~~Confidential~~

Helsinki, 26 August 2021

Addressees
Registrant of JS_30007-47-7 listed in the last Appendix of this decision

Date of submission of the dossier subject of a decision
20/05/2019

Registered substance subject to this decision, hereafter 'the Substance'
Substance name: 5-bromo-5-nitro-1,3-dioxane
EC number: 250-001-7
CAS number: 30007-47-7

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)

Based on Article 40 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below by ***4 September 2023***.

The requested information must be generated using the Substance unless otherwise specified.

A. Information required from the Registrants subject to Annex VIII of REACH

1. *In vitro* cytogenicity study in mammalian cells (test method: OECD TG 473) or *In vitro* micronucleus study (test method: OECD TG 487);

2. *In vivo* genetic toxicity study (Annex VIII, Section 8.4., column 2) to be selected according to the following specifications:

    a. If the results of the *in vitro* test requested under A.1 are **negative**:

    In vivo mammalian alkaline comet assay (test method: OECD TG 489) in rats, oral route, on the following tissues: liver, glandular stomach and duodenum;

    or

    Transgenic rodent somatic and germ cell gene mutation assay (test method: OECD TG 488 from 2020[1]) in transgenic mice or rats, oral route on the following tissues: liver and glandular stomach; duodenum must be harvested and stored for up to 5 years. Duodenum must be analysed if the results of the glandular stomach and of the liver are negative or inconclusive.

    b. If the results of the *in vitro* test requested under A.1 are **positive**:

    In vivo mammalian alkaline comet assay (test method: OECD TG 489) combined with *in vivo* mammalian erythrocyte micronucleus test (test method: OECD TG

[1] The updated OECD TG 488, adopted on 26 June 2020, is available on OECD website at https://www.oecd-ilibrary.org/docserver/9789264203907-en.pdf?expires=1596539942&id=id&accname=guest&checksum=D552783C4CB0FC8045D04C88EFFBFA66.

474) in rats, oral route. For the comet assay the following tissues shall be analysed: liver, glandular stomach and duodenum.

Your originally proposed test, an In vivo mammalian erythrocyte micronucleus test (EU B.12./OECD TG 474), using the Substance is rejected, according to Article 40(3)(d).

The reasons for the request(s) are explained in the following appendix entitled "Reasons to request information required under Annexes VIII of REACH".

Information required depends on your tonnage band

You must provide the information listed above for all REACH Annexes applicable to you, and in accordance with Articles 10(a) and 12(1) of REACH:

- the information specified in Annexes VII and VIII to REACH, for registration at 10-100 tpa;

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 testing and reporting requirements provided under the Appendix entitled "Requirements to fulfil when conducting and reporting new tests for REACH purposes". For references used in this decision, please consult the Appendix entitled "List of references".

Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. An appeal, together with the grounds thereof, has to be submitted to ECHA in writing. An appeal has suspensive effect and is subject to a fee. Further details are described under: http://echa.europa.eu/regulations/appeals.

Approved[2] under the authority of Christel Schilliger-Musset, Director of Hazard Assessment

[2] 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 A: Reasons to request information required under Annex VIII of REACH

This decision is based on the examination of the testing proposals you submitted and on scientific information submitted by third parties.

1. In vitro cytogenicity study in mammalian cells or In vitro micronucleus study

An *in vitro* cytogenicity study in mammalian cells or an *in vitro* micronucleus study is an information requirement under Annex VIII to REACH (Section 8.4.2.).

Further, ECHA guidance R.7a, section R.7.7.6.3 (p.568) specifies that "*In order to ensure the necessary minimum level of information is provided, at least one further test is required in addition to the gene mutation test in bacteria. This should be an in vitro mammalian cell test capable of detecting both structural and numerical chromosome aberrations.*" It is necessary to request an *in vitro* cytogenicity test as an additional test to further investigate the mutagenicity of the substance in accordance with the REACH integrated testing strategy. The obtained *in vitro* data will inform on the genotoxic concern(s) associated with the substance and help identify the most adequate follow-up *in vivo* study.

1.1. Information provided to fulfil the information requirement

You have submitted a testing proposal for an *in vivo* mammalian erythrocyte micronucleus test to be performed with the Substance to further investigate the mutagenicity of the substance.

However, no information from an *in vitro* cytogenicity study or an *in vitro* micronucleus study on the substance in mammalian cells is available in the dossier.

ECHA therefore considers that an appropriate *in vitro* cytogenicity or micronucleus study is necessary to further investigate the mutagenicity of the substance and to help identify the most adequate follow-up *in vivo* study.

For the following reasons ECHA further considers that the data provided in your dossier does not meet the conditions for adaptation of the information.

In the justification provided in your dossier you indicate that an "*in vitro cytogenicity study in mammalian cells or in vitro micronucleus study does not need to be conducted because adequate data from an in vivo cytogenicity test are available*". You provided the following studies with the Substance to support your justification:

i. *In vivo* micronucleus test, in mice, intraperitoneal route ([redacted], 1978), Reliability 2, no test guideline followed and non GLP; and
ii. *In vivo* micronucleus test, in mice, intraperitoneal route ([redacted], 1980), Reliability 2, no test guideline followed and non GLP.

Under Section 8.4.2., Column 2, first indent, Annex VIII to REACH, the study may be omitted "if *adequate data from an in vivo cytogenicity test are available*". ECHA Guidance[3] clarifies that the *in vivo* study must be either a micronucleus test or a chromosomal aberration test, performed according to OECD TG 474 or 475, respectively[4].

[3] ECHA Guidance R.7a, R.7.7.6.3, p.568
[4] ECHA Guidance R.7a, Table R.7.7–3, p.558

For the data from an *in vivo* cytogenicity test to be considered adequate, the *in vivo* study you submitted has to meet the requirements of OECD TG 474, and the specifications/conditions of this test guideline include:

a) The study must include a minimum of three doses/groups of treated animals, as well as a negative control group and a positive control group;
b) Each group must have a minimum of 5 analysable animals (the test can be performed in either sex);
c) The highest dose studied must be the maximum tolerated dose (MTD), i.e. the highest dose that is tolerated without evidence of toxicity (e.g. body weight depression or hematopoietic system cytotoxicity, but not death or evidence of pain, suffering or distress necessitating humane euthanasia). The highest dose can also be a dose that produces toxicity in the bone marrow (e.g. a reduction in the proportion of immature erythrocytes among total erythrocytes in the bone marrow or peripheral blood);
d) The proportion of immature among total (immature + mature) erythrocytes must be determined for each animal (by counting a total of at least 500 erythrocytes for bone marrow and 2000 erythrocytes for peripheral blood); and
e) At least 4000 immature erythrocytes per animal must be scored for the incidence of micronucleated immature erythrocytes.

The reported data for the *in vivo* studies you submitted did not include:

a) the appropriate number of doses;
b) a minimum of 5 animals per group;
c) a maximum studied dose that is a MTD or induces toxicity;
d) the analysis of the adequate number of cells; and
e) data on the proportion of immature erythrocytes among total erythrocytes and the mean number of micronucleated immature erythrocytes for each group of animals.

The information provided does not cover specifications/conditions required by OECD TG 474.

We note that in the dossier under the testing proposal, you indicate yourself that both of these studies i. and ii. above were "*not performed or documented adequately compared to modern methodologies.*"

Based on the above, the requirements of Section 8.4.2., Column 2, first indent, Annex VIII to REACH for an adaptation of the *in vitro* cytogenicity or micronucleus study information are not met.

1.2 Test design

Either the *in vitro* cytogenicity study in mammalian cells (test method OECD TG 473) or the *in vitro* micronucleus study (test method OECD TG 487) are considered suitable.

1.3 Outcome

Under Article 40(3)(c) of REACH, you are requested to carry out the additional test, as indicated above.

2. *In vivo* genetic toxicity study

Under Annex VIII Section 8.4., column 2 of REACH, the performance of an appropriate *in vivo* somatic cell genotoxicity study must be considered if there is a positive result in any of the *in vitro* genotoxicity studies in Annex VII or VIII.

Your dossier contains positive results for the *in vitro* gene mutation study in mammalian cells

which raise the concerns for gene mutations.

2.1 Information provided to fulfil the information requirement

You have submitted a testing proposal for an *in vivo* mammalian erythrocyte micronucleus test to be performed with the Substance.

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.

ECHA received third party information concerning the testing proposal during the third party consultation. This information is addressed below under section 2.2.

ECHA agrees that an appropriate *in vivo* follow up genotoxicity study is necessary to address the concerns identified *in vitro.*

2.2 Test selection

The positive *in vitro* results available in the dossier indicate a concern for gene mutation.

You proposed to perform the *in vivo* mammalian erythrocyte micronucleus test (OECD TG 474). However, ECHA notes that the proposed test is not appropriate to investigate effects on gene mutations *in vivo.*

According to the ECHA Guidance Chapter R.7a, Section R.7.7.6.3, either the transgenic rodent somatic and germ cell gene mutation assay ("TGR assay", OECD TG 488) or the *in vivo* mammalian alkaline comet assay ("comet assay", OECD TG 489) is suitable to follow up a positive *in vitro* result on gene mutation.

However, as also indicated in the third party information, you have not provided any information on chromosomal aberration (*in vitro*) but only a waiver referring to two *in vivo* studies on micronuclei formation in mouse bone marrow. The third party information notes that in the testing proposal you indicate that these existing two *in vivo* studies are not sufficiently reliable. Moreover, the third party notes that for the *in vitro* gene mutation study in mammalian cells available in the dossier, you have not provided information on the colony sizing.

As explained above, under Appendix A, section 1, the adaptation provided to waive the *in vitro* chromosomal aberration study does not meet the requirements of Section 8.4.2., Column 2, first indent, Annex VIII to REACH. Therefore, by this decision, ECHA also requests an *in vitro* chromosomal aberration test (for the reasons see above Appendix A.1.), which may raise a concern for chromosomal aberration in case of positive results.

In case there is also a concern for chromosomal aberration, you must combine the comet assay and the *in vivo* mammalian erythrocyte micronucleus test ("MN test", OECD TG 474) into a single study. The MN test is a mutagenicity test that provides evidence on *in vivo* chromosomal mutagenicity, as the study detects both structural and numerical chromosomal aberrations. The combined study can help reduce the number of tests performed and the number of animals used while addressing both chromosomal aberration and gene mutation.

Therefore, you must wait for the results of the *in vitro* test requested under A.1. and, depending on these results, to conduct either a) Comet assay or TGR, if the test results of request A.1 are negative; or b) Comet assay combined with MN test if the test results of request A.1 are positive. The deadline set in this decision allows for sequential testing.

2.3 Specification of the study design

*b) Comet assay or TGR assay (if the test results of request A.1 are **negative**)*

Comet assay:

According to the test method OECD TG 489, the test must be performed in rats. 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.

In line with the test method OECD TG 489, the test must be performed by analysing tissues from 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.

In case you decide to perform the comet assay, you may consider to collect the male gonadal cells from the seminiferous tubules in addition to the other aforementioned 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.

TGR assay:

According to the test method OECD TG 488, the test must be performed in transgenic mice or rats and the test substance is usually administered orally.

Based on the recent update[5] of OECD TG 488, you are requested to follow the new 28+28d regimen, as it permits the testing of mutations in somatic tissues and as well as in tubule germ cells from the same animals. This updated version provides for a transitional period for the new version. However, ECHA is aware that testing according to the updated OECD TG is already available from CROs and the new study design would provide meaningful germ cell data, so this decision requires the application of the new version.

According to the test method OECD TG 488, the test must be performed by analysing tissues from liver as slowly proliferating tissue and primary site of xenobiotic metabolism, glandular stomach and duodenum as rapidly proliferating tissue and site of direct contact.

[5] The updated OECD TG 488, adopted on 26 June 2020, is available on OECD website at https://www.oecd-ilibrary.org/docserver/9789264203907-en.pdf?expires=1596539942&id=id&accname=guest&checksum=D552783C4CB0FC8045D04C88EFFBFA66

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 mutagenicity at the site of contact in the gastro-intestinal tract. However, duodenum must be stored (at or below −70 ºC) until the analysis of liver and glandular stomach is completed; the duodenum must then be analysed only if the results obtained for the glandular stomach and for the liver are negative or inconclusive.

In case you decide to perform the TGR, you may consider to collect the male germ cells (from the seminiferous tubules) at the same time as the other tissues, in order to limit additional animal testing. According to the OECD 488, the tissues (or tissue homogenates) can be stored under specific conditions and used for DNA isolation for up to 5 years (at or below −70 ºC). This duration is sufficient to allow you or ECHA, to decide on the need for assessment of mutation frequency in the collected germ 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.

*c) Comet assay combined with MN test (if the test results of request A.1 are **positive**)*

According to the test method OECD TG 489, the test must be performed in rats. Therefore, the combined test (OECD TG 489 and OECD TG 474) must be performed in rats. Having considered the anticipated routes of human exposure and the need for adequate exposure of the target tissue(s) performance of the test by the oral route is appropriate.

In line with the test method OECD TG 489, the test must be performed by analysing tissues from 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.

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 [1]).

Germ cells
You may consider to collect the male gonadal cells from the seminiferous tubules in addition to the other aforementioned 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.

Reference:
[1] Bowen DE 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. *Muta Res*;722:7–19.

2.4 Outcome

Your testing proposal is rejected under Article 40(3)(d) of REACH. Under Article 40(3)(c) you are requested to carry out the additional test with the Substance, as specified above.

Appendix B: Requirements to fulfil when conducting and reporting new tests for REACH purposes

A. 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[6].

B. Test material

Before generating new data, you must agree within the joint submission on the chemical composition of the material to be tested (Test material) which must be relevant for all the registrants of the Substance.

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 variation in compositions reported by all members of the joint submission,
   - 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 and whether it is suitable for use by all members of the joint submission.

Technical instructions on how to report the above is available in the manual on How to prepare registration and PPORD dossiers[7].

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

Appendix C: Procedure

ECHA started the testing proposal evaluation in accordance with Article 40(1) on 6 July 2020.

ECHA held a third party consultation for the testing proposal(s) from 19 October 2020 until 3 December 2020. ECHA received information from third parties (see corresponding Appendix).

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 did not receive any comments within the commenting period.

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 D: List of references - ECHA Guidance[8] and other supporting documents

Evaluation of available information
Guidance on information requirements and chemical safety assessment, Chapter R.4 (version 1.1., December 2011), referred to as ECHA Guidance R.4 where relevant.

QSARs, read-across and grouping
Guidance on information requirements and chemical safety assessment, Chapter R.6 (version 1.0, May 2008), referred to as ECHA Guidance R.6 where relevant.

Read-across assessment framework (RAAF, March 2017)[9]

RAAF - considerations on multi-constituent substances and UVCBs (RAAF UVCB, March 2017)[10]

Physical-chemical properties
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Toxicology
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7c (version 3.0, June 2017), referred to as ECHA Guidance R.7c in this decision.

Environmental toxicology and fate
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7b (version 4.0, June 2017), referred to as ECHA Guidance R.7b in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7c (version 3.0, June 2017), referred to as ECHA Guidance R.7c in this decision.

PBT assessment
Guidance on information requirements and chemical safety assessment, Chapter R.11 (version 3.0, June 2017), referred to as ECHA Guidance R.11 in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.16 (version 3.0, February 2016), referred to as ECHA Guidance R.16 in this decision.

Data sharing
Guidance on data-sharing (version 3.1, January 2017), referred to as ECHA Guidance on data sharing in this decision.

[8] https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment
[9] https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across
[10] https://echa.europa.eu/documents/10162/13630/raaf_uvcb_report_en.pdf/3f79684d-07a5-e439-16c3-d2c8da96a316

OECD Guidance documents[11]
Guidance Document on aqueous–phase aquatic toxicity testing of difficult test chemicals – No 23, referred to as OECD GD 23.

Guidance document on transformation/dissolution of metals and metal compounds in aqueous media – No 29, referred to as OECD GD 29.

Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption – No 150, referred to as OECD GD 150.

Guidance Document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test – No 151, referred to as OECD GD 151.

[11] http://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm

Appendix E: Addressees of this decision and the corresponding information requirements applicable to them

You must provide the information requested in this decision for all REACH Annexes applicable to you.

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.