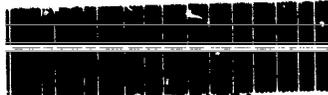


CODING FORMS FOR SRC INDEXING

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New Doc ID	FYI-OTS-0794-1081	Old Doc ID	
Date Produced	12/06/88	Date Received	07/26/94
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Submitting Organisation		BASF CORP	
Contractor			
Document Title		INITIAL SUBMISSION: LETTER FROM BASF CORP TO USEPA REGARDING ENCLOSED INFORMATION ON MORPHOLINE WITH ATTACHMENTS, DATED 12/06/88	
Chemical Category		MORPHOLINE	

BASF Corporation
Chemicals Division



INIT 07/26/94

BASF

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RECEIVED
FBI

FYT-0794-001081

December 6, 1988



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Contains No CBI

Ms. Roberta Wedge
Dynamac Corporation
The Dynamac Building
11140 Rockville Pike
Rockville, MD 20852

Dear Ms. Wedge:

Subject: Morpholine - Your request of September 19, 1988

In response to your request, BASF is submitting the following information on morpholine.

Production Information

Morpholine is manufactured at our production plant located in Geismar, Louisiana and is imported from BASF Aktiengesellschaft in West Germany.

It is produced by reacting diethylene glycol with ammonia. We estimate the U. S. morpholine market to be 18 M lbs./year. BASF's share is approximately 30% of the U. S. market.

Use Information

Major morpholine outlets are in the formulating of neutralizing agents for boiler water treatment chemicals and in the manufacturing of rubber chemicals. Secondary outlets include formulation of waxes and polishes as well as active ingredients (such as pharmaceuticals, biocides, etc.)

The following are attached:

1. The BASF Corporation Technical Bulletin for Morpholine (Attachment 1)
2. The BASF Corporation Material Safety Data Sheet
3. The BASF United Kingdom Ltd. Material Safety Data Sheet

0 0 0 3

Page 2
Ms. Roberta Wedge
Dynamac Corporation

Unpublished Toxicity Studies:

Results of active toxicity and ecotoxicity studies are cited in the above MSD sheets. In addition, BASF is submitting a study titled Evaluation of Morpholine in the In-Vitro Transformation of BALB/3T3 Cells With and Without Metabolic Activation. (Attachment 4)

BASF does not claim any of the above information confidential at this time. Please keep me advised of the status of the ITC review. If there are any further questions, please contact me.

Very truly yours,

BASF CORPORATION
Chemicals Division

Mary A. Klosowski

Mary A. Klosowski
Manager, Government Regulations

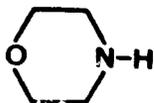
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Morpholine

Technical Bulletin

July 1987



CAS Reg. no.: 110-91-8
Molecular weight: 87.1 g/mol
Product no.: 664331

Appearance	colorless, hygroscopic liquid, free of suspended matter
-------------------------	---

Specification

Assay	99% min. (GC)
Impurities	ethylenediamine, 4-ethylmorpholine, water

Physical properties

Solubility	miscible with water and almost all common organic solvents, slightly soluble in aliphatic hydrocarbons
Boiling range	128-130°C
Freezing point	-5°C
Density d_{20}	1.000-1.002 g/cm ³
Weight/gal.	8.34 lbs
Flash point	31°C (closed cup)

Suggested applications

This chemical intermediate can be used in the production of pharmaceuticals, agricultural chemicals, dye intermediates, textile chemicals, paper chemicals, rubber chemicals, corrosion inhibitors, optical brighteners, emulsifiers, plasticizers, curing agents for epoxy resin, photographic chemicals and catalysts for polyurethane resins.

Important: While the information and data contained in this bulletin are presented in good faith and believed to be reliable, they do not constitute a part of our terms and conditions of sales unless specifically incorporated in our Order Acknowledgement. NOTHING HEREIN SHALL BE DEEMED TO CONSTITUTE A WARRANTY, EXPRESS OR IMPLIED, THAT SAID INFORMATION OR DATA ARE CORRECT OR THAT THE PRODUCTS DESCRIBED ARE MERCHANTABLE OR FIT FOR A PARTICULAR PURPOSE, OR THAT SAID INFORMATION, DATA OR PRODUCTS CAN BE USED WITHOUT INFRINGING PATENTS OF THIRD PARTIES.

Shipping

Available in tank trucks and non-returnable steel drums containing 450 lbs. net weight. DOT label required: Flammable Liquid.
Proper shipping name: Morpholine—UN 2054

Handling hazards

Morpholine is a flammable liquid that is corrosive to the skin and eyes. Inhalation of vapors irritates the respiratory tract. Refer to our Material Safety Data Sheet for information on toxicity, safety precautions, handling and disposal.

BASF Corporation
Chemicals Division
100 Cherry Hill Road
Parsippany, N.J. 07054
(201) 263-3400
800-526-1072

Intermediates & Fine Chemicals

BASF

DATA SHEET

PRODUCT NUMBER: 084331 Morpholine

SECTION I - IDENTIFICATION Registered Trademarks

TRADE NAME: Morpholine

CHEMICAL NAME: Morpholine

SYNONYMS: Tetrahydro-1,4-oxazine,
Diethylenimide Oxide

FORMULA: C₄H₈NO

CHEMICAL FAMILY: Secondary Alkyl Amine

MOL. WGT.: 87.1

SECTION II - INGREDIENTS

COMPONENT	CAS NO.	%	PEL/TLV - SOURCE
Morpholine	110-91-8	99	20 ppm Skin OSHA, ACGIH, 1982
SARA Title III Sect. 313: Not listed.			

SECTION III - PHYSICAL DATA

BOILING/MELTING POINT @ 760 mm Hg: 120°C/ -5°C	pH: 10.1 (5 g/l water)
VAPOR PRESSURE mm Hg @ 20°C: 13	Vapor Density: 3.0
SPECIFIC GRAVITY OR REL. DENSITY: 1.000	
SOLUBILITY IN WATER: Complete	
APPEARANCE: Hygroscopic Liquid	ODOR: Amine
	INTENSITY: N/A

SECTION IV - FIRE AND EXPLOSION HAZARD DATA

FLASH POINT (TEST METHOD): 31°C DIN 51755	AUTOIGNITION TEMP: 275°C/
FLAMMABILITY LIMITS IN AIR (% BY VOL)	LOWER: 1.8 UPPER: 10.8
EXTINGUISHING MEDIUM	Use water, alcohol foam, CO ₂ or dry chemical extinguishing media.
SPECIAL FIREFIGHTING PROCEDURES	Firefighters should be equipped with self-contained breathing apparatus and turnout gear. Water may be ineffective except to cool and dilute vapors.
UNUSUAL FIRE AND EXPLOSION HAZARDS	Dangerous, when exposed to heat or flames. Can react with oxidizing materials. ASTM D2155

EMERGENCY TELEPHONE NUMBER

CHENTREC 800-424-9300

201-316-3000

THIS NUMBER IS AVAILABLE DAYS, NIGHTS, WEEKENDS AND HOLIDAYS

0006

PRODUCT NUMBER: 004331 Morpholine

SECTION V - HEALTH DATA

TOXICOLOGICAL TEST DATA:

Morpholine
Rat, Oral LD50
Mouse, Inhalation LC50
Rabbit, Dermal LD50
Rabbit, Skin
Rabbit, Eyes

RESULT:

1050 mg/kg.
1320 mg/m3
500 mg/kg.
Severely irritating
Severely irritating

EFFECTS OF OVEREXPOSURE:

Morpholine is severely irritating to the eyes and skin. Overexposure to the vapors may cause respiratory irritation and transient eye irritation. Temporary foggy vision has been reported. Morpholine caused lacrimation, salivation and tremors in rats at lethal oral doses and in rabbits at lethal dermal doses. At sublethal levels, effects included lacrimation, irritation and inactivity. Repeated skin application has caused skin, liver and kidney injury in rabbits. Rats and guinea pigs exposed to 18000 ppm in air exhibited irritation of the eyes and respiratory tract. Repeated exposures caused lung, liver and kidney injury. In an industry-sponsored lifetime inhalation study, morpholine was not carcinogenic to rats at concentrations of 10, 50 and 150 ppm. Morpholine did cause ophthalmic and nasal lesions consistent with its irritating properties.

FIRST AID PROCEDURES:

Eyes-Immediately wash eyes with running water for 15 minutes. Get immediate medical attention.
Skin-Wash affected areas with water while removing contaminated clothing. Get immediate medical attention. Launder contaminated clothing before reuse.
Ingestion-If swallowed, DO NOT INDUCE VOMITING. Dilute with water or milk and get immediate medical attention. Never give fluids or induce vomiting if the victim is unconscious or having convulsions.
Inhalation-Move to fresh air. Aid in breathing, if necessary, and get immediate medical attention. ry, and get medical attention.

SECTION VI - REACTIVITY DATA

STABILITY: Stable.

CONDITIONS TO AVOID: Heat and flames.

CHEMICAL INCOMPATIBILITY: Oxidizing contaminants and mineral acids.

HAZARDOUS DECOMPOSITION PRODUCTS: CO CO2 and NOx.

HAZARDOUS POLYMERIZATION: Does not occur

CONDITIONS TO AVOID: N/A

CORROSIVE TO METAL: No

OXIDIZER: No

SECTION VII - SPECIAL PROTECTION

RESPIRATORY PROTECTION: Approved organic vapor respirator.

EYE PROTECTION: Goggles, face shield.

PROTECTIVE CLOTHING: Gloves, coveralls, apron, boots as necessary to prevent skin contact.

VENTILATION: Use local exhaust to control to P.E.L.

OTHER: Eyewash fountains and safety showers should be easily accessible.

PRODUCT NUMBER: 094331 Morpholine

SECTION VIII - ENVIRONMENTAL DATA

ENVIRONMENTAL TOXICITY DATA:

Aquatic toxicity rating, TLMS: 100-1000 ppm.
Minnow, 48 hour LC50: >800 mg/l.

SPILL AND LEAK PROCEDURES:

Spills should be contained, solidified, and placed in suitable containers for disposal in a RCRA licensed facility. This material is RCRA hazardous due to its properties.

HAZARDOUS SUBSTANCE SUPERFUND: No RQ (lbs):

WASTE DISPOSAL METHOD:

Incinerate in a RCRA licensed facility. Do not discharge into waterways or sewer systems without proper authority.

HAZARDOUS WASTE 40CFR261: Yes HAZARDOUS WASTE NUMBER: D 001

CONTAINER DISPOSAL:

Empty containers with less than 1 inch of residue may be landfilled at a licensed facility. Recommend crushing or other means to prevent unauthorized reuse. Other containers must be disposed of in a RCRA licensed facility.

SECTION IX - SHIPPING DATA

D.O.T. PROPER SHIPPING NAME (49CFR172.101-102) Morpholine		HAZARDOUS SUBSTANCE (49CFR CERCLA LIST) No
		REPORTABLE QUANTITY (RQ) N/A
D.O.T. HAZARD CLASSIFICATION (CFR172.101-102) PRIMARY Flammable Liquid		SECONDARY
D.O.T. LABELS REQUIRED (49CFR172.101-102) Flammable Liquid	D.O.T. PLACARDS REQUIRED (CFR172.504) Bulk Flammable 2084	POISON CONSTITUENT (49CFR172.203(K))
BILL OF LADING DESCRIPTION Morpholine--Flammable Liquid--UN 2084		
CC NO. 270	UN/NA CODE: 2084	

DATE PREPARED: 4 / 11 / 88

UPDATED: 8 / 15 / 88

WHILE BASF CORPORATION BELIEVES THE DATA SET FORTH HEREIN ARE ACCURATE AS OF THE DATE HEREOF, BASF CORPORATION MAKES NO WARRANTY WITH RESPECT THERETO AND EXPRESSLY DISCLAIMS ALL LIABILITY FOR RELIANCE THEREDN. SUCH DATA ARE OFFERED SOLELY FOR YOUR CONSID RATION, INVESTIGATION, AND VERIFICATION.

PRODUCT NUMBER: 884331 Morpholine

SECTION X - PRODUCT LABEL

Morpholine

DANGER: FLAMMABLE
CONTACT WITH THE EYES AND SKIN RESULTS IN SEVERE IRRITATION.
OVEREXPOSURE TO THE VAPORS MAY CAUSE RESPIRATORY IRRITATION, TRANSIENT EYE IRRITATION AND TEMPORARY BLURRED VISION.
REPEATED EXPOSURES CAUSED SKIN, LUNG, LIVER AND KIDNEY INJURY IN LABORATORY ANIMAL STUDIES. OTHER EFFECTS INCLUDED LACRIMATION, SALIVATION, TREMORS AND INACTIVITY.

Use with local exhaust to control to P.E.L. Wear an approved organic vapor respirator, goggles, face shield, gloves, coveralls, apron, boots and other protective clothing as necessary to prevent contact. Eyewash fountains and safety showers must be easily accessible.

FIRST AID:

Eyes-Immediately wash eyes with running water for 15 minutes.

Get immediate medical attention.

Skin-Wash affected areas with water while removing contaminated clothing. Get immediate medical attention. Launder contaminated clothing before reuse.

Ingestion-If swallowed, DO NOT INDUCE VOMITING. Dilute with water or milk and get immediate medical attention. Never give fluids or induce vomiting if the victim is unconscious or having convulsions.

Inhalation-Move to fresh air. Aid in breathing, if necessary, and get immediate medical attention.

IN CASE OF SPILLS OR LEAKS: Material is a RCRA-regulated product. Spills should be contained, absorbed and placed in suitable containers for disposal in a RCRA-licensed facility.

STORAGE AND HANDLING: Avoid contact with heat, sparks and open flame. Can react with oxidizing materials.

IN CASE OF FIRE: Use water fog, alcohol foam, CO2 or dry chemical extinguishing media. Firefighters should be equipped with self-contained breathing apparatus and turnout gear.

EMPTY CONTAINERS: All labeled precautions must be observed when handling, storing and transporting empty containers due to product residues. Do not reuse this container unless it is professionally cleaned and reconditioned.

DISPOSAL: Spilled material, unused contents and empty containers must be disposed of in accordance with local, state and federal regulations. Refer to our Material Safety Data Sheet for specific disposal instructions.

IN CASE OF CHEMICAL EMERGENCY: Call CHEMTREC day or night for assistance and information concerning spilled material, fire, exposure and other chemical accidents. 800-424-9300

ATTENTION: This product is sold solely for use by industrial institutions.

Refer to our Technical Bulletin and Material Safety Data Sheet regarding safety, usage, applications, hazards, procedures and disposal of this product. Consult your supervisor for additional information.

CAS No.: 110-81-8

Proper Shipping Name: Morpholine - UN 2054

Made in West Germany

Intermediates and Fine Chemicals

0887

PRODUCT SAFETY DATA SHEET

MSF United Kingdom Limited

MSF AS ref: C 20 20 **MSF/37**
Translation ref: 22/22/2 **Date: 21.2.86**
Revised 14 3 88



PRODUCT NAME: Morpholine

1.1 Chemical nature:

Morpholine
CAS No 110-91-8

1.2 Form: Liquid **1.3 Colour: Colourless** **1.4 Odour: Typical amine**

2 PHYSICAL DATA AND SAFETY DATA **Tested in accordance with:**

2.1 Change in physical state:

Freezing Range : -3°C (BS 523:1964)
Boiling Range (5-95ml): 128-130°C (DIN 51 751)

2.2 Density: (20°C) 1.0 g/cm³ (DIN 51 757)
Bulk density: kg/m³

2.3 Vapour pressure: (20°C) 10 mbar
(50°C) 60 mbar

2.4 Viscosity: (20°C) 2.3 mPa.s

2.5 Solubility in water: (°C) Miscible g/l
in (°C) g/l

2.6 pH (at 5 g/l H₂O): (20 °C) 10.1

2.7 Flash point: 51°C (DIN 51 755)

2.8 Ignition temperature: 275°C (DIN 51 794)

2.9 Explosive limits: Lower: 1.4% Upper: 13.0% v/v

2.10 Thermal decomposition: No decomposition below 330°C

2.11 Hazardous decomposition products:

2.12 Hazardous reactions: Exothermic reaction with acids

2.13 Additional information:

PRODUCT NAME: Morpholine

3. TRANSPORT **IMDG-Code:** 3.3 **UN-No:** 2.5A **ICAO/IATA-DCR:** 2054

RID/ADR: 3.31C

Other Information: May not be transmitted by post. MFAG 322, EMS 3-02

4. REGULATIONS

4.1 Information on labelling:

The product is included in the EEC list of dangerous substances (EEC Directive 76/907/EEC including the latest amendment, equivalent to the UK Classification Packaging and Labelling Regulations 1984, SI 1244) and must be labelled accordingly.

EC No. : 613-028-00-9
Hazard Symbol : Corrosive
Risk Phrases : 10-20/21/22-34
Safety Phrases: 23-26

Characterisation in relation to non perception : Safety Phrases: 23-36-26
The product is listed in Part 1A of the Approved List for the UK Classification Packaging and Labelling Regulations 1984 (SI 1244) for conveyance as a flammable liquid.

UK Emergency Action Code (Hazchem): 2F

4.2 Other Information:

The normal precautions for the handling of chemicals must be observed

Occupational Exposure Limit (OEL) : 20 ppm 70 mg/m³ long term
30 ppm 105 mg/m³ short term 1985

5 PROTECTIVE MEASURES, STORAGE AND HANDLING

5.1 Technical protective measures:

5.2 Personal protective equipment:	Respiratory protection:	Eye protection: Yes
	Hand protection: Yes	Other:

5.3 Industrial hygiene:

5.4 Protection against fire and explosion:

5.5 Disposal:

Incineration

Dilute aqueous solution can be treated in biological effluent treatment plants

PRODUCT NAME: Morpholine

6 MEASURES IN CASE OF ACCIDENTS AND FIRES

6.1 After spillage/leakage/gas leakage:

Soak up with absorbent material (e.g. sand, kieselguhr)
Wash away small quantities with plenty of water

6.2 Extinguishing media: Suitable: water spray (fog) CO₂
 foam Dry powder

Not to be used:

6.3 First Aid:

Remove contaminated clothing immediately
Wash affected skin with running water or better with vinegar or 5% acetic acid. Superior is Polyethylene Glycol 400 (*Lutrol E400). Avoid: Alcohol application.
Wash affected eyes for several minutes with running water with eyelids held open. Consult eye specialist.
After inhalation of vapour, remove patient to fresh air, artificial respiration keep warm, keep quiet, keep respiratory passages free.
In cases of loss of consciousness, position and keep patient in recovery position

6.4 Further Information:

7 Toxicity

Experience on Humans: Vapour/mist irritate the eyes, respiratory organs and skin.

LD50 oral (rat) : 1910 mg/kg

LD50 dermal (rabbit) : ca 500 mg/kg

LC50 inhalation (rat) : >42 mg/l/8 hr

Primary skin irritation (rabbit, BASF test): Corrosive.

Primary mucous membrane irritation (rabbit eye, BASF test): Corrosive.

Acute inhalation hazard (rat; test results dependent on toxicity and

volatility: No deaths occurred after 1 hour exposure to an atmosphere saturated with the substance at 20°C. Deaths occurred on longer exposures.

Special Properties: Danger of skin resorption. Continued resorption causes liver and kidney damage. With nitrites or nitrous acid, nitrosamines may form under specific conditions studies have shown that nitrosamines are carcinogenic in animal studies.

PRODUCT NAME: Morpholine

8 Ecology

Product should not be allowed to enter water courses without prior treatment

Fish Toxicity: Goldorfen test LC 50 >500 mg/l/48 hr

Degradability in water: Static test TOC >60%, readily degradable mainly by biological processes.

By careful addition to adapted biological effluent treatment plants, no adverse effects on the degradative activity of the activated sludge are expected.

WGK (German Water Hazard Classification): 2

Confidential

GENETICS ASSAY NO. 5797
LBI SAFETY NO. 7616

EVALUATION OF
MORPHOLINE
IN THE
IN VITRO TRANSFORMATION
OF BALB/3T3 CELLS WITH
AND WITHOUT METABOLIC
ACTIVATION ASSAY
801490

FINAL REPORT

SUBMITTED TO:

BASF AKTIENGESELLSCHAFT
BRUNCKSTRASSE 80
D-6700 LUDWIGSHAFEN
WEST GERMANY

SUBMITTED BY:

LITTON BIONETICS, INC.
5516 NICHOLSON LANE
KENSINGTON, MARYLAND 20895

LBI PROJECT NO. 21002

REPORT DATE: JULY, 1982

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proprietor's explicit permission.

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BIONETICS

PREFACE

This report contains a summary of the data compiled during the evaluation of the test compound. The report is organized to present the results in a concise and easily interpretable manner. The first part contains Items I-IX. Items I-IV provide sponsor and test article identification information, type of assay, and the protocol reference number. Item V provides the initiation and completion dates of the study. Item VI identifies the supervisory personnel. Item VII indicates the tables and/or figures containing the test results. The interpretation of the results is in Item VIII. Item IX provides the conclusion and evaluation.

The second part of the report describes the study design, which includes the materials and procedures employed in conducting the assay. This part of the report also contains evaluation criteria used by the study director, and any appendices.

All test and control results presented in this report are supported by raw data which are permanently maintained in the files of the Department of Molecular Toxicology or in the archives of Litton Bionetics, Inc., 5516 Nicholson Lane, Kensington, Maryland 20895.

Copies of the raw data will be supplied to the sponsor upon request.

The described study was performed in accordance with Good Laboratory Practice regulations except if noted to the contrary. To the best of the signer's knowledge there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study.



BIONETICS

- I. SPONSOR: BASF Aktiengesellschaft
- II. MATERIAL (TEST COMPOUND): GENETICS ASSAY NUMBER: 5797
- A. Identification: Morpholine
- B. Data Received: June 22, 1981
- C. Physical Description: Clear, colorless liquid
- III. TYPE OF ASSAY: In Vitro Transformation of Balb/3T3 Cells
With and Without Metabolic Activation
- IV. ASSAY DESIGN NUMBER: 482
- V. STUDY DATES:
- A. Initiation: June 15, 1981
- B. Completion: March 26, 1982
- VI. SUPERVISOR/ PERSONNEL:
- A. Study Director: John O. Rundell, Ph.D.
- B. Laboratory Supervisor: Edwin J. Matthews, Ph.D.
- VII. RESULTS:
- The results of the assay are presented in Tables 1 through 8 on pages 6 through 13.
- VIII. INTERPRETATION OF RESULTS:

The test material, morpholine, was soluble in culture medium at the tested concentration of 40.0 μ l/ml. An alkaline pH shift was observed at this concentration and therefore the stock solution was neutralized by addition of a volume of 5N HCl before dilutions were performed. After pH adjustment, a series of 18 test material dilutions, ranging from 8.0 μ l/ml to 0.061 nl/ml in two-fold dilution steps, was prepared for use in the preliminary cytotoxicity tests. Two clonal survival assays (preliminary cytotoxicity tests) were performed; one assay was conducted using Balb/c-3T3 cells alone and another was performed under activation conditions using Balb/c-3T3 - rat liver cell co-cultures. The 3T3 target cell survivals derived from these experiments were then used to select test material concentrations for use in the non-activation and activation transformation assays, respectively. The results of the cytotoxicity tests are shown in tables 1 and 2. Two independent non-activation cytotoxicity tests were performed. In the absence of rat

VIII. INTERPRETATION OF RESULTS: (Continued)

liver cells (Table 1), the relative survivals ranged between approximately 35.2% at 1000.0 nl/ml and near 90-118% over the 500.0 to 0.061 nl/ml range for Trial I. No survivors were observed at test material concentrations of 2000 nl/ml and higher. For Trial II (Table 1) the relative survivals ranged from 0.63% at 1000.0 nl/ml to nearly 90% to 132% over the 250.0 to 0.061 nl/ml range. As was observed for Trial I, no survivors were obtained at the dose of 2000.0 nl/ml and higher. As shown in Table 2, treatments of 3T3 cells in the presence of rat liver cells did not result in a significant shift in apparent test material cytotoxicity. Thus, the relative survivals ranged between 0.4% at 1000.0 nl/ml and near 85 to 117% for treatments with 125.0 to 0.061 nl/ml. No 3T3 cell survivors were observed for test material treatments of 2000.0 nl/ml and higher in the presence of rat liver cells. The activation and non-activation transformation assays are normally conducted using test material concentrations that result in between 10-20% and 100% survival and are considered to be most sensitive near 20% survival, since maximal transformation frequencies for a series of model compounds were obtained for treatments that caused survivals between 10% and 25%. Therefore, test material concentrations between 35.0 nl/ml and 1400 nl/ml and 15.0 nl/ml and 600.0 nl/ml (Trial II) and 17.5 nl/ml and 700 nl/ml corresponding to a survival range of approximately 100% to 20% (estimated graphically, see Tables 1 and 2), were selected for application in the two nonactivation trials and in the activation assay, respectively.

The results of the non-activation transformation assay trials are shown in Tables 3, 4, 5 and 6.

The negative control measures the frequency of the appearance of spontaneously transformed foci in the current assay. As shown in Table 3 (Trial I), one transformed focus was observed among the 16 negative control dishes. The absolute negative control frequency of 0.07 focus/dish was within the expected range of zero to 0.5 focus/dish. In contrast, a total of 87 transformed foci was observed among the 18 MCA-treated positive control dishes for an average frequency of 4.83 foci/dish (absolute). While the data shown in Table 3 do not contain any unusual values, dishes with large numbers of transformed foci (>10) have been observed in other experiments (see Trial II, Table 5). The appearance of large numbers of foci in a single dish, compared to other dishes in a set, is considered to be caused by the respreading of one or a small number of foci by mechanical disruption during the refeeding process that occurs twice weekly for the incubation period. The assumption that the appearance of dishes with high numbers of foci results from a technical rather than a biological cause is supported

VIII. INTERPRETATION OF RESULTS: (Continued)

by the finding that such dishes appear to occur randomly in all experimental data, in treated dishes, as well as in negative controls. If such data are included in this analysis of experiments, a marked skewing of the average frequency of foci occurs that is inappropriate for application of parametric tests for statistical significance. Analyses of the distribution patterns of the transformation assay data showed that the data closely fit a log-normal curve. Conversion of the raw data to the \log_{10} results in a normal distribution and the presence of a few dishes with high numbers of foci does not disturb this relationship. Bailey's modification of Student's t-test can then be used to avoid the assumptions (intrinsic in many parametric tests of significance) of equal variances and equal numbers of entries in the treatment and control sets. The results of these analyses of the present transformation assay data are shown in Table 4. Statistical analysis of the transformation assay data (see Tables 3 and 4) show that concentrations of test material from 35.0 to 1400.0 nl/ml were non-transforming to 3T3 cells in the absence of rat liver cell activation.

These conclusions, regarding the absence of test material transforming activity under non-activation conditions, were confirmed by an independent test. The results of this assay trial are shown in Tables 5 and 6. In this assay, a total of 3 transformed foci was observed among 37 negative control dishes for an average frequency of 0.08 focus/dish. In contrast, treatments with 5.0 $\mu\text{g/ml}$ MCA resulted in the appearance of a total of 117 foci for an absolute mean of 6.16 foci/dish. These negative and positive control results were similar to those observed for Trial I in that the spontaneous frequencies for the two assay trials were similar and the positive control frequencies attained the 95% confidence level of being significantly altered. As was observed for Trial I, none of the treatments with the test material resulted in a significant increase in the frequency of transformed foci (see Tables 5 and 6). Accordingly, in this trial, concentrations of test material from 15.0 to 600.0 nl/ml were found to be non-transforming to 3T3 cells.

Three activation assay trials were conducted; Trial I was completed on September 1, 1981, but was rejected for analysis because of failure of the assay to resolve the positive control treatment. A second activation assay trial was initiated on February 1, 1982, but could not be completed because of culture contamination. The basis for the failure of the first activated assay to resolve the positive control treatments was the appearance of significant areas of cellular hyperplasia in the negative control as well as among the several treatment groups. These hyperplastic areas were found to be the result of the proliferation of fibroblast-like cells contaminating the rat liver cell preparation at the level of approximately 1% of the plated cells.

VIII. INTERPRETATION OF RESULTS (Continued)

This rat liver cell contaminant-mediated hyperplasia resulted in the introduction of uncertainty in assay scoring, since their morphologies were similar to those observed for foci of transformed 3T3 cells. The third activation assay trial was, therefore, conducted using a modified protocol. The principle provisions of this protocol compared to those described in edition 3 of Protocol 482 include lethal x-irradiation of the rat liver cell population and the conduct of culture treatments without replating. The exact methodologies employed in Trial III are described in the accompanying Assay Design section.

The results of the third activation assay trial are shown in Table 7.

As previously described, the negative control measures the frequency of the appearance of spontaneously transformed foci. In this assay, one transformed focus was observed among the 19 negative control dishes. In contrast, the positive control treatment with 10.0 µg/ml cyclophosphamide induced a total of 7 foci among 20 dishes (Table 7). Log₁₀ analysis of these data (Tables 7 and 8) showed that the positive control frequency of 0.26 focus/dish was highly significant (p<.05) compared to the negative control and therefore, the sensitivity of this assay was normal.

The effect of test material treatments on 3T3 cell transformation under condition of rat liver cell co-cultivation is also shown in Tables 7 and 8. After log₁₀ analysis, the average number of foci/dish ranged from 0 at 17.5, 350.0 and 700.0 n1/ml to 0.12 at 175.0 n1/ml. Compared to the negative control, none of the frequencies of transformed foci observed for the test material treatment achieved the 95% confidence level of being significantly altered (Table 8). In addition, in agreement with the nonactivation results, no evidence of a dose-related response was observed and therefore, concentrations of test material of 17.5 to 700.0 n1/ml were evaluated as being non-transforming in the activation assay.

IX. CONCLUSIONS

The test material, Morpholine, did not induce significant increases in transformed foci over the tested concentration ranges of 15.0 to 1400.0 n1/ml and 17.5 to 700.0 n1/ml in the non-activation and activation assay, respectively. The tested concentrations corresponded to approximately 20% to nearly 100% survival in the non-activation and activation preliminary cytotoxicity tests. Therefore, the test material is considered to be inactive in the Balb/3T3 and Balb/3T3 rat liver cell-mediated in vitro cell transformation assays.



BIONETICS

SUBMITTED BY:


John O. Rundel, Ph.D.
Section Chief
In Vitro Carcinogenesis
Department of Molecular
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7/29/82
Date

REVIEWED BY:

for: 
David J. Brusick, Ph.D.
Director
Department of Molecular
Toxicology

7/29/82
Date

TABLE 1
TOXICITY TEST IN BALB 3T3 CELLS

CLIENT: BASF

DEPT ASSAY NO. 5797

DATE: June 25, 1981 (I)
August 25, 1981 (II)

TEST MATERIAL CODE: Morpholine

SOLVENT: Medium

<u>TEST COMPOUND DOSES TESTED (nl/ml)</u>	<u>AVERAGE NUMBER OF COLONIES/PLATE</u>		<u>% SURVIVAL RELATIVE TO CONTROL</u>	
	<u>Trial I</u>	<u>Trial II</u>	<u>Trial I</u>	<u>Trial II</u>
1. 8000.0	0	ND	0	ND
2. 4000.0	0	0	0	0
3. 2000.0	0	0	0	0
4. 1000.0	7.3	0.33	35.2	0.63
5. 500.0	45.7	15.0	92.6	28.8
6. 250.0	44.0	46.7	89.2	89.8
7. 125.0	48.0	59.7	97.4	114.8
8. 62.5	46.7	62.0	94.7	119.2
9. 31.3	47.7	54.7	96.7	105.2
10. 15.6	46.0*	57.0	93.3	109.6
11. 7.81	43.7	67.7	88.6	130.2
12. 3.91	41.7	50.7	84.5	97.5
13. 1.95	46.3	63.7	94.0	122.5
14. 0.977	47.3	60.7	96.0	116.7
15. 0.488	55.3	68.7	112.2	132.0
16. 0.244	58.3	68.0	118.3	130.8
17. 0.122	53.3	66.3	108.2	127.5
18. 0.061	52.7	54.0	106.8	103.8
19. Control (culture medium)	49.3	52.0	100.0	100.0

*Two cultures were scored at this dose.
ND = Dose not tested.



BIONETICS

TABLE 2

**RAT LIVER CELL-MEDIATED
TOXICITY TEST IN BALB 3T3 CELLS**

CLIENT: BASFDEPT ASSAY NO. 5797DATE: July 7, 1982TEST MATERIAL CODE: MorpholineSOLVENT: Medium

<u>TEST COMPOUND DOSES TESTED (n1/ml)</u>	<u>AVERAGE NUMBER OF COLONIES/PLATE</u>	<u>% SURVIVAL RELATIVE TO CONTROL</u>
1. 8000.0	0	0
2. 4000.0	0	0
3. 2000.0	0	0
4. 1000.0	0.3	0.4
5. 500.0	27.3	40.3
6. 250.0	51.7	76.4
7. 125.0	57.7	85.2
8. 62.5	58.7	86.7
9. 31.3	59.7	88.2
10. 15.6	53.0	78.3
11. 7.81	69.7	103.0
12. 3.91	74.0	109.3
13. 1.95	70.0	103.4
14. 0.977	74.3	109.7
15. 0.488	75.0	110.8
16. 0.244	79.0	116.7
17. 0.122	67.3	99.4
18. 0.001	71.0	104.9
19. Control (culture medium)	67.7	100.0

0022

TABLE 5
 SUMMARY OF DATA FROM TRANSFORMATION ASSAY
 TRIAL 11

CLIENT: BASF GENETICS ASSAY NO.: 5797 TEST DATE: September 18, 1991
 CLIENT'S COMPOUND CODE: Morpholine SOLVENT: Media 373 CLONE: 1-13, C-14

TEST DOSES TESTED	NUMBER OF FOCI PER DISH SCORED																				TOTAL NO. OF FOCI	NO. OF FOCI/DISH	ABSOLUTE/LOG ₁₀	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20				
Negative Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00 / 0.04	
(Culture Medium)	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	3			
Positive Control (MCA) 5.0 µg/ml	5	4	3	6	2	5	9	5	4	5	8	3	6	7	5	9	25	2	4	C	117		6.16 / 8.24	
Test Material	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	C	C	C	C	C	1		0.07 / 0.03	
15.0 nl/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	C	C	C	1		0.06 / 0.04	
75.0 nl/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	C	1		0.05 / 0.04
150.0 nl/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0 / 0
300.0 nl/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0 / 0
600.0 nl/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		0 / 0

C = Dish was contaminated and not scored.

TABLE 6

STATISTICAL ANALYSIS OF TRANSFORMATION ACTIVITY

CLIENT: BASF GENETICS ASSAY NO. 5797 DATE: September 15, 1981
 COMPOUND CODE: Morpholine SOLVENT: Culture Medium

Treatment Condition	Log ₁₀ Analysis* of Foci/Dish				t Statistic**	p Values
	Mean ± SD	n	SE			
Media Control	0.016	0.099	37	0.016	Control	Control
NCA 5.0 µg/ml	0.795	0.213	19	0.049	+15.093	p<.01
Test Material						
600.0 nl/ml	0	0	18	0	- 1.000	NS
300.0 nl/ml	0	0	20	0	- 1.000	NS
150.0 nl/ml	0.016	0.069	19	0.016	- 0.022	NS
75.0 nl/ml	0.018	0.073	17	0.018	+ 0.058	0.9<p<1.0
15.0 nl/ml	0.020	0.078	15	0.020	+ 0.146	.8<p<.9

*NOTE: Each raw data point was increased by 1.0 and converted to log₁₀ before statistical analysis was applied.

**NOTE: t-test equations:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{(\overline{SE}_1)^2 + (\overline{SE}_2)^2}}, \text{ and}$$

$$\text{degrees of freedom} = df = \frac{1}{\frac{\mu^2}{n_1 - 1} + \frac{(1 - \mu)^2}{n_2 - 1}}$$

$$\text{where } \mu = \frac{(SD_1)^2/n_1}{(SD_1)^2/n_1 + (SD_2)^2/n_2} = \frac{(SE_1)^2}{(SE_1)^2 + (SE_2)^2}$$

SD = Standard Deviation SE = Standard Error
 NS = Not Significant
 * = Excluded from analysis due to excessive toxicity.

**Bailey, Norman, T.J.: Statistical Methods in Biology. Wiley and Sons, Inc., N.Y., pg 50, 1959.

0026

TABLE 7

SUMMARY OF DATA FROM RAT LIVER
CELL-MEDIATED TRANSFORMATION ASSAY
TRIAL III

CLIENT: BASE GENETICS ASSAY NO.: 579Z TEST DATE: February 23, 1982
 CLIENT'S COMPOUND CODE: Morpholine SOLVENT: Medium 3T3 CLONE: 1-13, C-14

TEST	NUMBER OF FOCI PER DISH SCORED																				TOTAL NO. OF FOCI ABSOLUTE/LOG ₁₀	NO. OF FOCI/DISH
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Negative Control (Culture Medium)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	C	1	0.05 / 0.04
Positive Control CP, 10.0 µg/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	1	1	1	1	7	0.36 / 0.26
Test Material 17.5 n1/ml	0	0	C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 / 0
87.5 n1/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0.05 / 0.04
175.0 n1/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	C	C	3	0.17 / 0.12
350.0 n1/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	C	0	0 / 0
700.0 n1/ml	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 / 0

C = Dish was contaminated and not scored.
 CP = Cyclophosphamide

TABLE 8

STATISTICAL ANALYSIS OF TRANSFORMATION ACTIVITY

CLIENT: BSF GENETICS ASSAY NO. 5797 DATE: March 26, 1962
 COMPOUND CODE: Morpholine SOLVENT: Culture Medium

Treatment Condition	Log ₁₀ Analysis* of Foci/Dish				t Statistic**	P Values
	Mean ± SD	n	SE			
Media Control	0.016	0.069	19	0.016	Control	Control
CP 10µg/ml	0.099	0.160	20	0.036	+2.108	.02<p<.05
Test Material						
700.0 nl/ml	0	0	20	0	-1.000	NS
350.0 nl/ml	0	0	19	0	-1.000	NS
175.0 nl/ml	0.050	0.115	18	0.027	+1.502	.1<p<.2
87.5 nl/ml	0.015	0.067	20	0.015	+0.435	.6<p<.7
17.5 nl/ml	0	0	20	0	-1.000	NS

*NOTE: Each raw data point was increased by 1.0 and converted to log₁₀ before statistical analysis was applied.

**NOTE: t-test equations:

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{\sqrt{(\frac{SE_1}{n_1})^2 + (\frac{SE_2}{n_2})^2}}, \text{ and}$$

$$\text{degrees of freedom} = df = \frac{1}{\frac{\mu^2}{n_1-1} + \frac{(1-\mu)^2}{n_2-1}}$$

$$\text{where } \mu = \frac{(SD_1)^2/n_1}{(SD_1)^2/n_1 + (SD_2)^2/n_2} = \frac{(SE_1)^2}{(SE_1)^2 + (SE_2)^2}$$

SD = Standard Deviation SE = Standard Error
 NS = Not Significant
 * = Excluded from analysis due to excessive toxicity.

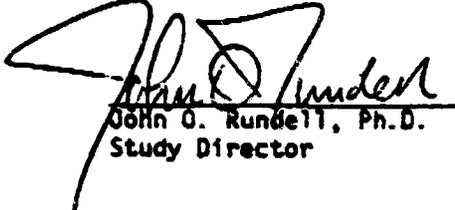
**Bailey, Norman, T.J.: Statistical Methods in Biology. Wiley and Sons, Inc., N.Y., pg 50, 1959.

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PROTOCOL CHANGES

The study design used in the conduct of the assay described in this report differed from the signed protocol. These changes are detailed in the attached Assay Design (No. 482), described in the present report, and summarized below:

1. Rat liver cells were lethally x-irradiated prior to their use in the activation transformation assay.
2. The activation transformation assay cultures were plated with 1×10^6 3T3 cells and after about 24 hours incubation, 1×10^6 x-irradiated rat liver cells were plated onto each 3T3 cell culture.
3. Co-culture treatments were initiated 3-4 hours after rat liver cell plating and continued for 48 hours. The 3T3 target cells were not trypsinized and replated; after treatment, the cultures were washed and incubation continued exactly as for the non-activation assay.
4. The assay evaluation criteria were changed so as to provide for direct analyses of experimental results in relation to the appropriate current negative controls. Statistical tests of significance were conducted on logarithmically transformed data using Bailey's modification of Student's t-test.


John O. Rundell, Ph.D. 7/29/87
Study Director Date

04029

ASSAY DESIGN (NO 482)

1. OBJECTIVE

This assay evaluates a test material for its ability to induce morphological transformation of BALB/c 3T3 cells in the presence and absence of metabolically active rat liver cells. Transformation of BALB/c 3T3 cells is recognized by dense, piled-up foci of altered cells superimposed on a monolayer of normal cells.

2. RATIONALE

BALB/c 3T3 mouse cells multiply in culture until a monolayer is achieved and then cease further division. These cells, if injected into immunosuppressed, syngeneic host animals, do not produce neoplastic tumors. However, cells treated in vitro with chemical carcinogens will give rise to foci of cellular growth superimposed on the cell monolayer. If these foci are picked from the cultures, grown to larger numbers and injected into animals, a malignant tumor will in most cases be obtained. Thus, the appearance of piled-up colonies in treated cell cultures is correlated with malignant transformation. The absolute correlation between morphological transformation and the tumorigenicity of isolated foci can be measured, for any given treatment, by isolation and transplantation of test-article-induced foci (see Protocol Nos. 486 and 483). Because tumorigenic cells also express the anchorage independent phenotype, the tumorigenic potential of morphological transformants can also be estimated by determining the ability to grow in soft agar (see Protocol No. 485).

3T3 cells have a limited capacity for metabolizing test materials and this capacity is known to vary considerably among cell cultures obtained from different clones. Freshly isolated lethally x-irradiated cells from rat liver retain a high metabolic activity and can be co-cultivated with 3T3 cells in order to achieve a cell-mediated activation of test materials. The rat liver cells attach to the culture dish, spread out and may form close membrane associations with the 3T3 cells. Metabolic products formed in the liver cells may then be effectively transported to the 3T3 cells and may cause them to transform.

3. MATERIALS

A. Indicator Cells

Clone 1-13 of BALB/c 3T3 mouse cells was obtained from Dr. Takeo Katunaga of the National Cancer Institute. Further subclones, selected for low spontaneous frequencies of foci formation, were used for assays. Stocks are maintained in liquid nitrogen, and laboratory cultures are checked periodically to ensure the absence

3. MATERIALS (Continued)

of mycoplasma contamination. Cultures are grown and passaged weekly in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum.

NOTE: The concentration of FBS used is determined for each lot prior to its use in the assay.

B. Metabolic Activation System -- Primary Rat Liver Cell Cultures

Primary rat liver cells are freshly isolated by enzyme perfusion of adult, male, Fischer 344 rat liver according to the technique described by G.H. Williams (1977). The metabolically active liver cells thus obtained are lethally x-irradiated (5000 r) and seeded in EMEM into each culture container to be used for the dose selection or the transformation assay 24 hours after the seeding of the 3T3 target cells.

C. Negative Control Articles

A negative control consisting of assay procedures performed on untreated cells were performed in all cases. If the test article is not soluble in growth medium, an organic solvent such as acetone or dimethylsulfoxide (DMSO) was used; the final concentration of solvent in the growth medium was 1% or less. Cells exposed to solvent in the medium are also be assayed as the solvent negative control to determine any effects on survival or transformation caused by the solvent alone. At least sixteen 60 mm dishes of the appropriate type of negative control were prepared for each assay.

D. Positive Control Article

A known carcinogen, such as 3-methylcholanthrene (MCA), was used as a positive control for the nonactivation transformation of 3T3 cells. At least sixteen 60 mm dishes were treated with the appropriate dose of positive control chemical for each assay.

Cyclophosphamide (CY) or dimethylnitrosamine (DMN) was used as a positive control for the transformation of 3T3 cells in the activation assay. CY and DMN are inactive or very weakly active as a transforming agent in 3T3 cultures in the absence of exogenous metabolic activation. Up to three groups of at least 16 dishes were treated with the appropriate doses of CY or DMN for each assay.

4. EXPERIMENTAL DESIGN

A. Dose Selection

The solubility of the test article in growth medium, DMSO, or other solvent was determined first. Fifteen dose levels of the test article were then chosen, starting with a maximum applied dose of 1 mg/ml

4. EXPERIMENTAL DESIGN (Continued)

a week. The dishes were examined by eye and microscope to determine the number of foci of transformed cells.

2. Activation Assay

The metabolic activation procedure used at LBI uses x-irradiated rat liver cells to mediate the biotransformation of test articles not metabolized by the 3T3 indicator cells and is based on an *in vitro* protocol developed at Litton Bionetics, Inc., (LBI), (Matthews, E.J., Guntakatta, M. and Rundell, J.O., 1981).

A series of 60 mm dishes were seeded with approximately 1×10^4 3T3 cells/dish and incubated for 24 hours. Then 1×10^5 primary rat hepatocytes were seeded into each 3T3 culture. After 3-6 hours incubation, the supernatant culture medium was removed and at least 16 dishes were treated for each of the following conditions: five doses of test article selected from the results of the Activation Cytotoxicity Assay; positive control (up to three concentrations); and negative control. The dishes were incubated for a 48-hour exposure period; the cells were then washed, refed and incubation continued for 4 weeks with refeeding twice a week. Assay scoring was done as described for the non-activation assay (4.B.1).

5. SCORING OF TRANSFORMED FOCI

At the end of the four-week incubation period, cultures of normal cells yield a uniformly stained monolayer of round, closely-packed cells. Transformed cells form a dense mass (focus or colony) that stains deeply (usually blue) and is superimposed on the surrounding monolayer of normal cells. The foci are variable in size.

Scored foci have several variations in morphological features. Most scored foci consist of a dense piling-up of cells and exhibit a random, criss-cross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci are composed of: 1) more rounded cells with little criss-crossing at the periphery but with necrosis at the center caused by dense piling-up of a large number of cells, or 2) foci without the necrotic center and large number of cells but which exhibit the criss-cross pattern of overlapping cells throughout most of the colony. Foci that have these characteristics and exceed 2 mm in diameter are scored +++ and those <2 mm in diameter are designated ++.

Some densely stained areas are not scored as transformed foci. These include focal areas where some piling-up of rounded cells has occurred but the random orientation of fibroblastic cells is not observed (+). Microscopic examination is employed for scored foci and in the final judgment of transformed character for any marginal foci.

4. EXPERIMENTAL DESIGN (Continued)

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Some densely stained areas are not scored as transformed foci. These include focal areas where some piling-up of rounded cells has occurred but the random orientation of fibroblastic cells is not observed (+). Microscopic examination is employed for scored foci and in the final judgement of transformed character for any marginal foci.



LITTON
BIONETICS

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CONFIRMATION OF TUMORIGENIC POTENTIAL OF ISOLATED MORPHOLOGICALLY TRANSFORMED FOCI

The BALB/3T3 transformation assay measures the ability of a test article to induce a specific type of cellular morphological alterations. This morphological damage is correlated with the expression of oncogenic potential among the population of morphologically altered (transformed) cells. Thus the appearance of morphologically transformed foci in treated cultures is thought to be the result of an inductive process constituting the first measurable step in the progression toward the in vitro cellular acquisition of the neoplastic phenotype. This hypothesis can be readily tested for specific test articles by measuring the coincidence between the expression of the anchorage independent (ar+) and/or tumorigenic phenotype among isolated test-article induced, morphologically-transformed foci. These confirmation studies are described in Protocol Nos. 486 (Isolation of Morphological Transformants), 485 (Growth in Soft Agar) and 483 (Confirmation of Transformant Tumorigenicity). Protocols and price quotations for these studies are available on request.

7. REFERENCES

Kakunaga, T.: A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from BALB/c 3T3. *Int. J. Cancer*, 12:463-473, 1973.

Bailey, Norman T.J.: *Statistical Methods in Biology*, Wiley and Sons, Inc., NY, page 50, 1959.

Williams, G.M.: Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture. *Cancer Res.*, 37:1845-1851, 1977.

Matthews, E.J., Guntakatta, M. and Rundell, J.O.: Unpublished Observation, 1981.

8. ASSAY ACCEPTANCE CRITERIA

The assay is considered acceptable for evaluation of the test results if the following criteria are met:

The negative control dishes consist of a contiguous monolayer of cells which may or may not contain transformed foci. The lack of contiguous sheet of cells indicates growth conditions too poor to allow the reliable detection of weak transforming agents.

The negative control transforming frequency does not exceed an average of about 2-3 foci/dish after \log_{10} analysis. Attempts are made to isolate and maintain cell stocks (subclones of BALB/3T3 1-13) with a very low spontaneous frequency of transformation.

8. ASSAY ACCEPTANCE CRITERIA (Continued)

- The positive control yields an average number of foci/dish that is significantly different from the negative control at the 95 or 99% confidence level.
- A minimum of 10 flasks per test condition are available for analysis. At least 3 dose levels of test substance are assayed.
- The dose range of test article assayed falls within the 10-100% survival range as determined by the preliminary toxicity test, which measures relative cloning efficiencies.

9. EVALUATION CRITERIA

The appearance of transformed foci is usually symmetric for any given treatment condition. However, large numbers of foci may appear at random in one or more dishes in a set resulting in skewing of the mean number of foci in that set. This skewing appears to be due to a technical rather than a biological cause and this conclusion is supported by the finding that dishes with high numbers of foci occur randomly in all experimental data, in treated dishes as well as in negative controls. The appearance of large numbers of foci in a dish, compared to other dishes in a set, is considered to be caused by mechanical disruption during the refeeding process that occurs twice weekly during the 4-week assay period. Recent analyses of the historical negative control results showed that when the data was converted to logarithmic form (base 10) a normal distribution was obtained and a few dishes with abnormally high numbers of foci (e.g.: >10) did not disturb this relationship. With the transformation assay data in a normal distribution, a t-test can be applied for determining statistical significance.

Bailey's modification of Student's t-test (Statistical Methods in Biology, Wiley and Sons, Inc., NY, page 50, 1959) will be used to determine whether the results for each treatment condition was significantly different from the experimental negative control ($\sim p \leq .05$ or $\sim p \leq .01$). The Study Director will evaluate the results of each treatment condition in relation to the observed activities of model compounds and will exercise scientific judgment in the evaluation of each test article. In general, a response at only one dose level just attaining the 95% confidence level will normally not be considered sufficient evidence for activity in this assay for activity in this assay. However, responses at one or more treatment levels attaining the 95% confidence level and exhibiting evidence of dose dependency will be considered as positive evidence of transforming activity and responses achieving the 99% confidence level over one or more test material treatments will be similarly interpreted.

Q.A. Inspection Statement
(reference 21 CFR 58.35(b)(7))

PROJECT 21008

LBI Assay No. 5797

TYPE of STUDY In vivo transformation with and without substrate activation

This final study report was reviewed by the LBI Quality Assurance Unit on 7/28/82. A report of findings was submitted to the Study Director and to Management on 7/28/82.

The short-term nature of this study precluded inspection while it was in process. The Quality Assurance Unit inspects an in-process study of this type approximately once per month to assure that no significant problems exist that are likely to affect the integrity of this type of study.

Mitchell S. Schaefer
Auditor, Quality Assurance Unit



BIONETICS

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CERTIFICATE OF AUTHENTICITY

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