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SCIENCE MEDICINES HEALTH

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Committee for Medicinal Products for Veterinary Use

CVMP assessment report regarding the request for an opinion under Article 30(3) of Regulation (EC) No. 726/2004

In relation to the potential risk for the consumer resulting from the use of diethanolamine as an excipient in veterinary medicinal products for food-producing species

Procedure no: EMEA/V/A/127

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1. Background information on the procedure

1.1. Request for CVMP opinion

On 7 March 2018 Belgium presented to the European Medicines Agency a request for an opinion in accordance with Article 30(3) of Regulation (EC) No. 726/2004 from the Committee for Medicinal Products for Veterinary Use (CVMP) on the potential risk for the consumer resulting from the use of diethanolamine as an excipient in veterinary medicinal products for food-producing species.

1.2. Steps taken during the referral procedure

- During the March 2018 CVMP meeting, the following was agreed:
  - Bruno Urbain was appointed rapporteur.
  - Gesine Hahn was appointed co-rapporteur.
  - The procedure started on 14 March 2018 and a list of questions was adopted.
- A public consultation was started on 16 March 2018 in order to provide stakeholders with the opportunity to input any information or data that they considered could be helpful to the CVMP in reaching its opinion. The deadline for the provision of information and comments was 14 May 2018.
- On 16 March 2018 a letter was sent to the marketing authorisation holders (MAHs) for veterinary medicinal products for food-producing species containing diethanolamine as an excipient informing them about the start of the procedure and including a list of questions as well as the official notification from Belgium to the CVMP/the Agency requesting an opinion in accordance with Article 30(3) of Regulation (EC) No 726/2004.
- The deadline for submission of responses by the MAHs was 14 May 2018.
- The rapporteur's assessment report was circulated to all CVMP members on 1 June 2018.
- The co-rapporteur's critique to the rapporteur's assessment report was circulated to all CVMP members on 11 June 2018.
- During the June 2018 CVMP meeting, the Committee considered the rapporteur's assessment report including the co-rapporteur's critique and agreed that no outstanding issues remained. The majority of the CVMP members indicated that they would support the (co-)rapporteur's conclusions.
- On 9 July 2018 the revised rapporteur's assessment report was circulated to all CVMP members.
- On 10 July 2018 the revised rapporteur's assessment report was forwarded to the MAHs.
- On 19 July 2018, the CVMP adopted an opinion in accordance with Article 30(3) of Regulation (EC) No. 726/2004.

2. Scientific discussion

2.1. Introduction

Diethanolamine is used as a solvent in various veterinary medicinal products authorised nationally in the majority European Union Member States. In January 2018 the CVMP removed diethanolamine from the list of substances considered as not falling within the scope of Regulation (EC) No. 470/2009, with regard to residues of veterinary medicinal products in foodstuffs of animal origin (also known as the 'out of scope' list). The decision was based on concerns relating to carcinogenicity and genotoxicity as diethanolamine has been shown to have carcinogenic potential in mice and the available genotoxicity data did not allow a conclusion to be drawn on the relevance of the findings for humans. The International Agency for Research on Cancer (IARC) has classified diethanolamine as possibly carcinogenic to humans.

The removal of diethanolamine from this 'out of scope' list meant that there were veterinary medicinal products for food-producing animals on the market that contained a substance for which the MRL status is not addressed.

While the CVMP had concluded that the continued inclusion of diethanolamine in the 'out of scope' list was not justified, it had not gone further in relation to quantifying the risks for the consumer, as such a comprehensive evaluation is not foreseen for consideration of (potential) 'out of scope' entries.

Therefore Belgium requested the CVMP to give an opinion on the risk for the consumer resulting from the use of diethanolamine as an excipient in veterinary medicinal products for food-producing species and, in relation to this, to present its view on the need for an MRL evaluation for the substance.

The questions raised by Belgium to the CVMP were as follows:

1. Can CVMP confirm whether diethanolamine is a DNA reactive carcinogen? In 2013 diethanolamine was reviewed and classified as possibly carcinogenic to humans (group 2B) by IARC. However, at that time IARC was unable to conclude on the mechanism of carcinogenicity.
2. If it is concluded that diethanolamine is a DNA reactive, does this mean that the risk to the consumer should be considered as unacceptable?
3. If it is concluded that diethanolamine is not DNA reactive, is it possible to establish a margin of exposure that would be acceptable from a consumer safety perspective? In relation to this question it is noteworthy that the previous entry in the 'out of scope' list included the following restriction: *"at doses up to 0.3 mg/kg bw/day"*.
4. On the basis of its scientific evaluation, does the CVMP consider that, to allow the use of diethanolamine in veterinary medicinal products for food producing animals, a full MRL evaluation is needed?

2.1.1. Information made available to CVMP

Further to the identification of the veterinary medicinal products for food-producing species containing diethanolamine authorised nationally in the EU, the concerned MAHs were invited to provide the following:

1. The qualitative and quantitative composition of the products concerned by the procedure.

2. Data which may be at their disposal regarding the genotoxic and carcinogenic potential of diethanolamine, and in particular:

    2.1. Data aimed at establishing the mechanism of carcinogenic activity of diethanolamine and whether the carcinogenic activity results from DNA reactivity of the substance;

    2.2. If it is considered that the substance is not DNA-reactive, data to establish a threshold for the carcinogenic activity of diethanolamine.

3. Any relevant data which may be at their disposal on the metabolism and residues in target animal species that would enable an estimation of the consumer exposure.

4. An expert comment on the data.

In the public consultation, stakeholders were asked to provide data requested in questions 2 and 3.

Some MAHs grouped themselves during the procedure (irrespective of company affiliation) in order to provide consolidated answers to the questions raised by CVMP.

By 14 May 2018, the Agency received a total of 17 responses to the CVMP list of questions from the concerned companies and one response to the public consultation.

Information in response to question 1 regarding the qualitative and quantitative composition of veterinary medicinal products containing diethanolamine was provided by all 17 MAHs who provided responses to the CVMP list of questions.

In response to questions 2 to 4 the majority of concerned MAHs (14) stated that they had no relevant data available.

Zoetis provided a publication in response to question 3.

Provet provided a short comment on toxicity, metabolism and use of their products, suggesting a non-genotoxic mode of action for carcinogenic effects in mouse liver due to choline deficiency:

> *"The National Toxicology Program (NTP) has yielded negative results in short-term genotoxicity studies. The available data indicate that diethanolamine induces mouse liver tumours by a non-genotoxic mode of action that involves its ability to cause choline deficiency. This effect on choline homeostasis is seen to occur only after a critical level of exposure to diethanolamine is attained (Leung HW, 2005)."*

Intervet International B.V. provided a data package consisting of literature data, answers to the four questions and an expert report regarding the genotoxic and carcinogenic potential of diethanolamine.

2.2. CVMP assessment of the risk for the consumer resulting from the use of diethanolamine in food-producing species

2.2.1. Discussion on carcinogenicity data

In 2000, diethanolamine had been classified by the International Agency for Research on Cancer (IARC) as not classifiable as to its carcinogenicity to humans (group 3); however, in 2013 IARC re-classified diethanolamine as possibly carcinogenic to humans (group 2B). This re-classification appears to have been largely based on proposed mechanisms of carcinogenicity pertaining to data obtained in a mouse 2-year dermal study by the US National Toxicology Program (NTP). In this 2-year dermal toxicity study in $B6C3F_1$ male and female mice (NTP TR 478, 1999), doses of 0, 40, 80 or 160 mg diethanolamine/kg bw were applied topically as 0, 22.5, 45 or 90 mg/ml solutions, for 5 days per week

over 103 weeks. The dose formulations were prepared by mixing diethanolamine with 95% ethanol, the approximate dose of ethanol being estimated as 1400 mg ethanol/kg bw.

The results indicated a significant increase in the incidence of hepatocellular neoplasms in all diethanolamine-treated groups (males and females), with a positive trend seen in the incidences of renal tubular adenoma at all doses in treated males. The control animals had an unusually high incidence of hepatic tumours. The overall incidence of hepatic tumours was 78% in control males and 66% in control females, with a hepatocellular carcinoma (HCC) incidence of 24% in control males.

The high incidence of hepatic tumours in control mice in the NTP dermal study is in contrast to the lower incidence of hepatic tumours reported by other authors. Chandra and Frith (1992) reported an incidence of hepatocellular adenomas/carcinomas in untreated $B6C3F_1$ male mice of 24.5% (including a HCC incidence of 9.5% in males). Konishi *et al*. (1992) reported a chronic oral toxicity study in $B6C3F_1$ mice administered triethanolamine orally, with a HCC incidence of 11% (low dose) and 10% (high dose) in treated males, and 16% in control male mice.

A 2-year dermal toxicity study was also performed in rats (NTP TR 478, 1999). Doses of 0, 16, 32 or 64 mg diethanolamine/kg were applied topically in 95% ethanol for 5 days per week over 103 weeks in males while females received 0, 8, 16 or 32 mg/kg bw. There was no increase in tumour incidence in treated groups compared to controls.

A MAH argued that the high incidence of hepatic tumours in both control and treated mice in the dermal study may have been influenced by the use of ethanol, a hepatic carcinogen in mice, and the high susceptibility of the mouse strain used ($B6C3F_1$).

It was postulated that diethanolamine, because of its irritating effect, increased the ethanol absorption. This can be considered plausible, but there are no data in the NTP report allowing to conclude on enhanced ethanol absorption.

On the other hand the effect of alcoholic beverages on the risk for human cancer was last evaluated in the IARC Monographs in 2012 (Volume 100E). Section 5. Evaluation, page 472, mentions:

> *"There is sufficient evidence in humans for the carcinogenicity of alcohol consumption. Alcohol consumption causes cancers of the oral cavity, pharynx, larynx, oesophagus, colorectum, liver (hepatocellular carcinoma) and female breast. Also, an association has been observed between alcohol consumption and cancer of the pancreas. For cancer of the kidney and non-Hodgkin lymphoma, there is evidence suggesting lack of carcinogenicity.{…}*
>
> *There is sufficient evidence in experimental animals for the carcinogenicity of ethanol."*

Section 3. Cancer in experimental animals; 3.1.2 Mouse, mentions:

> *"$B6C3F_1$ male and female mice received 2.5% or 5% of ethanol in drinking-water for 104 weeks. No significant difference in tumour incidence at any site was observed in females. There was a significant dose-related trend for the incidence of hepatocellular adenomas, and hepatocellular adenomas and carcinomas combined in male mice. The administration of 5% ethanol resulted in an increase in the incidence of hepatocellular adenomas ($P < 0.05$) and a marginal increase ($P = 0.056$) in the incidence of hepatocellular adenomas and carcinomas combined in male mice (NTP, 2004; Beland et al., 2005)."*

It is noteworthy that ethanol is not associated with kidney tumours in experimental animals (including mice). Therefore it is likely that renal tubular neoplasms observed with diethanolamine and coconut oil acid diethanolamine (NTP TR 479, 2001) may be attributed to the exposure to diethanolamine.

It was also argued that mice could have been contaminated with *Helicobacter hepaticus*, which may induce hepatitis and/or hepatic tumours and could have had an impact on the incidence of hepatocellular neoplasms observed in these studies (Hailey *et al*., 1998).

The sensitivity of the $B6C3F_1$ mouse strain to develop hepatocellular tumours is well known (OECD Guidance Document 116 on the design and conduct of chronic toxicity and carcinogenicity studies) and as such their usefulness for carcinogenicity studies is questionable. The interaction between diethanolamine and the vehicle ethanol is plausible although toxicokinetic data are lacking to corroborate the hypothesis. Ethanol is known to be absorbed into the normal intact skin and may reach the blood stream to be systemically distributed. Moreover the rate of absorption is higher through damaged skin (Lachenmeier, 2008). Diethanolamine has been shown to induce skin irritation and consequently ethanol absorption and thus systemic toxicity of ethanol is possibly enhanced in the higher dose group and this could explain an increased incidence in hepatocellular neoplasms. Moreover, ethanol also acts as a penetration enhancer for diethanolamine. As ethanol was also used as vehicle in the rat carcinogenicity study, it is expected that the skin penetration will have been enhanced in both studies. However, it is to be noted that the penetration of an aqueous solution of diethanolamine (37% w/w) through mouse skin has been shown to be approximately 10 and 20 times higher than through rat and human skin, respectively. Consequently, systemic diethanolamine levels are expected to be much higher in the mouse than in rat (or human) following dermal exposure, even in the presence of ethanol. The absence of hepatic findings in the rat could be explained by the considerably lower systemic exposure of rats to diethanolamine and the fact that the percutaneous absorption in rats is much lower than in mice. The NTP technical report on toxicity studies of diethanolamine administered topically and in drinking water to F344/N rats and $B6C3F_1$ mice corroborates this conclusion:

> *"Preliminary results of disposition studies of diethanolamine in rats revealed that only 16% of a dose of 27.5 mg/kg was absorbed when applied over a skin area of 2 cm^2; at lower concentrations, the percentage of the applied dose that was absorbed was further decreased (RTI, 1991). For comparison to the dermal toxicology studies in which the dose of diethanolamine was applied over an area of about 6 cm^2, the treatment in the dermal absorption study (27.5 mg/kg applied over 2 cm^2) is approximately equal to a skin application of 83 mg/kg. This dose of diethanolamine did not cause ulceration or inflammation of the skin at the site of application in the 2- or 13-week studies. It is likely that the uptake of diethanolamine is greater than 16% at doses that cause ulceration. Oral administration of ^{14}C-diethanolamine resulted in nearly complete absorption of radiolabel from the gastrointestinal tract, and the tissue distribution of radioactivity was comparable in rats after intravenous or gavage administration (RTI, 1991). Thus, at equivalent administered doses, internal levels of diethanolamine would be much lower in rats that are exposed by topical application to non-ulcerative doses than in those given diethanolamine in the drinking water. The limited dermal absorption of diethanolamine in rats probably was the major reason why toxicological effects were less prominent in rats exposed to equivalent total doses of diethanolamine by topical application than in those exposed via drinking water.*

In mice, approximately 60% of a dose of 81 mg/kg diethanolamine was absorbed when applied over a skin area of 1 cm^2 (RTI, 1991). This dose is approximately equal to a skin application of 162 mg/kg when applied over an area of about 2 cm^2, as was done in the dermal toxicology studies in mice. This dose of diethanolamine did not cause ulceration or inflammation of the skin. A greater percentage of applied diethanolamine was absorbed from mouse skin compared to rat skin, which may be due to the fact that mouse skin is thinner than rat skin. However, this comparison of diethanolamine absorption may not be entirely valid, because a larger dose was used in the mouse study, and the absorption of diethanolamine from rat skin increased with dose."

In conclusion, the dose dependent occurrence of hepatocellular carcinomas (and hepatoma) in the mouse carcinogenicity study cannot unequivocally be attributed to diethanolamine. Confounders including ethanol, mouse strain-specificity and differences in toxicokinetics between species cannot be disregarded.

It is likely that the use of ethanol as a vehicle increased the dermal absorption of diethanolamine and therefore the systemic availability of diethanolamine. It should be noted that the incidences of hepatocellular adenoma and of hepatocellular adenoma or carcinoma (combined) in all groups receiving diethanolamine and of hepatocellular carcinoma and hepatoblastoma in males receiving 80 and 160 mg diethanolamine/kg bw per day (mid and high dose level) were significantly increased compared to the vehicle (i.e. ethanol) treated controls. Additionally, size and multiplicity of neoplasms in diethanolamine treated animals were stated to be considerably greater than in the vehicle controls in the NTP report. It can be concluded that diethanolamine contributed to the increase in tumour incidence and has the potential to cause carcinogenic effects in the liver (of mice) once the systemic exposure to diethanolamine is sufficiently high.

The idea that diethanolamine has the potential to cause carcinogenic effects once systemic exposure is sufficiently high is also in line with the increased incidence of renal tubule adenomas in male mice at the highest tested dose and the fact that kidney tumours are not associated with exposure to ethanol.

$B6C3F_1$ mice are not considered as particularly sensitive to the development of renal tubule carcinoma. Renal effects (kidney nephropathy and tubular mineralisation) were also noted both in rats and mice, following oral (drinking water) and dermal administration.

Furthermore it should be considered that the exposure route relevant for consumer risk assessment is the oral route. Systemic availability of diethanolamine in rats after oral administration is considerably higher than after dermal administration – even in the presence of ethanol as vehicle for dermal administration. It can be assumed that this is also the case for mice and that oral exposure might principally lead to relevant systemic exposure levels with regard to tumour formation.

A published oral study (Konishi *et al.,* 1992) was also provided to support the evaluation of carcinogenic potential in mice. In this chronic oral study, $B6C3F_1$ mice received triethanolamine at 0%, 1% or 2% in drinking water (daily) over 82 weeks. It was ascertained that the triethanolamine contained 1.9% diethanolamine as a contaminant and that daily oral doses of up to 69 mg diethanolamine/kg were co-administered with triethanolamine in $B6C3F_1$ mice, in the absence of ethanol. No evidence of carcinogenic potential was demonstrated. However no conclusion can be drawn from that study regarding diethanolamine. Indeed while it was claimed that diethanolamine was a contaminant (1.9%) of triethanolamine, there was no actual control of the exposure to diethanolamine. Indeed no toxicokinetic data are available. Moreover the study report was drafted in 1991 while the

publication on diethanolamine contamination is dated 1982 and there is no information on when the study started. In the absence of toxicokinetic data and a contemporary certificate of analysis and a full study report, this study cannot be used to assess the carcinogenic activity of diethanolamine. It should also be noted that even considering the dosing by Konishi was correct, probably it was too low to develop toxic effect. Indeed in a 13-week study in mice, animals dosed 100 to 1700 mg/kg in drinking water did show multiple organ sites toxicity, particularly multiple hepatocyte changes (Melnick *et al*., 1994).

In conclusion, diethanolamine is considered a carcinogenic substance causing adenomas and carcinomas in the kidney and the liver of mice. The lack of carcinogenic effects in the dermal study in rats might be explained by a lower systemic exposure to diethanolamine due to a lower dermal absorption compared to that of mice and the use of a lower dose range in the rat carcinogenicity study.

Despite difficulties in interpretation that arise as a result of the use of ethanol as the vehicle the carcinogenic effects in kidney and liver of mice are considered relevant long-term effects of diethanolamine for consumer risk assessment. This is in line with the IARC assessment, which concluded that there is sufficient evidence in experimental animals for the carcinogenicity of diethanolamine.

2.2.2. Discussion of Question 1

Can CVMP confirm whether diethanolamine is a DNA reactive carcinogen? In 2013 diethanolamine was reviewed and classified as possibly carcinogenic to humans (group 2B) by IARC. However, at that time IARC was unable to conclude on the mechanism of carcinogenicity.

The genotoxicity of diethanolamine has already been reviewed by the Committee in 2017 in the scope of the re-evaluation of the inclusion of diethanolamine in the so-called 'out of scope' list. New information has been made available since that time and is now also considered (Comet assay, Beevers *et al.* 2015).

A summary of genotoxicity testing results is given below:

A. IN VITRO TESTS

a. Bacterial systems

i. Salmonella typhimurium

Diethanolamine (33 to 3,333 µg/plate) was not mutagenic in *Salmonella typhimurium* strain TA98, TA100, TA1535, or TA1537 when tested with a preincubation protocol in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver S9 (Haworth *et al.,* 1983).

ii. E. coli

Diethanolamine was negative in a reverse mutation assay (4000 µg/plate) in *E. coli* WP2 uvrA with and without metabolic activation (Dean *et al*., 1985).

b. Yeast

Diethanolamine was negative in a mitotic gene conversion assay in *Saccharomyces cerevisiae* in stationary and log-phase cultures with and without metabolic activation (Dean *et al*., 1985).

c. Mammalian cells

i. Mouse lymphoma assay

No induction of trifluorothymidine resistance was observed in L5178Y mouse lymphoma cells treated with diethanolamine with or without Aroclor 1254-induced male Fisher 344 rat liver S9 (NTP, 1999).

ii. Chinese hamster ovary cell cytogenetics.

Diethanolamine did not induce sister chromatide exchanges or chromosomal aberrations in cultured Chinese hamster ovary (CHO) cells, with or without Aroclor 1254-induced male Sprague-Dawley rat liver S9.

B. IN VIVO TESTS

a. Aneuploidy

Four-day old *Drosophila melanogaster* females were given 4% sucrose solutions containing 5, 10, 20, 40 or 80% diethanolamine for 24 hours; after a 2-hour recovery they were mated with 7-day old males. Diethanolamine induced similar increases in the frequencies of female non-disjunction (chromosome missegregation) in oocytes of the progeny at all concentrations (Munoz & Barnett 2013). It cannot be concluded whether this effect results from the interaction of diethanolamine with specific cellular targets involved in normal chromosomal segregation, from unspecific toxic actions on mature and maturing oocytes or both. The relevance of this study regarding transferability to humans is questionable since the study was performed in a non-mammalian species and pharmacokinetics and metabolism in invertebrates (insects) and mammalians are entirely different.

b. Mouse peripheral blood micronucleus

Peripheral blood samples taken from male and female mice dermally administered 80 to 1,250 mg diethanolamine/kg bw in 95% ethanol dermally for 13 weeks showed no increase in the frequency of micronucleated normochromatic erythrocytes (NTP, 1992).

c. Comet assay

No evidence of DNA damage was observed in the stomach or liver of male Sprague-Dawley Crl:CD (SD) rats treated with diethanolamine at 175, 350, or 700 mg/kg at 0, 24 and 45 hours by gavage (Beevers *et al.* 2015).

OECD guideline 489 describes the *in vivo* mammalian alkaline comet assay used as a method to measure DNA strand breaks in eukaryotic cells. The publication by Beevers *et al.* relates to the testing of diethanolamine in the alkaline comet assay as part of the JaCVAM (Japanese Centre for the Validation of Alternative Methods) comet validation study. The testing protocol that has been used is the validation study protocol version 14.2. This validation study is also referred to in OECD guideline 489.

Rats were dosed orally by gavage using dose volumes of 10 ml/kg with a formulation for animal dosing prepared by dissolving the diethanolamine in physiological saline. Ethyl methanesulfonate (200 mg/kg) was used as the positive control.

The highest dose tested for diethanolamine was determined to be the maximum tolerated dose (MTD) during a dose range-finding assessment in groups of 3 rats using the same dosing regimen and the comet experiments. The lower dose levels were equivalent to 25% MTD or 50% MTD. In practice, during dose range-finding, rats were treated with diethanolamine at dose levels of 500, 700, 1000, 1500 or 2000 mg/kg/day. Doses of 1000 mg/kg/day or higher resulted in the death of at least one

animal dosed per group. No clinical signs of toxicity were observed in animals dosed up to and including 700 mg/kg/day.

For the comet assay study, groups of 6 male rats per dose level were treated at 0, 24 and 45 hours, with necropsy and tissue sampling performed at 48 hours (i.e. 3 hours after final administration). There were no clinical sings of toxicity and no effects on body weight in any of the diethanolamine dose groups, nor in the vehicle or positive control group. Histopathology demonstrated a dose-related reduction in the level of glycogen vacuolation in the liver of rats treated with 350 or 700 mg/kg/day as well as dose-related increases in minor squamous cell hyperplasia and oedema in the same dose groups.

In both stomach and liver cells, diethanolamine did not induce increases in DNA damage at any dose level tested with all groups of animals showing % tail intensity values that were lower than the concurrent vehicle control. Rats dosed with ethyl methanesulfonate demonstrated DNA damage in both stomach and liver.

C. OTHER FINDINGS

a. Morphological transformation in SHE cells

Diethanolamine (10-500 µg/ml, 0.1-5.0 mM) induced morphological transformation in Syrian Hamster Embryo (SHE) cells cultured for 7 days in media containing 28µM (3 µg/ml) choline; however this effect was prevented by supplementation of the medium with excess choline (30 mM; 3.125 mg/ml). Diethanolamine also inhibited choline uptake and decreased phosphatidylcholine synthesis by these cells. The latter changes were also prevented by supplementation of the medium with 30 mM choline (Lehman-McKeeman & Gamsky, 2000). Therefore, the inhibition of choline uptake may induce alterations in gene expression. The SHE assay is not a classical genotoxicity test but can be used to investigate morphological transformation of cells and/or like in this case the possible underlying epigenetic mechanism (choline deficiency) of carcinogenic effects.

b. Choline deficiency

Primary cultures of hepatocytes isolated from $B6C3F_1$ mice were grown in the presence of diethanolamine (4.5 mM; 473 mg/ml) or in choline deficient medium (0.86 µM; 0.09 mg/ml) for 48 h and evaluated for DNA methylation status in GC-rich regions. Both diethanolamine and choline deficient treatments resulted in 54 regions of altered methylation, of which 43 and 49 regions were hypomethylation, respectively, and only one hypermethylation with each treatment (Bachman *et al*., 2006). The authors suggested that by inhibiting choline uptake into cells, diethanolamine may decrease the supply of S-adenosyl methionine, the main methyl donor for many methylation reactions, leading to hypomethylations in promotor regions of genes and consequent alterations in gene expression. The results support the hypothesis that diethanolamine acts by this mechanism to produce mouse liver tumours. However that study did not provide any evidence that diethanolamine acts as a direct DNA acting substance.

c. Liver tumours

DNA was isolated from sections of liver tumours obtained in the 2-year dermal study in $B6C3F_1$ mice and analysed for genetic alterations in β-catenin *Catnb* and H-*ras* genes. The frequency of *Catnb* mutations was 100% in hepatoblastomas, 32% in hepatocellular neoplasms from mice exposed to diethanolamine and 10% in hepatocellular neoplasms from controls. Genetic alteration in exon 2 of the *Catnb* gene included deletion mutations and point mutations that occurred at much higher frequencies in liver neoplasms from diethanolamine-exposed mice compared to controls (Hayashi *et al*., 2003).

Therefore a genotoxic mechanism of diethanolamine induced liver tumours could not be completely excluded. On the other hand the results did not provide a convincing evidence that diethanolamine is a direct acting genotoxin since it is well known that cells from tumour tissues exhibit various mutations due to accelerated cell proliferation (genetic instability). Moreover, the observed mutation could also be related to an indirect mechanism of choline depletion as β-catenin mutation has also been observed in hepatocellular carcinomas induced by a choline-deficient L-amino acid defined diet in rats (Tsujiuchi *et al*., 1999).

IARC considered that:

- *"a genotoxic mechanism is supported by the induction of aneuploidy in Drosophila and the elevated frequency of β-catenin Catnb genes in liver tumours induced by diethanolamine. However diethanolamine was not mutagenic in most in vitro systems and did not increase the frequency of micronuclei in exposed mice.";*
- *"there is weak evidence that a genotoxic mechanism is involved in the induction of liver tumours by diethanolamine."*

It has to be clearly stated that genotoxicity tests conducted according to the recommended standard test battery are considered unequivocally negative. This is true for the standard *in vitro* tests (Ames, mouse lymphoma, chromosome aberration) as well as for the *in vivo* micronucleus test in mice. It is the CVMP view that the overall weight of evidence from negative genotoxicity tests performed in standardised systems, especially considering the *in vivo* mammalian micronucleus test and the recent *in vivo* comet assay (the latter was not considered by IARC), clearly outweighs the evidence of research work performed with non-standardised systems. It can thus be concluded that diethanolamine is unlikely to be DNA reactive.

Induction of choline deficiency has been proposed as the means by which diethanolamine induces liver neoplasms in mice. This hypothesis would support an epigenetic mode of action. Literature data suggest that this mechanism could also be relevant for the mouse kidney tubule adenoma/carcinomas. In a study in which male Sprague Dawley rats were fed a choline deficient diet for 6 days, followed by a normal diet for up to 119 days, acute renal lesions consisting of tubular epithelial cell necrosis were observed immediately after being fed a choline-deficient diet (Keith and Tryphonas, 1978). Chronic renal lesions consisting of interstitial nephritis characterised by fibrosis and scarring were observed 28-119 days after being fed the choline-deficient diet. The proximal convoluted tubule was most severely affected. Hepatic lesions were also observed.

Irrespective of whether diethanolamine can be considered a genotoxic carcinogen or a non-genotoxic carcinogen the possibility cannot be excluded that diethanolamine under specific conditions such as low pH in the stomach or food processing (heat) can be possibly converted to the known genotoxic carcinogen N-nitrosodiethanolamine (NDELA). Nitrosamine formation *in vivo* is thought to occur as a result of a non-enzymatic reaction between a secondary amine and nitrosating agents nitrate/nitrite in the acidic environment of the stomach resulting in an unacceptable consumer risk from ingestion of diethanolamine containing food.

Summary and conclusions

It can be concluded that diethanolamine is unlikely to be a DNA reactive carcinogen.

However the possibility cannot be excluded that diethanolamine under specific conditions including those that occur in the gastrointestinal tract, can be converted to the known genotoxic carcinogen N-nitrosodiethanolamine (NDELA).

CVMP response to question 1 from Belgium:

Question:

Can CVMP confirm whether diethanolamine is a DNA reactive carcinogen? In 2013 diethanolamine was reviewed and classified as possibly carcinogenic to humans (group 2B) by IARC. However, at that time IARC was unable to conclude on the mechanism of carcinogenicity.

CVMP response:

The overall weight of evidence from negative genotoxicity tests performed in standardised systems, especially considering the *in vivo* mammalian micronucleus test and the comet assay, clearly outweighs the research work performed with non-standardised systems. It can be concluded that diethanolamine is unlikely to be a DNA reactive carcinogen.

Irrespective of this conclusion, the possibility cannot be excluded that diethanolamine, under specific conditions such as low pH in the stomach or during food processing (heat), can be converted to the known genotoxic carcinogen N-nitrosodiethanolamine (NDELA). Nitrosamine formation *in vivo* is thought to occur as a result of a non-enzymatic reaction between a secondary amine and nitrosating agents nitrate/nitrite in the acidic environment of the stomach resulting in an unacceptable consumer risk from the ingestion of diethanolamine containing food.

2.2.3. Discussion of Question 2

If it is concluded that diethanolamine is a DNA reactive, does this mean that the risk to the consumer should be considered as unacceptable?

On the basis of the discussion under section 2.2.2. it can be concluded that diethanolamine is unlikely to be a DNA reactive carcinogen (see section 2.2.2. above). Therefore this question is no longer relevant.

2.2.4. Discussion of Question 3

If it is concluded that diethanolamine is not DNA reactive, is it possible to establish a margin of exposure that would be acceptable from a consumer safety perspective? In relation to this question it is noteworthy that the previous entry in the 'out of scope' list included the following restriction: "at doses up to 0.3 mg/kg bw/day".

Substances and metabolites that may cause cancer by mechanisms other than direct interaction with DNA can be assumed to have threshold based mechanisms of action. If such substances are to be used in veterinary medicines for food producing animals, NO(A)ELs/BMDLs (no-observed-(adverse)-effect-levels; lower bound of the benchmark dose confidence interval) should be established for the relevant effects in appropriately justified studies and an acceptable daily intake (ADI) established.

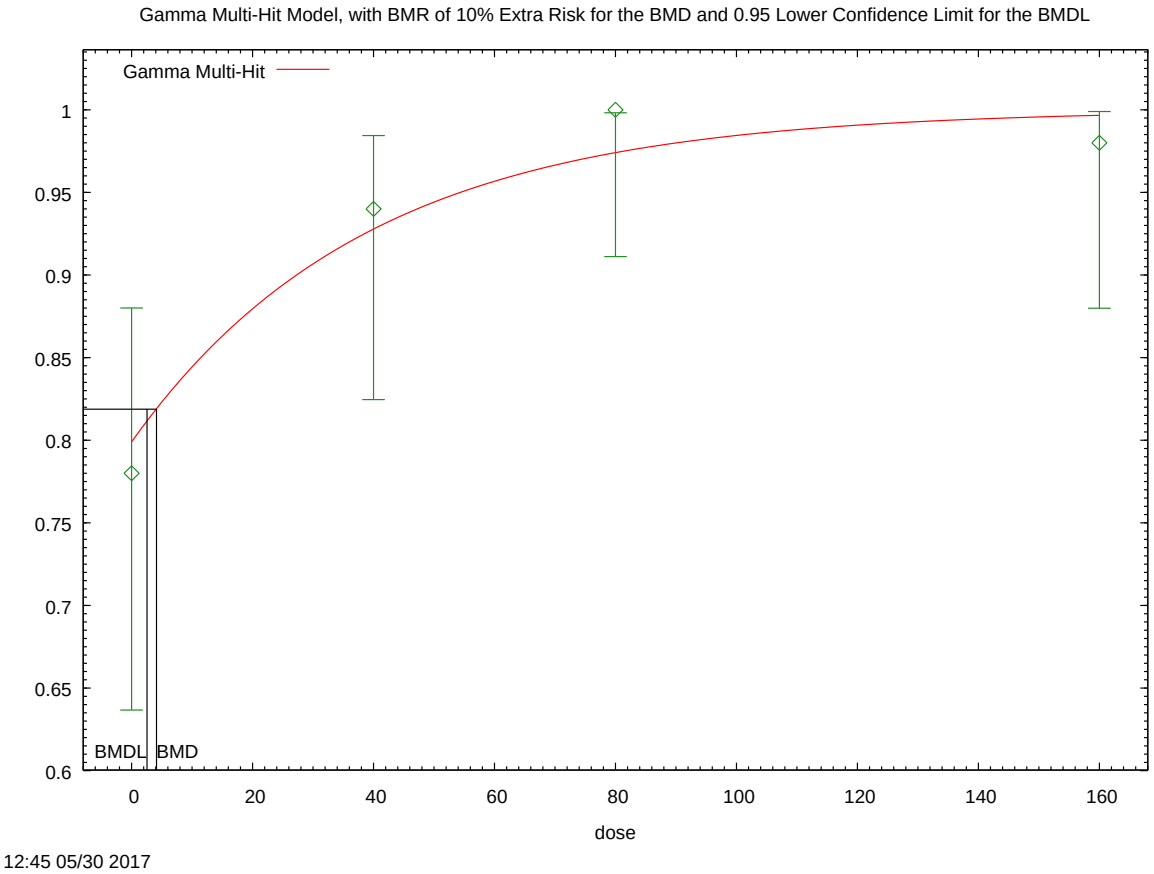
In the hypothesis that diethanolamine is not DNA reactive, the mouse carcinogenicity study should be considered. No NOAEL can be established from that study. However it is possible to derive a $BMDL_{10}$ using the EPA software BMDS 2.6.0.1 from the dataset of the NTP study TR 478 (https://ntp.niehs.nih.gov/ntp/htdocs/lt_rpts/tr478.pdf, see table 13).

$BMDL_{10}$ (mg/kg) for liver tumours in mice (dermal exposure)

	Male	Female
Hepatocellular adenoma, multiple	5.33	4.12
Hepatocellular adenoma (includes multiple)	5.35	2.98
Hepatocellular carcinoma, multiple	29.16	16.98
Hepatocellular carcinoma (includes multiple)	12.14	7.02
Hepatocellular adenoma or carcinoma (includes multiple)	2.55	failed
Hepatoblastoma	62.44	188
Hepatocellular adenoma or carcinoma or hepatoblastoma (includes multiple)	**2.55**	failed

The $BMDL_{10}$ for renal tubule adenoma in male mice is 69 mg/kg bw.

The lowest $BMDL_{10}$ is 2.55 mg/kg (see figure below for hepatocellular adenoma or carcinoma or hepatoblastoma):



A permissible daily exposure (PDE) can be derived based on the $BMDL_{10}$ by applying the following uncertainty factors:

$BMDL_{10}$ to "No effect level":	2
Route-to-route extrapolation:	3
Duration of the study:	1
Interspecies factor:	10
Intraspecies factor:	10
Severity of effect (non-genotoxic carcinogen):	10
Overall uncertainty factor:	**6000**

$$\text{PDE} = \frac{\text{BMDL}_{10}}{\text{Overall uncertainty factor}}$$

PDE = 0.425 µg/kg bw per day

= 25.5 µg per day for a 60 kg person.

Since the choline hypothesis could possibly explain the β-catenin mutations and tumour formation observed in the liver and kidney of mice, an alternative could be to calculate a PDE on the basis of the NOAEL for effects on choline (10 mg/kg) obtained from a study in mice involving 4-week dermal administration of diethanolamine under same circumstances as in the mouse carcinogenicity study (Lehman-McKeeman *et al.,* 2002). Briefly, $B6C3F_1$ mice were dosed dermally with diethanolamine in 95% ethanol for 4 weeks (5 days/week). Control animals were either not dosed or dosed with 95% ethanol only. The pattern of changes observed in choline metabolites after diethanolamine treatment was very similar to that observed in choline-deficient mice, and the NOAEL for diethanolamine-induced changes in choline homeostasis was 10 mg/kg/day

The reactions were dose-dependent and reversible. Dermal application of 95% ethanol decreased the levels of hepatic betaine, the oxidation product of choline, suggesting that use of ethanol as a vehicle for dermal application of diethanolamine could exacerbate the biochemical effects of diethanolamine.

A permissible daily exposure can be derived based on the NOAEL in line with the method described in the Guideline on setting health based exposure limits for use in risk identification in the manufacture of different medicinal products in shared facilities (EMA/CHMP/CVMP/SWP/169430/2012).

The following uncertainty factors are applied:

No established NOEL:	1
Route-to-route extrapolation:	3
Duration of the study:	10
Interspecies factor:	10
Intraspecies factor:	10
Severity of effect (non-genotoxic carcinogen):	10
Overall uncertainty factor:	**30000**

$$\text{PDE} = \frac{\text{NOAEL x Weight Adjustment}}{\text{Overall uncertainty factor}}$$

PDE = 0.333 µg/kg bw per day

= 20 µg per day for a 60 kg person.

To derive an estimate for consumer exposure the maximum dose of diethanolamine of 0.3 mg/kg bw/day (=300 µg/kg bw per day) from the previous entry in the 'out of scope' list was used. Based on a single treatment and using the standard food basket of 2 kg (0.5 kg meat and 1.5 l milk), this would lead to a consumer exposure of 2 kg x 300 µg diethanolamine/kg bw / 60 kg = 10 µg diethanolamine/kg bw.

For products for which repeated treatments are recommended, the consumer exposure would be 20 µg diethanolamine/kg bw and 30 µg diethanolamine/kg bw for 2 times/days treatment and 3 times/days treatment, respectively.

Comparing the PDEs to the consumer exposure estimate of 10 µg/kg indicates that residues in food even after single treatment may be well above (30 times or 23.5 times depending on the PDE) the limit that could be accepted. Residue concentrations after repeated treatments may be even higher.

These considerations are based on the assumptions of complete absorption, even distribution of diethanolamine in the body of treated animals, and no elimination. However, for injection sites and also for liver tissues these assumptions might not represent the worst case.

Indeed, the consumer exposure estimate would be much higher if residue concentrations in injection site tissues from animals treated intramuscularly or subcutaneously are taken into account. If e.g. an animal of 100 kg bw is treated with 0.3 mg diethanolamine/kg bw, this would result in an amount of 30 mg diethanolamine in an injection site, which is far above the PDE of 20–25.5 µg/person. Ingestion of even small parts of an injection site would lead to an unacceptable risk for the consumer.

Furthermore, according to a paper by Mathews *et al*. (1997) diethanolamine can accumulate in liver and kidney tissues, which might also lead to higher consumer exposure than calculated above based on the assumption of even distribution of diethanolamine in the body.

No refinements of the above calculation can be made, as no data on diethanolamine concentrations in target animal tissues are available. In order to reduce the theoretical exposure estimate, data would be needed showing that extensive metabolism and/or excretion leads to formation of toxicologically irrelevant (harmless) substances and/or a decrease in diethanolamine concentrations in edible tissues and milk within short time frames.

Summary and conclusions

From the mouse carcinogenicity study no NOAEL can be established. However it is possible to derive a $BMDL_{10}$ of 2.55 mg/kg bw per day for liver tumours and to derive a permissible daily exposure (PDE) of 0.425 µg/kg bw per day applying an overall uncertainty factor equal to 6000. In an alternative worst case scenario a PDE equal to 0.333 µg/kg bw per day may be calculated on the basis of the NOAEL for effects on choline (10 mg/kg) obtained from a study in mice involving a 4-week dermal administration of diethanolamine and applying an uncertainty factor of 30000.

The lowest calculated PDE of 0.333 µg/kg bw compared to a worst-case estimate for consumer exposure indicates an unacceptable risk to the consumer even after a single administration of diethanolamine containing products. Consumer exposure estimates and the resulting risk increase if repeated treatments or injection site consumption are considered.

In conclusion, the margin of exposure that is established based on the PDE and the worst case exposure estimate is not acceptable. In the absence of residue data in target species demonstrating that carcinogenic residues are below the PDE, worst case scenario calculations indicate that consumer exposure to residues of diethanolamine would represent an unacceptable risk.

CVMP response to question 3 from Belgium:

Question:

If it is concluded that diethanolamine is not DNA reactive, is it possible to establish a margin of exposure that would be acceptable from a consumer safety perspective? In relation to this question it is noteworthy that the previous entry in the 'out of scope' list included the following restriction: *"at doses up to 0.3 mg/kg bw/day"*.

CVMP response:

From the mouse carcinogenicity study no NOAEL can be established. However it is possible to derive a $BMDL_{10}$ of 2.55 mg/kg bw per day for liver tumours and to derive a permissible daily exposure (PDE) of 0.425 µg/kg bw per day applying an overall uncertainty factor equal to 6000. Alternatively a PDE of 0.333 µg/kg bw per day may be calculated on the basis of the NOAEL for effects on choline (10 mg/kg) obtained from the study in mice involving 4-week dermal administration of diethanolamine and applying an uncertainty factor of 30000.

The lowest calculated PDE of 0.333 µg/kg compared to a worst-case estimate for consumer exposure indicates an unacceptable risk to the consumer even after a single administration of diethanolamine containing products. Consumer exposure estimates and the resulting risk increase if repeated treatments or injection site consumption are considered.

In conclusion, the margin of exposure that is established based on the PDE and the worst case exposure estimate is not acceptable. In the absence of residue data in target species demonstrating that carcinogenic residues are below the PDE, worst case scenario calculations indicate that consumer exposure to residues of diethanolamine would represent an unacceptable risk.

2.2.5. Discussion of Question 4

On the basis of its scientific evaluation, does the CVMP consider that, to allow the use of diethanolamine in veterinary medicinal products for food producing animals, a full MRL evaluation is needed?

Diethanolamine is considered carcinogenic but not DNA reactive. Consequently it would, in principle, be possible to establish a PDE and include an entry for the substance in Regulation 37/2010 to ensure that the PDE is not exceeded. However, considering the intake of diethanolamine residues via food from treated animals, there is a concern over the potential formation of N-nitrosodiethanolamine (NDELA), particularly in the acidic environment of the stomach after oral exposure or during food processing (heat). It would have to be shown that this mechanism is not relevant under the conditions in the human gastrointestinal tract as well as in food processing.

From carcinogenicity data in mice, a PDE has been estimated. However in the absence of residue data in the target species and based on worst-case exposure scenarios, the margin of exposure that can be established is not acceptable. With appropriate data, refinement of the consumer exposure estimates might be possible. In principle such data might aim to demonstrate the absence of oral bioavailability or the absence of residues in food. From the available information in rats, it is known that oral bioavailability is relatively high. Therefore the only possibility for refinement would be based on demonstrating the absence of residues of concern in the target species. As consumption of an injection site containing residues represents one of the worst case exposure scenarios this would need to be taken into account in any refined calculations.

The possibility to reduce consumer exposure by decreasing the diethanolamine concentrations in the concerned products may also be considered. However, an MRL evaluation would still be needed in order to demonstrate that consumer exposure is acceptably low.

In conclusion, an MRL evaluation would be needed to address the above issues. All other standard parts of the MRL dossier would also need to be addressed.

Summary and conclusions

It would in principle be possible to undertake an MRL evaluation to consider the possibility of an entry for diethanolamine in Table 1 of Commission Regulation (EU) No 37/2010. However, the applicant would need to provide the necessary dossier, including residue depletion data in edible tissues and milk, showing that residues of concern (i.e. consumer exposure) do not occur at levels that would lead to exposure greater than the PDE. Residue data in food derived from treated animals would be needed showing that carcinogenic residue concentrations are much lower than estimated in worst case exposure scenarios. In addition, the consumption of an injection site containing residues would need to be taken into account. The potential formation of nitrosamines would also need to be addressed.

Substitution of diethanolamine in veterinary medicinal products or the possibility to reduce consumer exposure via reducing the diethanolamine concentrations in the concerned products may also be considered (in the latter case there would still be the need for an MRL evaluation to demonstrate that consumer exposure would be acceptably low).

If diethanolamine is to be further used in veterinary medicinal products for food producing species, a MRL evaluation according to Regulation (EC) No. 470/2009, additionally addressing possible nitrosamine formation would be needed.

CVMP response to question 4 from Belgium:

Question:

On the basis of its scientific evaluation, does the CVMP consider that, to allow the use of diethanolamine in veterinary medicinal products for food producing animals, a full MRL evaluation is needed?

CVMP response:

It would in principle be possible to undertake an MRL evaluation to consider the possibility of an entry for diethanolamine in Table 1 of Commission Regulation (EU) No 37/2010. However, the applicant would need to provide the necessary dossier, including residue depletion data in edible tissues and milk, showing that residues of concern (i.e. consumer exposure) do not occur at levels that would lead to exposure greater than the PDE. Residue data in food derived from treated animals would be needed showing that carcinogenic residue concentrations are much lower than estimated in worst case exposure scenarios. In addition, the consumption of an injection site containing residues would need to be taken into account. The potential formation of nitrosamines would also need to be addressed.

If diethanolamine is to be further used in veterinary medicinal products for food producing species, a MRL evaluation according to Regulation (EC) No. 470/2009, additionally addressing possible nitrosamine formation, would be needed.

3. Overall summary of the scientific evaluation

Diethanolamine is used as a solvent in various veterinary medicinal products authorised nationally in the majority European Union Member States. In 2013, IARC classified diethanolamine as possibly carcinogenic to humans (group 2B) and concluded that there is sufficient evidence in experimental animals for the carcinogenicity of diethanolamine. This classification was based on carcinogenicity findings from a 2-year dermal study in mice conducted by the US NTP where diethanolamine was applied topically at doses of 40, 80 or 160 mg/kg bw diluted in 95% ethanol, for 5 days per week over 103 weeks. All doses of diethanolamine were considered to have caused significant increases in hepatocellular neoplasms in male and female mice, with a positive trend seen in the incidences of renal tubular adenoma at all doses in treated males. IARC noted that "tumours of the kidney and hepatoblastomas are rare spontaneous neoplasms in experimental animals".

One marketing authorisation holder considered the NTP dermal study to have been confounded by the co-administration of ethanol which was used as the vehicle for diethanolamine application, as ethanol has been shown to be carcinogenic and may enhance and/or potentiate biochemical interactions of other substances.

A further MAH considered that diethanolamine induces mouse liver tumours by a non-genotoxic mode of action that involves its ability to cause choline deficiency.

The CVMP considered that the dose dependent occurrence of hepatocellular carcinoma (and hepatoma) in the mouse carcinogenicity study cannot unequivocally be attributed to diethanolamine and confounders including ethanol, mouse strain-specificity and differences in toxicokinetics between species cannot be disregarded. However, incidences of hepatocellular adenoma and of hepatocellular adenoma or carcinoma (combined) in all groups receiving diethanolamine and of hepatocellular carcinoma and hepatoblastoma in males were significantly increased compared to vehicle (i.e. ethanol) treated controls. Additionally, size and multiplicity of neoplasms in diethanolamine treated animals were greater than in the vehicle controls.

Furthermore, ethanol is not associated with kidney tumours in experimental animals (including mice). Therefore observed renal tubular neoplasms in mice after treatment with diethanolamine in ethanol are considered relevant for consumer risk assessment.

Overall it can be concluded from the NTP studies that there is evidence that diethanolamine is carcinogenic in mice. The lack of carcinogenicity in rats may be explained by use of a lower dose range in the carcinogenicity study and a lower dermal absorption of diethanolamine compared to mice leading to a lower systemic exposure to diethanolamine.

Regarding genotoxicity, based on the available information, it can be concluded that diethanolamine is not likely to be a DNA reactive carcinogen. Regarding the mechanism of carcinogenicity, induction of choline deficiency has been proposed as the means by which diethanolamine induces liver neoplasms in mice. This hypothesis would support an epigenetic mode of action.

On the other hand, there is a concern over the potential formation of N-nitrosodiethanolamine, a strong DNA reactive carcinogen in experimental animals, which may be produced from diethanolamine in the acidic conditions of the stomach or during food processing (heat).

Regarding consumer safety the margin of exposure that is established based on the PDE and the worst case exposure estimate is not acceptable for the consumer. In the absence of residue data in target species demonstrating that carcinogenic residues are below the PDE, worst case scenario calculations indicate that consumer exposure to residues of diethanolamine would represent an unacceptable risk.

If diethanolamine is to be further used in veterinary medicinal products for food producing species, a MRL evaluation according to Regulation (EC) No 470/2009, additionally addressing possible nitrosamine formation would be needed.

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