photoirritation, or photosensitization effects were noted. Dermal penetration of ensulizole in human volunteers, following application of a cosmetic gel formulation, ranged from 0.11% to 0.26% of the applied dose, whereas the maximum oral absorption found in rats was 4%. There was no evidence of ensulizole bioaccumulation in rat *in vivo* studies as expected based on its high water solubility and low octanol/water partition coefficient.

It is worth noting that the form of ensulizole, i.e. salt or weak acid, used in studies was provided where available. The salts, namely sodium and to a lesser extent potassium and TEA, are the most common water-soluble forms of ensulizole. In experiments using an aqueous vehicle or ethanol, even when not stated, it is likely a salt form of ensulizole was

used. Nonetheless, the form, acid or salt, is not always explicitly stated. As a weak acid, ensulizole has poor water solubility and, theoretically, greater bioavailability. In the majority of studies, the salt form has been used because of its water solubility. Without question, the form used in sunscreen finished products is the salt, i.e. water-soluble form. However, this difference between salt and weak acid forms of ensulizole has an effect on physiochemical properties and possibly bioavailability.

Ensulizole was nonirritant, non-(photo)sensitizing, and non-phototoxic in a broad array of standard nonclinical (e.g. OECD TG 404, 405, and 406) and *in vitro* (e.g. OECD TG 432) studies, as well as several non-GLP investigations of phototoxicity, skin sensitization, and dermal photosensitization.

Ensulizole has demonstrated low acute mammalian toxicity via the oral, dermal, and intraperitoneal routes and low subchronic repeated-dose toxicity. Subchronic (3-month) NOAELs were identified at 100 (dermal rabbit) and 1000 (oral rat OECD TG 408 study) mg/kg/day, the highest doses tested, respectively.

In developmental toxicity studies, ensulizole was not embryotoxic, neither did it show any developmental toxicity up to the highest tested doses. No effects of ensulizole on the reproductive organs were observed in available oral or dermal repeated-dose subchronic toxicity studies. There were no single or multigeneration reproductive toxicity studies available for ensulizole. The results from *in silico*, *in vitro*, and *in vivo* studies suggest a lack of effects or endocrine activity of ensulizole on the EATS systems.

Ensulizole has caused neither gene mutations nor chromosomal aberrations in the presence or absence of metabolic activation in various *in vitro* mutagenicity studies in bacterial or mammalian cell systems. The substance has also shown no evidence of photogenotoxic potential. Although no *in vivo* genotoxicity studies were available, the complete absence of evidence for any effects in well-conducted *in vitro* studies of gene mutation and clastogenicity support the conclusion that ensulizole is unlikely to be genotoxic. Though a long-term carcinogenicity study on ensulizole has not been performed, carcinogenicity is not anticipated based on the negative *in vitro* genotoxicity data, lack of relevant effects on the androgen or estrogen system or immune system, and the empirical evidence from repeated-dose and developmental toxicity studies (including no histopathological changes indicative of cytotoxicity, increased cell proliferation, or cancer precursor lesions in evaluated organs).

During the past two decades, some suggestions were made that the outcome of carcinogenicity studies can be predicted such that actual conduct of long-term animal studies is not necessary if one has information on whether the material is a direct-acting DNA mutagen, causes toxicity in any organ, causes cell proliferation, is cytotoxic, causes hormonal perturbations, or if one has results from relevant QSAR analyses or omics studies (Cohen 2004; MacDonald 2004; Van der Laan et al. 2016; Woutersen et al. 2016; Cohen et al. 2019; Doe et al. 2019). In a recent evaluation, no evidence of biologically relevant carcinogenic mode of action for ensulizole was identified (Cohen et al. 2025). In addition, from the retrospective analysis of the various datasets by regulators as well as industry associations, it was concluded that based on pharmacology, genotoxicity, and chronic toxicity data (usually present at the end of phase 2 in the development of a new pharmaceutical), the outcome of the 2-year rat carcinogenicity study can be predicted with reasonable assurance at the two extremes of the spectrum. Negative predictions can be made when predictive carcinogenic signals are absent and positive predictions can be made when such signals are present. In between, a category of compounds remains for which the outcome of the carcinogenicity studies cannot be predicted with sufficient certainty (ICH 2016).

In support of this conclusion, Woutersen et al. (2016) and Van der Laan et al. (2016) evaluated whether preneoplastic lesions in subchronic toxicity studies could predict outcomes in chronic carcinogenicity studies for 163 non-genotoxic chemicals. Although 75% of the 148 compounds that were negative for preneoplastic lesions in subchronic studies were also negative in the carcinogenicity studies, the predictivity was improved to 97% when relevance of animal tumors was taken into account (Van der Laan et al. 2016; Woutersen et al. 2016). The authors concluded that their results "support the concept that chemicals showing no histopathological risk factors for neoplasia in a subchronic study in rats may be considered non-carcinogenic and do not require further testing in a carcinogenicity study" (Woutersen et al. 2016). The application of such considerations to the assessment of the carcinogenic potential of organic UV filters, such as ensulizone, is discussed in more detail in Cohen et al. (2025).

Recent postmarketing safety studies were identified for ensulizole. In an assessment of 402 patients with suspected clinical photosensitivity, sunscreens containing ensulizole (1% in water; 10% in petrolatum) were associated with one allergic and seven photoallergic reactions collectively, which were considered rare incidences by the study authors. In a prospective study investigating suspected PACD in 30 centers across 12 European countries, there were no such reactions reported in any subjects related to ensulizole (10% in petrolatum). In a double-blind randomized study, photopatch testing was conducted using 2%–10% ensulizole in petrolatum according to the European consensus methodology, which produced no irritation reactions in investigated subjects. In a retrospective study conducted in Portugal that evaluated 83 patients, only one positive reaction related to ensulizole was reported (10% in petrolatum). In a retrospective multicenter study conducted in Italy that evaluated 1082 patients, no photoallergic reactions were reported in any subject treated with ensulizole (10% in petrolatum). In a study conducted by the contact dermatitis clinic of University Hospitals Cleveland Medical Center (Cleveland, Ohio), two cases of facial dermatitis in users of ensulizole-containing facial moisturizers were identified. Only a weak (questionable) reaction was noted on patch testing; however, strong reactions were noted after additional UVA exposure. In neither case was there a positive reaction to any sunscreen present in the North American photopatch series, suggesting a lack of any cross-reaction patterns. None of these occasionally reported local skin reactions to ensulizole-containing sunscreen formulations were associated with systemic toxicity.

Based on the available data, the NOAEL of 1000 mg/kg/day for general toxicity from an oral OECD TG 408 study was used to calculate an MoS. In the current safety evaluation, three exposure scenarios were considered: high (maximum amount based on FDA guidance for industry; four full-body applications), medium (American Academy of Dermatology's recommended application amount for sunscreen), and low (non-recreational use; facial product). Conservatively estimated systemic doses of 0.17, 0.049, and 0.006 mg/kg/day were calculated for these respective scenarios, resulting in calculated MoS values of 235, 816, and 6667, respectively, for ensulizole based on general toxicity. Therefore, the available data show that ensulizole is safe and poses no human health risks when used in sunscreen products at concentrations up to 4%, which is consistent with existing regulatory safety acceptance and approval of the compound in the United States, Canada, and Australia.

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Author contribution

The roles of all the authors in the preparation of the manuscript were as follows: K.G. Norman: investigation, writing—review and editing, project administration; L. Kaufman: investigation, writing—original draft, writing—review and editing, visualization; P. Griem: writing—original draft, writing—review and editing; L. Loretz: conceptualization; methodology, investigation; writing—original draft; A. Kowcz: conceptualization; writing—review and editing, supervision, project administration, funding acquisition; S.M. Cohen: conceptualization, methodology, investigation, writing—review and editing, supervision; A.R. Scialli: conceptualization, methodology, investigation, writing—review and editing; A.R. Boobis: conceptualization, methodology, investigation, writing—review and editing; D. Jacobson-Kram: conceptualization, methodology, investigation, writing—review and editing; R. Schoeny: conceptualization, methodology, investigation, writing—review and editing; T.J. Rosol: conceptualization, methodology, investigation, writing—review and editing; G.M. Williams: conceptualization, methodology, investigation, writing—review and editing; N.E. Kaminski: conceptualization, methodology, investigation, writing—review and editing; F.P. Guengerich: conceptualization, methodology, investigation, writing—review and editing; J.F. Nash: conceptualization, methodology, investigation, writing—original draft; writing—review and editing, supervision.

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Supplementary material

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