~~Confidential~~

Helsinki, 23 May 2024

Addressee(s)
Registrant of Reaktiv-Orange DYPR 1466 as listed in Appendix 3 of this decision

Date of submission of the dossier subject to this decision
02 December 2022

Registered substance subject to this decision ("the Substance")
Substance name: Lithium, sodium 2-(4-chloro-6-cyanoamino-1,3,5-triazin-2-ylamino)-5-hydroxy-6-(2-methoxy-5-(sulfatoethanesulfonyl)phenylazo)naphthalene-1,7-disulfonate
EC/List number: 440-050-4

Decision number: Please refer to the REACH-IT message which delivered this communication (in format TPE-D-XXXXXXXXXX-XX-XX/F)

DECISION ON TESTING PROPOSAL(S)

Under Article 40 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below by ***30 November 2026***.

Requested information must be generated using the Substance unless otherwise specified.

Information required from all the Registrants subject to Annex VII of REACH

1. In vivo mammalian alkaline comet assay (triggered by Annex VII, Section 8.4., Column 2; test method: OECD TG 489) combined with in vivo mammalian erythrocyte micronucleus test (test method: OECD TG 474) in rats, or if justified, in mice, oral route.
   - For the comet assay the following tissues must be analysed: liver, glandular stomach and duodenum.
   - For the micronucleus test centromere staining must be performed *if* the substance induces an increase in the frequency of micronuclei in the OECD TG 474.

The reasons for the decision(s) are explained in Appendix 1.

Information required depends on your tonnage band

You must provide the information listed above for all REACH Annexes applicable to you in accordance with Articles 10(a) and 12(1) of REACH. The addressee(s) of the decision and their corresponding information requirements based on registered tonnage band are listed in Appendix 3.

You are only required to share the costs of information that you must submit to fulfil your information requirements.

How to comply with your information requirements

To comply with your information requirements, you must submit the information requested by this decision in an updated registration dossier by the deadline indicated above. You must also **update the chemical safety report**, where relevant, including any changes

to classification and labelling, based on the newly generated information.

You must follow the general requirements for testing and reporting new tests under REACH, see Appendix 4.

Appeal

This decision, when adopted under Article 51 of REACH, may be appealed to the Board of Appeal of ECHA within three months of its notification to you. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Authorised[1] under the authority of Mike Rasenberg, Director of Hazard Assessment

Appendix 1: Reasons for the decision
Appendix 2: Procedure
Appendix 3: Addressees of the decision and their individual information requirements
Appendix 4: Conducting and reporting new tests under REACH

[1] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

Appendix 1: Reasons for the decision

Contents

Reasons related to the information under Annex VII of REACH

1. In vivo mammalian alkaline comet assay combined with in vivo mammalian erythrocyte micronucleus test

1 Under Annex VII, Section 8.4., Column 2, an appropriate *in vivo* mammalian somatic cell genotoxicity study as referred to in Annex IX, point 8.4.4, must be performed in case of a positive result in any of the *in vitro* studies referred to in Annex VII, Section 8.4. The *in vivo* study must address the concerns raised by the *in vitro* study results, i.e. the chromosomal aberration concern or the gene mutation concern or both, as appropriate.

1.1. Triggering of the information requirement

2 Your dossier contains positive results for the *in vitro* gene mutation study in bacteria (OECD TG 471, 1999) and the *in vitro* chromosomal aberration test (OECD TG 473, 1999) which raise the concerns for gene mutations and chromosomal aberrations.

1.2. Information provided to fulfil the information requirement

3 You have submitted a testing proposal for an *in vivo* mammalian alkaline comet assay to be performed with the Substance.

1.3. Rejection of existing in vivo study

4 ECHA notes that your dossier contains an *in vivo* micronucleus test (OECD TG 474, 2000) that you consider as a follow-up *in vivo* study to investigate the concern identified in the *in vitro* the chromosomal aberration test (OECD TG 473, 1999).

5 However, for the following reasons this *in vivo* MN test does not meet the specifications of the test guideline and therefore cannot be considered an adequate study to meet the information requirement.

6 To fulfil the information requirement, the study must comply with the OECD TG 474 (Article 13(3) of REACH). Therefore, several specifications must be met, in particular the study must include a minimum of three dose level groups of treated animals. The only exception where only one group of treated animals can be accepted is in the case of a limit test. The OECD TG 474 considers the 'Limit test' of 2000 mg/kg body weight acceptable only in case the study does not produce observable toxic effects and genotoxicity is not expected for the substance.

7 The provided study (OECD TG 474, 2000) included only one group of treated animals (i.e., less than three groups) at one dose level of 2000 mg/kg bw/day.

8 Further, you did not demonstrate that genotoxicity would not be expected. By contrast the Substance induced a clastogenic effect in the available *in vitro* CA test (OECD 473, 1999).

9 In your comments to the draft decision, you mentioned [two] *"reasons why the positive result of the in-vitro [chromosomal aberration] test was considered to be a false positive result"*.

10 First, you indicated that *"in the chromosomal aberration test, the test compound induced an increase in the number of chromosome aberrations in the absence of S9-mix at the two highest concentrations at cytotoxic concentrations only"*.

11 However, in your IUCLID dossier, it is indicated that *"Survival was reduced in a dose-related manner reaching 68.7% of the solvent control value at the highest concentration [5000 µg/mL] without S9-mix"*. This level of survival is acceptable as it is higher than the threshold

value of 50% recommended by the OECD TG 473, so we conclude that the positive result of the *in vitro* chromosomal aberration test is reliable.

12 Second, you mentioned *that "The in-vitro chromosome aberration test was carried out in V79 cells. Kirkland D et al (2005) demonstrated an extremely high false-positive rate for in-vitro clastogenicity tests, particularly in mammalian cell tests, when compared to rodent carcinogenicity study results ([redacted] (2008))"*.

13 However, we note that the article by Kirkland compared the outcomes of *in vitro* clastogenicity tests in mammalian cells and of rodent carcinogenicity study, while the current decision addresses the *in vivo* genotoxicity endpoint. Moreover, you have not provided documentation as to why this information is relevant for your Substance.

14 In your comments you further developed why you consider that *"no positive result in the in-vivo MNT was expected"*: *"vinyl sulfone substances result in false positive test results in in-vitro tests for clastogenicity"*; *"these chemical agents react via the Michael addition reaction"* and *"are known to deplete glutathione in in-vitro test systems"*; *"in-vitro systems have very low levels of glutathione"* and *"this is not the case in the in-vivo test system, where glutathione is present in adequate amount"*.

15 However, you have not provided any experimental evidence that glutathione depletion was the only mechanism responsible for the positive effect observed in the *in vitro* chromosomal aberration study (OECD TG 473, 1999).

16 Therefore, the information provided does not cover the specifications(s) required by the OECD TG 474, and consequently information needs to be generated to fill the data gap.

1.4. Considerations of alternative methods

17 ECHA requested your considerations for alternative methods to fulfil the information requirement for Genetic toxicity *in vivo*. You provided your considerations concluding that there were no alternative methods which could be used to adapt the information requirement(s) for which testing is proposed. ECHA has taken these considerations into account.

1.5. Conclusion on the need for the tests

18 Having regard of all the above, ECHA agrees that an appropriate *in vivo* follow up genotoxicity study is necessary to address the concern(s) identified *in vitro*.

1.6. Test selection

19 The positive *in vitro* results available in the dossier indicate a concern for both chromosomal aberration and gene mutation.

20 The available MN test (OECD TG 474, 2000, with only one dose tested) cannot be considered adequate to investigate the chromosomal aberration concern identified in the *in vitro* test (OECD TG 473, 1999).

21 In case a substance induces both *in vitro* concerns for gene mutation and chromosomal aberration, and there is no adequate *in vivo* genotoxicity data available, the registrant needs to perform a combination of the comet assay (OECD TG 489) and the micronucleus test (OECD TG 474).

22 The *in vivo* mammalian alkaline comet assay ("comet assay", OECD TG 489) can be combined with an *in vivo* mammalian erythrocyte micronucleus test ("MN test", OECD TG 474) in a single study (see OECD TG 489 para. 33; OECD TG 474 para. 37c; Guidance on IRs & CSA, Section R.7.7.6.3). While the comet assay can detect primary DNA damage that may lead to gene mutations and/or structural chromosomal aberrations, the MN test can

detect both structural chromosomal aberrations (clastogenicity) and numerical chromosomal aberrations (aneuploidy). A combined study will thus address both the identified concerns for chromosomal aberration as well as gene mutation.

23 The combined study, together with the results of the *in vitro* mutagenicity studies, can be used to make definitive conclusions about the mechanism(s) inducing *in vivo* mutagenicity and lack thereof. Furthermore, the combined study can help reduce the number of tests performed and the number of animals used while addressing (structural and numerical) chromosomal aberrations as well as gene mutations.

24 Therefore, the comet assay combined with the MN test is the most appropriate study for the Substance.

1.7. Specification of the study design

25 You proposed testing in the rat. According to the test method OECD TG 489, rats are the preferred species. Other rodent species can be used if scientifically justified. According to the test method OECD TG 474, the test may be performed in mice or rats. Therefore, the combined study must be performed in rats, or if justified, in mice.

26 You proposed testing by the oral route. Having considered the anticipated routes of human exposure and adequate exposure of the target tissue(s) performance of the test by the oral route is appropriate.

27 In line with the test method OECD TG 489, the test must be performed by analysing tissues from the liver as primary site of xenobiotic metabolism, glandular stomach and duodenum as sites of contact. There are several expected or possible variables between the glandular stomach and the duodenum (different tissue structure and function, different pH conditions, variable physico-chemical properties and fate of the Substance, and probable different local absorption rates of the Substance and its possible breakdown product(s)). In light of these expected or possible variables, it is necessary to analyse both tissues to ensure a sufficient evaluation of the potential for genotoxicity at the site of contact in the gastro-intestinal tract.

28 The combination of OECD TGs 489 and 474 should not impair the validity of and the results from each individual study. Careful consideration should be given to the dosing, and tissue sampling for the comet analysis alongside the requirements of tissue sampling for the mammalian erythrocyte micronucleus test (see OECD TG 489, e.g. Bowen et al. 2011[2]).

1.7.1. Assessment of aneugenicity potential

29 If the result of the *in vivo* MN test is positive, i.e. your Substance induces an increase in the frequency of micronuclei, you must assess the aneugenic potential of the Substance. In line with the OECD TG 474 (paragraph 42), you should use one of the centromere labelling or hybridisation procedures to determine whether the increase in the number of micronuclei is the result of clastogenic events (i.e. micronuclei contain chromosome fragment(s)) and/or aneugenic events (i.e. micronuclei contain whole chromosome(s)).

1.7.2. Investigation of target tissue exposure

30 The applicable test method OECD TG 474 states that *"If there is evidence that the test substance(s), or its metabolite(s), will not reach the target tissue, it may not be appropriate to use this test".* Additionally, a negative test result can be considered reliable only if *"Bone marrow exposure to the test substance(s) occurred".*

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[2] Bowen D.E. et al. (2011), Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the comet assay and the flow-cytometric peripheral blood micronucleus test. Mutation Research 722 7–19.

31 In your comment to the draft decision, you *"do not consider it necessary to collect blood samples to demonstrate exposure, as this is evident from the results of the 28-day repeated dose oral gavage study in rats ([redacted] (1999)). […] In this study, the urine of most high-dose animals was discoloured light pink to salmon pink, […] in the high dose group the serum of all animals was discoloured dark salmon; in the intermediate dose group, the serum of all males and most females was discoloured salmon. At necropsy, animals of the intermediate and/or high dose groups showed reddish discoloured skin, adipose tissue, kidneys, testes, epididymes, stomach, and small intestines. Hence, exposure of animals to the test substance could be shown at dose levels of 250 and 1000 mg/kg bw/day in skin, stomach, and small intestines, serum, adipose tissue, testes, epididymes, kidneys, and urine. Further proof of exposure is not deemed necessary".*

32 We agree that the available data shows that the orange-red colour of the substance is present in the serum, tissues and urine of SD rats after 28-day of oral gavage, which is a proof of systemic availability and exposure of the bone marrow. Therefore, the request to *"take blood samples at appropriate times and measure plasma levels of the Substance and/or its metabolites, unless exposure of the bone marrow can be demonstrated through other means"* was removed from the decision.

1.7.2.1. Germ cells

33 You proposed not to collect germ cells as the OECD TG 489 is not validated for germ cell investigation.

34 However, you may still consider collecting the male gonadal cells from the seminiferous tubules in addition to the other tissues in the comet assay, as it would optimise the use of animals. You can prepare the slides for male gonadal cells and store them for up to 2 months, at room temperature, in dry conditions and protected from light. Following the generation and analysis of data on somatic cells in the comet assay, you should consider analysing the slides prepared with gonadal cells. This type of evidence may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation.

35 In your comments to the draft decision, you indicated that you *"still [do] not consider it meaningful to collect male gonadal cells because – as the guideline states – the standard alkaline comet assay as described in OECD TG 489 is not considered appropriate to measure DNA strand breaks in mature germ cells for various reasons. According to the guideline, it should also be considered that "gonads contain a mixture of somatic and germ cells. For this reason, positive results in whole gonad (testis) are not necessarily reflective of germ cell damage""*. You also quoted Gajski et al. (2021), who concluded that *"in sperm the DNA is differently packed than in somatic cells",* and you consider that *"the standard protocol of the comet assay needs to be adapted when it is applied to sperm"*. Moreover, you remind that *"Dirven et al. (2023) developed an approach to distinguish specific germ cells from other cells of the testicle to provide germ cell-specific DNA damage level assessments. This approach would have to be validated and included into TG 489 to produce reliable data on germ cell DNA damage"*.

36 ECHA clarifies that the collection of male gonadal cells in the comet assay i) is only a recommendation, and ii) is not aimed at investigating genotoxic effects specifically in mature germ cells but on the mixture of somatic and germ cells. As specified in paragraph 10 of OECD TG 489, the inclusion of such examination may bring relevant information for the overall assessment of germ cell mutagenicity, for instance with respect to gonad exposure to the Substance and/or its metabolites. Furthermore, the feasibility of the

analysis of cells from the gonads has been demonstrated in the literature (Speit et al, 2009[3]; Zheng and Olive, 1997[4]; Cordelli et al, 2003[5]; Dirven et al., 2023[6]).

1.8. Outcome

37 Under Articles 40(3)(b) and (c), your testing proposal is accepted under modified conditions, and you are requested to carry out the additional test with the Substance, as specified above.

[3] Speit, G, M. Vasquez, A. Hartmann (2009), The comet assay as an indicator test for germ cell genotoxicity, Mutation Research, Vol. 681/1, pp. 3-12

[4] Zheng, H., P.L. Olive (1997), Influence of oxygen on radiation-induced DNA damage in testicular cells of C3H mice, International Journal of Radiation Biology, Vol. 71/3, pp. 275-282

[5] Cordelli, E. et al. (2003), Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation, Journal of Radiation Research, Vol. 160/4, pp. 443-451

[6] Dirven, Y., Eide, D.M., Henriksson, E.W., Hjorth, R., Sharma, A.K., Graupner, A. et al. (2023) Assessing testicular germ cell DNA damage in the comet assay; introduction of a proof-of-concept. Environmental and Molecular Mutagenesis, 64(2), 88–104. https://doi.org/10.1002/em.22527

References

The following documents may have been cited in the decision.

Guidance on information requirements and chemical safety assessment (Guidance on IRs & CSA)

Chapter R.4 Evaluation of available information; ECHA (2011).
Chapter R.6 QSARs, read-across and grouping; ECHA (2008).
Appendix to Chapter R.6 for nanoforms; ECHA (2019).
Chapter R.7a Endpoint specific guidance, Sections R.7.1 – R.7.7; ECHA (2017).
Appendix to Chapter R.7a for nanomaterials; ECHA (2017).
Chapter R.7b Endpoint specific guidance, Sections R.7.8 – R.7.9; ECHA (2017).
Appendix to Chapter R.7b for nanomaterials; ECHA (2017).
Chapter R.7c Endpoint specific guidance, Sections R.7.10 – R.7.13; ECHA (2017).
Appendix to Chapter R.7a for nanomaterials; ECHA (2017).
Appendix R.7.13-2 Environmental risk assessment for metals and metal compounds; ECHA (2008).
Chapter R.11 PBT/vPvB assessment; ECHA (2017).
Chapter R.16 Environmental exposure assessment; ECHA (2016).

Guidance on data-sharing; ECHA (2017).
Guidance for monomers and polymers; ECHA (2023).
Guidance on intermediates; ECHA (2010).

All guidance documents are available online: https://echa.europa.eu/guidance-documents/guidance-on-reach

Read-across assessment framework (RAAF)

RAAF, 2017 Read-across assessment framework (RAAF); ECHA (2017)
RAAF UVCB, 2017 Read-across assessment framework (RAAF) – considerations on multi- constituent substances and UVCBs); ECHA (2017).

The RAAF and related documents are available online:
https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across

***OECD Guidance documents* (OECD GDs)**

OECD GD 23 Guidance document on aquatic toxicity testing of difficult substances and mixtures; No. 23 in the OECD series on testing and assessment, OECD (2019).
OECD GD 29 Guidance document on transformation/dissolution of metals and metal compounds in aqueous media; No. 29 in the OECD series on testing and assessment, OECD (2002).
OECD GD 150 Revised guidance document 150 on standardised test guidelines for evaluating chemicals for endocrine disruption; No. 150 in the OECD series on testing and assessment, OECD (2018).
OECD GD 151 Guidance document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test; No. 151 in the OECD series on testing and assessment, OECD (2013).

Appendix 2: Procedure

ECHA received your testing proposal(s) on 16 December 2022 and started the testing proposal evaluation in accordance with Article 40(1).

ECHA held a third-party consultation for the testing proposal(s) from 3 May 2023 until 19 June 2023. ECHA did not receive information from third parties.

ECHA followed the procedure detailed in Articles 50 and 51 of REACH.

ECHA notified you of the draft decision and invited you to provide comments.

ECHA took into account your comments and partially amended the request.

The deadline of the decision is set based on standard practice for carrying out OECD TG tests. It has been exceptionally extended by 12 months from the standard deadline granted by ECHA to take into account currently longer lead times in contract research organisations.

ECHA notified the draft decision to the competent authorities of the Member States for proposals for amendment.

As no amendments were proposed, ECHA adopted the decision under Article 51(3) of REACH.

Appendix 3: Addressee(s) of this decision and their corresponding information requirements

In accordance with Articles 10(a) and 12(1) of REACH, the information requirements for individual registrations are defined as follows:

- the information specified in Annex VII to REACH, for registration at 1-10 tonnes per year (tpa), or as a transported isolated intermediate in quantity above 1000 tpa.

Registrant Name	Registration number	Highest REACH Annex applicable to you
[redacted]	[redacted]	[redacted]

Where applicable, the name of a third-party representative (TPR) may be displayed in the list of recipients whereas ECHA will send the decision to the actual registrant.

Appendix 4: Conducting and reporting new tests for REACH purposes

1. Requirements when conducting and reporting new tests for REACH purposes

1.1. Test methods, GLP requirements and reporting

(1) Under Article 13(3) of REACH, all new data generated as a result of this decision must be conducted according to the test methods laid down in a European Commission Regulation or to international test methods recognised by the Commission or ECHA as being appropriate.

(2) Under Article 13(4) of REACH, ecotoxicological and toxicological tests and analyses must be carried out according to the GLP principles (Directive 2004/10/EC) or other international standards recognised by the Commission or ECHA.

(3) Under Article 10(a)(vi) and (vii) of REACH, all new data generated as a result of this decision must be reported as study summaries, or as robust study summaries, if required under Annex I of REACH. See ECHA Practical Guide on How to report robust study summaries[7].

(4) Under the introductory part of Annexes VII/VIII/IX/X to REACH, where a test method offers flexibility in the study design, for example in relation to the choice of dose levels or concentrations, the chosen study design must ensure that the data generated are adequate for hazard identification and risk assessment.

1.2. Test material

(1) Selection of the Test material(s)
The Test Material used to generate the new data must be selected taking into account the following:
- the boundary composition(s) of the Substance,
- the impact of each constituent/ impurity on the test results for the endpoint to be assessed. For example, if a constituent/ impurity of the Substance is known to have an impact on (eco)toxicity, the selected Test Material must contain that constituent/ impurity.

(2) Information on the Test Material needed in the updated dossier
- You must report the composition of the Test Material selected for each study, under the "Test material information" section, for each respective endpoint study record in IUCLID.
- The reported composition must include all constituents of each Test Material and their concentration values and other parameters relevant for the property to be tested.

This information is needed to assess whether the Test Material is relevant for the Substance.

Technical instructions on how to report the above is available in the manual on How to prepare registration and PPORD dossiers[8].

[7] https://echa.europa.eu/practical-guides
[8] https://echa.europa.eu/manuals