Original Article

www.ToxicolRes.org
Toxicological Research

Toxicol. Res. Vol. 34, No. 2, pp. 111-125 (2018)
https://doi.org/10.5487/TR.2018.34.2.111

Open Access

Permitted Daily Exposure for Diisopropyl Ether as a Residual Solvent in Pharmaceuticals

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Abstract

Solvents can be used in the manufacture of medicinal products provided their residual levels in the final product comply with the acceptable limits based on safety data. At worldwide level, these limits are set by the "Guideline Q3C (R6) on impurities: guideline for residual solvents" issued by the ICH. Diisopropyl ether (DIPE) is a widely used solvent but the possibility of using it in the pharmaceutical manufacture is uncertain because the ICH Q3C guideline includes it in the group of solvents for which "no adequate toxicological data on which to base a Permitted Daily Exposure (PDE) was found". We performed a risk assessment of DIPE based on available toxicological data, after carefully assessing their reliability using the Klimisch score approach. We found sufficiently reliable studies investigating subchronic, developmental, neurological toxicity and carcinogenicity in rats and genotoxicity *in vitro*. Recent studies also investigated a wide array of toxic effects of gasoline/DIPE mixtures as compared to gasoline alone, thus allowing identifying the effects of DIPE itself. These data allowed a comprehensive toxicological evaluation of DIPE. The main target organs of DIPE toxicity were liver and kidney. DIPE was not teratogen and had no genotoxic effects, either *in vitro* or *in vivo*. However, it appeared to increase the number of malignant tumors in rats. Therefore, DIPE could be considered as a non-genotoxic animal carcinogen and a PDE of 0.98 mg/day was calculated based on the lowest No Observed Effect Level (NOEL) value of 356 mg/m^3 (corresponding to 49 mg/kg/day) for maternal toxicity in developmental rat toxicity study. In a worst-case scenario, using an exceedingly high daily dose of 10 g/day, allowed DIPE concentration in pharmaceutical substances would be 98 ppm, which is in the range of concentration limits for ICH Q3C guideline class 2 solvents. This result might be considered for regulatory decisions.

***Key words*:** Diisopropyl ether, Residual solvent, ICH Q3C guideline, Permitted daily exposure, Risk assessment

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List of abbreviations: AFC, antibody-forming cell; AIFA, italian medicines agency; API, active pharmaceutical ingredient; DEE, diethyl ether; DIPE, diisopropyl ether; ECHA, european chemicals agency; EMA, european medicines agency; EPA, united states environment protection agency; ETBE, ethyl tert-butyl ether; FOB, functional observational battery; G/DIPE, gasoline/DIPE; GC, gas chromatography; GD, gestation day; GFAP, glial fibrillary acidic protein; GLP, good laboratory practice; HSDB, hazardous substances data bank; ICH EWG, ICH expert working group; ICH, international conference on harmonisation of technical requirements for registration of pharmaceuticals for human use; IRIS, integrated risk information system; LD, lactation day; MeSH, medical subject heading; MTBE, methyl tert-butyl ether; NIOSH, national institute for occupational safety and health; NOEL, no observed effect level; NTP, U.S. national toxicology program; OECD, organisation for economic co-operation and development; PDE, permitted daily exposure; REACH, registration, evaluation, authorisation and restriction of chemicals; SCE, sister chromatid exchange; sRBC, sheep red blood cells; TAME, tert-amyl methyl ether.

pISSN: 1976-8257 eISSN: 2234-2753

INTRODUCTION

Diisopropyl ether (DIPE, also known as isopropyl ether; CAS number 108-20-3) is a widely used solvent included in the High Production Volume Chemical list issued by the Organisation for Economic Co-operation and Development (OECD) (1). Due to its physicochemical properties, DIPE is effective for the extraction of polar or mid-polar compounds. In the pharmaceutical industry, DIPE is used in the manufacture of Active Pharmaceutical Ingredients (APIs), mainly as an extractant and for purification by crystallization of several compounds, particularly steroids (2,3).

Solvents can be used in the manufacture of medicinal products provided their residual levels in the finished product comply with the acceptable limits set by regulatory agencies based on safety data. At worldwide level, the acceptable limits of residual solvents are defined in the "Guideline Q3C (R6) on impurities: guideline for residual solvents" issued by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (4). The objective of this guideline is to recommend acceptable amounts for residual solvents in pharmaceuticals for the safety of the patient. The ICH Q3C Guideline classifies solvents in one of the following three classes: solvents to be avoided (class 1); solvents to be limited (class 2), for which a Permitted Daily Exposure (PDE) should be established; solvents with low toxic potential (class 3).

The ICH Q3C guideline includes DIPE in a further group of solvents for which "no adequate toxicological data on which to base a Permitted Daily Exposure (PDE) was found". The use of these solvents is theoretically possible upon justification for their residual levels. In practice, this situation creates a state of uncertainty regarding the regulatory status of DIPE and the possibility of using it in pharmaceutical manufacture.

The first version of ICH Q3C guideline was finalised in July 1997 but since then new studies on DIPE toxicity have been produced, which should make it possible to perform a risk assessment of DIPE. This assessment may contribute to regulatory decisions on the classification of DIPE according to the ICH Q3C guideline, thus helping resolving the uncertainty about its possible use. Revision of classification is in agreement with the principles laid down in the part II of the ICH Q3C guideline for which PDEs could be calculated/updated as new reliable safety data become available.

The aim of the present work was to perform a risk assessment of DIPE based on available toxicological data, whose reliability is confirmed in agreement with the recommendations of the ICH Q3C guideline. According to the guideline, toxicological data used for risk assessment should derive from studies adopting adequately sound protocols such as those described for example by OECD or the United States Environment Protection Agency (EPA) guidelines. It is therefore essential to evaluate accurately the reliability of the available toxicological data. We assessed the compliance of retrieved toxicological data with existing guidelines and evaluated the data reliability using the Klimisch score approach, which was originally proposed by Klimisch and co-workers (5) and is now widely accepted. This approach has also been adopted by the European Chemicals Agency (ECHA) for assessing the data provided to fulfil the obligations of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation. Once determined the lowest No Observed Effect Level (NOEL) value based on available data, we derived the PDE value in agreement with the ICH Q3C guideline.

MATERIALS AND METHODS

Data search. We conducted a literature search from the PubMed database using the Medical Subject Heading (MeSH) term 'Diisopropyl ether' combined with the MeSH terms 'Toxicity', 'Toxicity tests', 'Toxicity test, subacute', 'Toxicity test, subchronic', 'Toxicity test, chronic', 'Mutagenicity tests'. Toxicokinetic data were searched by using the MeSH term 'Diisopropyl ether' combined with the MeSH term 'Toxicokinetics'. Data on the metabolism of structurally related ethers were searched by using the MeSH term "Ether/metabolism" (or similar for specific classes of ethers, such as methyl ethers). We also searched Google and Google Scholar using the same terms as above. Once found a relevant paper in PubMed, the search was extended to related articles. References cited in relevant articles were also evaluated. Finally, toxicological data were also searched in the most relevant databases (Toxnet, ECHA, National Institute for Occupational Safety and Health).

Assessment of data reliability. The reliability of retrieved data was assessed according to Klimisch and coworkers approach (5), which is based on compliance with generally valid and/or internationally accepted testing guidelines; agreement with the principles of Good Laboratory Practice (GLP); completeness and scientific soundness of the documentation. Klimisch scores are shown in Table 1. The GLP status of several retrieved published *in vitro* and *in vivo* toxicity studies is unknown. However, it is important to note that studies not complying with GLP may nevertheless be considered reliable according to the Klimisch score approach (i.e., having a score 1 or 2).

In assigning the Klimisch score, we also followed the recommendations of the ECHA guidance on information requirements and chemical safety assessment. For each toxicological study, we verified the compliance to testing

Table 1. Klimisch scores

Score	Reliability	Description
1	Reliable without restrictions	Studies or data [...] generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline [...] or in which all parameters described are closely related/comparable to a guideline method.
2	Reliable with restrictions	Studies or data [...] (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.
3	Not reliable	Studies or data [...] in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g. unphysiological pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgment.
4	Not assignable	Studies or data [...] which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.).

guidelines issued by EPA or OECD. Only data with a Klimisch score 1 or 2 were considered for the risk assessment.

Calculation of PDE and acceptable limits. PDE (mg/day) for DIPE was calculated in agreement with the following ICH Q3C guideline Appendix 3 formula:

PDE (mg/day) = NOEL (mg/kg/day) × weight adjustment ÷ (F1 × F2 × F3 × F4 × F5) (modifying factors)

We used the lowest value among all NOEL values derived from published data. Because in all selected animal studies exposure to DIPE occurred through inhalation (with the exception of the carcinogenicity study), the inhaled concentration was transformed into a weight/weight dose. Therefore, the inhaled concentration was first converted from ppm to mg/m^3 (mg/m^3 = ppm × molecular weight/24.45, based on the following conditions: temperature 25°C; pressure 1 atmosphere). In order to calculate the absorbed dose, this value was multiplied for the respiratory volume (0.29 m^3/day) in the rat (the species used in all relevant studies), assuming a complete bioavailability and the dose was then divided by the reported weight:

Concentration (mg/m^3) × 0.29 m^3/day ÷ W (kg) = absorbed dose (mg/kg).

To calculate the NOEL, the absorbed dose value was corrected for discontinuous exposure (6 hr/day for 5 days/week) and converted into a continuous one:

Absorbed dose (mg/kg) × 6 hr ÷ 24 hr × 5 day/7 day = NOEL (mg/kg/day).

PDE was obtained by multiplying the NOEL by 50 kg (weight adjustment) and dividing this value by the appropriate modifying factors. According to the ICH Q3C guideline, the modifying factors are as follows. F1 is a factor to account for extrapolation between species. Its value depends on the species used in the study (for rat, F1 = 5). F2 is a factor of 10 to account for variability between individuals. F3 is a variable factor to account for extrapolation from short-term toxicity studies to lifetime exposure. F4 is an additional factor that may be applied in cases of severe toxicity, e.g., non-genotoxic carcinogenicity, neurotoxicity or teratogenicity. F5 is a further variable factor that may be applied if the NOEL was not established.

The acceptable residual concentration (ppm) in an API was calculated as:

Concentration = 1,000 × PDE/dose, where PDE is expressed as mg/day and the dose as g/day.

RESULTS

The selected toxicity studies for DIPE risk assessment are summarised in Table 2.

Six recently published studies thoroughly characterised the toxicity of gasoline combined with oxygenated fuel additives, including DIPE (6-11). In these studies, a control group exposed to gasoline alone was used (in addition to controls). This protocol allowed identifying the effects of DIPE and using these mixture studies in risk assessment.

Data on toxicokinetic or metabolism of DIPE were not available. However, we found data on the metabolism of substances structurally related to DIPE, i.e., low molecular weight aliphatic ethers such as diethyl ether (DEE), methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE) and tert-amyl methyl ether (TAME).

Table 2. Summary of the selected toxicity studies for DIPE risk assessment

Type of study	Substance tested	Species number, duration sex/group	Administration route doses	NOEL	Reference	Klimisch score
Subchronic and developmental toxicity	DIPE	Rat Subchronic: 14/sex/group 13 weeks (6 hr/day, 5 day/week) Developmental: 22 female/group 6-15 gestation days (6 hr/day)	Inhalation Subchronic: 480; 3,300; 7,100 ppm Developmental: 430; 3,095; 6,745 ppm	Subchronic: 480 ppm based on an increase in weight of liver and kidney at 3,300 and 7,100 ppm Developmental: 430 ppm based on an increase in the incidence of rudimentary 14th ribs at 3,095 and 6,745 ppm	10	2
Subchronic neurotoxicity	DIPE	Rat 10/sex/group 13 weeks	Inhalation 450; 3,250; 7,060 ppm	3,250 ppm based on a slight decrease in motor activity observed in females exposed to 7,060 ppm	17	2
Genotoxicity *in vitro*	DIPE	*S. typhimurium*, *E. coli*, *S. cerevisiae*, Rat liver RL4 cell line	*In vitro*	NA	19	2
Carcinogenicity	DIPE	Rat 100/sex/group 78 week (4 day/week)	Oral 250; 1,000 mg/kg	250 mg/kg based on an increase in total malignant tumors at both doses	24	2
Subchronic toxicity	G/DIPE	Rat 10 or 20 sex/group 13 weeks + 4 weeks recovery	Inhalation 2,000; 10,000; 20,000 mg/m^3	10,000 mg/m^3 based on the reticulocyte count	6	1
Subchronic neurotoxicity	G/DIPE	Rat 5/sex/group 13 weeks	Inhalation 2,000; 10,000; 20,000 mg/m^3	20,000 mg/m^3 due to the lack of effect on investigated parameters	7	1
Developmental toxicity	G/DIPE	Rat 25 female/group 5-20 gestation days	Inhalation 2,000; 10,000; 20,000 mg/m^3	Maternal: 2,000 mg/m^3 based on a transient reduction in weight gain at 10,000 or 20,000 mg/m^3 Fetal: 10,000 mg/m^3 based on decreased foetal body weight	8	1
Reproductive toxicity	G/DIPE	Rat 26/sex/group Female and male prior to mating: 10 weeks and during the 14-day mating period. Mated females: - 0-19 gestation day - 5-28 lactation day	Inhalation 2,000; 10,000; 20,000 mg/m^3	Parental: 2,000 mg/m^3 based on increased liver weights in the mid and high dose groups Offspring: 20,000 mg/m^3 due to the lack of effect on reproductive parameters	9	1

Table 2. Continued

Type of study	Substance tested	Species number, duration sex/group	Administration route doses	NOEL	Reference	Klimisch score
Immunotoxicity	G/DIPE	Rat 5 female/group 4 weeks	Inhalation 2,000; 10,000; 20,000 mg/m^3	10,000 mg/m^3 based on decrease in IgM antibody forming cell response to the T-dependent antigen sheep red blood cells at 20,000 mg/m^3	10	1
Genotoxicity *in vivo*	G/DIPE	Rat 5/sex/group 4 weeks	Inhalation 2,000; 10,000; 20,000 mg/m^3	20,000 mg/m^3 due to the lack of effect on micronucleated immature erythrocytes and sister chromatid exchange	11	1

DIPE: diisopropyl ether. G/DIPE: vapour mixture of Gasoline and diisopropyl ether. NOEL: no observed effect level. NA: not applicable.

DIPE studies.

• ***Subchronic toxicity:*** Dalbey and Feuston (12) investigated the subchronic toxicity of DIPE (purity: 92%) in Sprague-Dawley-derived rats [Tac:N(SD)fBR]. Three groups of rats (14/sex) were exposed to DIPE at concentrations of 480, 3,300, or 7,100 ppm for 13 weeks in 1-m^3 chambers. Gas chromatography (GC) analysis of samples of air from the chambers was routinely performed and showed that DIPE represented 91~92% of the total vapours. The remaining components were identified by GC/mass spectrometry. Airflow was controlled and provided at least 12 air changes/hr. The Authors used both an unexposed and a sham-exposed control groups. During the course of experiment, body weights were recorded weekly and clinical signs daily. Blood samples were taken following the last exposure for haematological and serum chemistry analysis. All animals were necropsied. The following organs of each animal were weighed: adrenals, kidney, spleen, brain, liver, testes, epididymides, ovaries, thymus, heart, prostate, and uterus. The following tissues were processed from all sham-exposed and high-dose animals: adrenals, ovaries, sternum, pancreas, brain (three sections), salivary gland, eye, optic nerve, spleen, heart, stomach, colon, testes, duodenum, thymus, kidneys, thyroid, liver, lymph nodes, lung (left lobe), nasal turbinates, thigh muscle, skin (six sites), urinary bladder, sciatic nerve, seminal vesicles, preputial glands, and any gross lesions. Histopathology was carried out on liver and kidney of male rats exposed to 3,300 ppm. The morphology and number of sperm were evaluated in epididymes in both control groups and the high-concentration group. The left testis was weighed and used for determination of the number of testicular spermatids.

DIPE induced no change in clinical signs. DIPE did not adversely affect haematology and serum chemistry endpoints with the exception of a 30% increase in cholesterol, observed only in males exposed to the highest concentration (7,100 ppm). Exposure to DIPE 3,300 and 7,100 ppm caused a dose-related increase in the liver weight, in both sexes. At 7,100 ppm, hypertrophy of liver cells was observed in males only. Kidney weight was increased in males exposed to DIPE 3,300 and 7,100 ppm, and in females exposed to 7,100 ppm. Microscopic examination revealed an increase in hyaline droplets in the proximal convoluted tubules only in males exposed to 7,100 ppm. No other exposure-related changes were observed microscopically. The numbers of sperm and spermatids were not affected by exposure to DIPE. At 480 ppm, DIPE caused no change in any of the parameters investigated.

The GLP status of the study is unknown. However, the study is well documented and essentially complies with the EPA Guideline on 90-Day Inhalation Toxicity (13) with relatively minor deviations, which included a lower number of animals per experimental group (14 instead of recommended 20), not measuring the blood clotting potential and lack of ophthalmological examination. We assigned a Klimisch score 2 to this study.

• ***Developmental toxicity:*** Dalbey and Feuston (12) also investigated the developmental toxicity of inhaled DIPE. Sprague-Dawley rats (22 female/group) were exposed on 6-15 gestation days (GD) to DIPE (purity: 92%) 430, 3,095, or 6,745 ppm and sacrificed on day 20. There were also two control groups: untreated and sham-exposed. Body weights and food consumption were recorded on days 0, 6, 13, 16, and 20. The same haematology and serum chemistry parameters as in the subchronic study were evaluated. All organs were examined grossly. The number of corpora lutea per ovary and the gravid uterine weight were recorded. Uterine contents were examined and the

numbers of implantation sites, early and late resorptions, and live and dead foetuses were also recorded. Fetuses were weighed and grossly examined for external anomalies, after which fetuses of each litter were equally distributed between two groups and examined for visceral anomalies after fixing in Bouin's solution and for skeletal anomalies after fixing in 95% ethanol.

Dams exposed to the highest concentration of 6,745 ppm had a significant reduction in body weight gain and decrease in food consumption. No treatment-related effects were noted at the time of macroscopic examination. Exposure to DIPE did not influence any parameter of reproduction and fetal development. A statistically significant increase in the incidence of rudimentary 14th ribs was observed at 3,095 and 6,745 ppm. All of the observed 14th ribs were rudimentary except for two fetuses from each of the mid- and high-dose groups that had either bilateral short 14th ribs or bilateral short and rudimentary 14th ribs. The biological significance of rudimentary ribs has long been debated but the toxicological relevance of such findings is still uncertain (14). The Authors of the study concluded that this effect did not represent a conclusive evidence of developmental toxicity. There was no apparent toxicity, either maternal or fetal, at the lowest exposure concentration, 430 ppm.

The study met the requirements of the OECD guideline 'Prenatal Developmental Toxicity Study' (15) and the EPA guideline for prenatal developmental toxicity studies (16). Its GLP status is unknown. We assigned a Klimisch score 2 to this study.

• ***Subchronic neurotoxicity:*** Rodriguez and Dalbey (17) investigated neurotoxicity in rats using a functional observational battery (FOB), automated motor activity, and neuropathology assessment. Sprague-Dawley rats were exposed for 13 weeks by inhalation to DIPE (purity ~92%) 0, 450, 3,250, or 7,060 ppm (10/sex/group). Exposure methods and monitoring were the same as those for the subchronic study (12). Sham-exposed and exposed rats were housed continuously and individually in 1-m^3 inhalation chambers except during behavioural testing. The FOB consisted of initially observing home-cage positioning, posture, and reaction to removal from the cage. This was followed by evaluation for exophthalmus/palpebral closure, lacrimation, salivation, pupillary response, palpebral reflex, and pinna reflex (scored by the response of the animal as the hair on the inside of the pinna was touched). The animals were then observed for open field behaviour. Reactions to the approach of a pencil, finger snap, and tail pinch were ranked and recorded. Finally, fore- and hind limb grip strength was measured. The FOB was repeated following 2, 4, 8, and 13 weeks of exposure, motor activity was determined following 4, 8, and 13 weeks of exposure. Automated motor activity was assessed for 30 min in figure-8 mazes after the completion of the FOB. Body weights were recorded weekly and signs of toxicity daily, prior to each exposure. Following the final determination of motor activity, brain, spinal cord, gasserian and dorsal root ganglia, and sciatic nerve of six animals/group/sex exposed to 0 or 7,060 ppm DIPE were removed, processed for embedding in paraffin or glycol methacrylate and sectioned for microscopic pathologic evaluation. Few statistically significant changes in some FOB endpoints were found at weeks 2 and 4 but with no dose-relationship. The pinna reflex (scored by the response of the animal as the hair on the inside of the pinna was touched) was reduced compared to controls at week 2 only in the low dose group. The unperturbed activity level was lower than in controls in the low- and high-dose females at week 4. In females, exposure to highest DIPE concentration was related to a slight decrease (−15 to −20%) in motor activity in the figure-8 maze (statistically significant at week 4). In contrast, motor activity at week 8 was slightly increased in females exposed to DIPE 450 ppm. Microscopic examination revealed no apparent change of tissues from the nervous system.

The study complied with the EPA Guideline for neurotoxicity screening (18). The GLP status is unknown but the study is well documented. We therefore assigned a Klimisch score 2 to the study.

• ***Genotoxicity in vitro:*** Genotoxicity of DIPE (purity > 98.5%) was investigated *in vitro* by Brooks and coworkers (19) performing bacterial mutation assays, a yeast assay for mitotic gene conversion, and assays for chromosome damage in cultured mammalian cells. For bacterial mutation assays, the following strains of S. typhimurium (TA1535, TA1537, TA1538, TA98 and TA100) and E. coli (WP2 and WP2 uvr A, WP2 uvrApKM 101) were used at a maximum DIPE concentration of 8,000 μg/mL. These strains are in agreement with the combination of strains recommended by the OECD guideline for bacterial reverse mutation test in order to detect most types of mutagens (20). Methods were overall similar to those of the OECD guideline. However, the paper does not report information on cytotoxicity or the presence of precipitate in the cultures. The yeast assay for mitotic gene conversion used S. cerevisiae JD1, heteroallelic at the histidine-4 and tryptophan-5 loci, which is one of the strains listed in the relevant OECD guideline 481 (21). The number of replicates and exposure concentrations (maximum DIPE concentration 5 mg/mL), the use of positive controls, and the performance of the test were in agreement with the OECD 481 guideline. The Authors state that the final experimental conditions were based on the results of a first experiment, taking into account the effect of the test material on cell viability. The assays for chromosome damage in cultured mammalian cells used rat liver RL4 cell line (this epithelial-type cell line was derived in the same laboratory in which the genotoxicity study was performed). In this assay,

metabolic activation by S9 was not used because the RL4 cells are metabolically competent. The conditions of the test, including the selection of the exposure concentrations (based on growth inhibition results, maximum DIPE concentration 1,200 μg/mL), the use of positive controls and the performance of the test were in agreement with the OECD 473 guideline (22).

DIPE did not induce reverse gene mutation in bacteria, mitotic gene conversion in yeast or chromosome damage in mammalian cells, thus DIPE has no potential to cause point mutations neither to be clastogen/aneugenic.

Recommendations on genotoxicity testing and data interpretation for pharmaceuticals intended for human use are laid down in the ICH S2(R1) guideline (23). For the bacterial mutation assay, the ICH S2(R1) protocol recommendations are the same as those of the OECD guideline (21). The other two cytogenetic assays used with DIPE are currently not included in the core *in vitro* test battery reported in ICH S2(R1) guideline. In particular, the mitotic gene conversion in yeast has been now superseded by *in vitro* mammalian cell systems including metaphase chromosome aberration/micronucleus/mouse lymphoma L5178Y cell thymidine kinase gene mutation assays. In addition, *in vitro* metaphase chromosome analysis is now preferably detected in peripheral blood. However, the rat liver cell line used for DIPE screening can be considered adequate for detection of structural and numerical chromosome aberrations also considering the inherent presence of metabolic activation systems in the cell line selected. No explicit information on cytotoxicity was reported in the study, on which basis the maximum concentration to be tested should be selected, but it can be reasonably assumed that no toxic concentration levels have been assayed in the wide range tested and that the negative result is sufficiently reliable. Although GLP status of genotoxicity studies is unknown, overall, the genotoxicity study report of Brooks and co-workers was considered reliable with restrictions (Klimisch score 2).

• ***Carcinogenicity:*** The research group of the Ramazzini Foundation investigated DIPE carcinogenicity in Sprague-Dawley rats (24). DIPE (purity > 98%) was administered by gavage in 1 mL olive oil solution at concentrations of 0, 250 or 1,000 mg/kg body weight to groups of 100/sex rats. DIPE was administered daily, 4 days weekly, for 78 weeks. The animals were then maintained under control conditions until spontaneous death. The study ended after 163 weeks of treatment with the death of the last animal at 171 weeks of age. Body weights, water and food consumption, status and behaviour of animals were examined daily, clinical examination for gross changes every 2 weeks. Blood or urine were not analysed during the study. Histopathology was performed on most of the organs recommended by the OECD guideline 451 for carcinogenicity studies (25) and on any other organ or tissue with pathologic lesions. No significant differences were observed in food consumption, body weight, behaviour, or non-oncological pathological changes between DIPE-treated and control animals. A decrease in survival was observed in DIPE-treated males versus controls in the period between the 56th and 88th week of age.

In DIPE-treated rats, an increase in total malignant tumors was observed in both males and females at both doses but with no clear dose-response relationship. In particular, DIPE treatment was linked to an increase in the incidence of carcinomas of the ear duct, an increase in the incidence of glial malignant tumors of the brain, an increase of hemolymphoreticular neoplasias, a slight increase in malignant sarcomas of the uterus and vagina. The onset of some interstitial cell adenomas of the testis was also noted in the treated group.

The validity of the findings of some carcinogenicity studies conducted by the research group of the Ramazzini Foundation has been questioned (26-28). Criticisms focused on the general study protocol, which differs from those of international risk assessment guidelines. Particular causes of concern have been the retention of test animals until death and the use of non pathogen-free conditions, the high background incidence of chronic inflammatory changes in vital organs and tissues, and the uncertainty about the correctness of the diagnoses of some tumour types. In the DIPE study (as in other cancer studies performed by the Ramazzini Foundation), rats were observed at necroscopy after natural death occurring at approx. 3 years of age (DIPE treatment covered half of this period) while cancer bioassays are usually terminated at 2 years. Termination at 2 years allows maximizing the number of control and treated animals available at the same age for comparisons. In addition, it minimizes late-developing background tumors that may limit the ability to detect chemical-induced effects. On the other hand, the longer duration of the Ramazzini Foundation studies may allow detecting late-stage or late-developing cancers. As noted by Gift and co-workers (29), the longer duration of the Ramazzini Foundation studies has been important for the detection of later-occurring tumors for a number of chemicals. Recently, the U.S. National Toxicology Program (NTP) and EPA sponsored a comprehensive review of the Ramazzini Foundation studies by an expert team (29). The study group reviewed the procedures and tumor diagnoses of the Ramazzini Foundation, and examined evidence for a number of issues raised regarding the studies performed by the Foundation. The conclusion was that Ramazzini Foundation results are generally consistent with those of the NTP and other laboratories and that these studies may be informative for health risk assessment when reviewed on a case-by-case basis. There are doubts with regard to lymphoma/leukemia diagnoses, because the types of lymphomas reported in exposed groups have also been observed in older untreated Sprague-Dawley rats in the Ramazzini Founda-

tion colony, and the fraction of Ramazzini Foundation control groups with a lymphoma/leukemia rate > 10% has increased with time (29). In addition, according to the NTP review of the Ramazzini Foundation studies, for lymphomas and leukemias pathological determinations via light microscopy evaluations are problematic, especially when confounded by infiltrates from an infectious disease (30). On the other hand, an increase in lymphomas/leukemias was only found in a minority of the Ramazzini Foundation studies (~5%) (29). Moreover, results of the Ramazzini Foundation studies were consistent with other studies for some chemicals (especially those metabolized to formaldehyde). Therefore, available data do not support a generalized incorrect attribution of lymphomas/leukemias to test substances in the Ramazzini Foundation studies. Concerning GLP, the Ramazzini Foundation Authors claimed that experiment was GLP compliant. Concordantly, Gift and co-workers (29) noted that independent reviews and available Ramazzini Foundation documentation suggested that quality control procedures associated with GLP were in place at the Ramazzini Foundation. In particular, in the cited NTP review of the Ramazzini Foundation studies (30), the Authors reported "very organized, clean facilities" and that "standard operating procedures, GLP documents, and necropsy records were within GLP expectations" after a tour of the Ramazzini Foundation laboratory and archives.

Deficiencies of the DIPE study were the following: a) only 2 doses were investigated instead of the 3 dose levels recommended by the OECD 451 guideline (25) and other international guidelines; b) survival-adjusted analyses (tumor rates and trend analyses) were not performed even though data suggested that survival may differ between treated and control groups; c) a complete analysis and stability data of the test substance or formulation were not provided. Overall considered, we concluded that these methodological deficiencies are not sufficient to invalidate the study findings and evaluated the Ramazzini Foundation study as reliable with restrictions (Klimisch score 2).

Studies with gasoline/DIPE mixtures. The Research Group of the American Petroleum Institute performed a series of studies investigating the toxicity of gasoline containing fuel oxygenate additives, including DIPE. The studies included evaluation of subchronic toxicity, neurotoxicity, genotoxicity, immunotoxicity, reproductive and developmental toxicity in rats. In all studies, the animals were exposed to 0 (control), 2,000, 10,000 or 20,000 mg/m^3 of gasoline alone or gasoline/DIPE (G/DIPE) vapour (generation and composition is described in a separate paper [31]). DIPE represented 17.8% of the G/DIPE vapour (31). The Authors affirm that these studies were conducted in accordance with EPA GLP (32).

• ***Subchronic toxicity:*** CD (Crl: CD@ IGS BR) albino rats (10 or 20 sex/group) were exposed to gasoline or G/DIPE vapour for 13 weeks (6). During each exposure, measurements of airborne concentrations were performed in the animals' breathing zone at least 4 times. In addition, samples were collected weekly and analysed by GC to characterize at least 18 major components. At the end of the exposure period, recovery (4 weeks) was studied in control and high dose rats. During the exposure period, rats were observed for mortality and signs of severe toxic or pharmacologic effects, body weight and food consumption were monitored. All animals received ophthalmoscopic examinations pre-test and at study termination. Haematological and clinical chemistry analyses were performed on blood samples collected after 4 and 13 weeks of exposure. Necropsies were conducted on 10/sex/group, and on all of the recovery group animals. Organs were trimmed and weighed, tissues were excised and preserved for histopathology in agreement with the recommendations of the OECD guideline for subchronic inhalation toxicity (33).

The results showed that G/DIPE caused no significant change in the examined endpoints with the exception of mild haematological changes. In males, there was a decrease (21~23% in the high dose group) in reticulocyte counts, which was dose-related. The decrease was reversible in the low- and mid- but not in the high-concentration exposed group. In females, there were a decrease (−35%) in monocytes but only in the high concentration group. Increased kidney weight and light hydrocarbon nephropathy were observed in treated male rats exposed to gasoline alone, G/DIPE or gasoline mixed with other oxygenate additives. These changes were reversible or nearly reversible after 4 weeks recovery. The microscopic lesions observed were consistent with changes associated with accumulation of alpha-2 microglobulin within epithelial phagolysosomes. It is widely recognized that hyaline droplet nephropathy following exposure to chemicals inducing excessive accumulation of alpha 2u-globulin is unique to male rats and is considered not to be relevant to humans (34) as also acknowledged by the EPA (35). Based on these results, the Authors considered the G/DIPE 10,000 mg/m^3 exposure level as the NOEL. This conclusion can be shared taking into account that the decrease in reticulocyte counts in males exposed to G/DIPE 2000 and 10,000 mg/m^3 was very mild and transient.

The study protocol, conduct and report were compliant with the OECD guideline 413 (33). The study was judged to be reliable without restrictions (Klimisch score 1).

• ***Neurotoxicity:*** O'Callaghan *et al*. (7) investigated neurotoxicity of G/DIPE. Sprague-Dawley rats (5/sex/group) were exposed to DIPE or G/DIPE following the same protocol as in the subchronic toxicity study. FOB and motor activity were conducted once before initiation of exposures and again during the 4th, 8th and 13th week of exposures. Evaluations were conducted on non-exposure days at least 16 hr post-exposure. Neuropathology

was examined in 10 rats (5 males and 5 females) exposed to the highest concentration. After perfusion and fixation, the brain, eye with optic nerve, spinal cord, trigeminal ganglia, dorsal root ganglia, dorsal and ventral root fibers, lungs and trachea were processed by standard techniques, embedded in paraffin and sectioned at approximately 6 microns. Peripheral nerves were post-fixed in 1% osmium tetroxide, processed and embedded in epoxy resin, sectioned at approximately two microns and stained with toluidine blue. Changes in glial fibrillary acidic protein (GFAP) levels in areas of the brain were examined by immunoassay. GFAP is a biomarker of neurotoxicity and the assay may reveal effects at doses below those causing light microscopic evidence of cell loss or damage (36).

The results showed some statistically significant changes in the motor activity. However, these changes were inconsistent and not dose-related. G/DIPE did not induce statistically significant changes in any of the FOB measures, in brain measurements or weights, GFAP levels. No microscopic changes attributable to test substance effect were observed in brain, spinal cord, eyes, peripheral nerves, or ganglia among the high dose exposed animals. These results show that exposure to G/DIPE cause no significant neurotoxicity.

The study complied with the EPA guideline for neurotoxicity screening (18). The study also met the requirements of the OECD guideline on neurotoxicity studies in rodents (37). The study was therefore judged to be reliable without restrictions (Klimisch score 1).

• ***Developmental toxicity:*** Roberts *et al*. (8) investigated the developmental toxicity of G/DIPE. Sprague-Dawley female rats (25/group) were exposed on GDs 5-20. Dams were sacrificed by CO_2 asphyxiation on GD 21. Clinical signs, body weights and food consumption were monitored throughout the study. A gross necropsy was performed on all confirmed-mated females. Uterine weights with ovaries attached were recorded at the time of necropsy, uterine contents were examined, corpora lutea and the numbers and locations of implantation sites, early and late resorptions, and live and dead (alive or dead in utero) fetuses were counted. Fetuses were weighed and examined externally for gross malformations and variations. The viscera of approximately one-half of the fetuses of each litter were examined by fresh dissection. Sections of the Bouin's-fixed fetal heads were examined for the presence of abnormalities. The remaining live fetuses (alive in utero) were examined for the presence of bone and cartilage malformations and ossification variations. Evaluations of dams and foetuses were blinded.

All rats survived to study termination. No significant clinical signs were observed during the study. The number of pregnant animals was similar to controls. Exposure to G/DIPE 10,000 or 20,000 mg/m^3 caused a transient reduction in maternal weight gain during the early days of the exposure period and was partially resolved by GD 21. Exposure to G/DIPE 10,000 or 20,000 mg/m^3 also caused a decrease in food consumption that did not exceed 10%. These maternal effects were not observed in the groups exposed to gasoline alone. Implantation sites, resorptions, mean litter size, corpora lutea, fetal number of viable fetuses and fetuses per litter were comparable to controls. Fetal body weight in the G/DIPE 20,000 mg/m^3 group litters was statistically significantly lower for females (5%) and combined sexes (3.3%) compared to controls. Fetal body weight was also lower in the groups exposed to gasoline alone but the Authors of the study considered this as a spurious finding because fetal weights in all gasoline exposure groups were within the range of the laboratory control groups and no dose-response occurred. It should be also taken into account that a decreased body weight was observed in the rats exposed to G/DIPE or G/TAME but not to other gasoline/oxygenate additive mixtures. We therefore concluded that the decrease in fetal body weight observed with G/DIPE should be attributed to DIPE. Exposure to G/DIPE did not induce significant increases in fetal external variations, visceral variations or malformations or skeletal malformations. Based on these results, the Authors established a maternal NOEL value of 2,000 mg/m^3. Developmental NOEL was established at 10,000 mg/m^3 due to the decreased fetal body weight found in the 20,000 mg/m^3 exposure group.

The study met the requirements of the relevant OECD (15) and EPA (16) guidelines and was judged to be reliable without restrictions (Klimisch score 1).

• ***Reproductive toxicity:*** Reproductive toxicity was assessed in a one-generation study in rats (9). Sprague Dawley rats (26/sex/group) were exposed 7 days/week for one generation. Parental males and females received 70 consecutive days (10 weeks) of exposure prior to mating and continued to be exposed during the 14-day mating period. Mated females were exposed daily from gestation day (GD) 0 through GD 19, not exposed after GD 19 through lactation day (LD) 4, and again exposed from LD 5 until weaning on LD 28. Pups were observed for sex, number of live and dead pups and pup abnormalities. Pups dead at delivery were identified as stillborn or live-born found dead based on the evaluation of lung floatation. Thereafter litters were observed twice daily and litter size was recorded daily. Pups were examined and weighed up to LD 28, when they were terminated. Macroscopic examinations were performed on up to three randomly selected pups/sex/litter on LD 28 including identification of any structural abnormalities or pathological changes. All remaining pups were examined for external abnormalities and sacrificed. Brain, spleen and thymus gland were weighed from one randomly selected pup/sex/litter. All parental male animals were sacrificed during the lactation period for a total of 16~20 weeks of exposure and all parental

females were sacrificed on their LD 28. Selected organs were weighed and organ/body weight and organ/brain weight ratios calculated. Macroscopic examinations were performed on all parental rats and histological evaluations of the tissue samples from the organs of rats in the control and 20,000 mg/m^3 groups were performed. Reproductive organs from all male and bred female rats in control and high dose groups were evaluated. Examination of all parental females included a vaginal smear at time of necropsy to determine stage of estrus and a count of uterine implantation scars if present. Ovary histopathology included evaluation of the primordial follicle population, number of growing follicles and corpora lutea. Right testes and right epididymis from each animal were removed intact, weighed and fixed. Sperm evaluations included motility, testicular homogenization-resistant sperm and cauda epididymal sperm count and sperm morphology.

In parental males, G/DIPE 10,000 and 20,000 mg/m^3 caused an increase in the weight of liver, epididymides, and seminal vesicles/coagulating gland. The changes in male organ weights were considered as minor by the Authors and did not occur in a dose-responsive manner. The Authors concluded that these effects were unlikely to be due to exposure or of toxicological relevance. No remarkable histopathological change was found. G/DIPE had no effect on litter size (pups/litter, pups born dead/litter), number of implantation sites/litter, pup birth weight, offspring survival, or sex ratio. Male and female fertility or reproductive performance, oestrus cyclicity and semen parameters were comparable between exposed and control groups. As found also in the subchronic toxicity study with G/DIPE (6), exposure to gasoline alone or mixed with DIPE caused increases in male kidney weights and microscopic evidence of light hydrocarbon nephropathy. The parental NOEL was 2,000 mg/m^3 based on increased liver weights in the mid and high dose groups. Reproductive NOEL was 20,000 mg/m^3 due to the lack of effect on reproductive parameters.

The study protocol and conduct complied with the OECD on one-generation reproduction toxicity study (38). The Authors claimed that their study met the requirements of the relevant EPA guideline (39), which may be correct but with the limitation that this was a one-generation study whereas the guideline concerns two-generation studies. We assigned a Klimisch score 1 to the study.

• ***Immunotoxicity:*** White and co-workers (10) investigated immunotoxicityin female Sprague Dawley rats (5/group). They used the IgM antibody-forming cell (AFC) response to the T-dependent antigen sheep red blood cells (sRBC), also known as the plaque assay. Animals were exposed for 4 weeks and immunized by intravenous injection of sRBC, four days prior to sacrifice. After sacrifice, spleens were removed, weighed and processed for determination of IgM antibody response using a modified haemolytic plaque assay of Jerne. Cells/spleen, AFCs/10^6 spleen cells, and AFC/spleen were determined.

In rats exposed to the lowest G/DIPE level of 2,000 mg/m^3 there was a statistically significant increase in the spleen relative weight of 21%. However, this effect was not dose dependent and was not considered by the Authors to be biologically significant. There was no effect on the relative weight of the thymus. G/DIPE had no effect on spleen cell numbers but induced a decrease in IgM antibody forming cell response to the T-dependent antigen sRBC which was statistically significant with the highest exposure level of 20,000 mg/m^3 (−63% as compared to the control group). The NOEL was therefore 10,000 mg/m^3. In the same study, a similar effect of depression of the humoral immune response was also observed following exposure to gasoline/ethanol and gasoline/ETBE, but not following exposure to gasoline alone, gasoline/MTBE, gasoline/TAME or gasoline/t-butyl alcohol.

The study was performed in agreement with the recommendations of the EPA guideline on Immunotoxicity (40) and was considered reliable without restrictions (Klimisch score 1).

• ***Genotoxicity in vivo:*** Genotoxicity was investigated in Sprague Dawley rats (5/sex/group) exposed for 4 weeks (6 hr/day, 5 days/week) using the bone marrow micronucleus and the sister chromatid exchange (SCE) tests (11). Rats dosed with cyclophosphamide were used as positive control.

G/DIPE caused no significant increases in of micronucleated immature erythrocytes nor in SCE.

Test procedures were in accordance with the relevant EPA guidelines (41,42). The study was considered to be reliable without restrictions (Klimisch score 1).

Metabolism. We found no study investigating the metabolism of DIPE but retrieved several studies investigating the metabolism of structurally related compounds, namely aliphatic ethers with low molecular weight. Bernauer and co-workers (43) investigated the metabolic pathways of MTBE and ETBE in rats following exposure to the ^{12}C- and ^{13}C-labeled ethers. The product of O-dealkylation of both ethers, tert-butyl alcohol (tert-butanol) was found in the urine as a minor metabolite. Compounds deriving from further oxidation of tert-butanol, 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate, were major metabolites found in the urine along with small amounts of ^{13}C. The Authors also investigated the biotransformation of the initial metabolite of the ethers, tert-butanol. They found that 2-methyl-1,2-propanediol, 2-hydroxyisobutyrate and tert-butanol sulphate were the major urinary metabolites, while minor metabolites were [^{13}C]-acetone, tert-butanol, and tert-butanol glucuronide. Based on these findings the following metabolic pathway was proposed. The ethers undergo oxidative dealkylation to form tert-butanol; tert-butanol can undergo conjugations

reactions or be oxidized to 2-methyl-1,2-propanediol; the latter can be further oxidized to 2-hydroxyisobutyrate. Amberg and co-workers subsequently found that following exposure to MTBE or ETBE, 2-hydroxyisobutyrate was the major, and tert-butanol and 2-methyl-1,2-propane diol the minor urinary metabolites, both in rats and humans (44). These findings are consistent with those of Bernauer and co-workers (43) and indicate that the metabolic pathway in humans is the same as in rats. Shamsipur and co-workers (45) characterized the metabolic biotransformation of MTBE by human CYP2A6. Their results indicated that the first measurable metabolite was tert-butanol. Other identified metabolites were tert-butoxy methanol, 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate. Analysis of the culture media also revealed the presence of formaldehyde, acetone and acetaldehyde (which according to the Authors may also be an artefact). The proposed metabolic pathway in presence of CYP2A6 is therefore the following: MTBE first undergoes oxidative dealkylation to form tert-butoxy methanol, which spontaneously dismutates to tert-butanol and formaldehyde; tert-butanol is oxidized to 2-methyl-1,2-propanediol, which is further oxidized to 2-hydroxyisobutyrate. To explain the presence of acetone, the Authors hypothesize that 2-hydroxyisobutyrate degrades to 2-propanol, which is then oxidized to acetone. Therefore, the oxidizing metabolic pathway previously proposed by Bernauer and co-workers (43) was essentially confirmed even though with additional steps.

Overall, all available studies indicate that oxidative O-dealkylation is the first metabolic step shared by alkyl ethers, resulting in the formation of an alcohol and a carbonyl compound, which can be further oxidized. In the case of DIPE, the first metabolites formed from oxidative O-dealkylation should be isopropanol and acetone. The major metabolic pathway of isopropanol is oxidation to acetone, catalysed mainly by alcohol dehydrogenase. Acetone can be further oxidized to acetol (hydroxyacetone) and methylglyoxal and subsequent conversion to glucose (46), a process that also occurs in man. An alternative pathway may occur which involves the conversion of acetol to L-1,2-propanediol and subsequent conversion of L-1,2-Propanediol to L-lactaldehyde, and of L-lactaldehyde to L-lactic acid (46). Other metabolites may also be formed, which are converted to glucose and other products of intermediary metabolism.

In conclusion, available rat and human data suggest that biotransformation of DIPE should not lead to reactive toxic metabolites.

Other data.

• ***Human data:*** The DIPE full record in the Hazardous Substances Data Bank (HSDB) reports the results of short-term human exposure to DIPE under controlled conditions. Exposure to 500 ppm over a 15 min period caused no irritation, but irritation of the eyes and nose was noted at 800 ppm for 5 min with some respiratory discomfort (47).

• ***Occupational exposure limits:*** For the National Institute for Occupational Safety and Health (NIOSH), the 10 hr time-weighted average is 500 ppm.

NOELs.

• ***Subchronic toxicity:*** DIPE caused no change in any of the parameters investigated at a concentration of 480 ppm (12), corresponding to 2,006 mg/m^3. For G/DIPE the NOEL was 10,000 mg/m^3 in the main subchronic study (6) based on the reticulocyte count data. Because DIPE was 17.8% of the generated vapour, this value corresponds to DIPE 1,780 mg/m^3. An increase in liver weights was found in reproductive toxicity study in mid and high dose G/DIPE groups (with a NOEL of 2,000 mg/m^3, corresponding to DIPE 356 mg/m^3) (9) but not in the main subchronic toxicity study. Exposure to DIPE alone caused an increase in liver weight but only at 13,794 and 29,678 mg/m^3 (3,300 and 7,100 ppm). The reliability of the NOEL value found in the reproductive toxicity study is therefore uncertain.

• ***Developmental toxicity:*** Exposure to DIPE did not influence any parameter of reproduction and foetal development (12). A statistically significant increase in the incidence of rudimentary 14th ribs was observed at 3,095 and 6,745 ppm but the biological significance and toxicological relevance of such findings is still uncertain. Maternal toxicity was found at 6,745 ppm because dams exposed to this concentration had a significant reduction in body weight gain and a decrease in food consumption. There was no apparent toxicity, either maternal or foetal, at the lowest exposure concentration, 430 ppm (1,797 mg/m^3), which is therefore the developmental NOEL. The results of the G/DIPE developmental toxicity study (8) support these findings because exposure to G/DIPE 10,000 or 20,000 mg/m^3 (but not to gasoline alone) caused a transient reduction in maternal weight gain and slightly decreased food consumption. G/DIPE 20,000 mg/m^3, but not the lower concentrations, also decreased the foetal body weight. Maternal and developmental NOEL for G/DIPE were therefore established at 2,000 and 10,000 mg/m^3, respectively, corresponding to a DIPE concentration of 356 and 1,780 mg/m^3.

• ***Reproductive toxicity:*** Only data obtained with G/DIPE area available (9). They indicate that reproductive NOEL is 20,000 mg/m^3 (3,560 mg/m^3 of DIPE) due to the lack of effect on reproductive parameters.

• ***Neurotoxicity:*** Exposure to DIPE (17) or G/DIPE (7) did not induce any observable change of tissues from the nervous system. There was no clear evidence of neurobehavioral changes induced by DIPE, with the possible exception of a slight decrease in motor activity observed

in females exposed to the highest DIPE concentration (7,060 ppm). We therefore established the mid exposure value of 3,250 ppm as the NOEL value for neurotoxicity. Exposure to G/DIPE caused no change in any of parameters investigated, taking into account that the transient changes observed in the motor activity were inconsistent. Therefore, the NOEL was established at 20,000 mg/m^3.

• ***Immunotoxicity:*** There are no data for DIPE alone. G/DIPE had no effect on the spleen cell numbers but induced a decrease in IgM antibody forming cell response to the T-dependent antigen sRBC which was statistically significant with the highest exposure level of 20,000 mg/m^3 (−63% as compared to the control group). A similar effect of depression of the humoral immune response was not observed with gasoline alone and can therefore attributed to DIPE. The NOEL was 10,000 mg/m^3 (DIPE concentration 1,780 mg/m^3) (10).

PDE and acceptable limits. Both *in vitro* and *in vivo* genotoxicity studies convincingly showed that DIPE is not genotoxic. The lack of a genotoxic effect is also corroborated be the metabolism data of structurally related ethers, which indicated that the metabolic pathway of such compounds do not lead to the formation of reactive metabolites. In light of its carcinogenic potential, DIPE can be considered as a non-genotoxic carcinogen, and a PDE can therefore be calculated according to the ICH Q3C guideline (Appendix 3), using the following formula:

$$\text{PDE (mg/day)} = \text{NOEL (mg/kg/day)} \times \text{weight adjustment} \div (F1 \times F2 \times F3 \times F4 \times F5)\ \text{(modifying factors)}$$

The lowest among all NOEL values was 356 mg/m^3, i.e., the maternal NOEL in the developmental toxicity study with G/DIPE (8). The calculated absorbed dose was divided by the maternal rat weight (0.374 kg) reported in the study (8) and converted from discontinuous to a continuous exposure. The resulting NOEL was 49 mg/kg/day, which was then multiplied for the weight adjustment factor (50 kg) and divided by the following modifying factors: F1 = 5 (for extrapolation from rats to humans); F2 = 10 (for inter-individual variability); F3 = 5 (for a 3-month study in rodents); F4 = 10 (for carcinogenic activity). Since the no effect level was established, no F5 was considered. Thus, we derived a PDE value for DIPE of 0.98 mg/day, which is in the range of the PDE values for ICH Q3C guideline class 2 solvents.

Acceptable limits in a substance for pharmaceutical use can be calculated according to the option 1 for class 2 solvents of the ICH Q3C guideline:

$$\text{Concentration (ppm)} = 1{,}000 \times \text{PDE} \div \text{dose (g/day)}$$

In a worst-case scenario, using an exceedingly high daily dose of 10 g/day, the allowed DIPE concentration in the drug substance would be 98 ppm, which is in the range of the concentration limits for ICH Q3C guideline class 2 solvents.

DISCUSSION

Literature search retrieved several toxicological *in vivo* and *in vitro* studies carried out using either DIPE alone or G/DIPE. Combined, these data allowed a comprehensive toxicological evaluation of DIPE.

All studies but the carcinogenicity one used inhalation exposure. Although we found no information on the inhaled bioavailability of DIPE, organic solvents have high inhalation bioavailability values (48) and inhalation route in rat showed to be an appropriate method to assess the occupational hazards related to volatile solvents (49). Therefore, it can be reasonably assumed that the inhaled bioavailability is similar to that after oral intake. Concordantly, Amberg and co-workers (50) estimated that the absorbed dose of a compound structurally similar to DIPE, MTBE, was > 80% and similar after both oral and inhalation exposure in man. Therefore, we think that inhalation study data can be used for risk assessment of residual solvents in pharmaceuticals. Accordingly, the ICH Expert Working Group (ICH EWG) used studies in which tetrahydrofuran and N-methylpyrrolidone were administered by inhalation for calculating the PDE values for these solvents (4, Appendix 3 part II and part III). The array of toxicological studies used by ICH EWG in the re-evaluation of the PDEs for tetrahydrofuran and N-methylpyrrolidone was similar to that used for DIPE. We therefore think that the available data are adequate for a risk assessment of DIPE according to the principles of the ICH Q3C guideline. In addition, the Research Group organized by the American Petroleum Institute performed a series of highly reliable (Klimisch score 1) studies investigating the toxicity of G/DIPE mixtures. Because in each of these mixture studies the effect of gasoline alone was also evaluated, it was possible to extrapolate the toxic effects due to DIPE itself.

The main target organs of DIPE toxicity are the liver and the kidney. In the liver, DIPE caused a dose-related increase in weight (in both sexes) and hypertrophy of liver cells (in males only). Kidney weight also tended to increase mildly (+7 to +13%) in both sexes. Droplet nephropathy was found in males only and the lesions observed were consistent with changes associated with accumulation of alpha-2 microglobulin within epithelial phagolysosomes. It is known that these findings are not relevant to humans because hyaline droplet nephropathy linked to accumulation of alpha 2u-globulin is unique to male rats (34).

Exposure to G/DIPE but not to gasoline alone caused a mild decrease (−22%) in reticulocyte count in males exposed to the highest concentration. This effect might indicate that bone marrow is also a target tissue. G/DIPE at the

highest concentration also decreased the IgM antibody forming cell response to the T-dependent antigen sRBC (−63% in the group exposed to the highest G/DIPE concentration, no effect in the lower concentration groups) but did not decrease the spleen cell number. This immunotoxic effect was also observed with G/ethanol and G/ETBE.

DIPE was essentially devoid of any neurotoxicity because exposure to DIPE alone or G/DIPE did not induce any observable change of tissues from the nervous system. Slight changes in neurobehavioral endpoints appeared to be not related to exposure and inconsistent. Furthermore, the biological significance of these subtle, reversible changes is uncertain.

In pregnant rats, DIPE (both alone and as G/DIPE mixture) caused a dose-dependent decrease in weight gain and food consumption. Whether or not these maternal effects are related to a decrease in the body weight of foetuses is uncertain because a decrease in foetal weight (−3%) was apparently found with G/DIPE but not with DIPE alone. The available data allow defining a DIPE concentration devoid of any teratogen effect. Following exposure to DIPE alone, a statistically significant increase in the incidence of rudimentary 14th ribs was observed at 3,095 (12,937 mg/m^3) and 6,745 ppm (28,194 mg/m^3). The toxicological relevance of such findings is uncertain (14). However, no increase was found at 430 ppm (1,797 mg/m^3). Concordantly, exposure to G/DIPE 20,000 mg/m^3 (corresponding to DIPE 3,560 mg/m^3) caused no increase in variations or malformations. Data obtained with G/DIPE indicate that a lack of effect on reproductive parameters at the highest tested concentration (corresponding to DIPE 3,560 mg/m^3).

With regard to the carcinogenic potential, available data allow to exclude that DIPE is genotoxic, as shown *in vitro* with DIPE alone (19) and *in vivo* with G/DIPE (11). Belpoggi and co-workers (24) found that exposure to DIPE was associated to an increase in total malignant tumors. We discussed above the issues concerning the reliability of this study. We finally decided to consider the study reliable with restrictions (and therefore introduced a F4 factor in the calculation of PDE, as recommended by the ICH Q3C guideline in case of non-genotoxic carcinogens) but doubts on the reliability may persist. The main concern refers to the diagnosis of lymphoma/leukemia, which led the EPA to the decision of not relying on data from the Ramazzini Foundation on lymphomas and leukaemias in Integrated Risk Information System assessments. In the DIPE study, the most frequent and most increased in treated groups cancer endpoints were actually lymphoma and leukaemia, and it is possible that exclusion of these endpoints from the total would lead to different results for total malignant tumors. If the results indicating that DIPE has a carcinogenic effect are considered unreliable, the PDE would increase to 9.8 mg/day.

We could not find any study investigating the metabolism of DIPE. However, based on available data on the metabolism in rat and human of structurally related ethers we propose a metabolic pathway for DIPE leading to formation of isopropyl alcohol (isopropanol) and acetone. These two compounds can be further metabolized but this should not lead to reactive toxic metabolites.

In conclusion, we calculated a PDE of 0.98 mg/day for DIPE based on sufficiently reliable toxicity data. This PDE value is in the range of those of ICH Q3C class 2 solvents. This result might be considered for future regulatory decisions.

CONFLICT OF INTEREST

L. Romanelli and M.G. Evandri are experts of the European Medicines Agency (EMA).

The authors report no conflicts of interest. The views expressed in this article are those of the authors and do not reflect official views or policy of the Italian Medicines Agency (AIFA) or the EMA.

Received September 13, 2017; Revised January 29, 2018; Accepted February 5, 2018

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