# Annex I to the CLH report

# Proposal for Harmonised Classification and Labelling

**Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2**

# International Chemical Identification:

## *2-[ethyl[3-methyl-4-[(5-nitrothiazol-2-yl)azo]phenyl]amino]ethanol*

**EC Number:** **271-183-4**

**CAS Number:** **68516-81-4**

**Index Number:** **-**

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# 1 HEALTH HAZARDS

## 1.1 Skin sensitisation

### 1.1.1 Animal data

#### 1.1.1.1 LLNA

***Study reference:***

Betts C.J., Dearman R.J., Kimber I., and Maibach H.I. (2005): Potency and risk assessment of a skinsensitizing disperse dye using the local lymph node assay. Contact dermatitis 52 (5), 268-272. DOI: 10.1111/j.0105-1873.2005.00578.x

***Detailed study summary and results:***

Betts and colleagues (Betts et al., 2005) performed an LLNA according to the standard protocol described in (Kimber and Basketter, 1992). C.I. Disperse Blue 106 (DB106), 87 % pure, was supplied by the Ecological and Toxicological Association of Dye and Organic Pigments Manufacturers (ETAD) via Yorkshire Chemicals PLC, Leeds. Dinitrochlorobenzene (DNCB, CAS: 97-00-7), 98.9% pure, was obtained from Sigma Chemicals (Poole, Dorset, UK).

For the studies, young adult male CBA/Ca mice (Harlan, Bicester, Oxfordshire, UK), 8–12 weeks of age, were used. Mice were housed in metal cages with a 12 hours light/dark cycle. The ambient temperature was maintained at 22 ± 3°C and relative humidity was 50 ± 20 %. Food (SDS PCD pelleted diet; Special Diets Services, Witham, UK) and water were available *ad libitum*.
Initially, a standard LLNA was conducted to investigate if DB106 has an inherent skin sensitisation potential. Therefore, (relatively high) concentrations of 1 %, 3 % and 10 % of DB106 formulated in the vehicle dimethyl formamide (DMF; BDH, VWR, Poole, Dorset, UK), incorporating the highest non-toxic concentration achievable in this vehicle, were used. Stimulation indices (SI) are shown in Table 1 (Experiment 1). These data demonstrate clearly that DB106 possesses skin-sensitising activity, with all concentrations of the test substance stimulating vigorous LNC proliferation. The authors suppose that with the used dose range maximal proliferation has been achieved resulting in a lack of a dose–response relationship.
Furthermore, the authors investigated different vehicles and found out that exposure of control mice to the vehicle dimethyl sulfoxide (DMSO) provoked somewhat higher levels of thymidine incorporation than those induced by application of DMF vehicle. However, despite the increase in background thymidine incorporation the authors observed that topical application of DB106 dissolved in DMSO stimulated marked proliferative responses.
For the main LLNA, four mice per dose group were exposed topically on the dorsum of both ears to 25 µl with concentrations of 0.25, 0.05, 0.025, 0.1, 0.01, and 0.005 % of DB106 or to the same volume of the vehicle (DMSO) alone, daily for three consecutive days. The sensitising potency of DNCB (0.01–0.25 % in DMSO) was measured concurrently. "*5 days after the initiation of exposure, all mice were injected intravenously via the tail vein with 20 µCi of [3H] methyl thymidine (3H-TdR; specific activity 2 Ci/mmol;* […] *in 250 µl of phosphate-buffered saline (PBS). 5 hr later, mice were killed, and the draining auricular lymph nodes were excised* [from each mouse ear] *and pooled for each experimental group* (pooled treatment group approach). *A single-cell suspension of* [lymph node cells] *LNCs was prepared by gentle mechanical disaggregation through 200-mesh stainless-steel gauze. Cells* [LNCs] *were washed twice with an excess of PBS and precipitated in 5 % trichloroacetic acid (TCA) at 4 °C. Approximately 12 hr later, pellets were resuspended in 1 ml of 5 % TCA and transferred to 10 ml of scintillation fluid* […]. *Incorporation of* $^{3}$*HTdR was measured by β-scintillation counting as disintegrations per minute (dpm) per node for each experimental group. In each case, a stimulation index (SI) relative to the concurrent vehicle-treated control*

*value was derived."* EC3-values (SI of 3 relative to concurrent vehicle treated controls) were calculated by linear interpolation of dose–response data.

Results for the main study (Experiment 2 and 3) are shown in Table 1.

Table 1: Local lymph node assay responses for DB106 and DNCB

| | **Disperse Blue 106 [dpm/node (SI)]** | | | **DNCB [dpm/node (SI)]†** |
|---|---|---|---|---|
| **Concentration (% w/v)** | **Experiment 1*** | **Experiment 2†** | **Experiment 3†** | |
| 0 | 582 (1) | 924 (1) | 885 (1) | 816 (1) |
| 0.005 | Not done | Not done | 753 (0.9) | Not done |
| 0.01 | Not done | 2352 (2.6) | Not done | 1991 (2.4) |
| 0.025 | Not done | 5031 (5.5) | 4561 (5.2) | 3458 (4.2) |
| 0.05 | Not done | 6073 (6.6) | 8291 (9.4) | 5981 (7.3) |
| 0.1 | Not done | 7590 (8.2) | 8071 (9.1) | 10085 (12.4) |
| 0.25 | Not done | 8483 (9.2) | Not done | 11971 (14.7) |
| 1 | 7889 (13.6) | Not done | Not done | Not done |
| 3 | 9283 (16.0) | Not done | Not done | Not done |

*DMF vehicle
†DMSO vehicle

### 1.1.1.2 Guinea pig maximisation test, slightly modified FCA method

***Study reference:***

Hausen B.M. and Menezes Brandao F. (1986): Disperse blue 106, a strong sensitizer. Contact dermatitis 15 (2), 102-103. DOI: 10.1111/j.1600-0536.1986.tb01294.x

***Detailed study summary and results:***

The sensitisation potential of DB106 was investigated using a slightly modified FCA method (Hausen and Brandão, 1986) according to a protocol published in (Hausen and Schmalle, 1985). DB106 was supplied by an Italian manufacturer as well as by a German chemical company (no further information) and was purified using preparative thin-layer chromatography plates (solvent system ethyl acetate-chloroform (4+1)).
On days 1, 5 , and 9, 10 guinea pigs (Pirbright white strain) were intradermally injected with 6 x 0.1 ml of an emulsion containing the dye dissolved in 3 ml FCA and 3 ml of N. saline, in a semicircular arc in the shoulder area from the left to the right paw (Hausen and Schmalle, 1985). The authors used 9 mg of the pure dye per animal for the whole procedure (This corresponds to 900 mg dye in 3 ml FCA and 3 ml of N. saline for 10 guinea pigs and results in a 1.5 % (w/v) dye emulsion for intradermal induction). Control animals were treated with an emulsion of FCA and equal amounts of N. saline alone. Eleven days after the end of the sensitisation procedure, challenge was performed by topical application of subirritant doses (1 %, 0.3 %, and 0.1 %) of the dye (The threshold of irritation was determined at a concentration of 10 % in acetone). Readings were performed after 24, 48, and 72 hours.
The authors reported that "*reactions obtained on challenge with dilutions of 1 %, 0.3 %, and 0.1 %* [of DB106] *were so strong that no reading could be made because the whole flank of the animals became extremely red and swollen*". One week later, after lesions disappeared, further epicutaneous tests with an additional dilution (0.001 %) were performed on the opposite flank. Results are shown in Table 2, one animal died during the experiment due to other causes.

Table 2: Results for sensitisation due to treatment with DB106 using a slightly modified FCA method (challenge concentration 0.001 %)

| | +++ | ++ | + | (+) | - |
|---|---|---|---|---|---|
| **24 h** | 6 | 3 | - | - | - |
| **48 h** | 7 | 2 | - | - | - |
| **72 h** | 6 | 3 | - | - | - |

### 1.1.1.3 "Biphasic LLNA"

***Study reference:***

Ahuja V., Platzek T., Fink H., Sonnenburg A., and Stahlmann R. (2010): Study of the sensitising potential of various textile dyes using a biphasic murine local lymph node assay. Archives of toxicology 84 (9), 709-718. DOI: 10.1007/s00204-010-0566-0

***Detailed study summary and results:***

Ahuja et al. (2010) conducted an LLNA including a "*biphasic or sensitization-challenge protocol*" to investigate the sensitising potential of DB106. Disperse Blue 106 (CAS no. 68516-81-4) was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. The test solutions were prepared freshly for each application in the vehicle dimethyl sulfoxide. The highest tested concentration was selected based on the solubility in DMSO.

The authors used female BALB/c mice (age: 7 week at the start of the experiment; weight: 18–22 g). Mice were kept in Macrolon® cages at room temperature (23 ± 1°C) and the relative humidity was 45–55 %, with a 12 hours light/dark cycle. Pellet feed and tap water were available *ad libitum*.

For the modified LLNA, at least seven (max. 10) mice were shaved over a surface of approximately 2 $cm^2$ on their backs and treated with 50 µl of test solution once daily for three consecutive days. "*Animals remained untreated on days 4-14. On days 15-17, mice were treated with 25 µl of the test solution on the dorsum of both ears.* Mice were killed on day 19 with *deep CO2 anaesthesia, the lymph nodes prepared and various endpoints analysed. The results were compared to a control group (n = 20) treated with the vehicle alone.*

*The endpoints analysed included ear thickness, ear biopsy weight, lymph node weight, lymph node cellularity and phenotypic determination of lymphocyte subsets. The ear thickness (mm) was measured with a spring-loaded micrometer […]. A section was taken from both ears with a punch of 6 mm diameter and weighed (mg). The draining auricular lymph nodes were excised and weighed (mg). The single cell suspension from a single lymph node was prepared by gentle mechanical disaggregation through stainless steel mesh filter, suspended in 2 ml phosphate-buffered saline […] and counted (million per lymph node) using the automated cell counter Sysmex F 820 […]."* Results for the cell count increase, ear thickness, and ear-punch weight (% of vehicle control) are summarised in Table 3.

For phenotypic determination of lymphocyte subsets, *"cells were stained using fluorochrome-conjugated antibodies. Antibodies used were phycoerythrin (PE)-conjugated rat anti-CD8a, fluorescein isothiocyanate (FITC) and PE-conjugated rat anti-CD4, FITC-conjugated rat antiCD45R/B220, PE-conjugated rat anti-CD19, FITC-conjugated hamster anti-CD69, PE-conjugated rat anti-CD1A […]. CD4 and CD8 are T-cell markers characterising T-helper and T-cytotoxic cells, respectively. CD45R, also known as B220, and CD19 are B-cell markers. The murine MHC-II corresponds to 1A. CD69, also known as "very early antigen", is an indicator of lymphocyte activation. Flow cytometry was done to characterise the activation of T-helper cells (CD4+/CD69+) and B cells (CD45+/1A+). Cell suspension from the lymph node of each animal ($10^6$ cells) was incubated with antibody in the dark at 4°C for 30 min and then washed twice in PBS and 0.02% sodium azide […]. Flow cytometry was performed with a FACScan flow cytometer […]. 1 x $10^4$ lymphocytes were counted per sample. Data were analysed using Winlist 5.0 software […].*

*Mean values were calculated for the lymph node cellularity, lymph node weight and lymphocyte sub-population of each animal. Results from the treated group were compared to those obtained from vehicle-treated control animals using one-way ANOVA followed by post hoc Dunnett test. Statistical analysis was done with the software SPSS 10.0 […]."*

Analysis show a significant decrease in CD4+ and CD8+ cells and an increase in CD19+, CD45+, CD45+/1A+, and CD4+/CD69+ cells after treatment with DB106, compared to vehicle control (Table 4).

Table 3: Lymph node cell-count increase, ear thickness, and ear-punch weight measurement (% of vehicle control) of mice treated with DB106 in a biphasic LLNA

| **Dye** | **Cell-count increase** | | | | | | **Ear thickness** | | | | | | **Ear-punch weight** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Concentration (%)** | | | | | | | | | | | | | | | | | |
| | **30** | **10** | **3.0** | **0.3** | **0.03** | **0.003** | **30** | **10** | **3.0** | **0.3** | **0.03** | **0.003** | **30** | **10** | **3.0** | **0.3** | **0.03** | **0.003** |
| DB106 | 174 | n. d. | 124 | 82 | 79 | 37 | 26 | n. d. | 13 | 17 | 9 | - | 22 | n. d. | 15 | 17 | 12 | 4* |

- Concentrations not tested in LLNA

* No significant increase at $p<0.05$ (t-test) between vehicle control and treated animals

Table 4: Phenotypic analysis (% positive cell population, mean ± SD) of the different epitope markers on lymphocytes obtained from lymph nodes of mice treated with DB106 in a biphasic LLNA

| | **CD4+** | **CD8+** | **CD19+** | **CD45+** | **CD45+/1A+** | **CD4+/CD69+** |
|---|---|---|---|---|---|---|
| **DB106 30%** | 39.6 ± 3.3* | 17.4 ± 2.4* | 26.8 ± 3.5* | 36.7 ± 3.6* | 32.5 ± 5.1* | 14.6 ± 2.3* |
| **DB106 3%** | 38.4 ± 3.5* | 16.6 ± 3.6* | 39.0 ± 3.0* | 40.4 ± 2.5* | 40.7 ± 8.7* | 14.0 ± 1.5* |
| **DB106 0.3%** | 38.5 ± 3.7* | 19.0 ± 2.2* | 36.4 ± 5.0* | 34.0 ± 4.4 | 31.1 ± 4.1* | 12.1 ± 1.1* |
| **DB106 0.03%** | 36.0 ± 1.6* | 16.3 ± 1.6* | 41.1 ± 2.6* | 38.3 ± 3.4* | 34.9 ± 2.8* | 9.8 ± 0.78 |
| **DB106 0.003%** | 40.4 ± 1.2* | 15.1 ± 1.3* | 34.1 ± 2.1* | 31.3 ± 2.0 | 31.3 ± 1.6* | 9.9 ± 0.82 |
| **Vehicle control** | 47.7 ± 4.3 | 20.8 ± 2.1 | 20.1 ± 2.5 | 31.4 ± 4.0 | 21.4 ± 3.4 | 10.3 ± 1.3 |

* Significant difference at $P < 0.05$ (one-way ANOVA followed by post hoc Dunnett test) between vehicle control and treated mice

## 2 REFERENCES


Ahuja V., Platzek T., Fink H., Sonnenburg A., and Stahlmann R. (2010): Study of the sensitising potential of various textile dyes using a biphasic murine local lymph node assay. Archives of toxicology 84 (9), 709-718. DOI: 10.1007/s00204-010-0566-0

Betts C.J., Dearman R.J., Kimber I., and Maibach H.I. (2005): Potency and risk assessment of a skin-sensitizing disperse dye using the local lymph node assay. Contact Dermatitis 52 (5), 268-272. DOI: 10.1111/j.0105-1873.2005.00578.x

Hausen B.M. and Brandão F.M. (1986): Disperse Blue 106, a strong sensitizer. Contact Dermatitis 15 (2), 102-103. DOI: 10.1111/j.1600-0536.1986.tb01294.x

Hausen B.M. and Schmalle H.W. (1985): Structure-activity aspects of 4 allergenic sesquiterpene lactones lacking the exocyclic alpha-methylene at the lactone ring. Contact Dermatitis 13 (5), 329-332. DOI: 10.1111/j.1600-0536.1985.tb02588.x

Kimber I. and Basketter D.A. (1992): The murine local lymph node assay: a commentary on collaborative studies and new directions. Food Chem Toxicol 30 (2), 165-169. DOI: 10.1016/0278-6915(92)90153-C