CONTENTS

CONTENTS (continued)

This proposal, including front page, contains 7o pages.

ABBREVIATIONS (a,b,c)

approx.	: approximately
AA	: amino acid
Apr.	: April
Aug.	: August
bidist.	: bidistilled
cig.	: cigarette
Dec.	: December
DIN	: Publication of the German Committee of Standards
DMSO	: dimethyl sulfoxide
eds., Eds.	: editors
EDTA	: ethylenediaminetetraacetic acid
e. g.	: for example
etc.	: and so on
Feb.	: February
Fr.	: Friday
x g	: centrifugal force in terms of the constant of gravitation (1 x g = 9.81 m/s2)
.GT.	: greater than
HGPRT+	: hypoxanthine-guanine phosphoribosyl transferase positive
HGPRT-	: hypoxanthine-guanine phosphoribosyl transferase negative
i. e.	: that is
IMC	: inhibition of metabolic cooperation
Jan.	: January
Jul.	: July
Jun.	: June

(a) in addition to those, which are explained immediately on the same page
(b) Units of measure are given in accordance with SI-norms (Système International d'Unités).
(c) abbreviations of 7 EVALUATION AND REPORTING and 8 FORMS not included

ABBREVIATIONS (continued)

KW	: calendar week (Kalenderwoche)
.LT.	: less than
Mar.	: March
MC	: metabolic cooperation
Mo.	: Monday
MWSC	: mainstream whole smoke condensate
MWSC-I	: mainstream whole smoke condensate collected with an impaction trap
NEAA	: non-essential amino acid
no., No., NO.	: number
Nov.	: November
Oct.	: October
pH	: negative decadic logarithm of hydrogen-ion concentration
PM	: Philip Morris
PT	: preliminary title
QA	: Quality Assurance
Sa.	: Saturday
Sep.	: September
Su.	: Sunday
6-TG	: 6-thioguanine
Th.	: Thursday
TPA	: 12-O-tetradecanoylphorbol 13-acetate
TPM	: total particulate matter
Tu.	: Tuesday
U	: unit(s)
We.	: Wednesday
WSC-I	: whole smoke condensate collected with an impaction trap

1 SUMMARY

In the present study the influence of "PHENOL" and "CATECHOL" on cell to cell communication in the metabolic cooperation (MC) assay with Chinese hamster V79 cells will be investigated.

The study includes 3 negative and 8 positive control groups. Negative controls will be untreated, treated with 5 grams/liter dimethyl sulfoxide (DMSO, solvent control) and with o.16x1oE-2 millimoles/liter equivalent to o.58 milligrams/liter phorbol. Positive controls will be treated with 12-O-tetradecanoylphorbol 13-acetate (TPA) and mainstream whole smoke condensate (MWSC-I) (a) of the standard reference cigarette type 2R1 respectively. TPA will be administered at the doses 1.6x1oE-6, 1.6x1oE-5, 1.6x1oE-4 and 1.6x1oE-3 millimoles/liter equivalent to o.oo1, o.o1, o.1 and 1 milligram/liter. MWSC-I will be administered at o.oo1, o.o1, o.1 and 1 milligram dry condensate/liter.

The test substance phenol will be administered at 1.1x1oE-4, 1.1x1oE-3, o.o11 and o.11 millimoles/liter equivalent to o.o1, o.1, 1 and 1o milligrams/liter. Catechol will be administered at 9.ox1oE-5, 9.ox1oE-4, 9.ox1oE-3 and o.o9 millimoles/liter equivalent to o.o1, o.1, 1 and 1o milligrams/liter.

For each group 1o cocultures of V79 wild type and mutant cells and 1o monocultures of mutant cells will be set up. In the cocultures 4x1oE5 V79 wild type and 1oE2 V79 mutant cells will be seeded per dish. In the monocultures 1oE2 mutant cells will be seeded per dish. 4 hours after seeding the medium will be aspirated and 6-thioguanine (6-TG) selection medium containing DMSO, reference or test substance will be added. On day 4 after seeding the medium

(a) MWSC-I: mainstream smoke condensate collected with an impaction trap

will be replaced by 6-TG selection medium without DMSO, reference or test substance. On day 8 after seeding colonies will be fixed with methanol and stained with Giemsa. Colonies will then be counted visually.

The study will be performed in 2 experiments, each experiment being performed on a separate day and consisting of 5 cocultures and 5 monocultures per group and dose.

Prior to the MC assay a cytotoxicity assay will be performed with the same substances and doses as described above. Of MWSC-I the additional dose of 1o milligrams/liter will be administered. 3 monocultures of 1oE2 V79 wild type and 1oE2 V79 mutant cells per group and dose will be set up. The procedure will be the same as described for the MC assay. The medium, however, used in the cytotoxicity assay contains no 6-TG.

I N B I F O
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2 RESPONSIBILITY

Study Director and Experimental Conduct:

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Study Codirector:

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Biologist (Diplombiologe) and Biochemist

Analytical Chemistry:

Dr.rer.nat. M. Speck
Chemist (Diplomchemiker)

Quality Assurance:

E. Römer
Biologist (Diplombiologe)

3 INTRODUCTION

Metabolic cooperation is the transfer of small metabolites between cells which is associated with physical contact between the donor and the recipient cell (Subak-Sharpe et al., 1969). MC can be visualized as the colony forming ability, when Chinese hamster V79 wild type (hypoxanthine-guanine phosphoribosyl transferase positive, HGPRT+) cells are seeded together with Chinese hamster V79 mutant (HGPRT-) cells in a medium containing 6-TG. Besides its function in the "Salvage" pathway to synthesize inosine 5'-monophosphate by phosphorylation of hypoxanthine, the enzyme HGPRT phosphorylates also directly guanine to guanine-5'-monophosphate. 6-TG is incorporated into nucleotides (see FIGURE A) instead of guanine causing an inhibition of the proliferation and cell death of HGPRT+ cells in medium containing 6-TG. V79 mutant (HGPRT-) cells proliferate in medium containing 6-TG. When an excess number of V79 wild type cells are cocultured with V79 mutant cells the number of surviving V79 mutant cells is reduced. This reduction of mutant survival is attributable to MC between V79 wild type and mutant cells. The extent of this reduction is dependent on the number of V79 wild type cells (Yotti et al., 1979).

Yotti et al. (1979) reported that the potent tumor promoter TPA inhibits the MC between V79 wild type and mutant cells. Nearly at the same time Murray and Fitzgerald (1979) published the observation of the inhibition of MC (IMC) by TPA in cocultures of epidermal and 3T3 cells, determined by the transfer of the label from radioactively prelabeled cells to nonprelabeled recipient cells. Since this time several substances of different chemical classes were assayed in the MC assay and a correlation between substances with MC inhibiting activity and the tumor promoting

purine synthesis

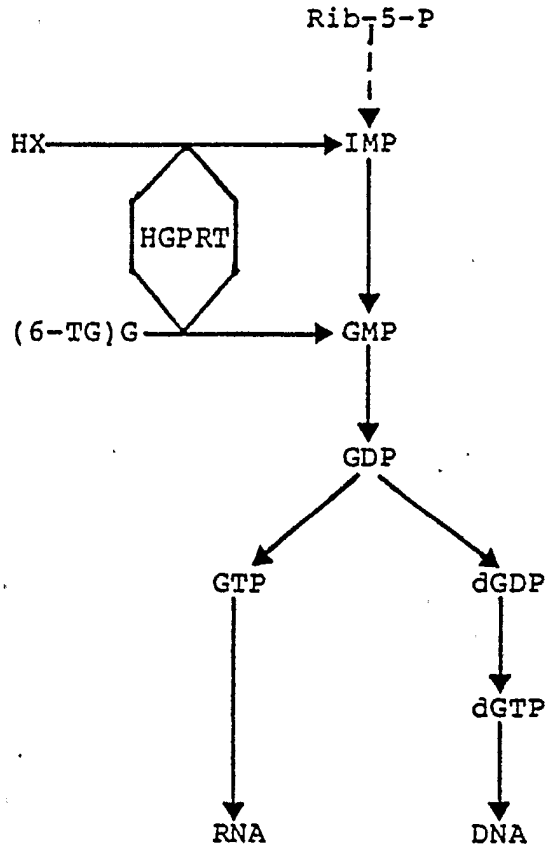


FIGURE A

INHIBITION OF NUCLEIC ACID SYNTHESIS BY 6-THIOGUANINE (6-TG)

Reference: J. Paul, Zell- und Gewebekulturen, Walter de Gruyter (1980)

Remarks:
dGDP : deoxyguanosine diphosphate
dGTP : deoxyguanosine triphosphate
DNA : deoxyribonucleic acid
G : guanine
GDP : guanosine diphosphate
GMP : guanosine monophosphate
GTP : guanosine triphosphate
HGPRT : hypoxanthine-guanine phosphoribosyl transferase
HX : hypoxanthine
Rib-5-P: ribose-5-phosphate
RNA : ribonucleic acid

activity as detected in long-term tests, was established. Examples of substances, which inhibit MC are a series of phorbol esters (Yotti et al., 1979), DDT and Lindane (Tsushimoto et al., 1982a), teleocidin (Jone et al., 1982), dinitrofluorobenzene (Warren et al., 1981), benzoyl peroxide (Slaga et al., 1981), specific congeners of polybrominated biphenyls (Trosko et al., 1981, Tsushimoto et al., 1982b) and polychlorinated biphenyls (Tsushimoto et al., 1982c), saccharin (Trosko et al., 198o), kepone and mirex (Tsushimoto et al., 1982d) bile acids (Noda et al., 1981) and oleic acid and anthralin (Trosko et al., 1982). The promotors estrogen, phenobarbital and sodium deoxycholate, however, did not inhibit MC and therefore did not fit into the mentioned correlation ("false negatives"). On the other side concanavalin A and chaetoglobosin A (Umeda et al., 198o), 2 substances which obviously modulate the function of the cell membrane, but which are not known to be tumor promotors, inhibited the MC ("false positives"). It seems to be reasonable that substances which influence the cell membrane function may also be active in the IMC, because cell to cell contact i. e. formation of gap junctions is a necessary prerequisite for MC.

In the present study the influence of phenol and catechol on MC in V79 cells will be investigated. The study includes 3 negative and 8 positive control groups. Negative controls will be untreated, treated with 5 grams/liter DMSO (solvent control) and with o.16x1oE-2 millimoles/liter equivalent to o.58 milligrams/liter phorbol. Positive controls will be treated with TPA and MWSC-I of the standard reference cigarette type 2R1 respectively. TPA will be administered at the doses 1.6x1oE-6, 1.6x1oE-5, 1.6x1oE-4 and 1.6x1oE-3 millimoles/liter equivalent to o.oo1, o.o1, o.1 and 1 milligram/liter. MWSC-I will be administered at o.oo1, o.o1, o.1 and 1 milligram dry condensate/liter.

The test substance phenol will be administered at 1.1x1oE-4, 1.1x1oE-3, o.o11 and o.11 millimoles/liter equivalent to o.o1, o.1, 1 and 1o milligrams/liter. Catechol will be administered at 9.ox1oE-5, 9.ox1oE-4, 9.ox1oE-3 and o.o9 millimoles/liter equivalent to o.o1, o.1, 1 and 1o milligrams/liter. Each concentration of each test substance will be called 1 group.

For each group 1o cocultures of V79 wild type and mutant cells and 1o monocultures of mutant cells will be set up. In the cocultures 4x1oE5 V79 wild type and 1oE2 V79 mutant cells will be seeded per dish. In the monocultures 1oE2 mutant cells will be seeded per dish. 4 hours after seeding the medium will be aspirated and 6-TG selection medium containing DMSO, reference or test substance will be added. On day 4 after seeding the medium will be replaced by 6-TG selection medium without DMSO reference or test substance. On day 8 after seeding colonies will be fixed with methanol and stained with Giemsa. Colonies will then be counted visually.

The experiment will be performed in 2 experimental parts, each part being performed on a separate day and consisting of 5 cocultures and 5 monocultures per group and dose.

Prior to the MC assay a cytotoxicity assay will be performed with the same substances and doses as described above. Of MWSC-I the additional dose of 1o milligrams/liter will be administered. 3 monocultures of 1oE2 V79 wild type and 1oE2 V79 mutant cells per group and dose will be set up. The procedure will be the same as described for the MC assay. The medium, however, used in the cytotoxicity assay contains no 6-TG.

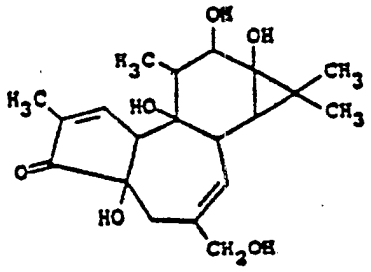
4 SUBSTANCES EXAMINED

4.1 Reference Substances

4.1.1 Phorbol

Synonyms: 4beta, 9alpha, 12beta, 13alpha, 2o-Pentahydroxy-tiglia-1,6-Dien-3-One, 4betaphorbol

Structure:



Chemical Abstracts Registration Serial No.: 17673-25-5

Data compilation: Merck Index 9 (198o)

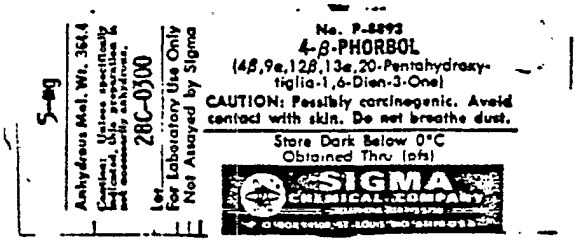
Source: no. P-8893, Sigma Chemie GmbH, D-8o28 Taufkirchen

Date of receipt at INBIFO: 1o.Jan.83

INBIFO substance no.: S2869A

Shipment container: colorless glass ampul in metal cylinder

Description on container:



Amount:	5 mg
Storage:	at minus 2o degrees centigrade, protected from light
Physical and chemical properties	
Relative molecular mass:	364.4
Appearance:	white powder
Odor:	-
Melting point (degrees centigrade):	dependent on crystalline forms (162 to 163, 233 to 234 and 249 to 25o degrees centigrade)
Boiling point (degrees centigrade):	-
Vapor pressure:	-
Density:	-
Solubility:	soluble in water and some polar solvents
Purity (o/o):	.GT.98
Stability	
Acid:	-
Alkali:	-
Heat:	-
Light:	-
Infrared spectrum:	-
Absorption maxima:	235 nm, 334 nm
Administrative regulations:	-

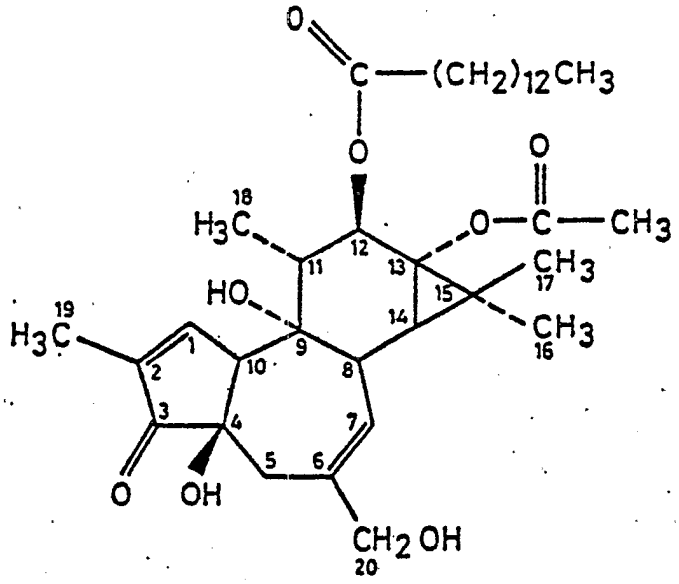
4.1.2 12-O-tetradecanoylphorbol 13-acetate (TPA)

Synonyms:

5-(3,4)benz(1,2-e)azulen-5-one,1,1a-alpha,1b-beta,4,4a,7a-alpha, 7b,8,9,9a-decahydro-4a-beta,7b-alpha, 9-beta, 9a-alpha-tetrahydroxy-3-(hydroxymethyl)-1,1,6,8-alpha-tetramethyl-9a-acetate-9-myristate

myristic acid,
13-O-acetylphorbol 12-myristate,
phorbol 12-myristate 13-acetate,
phorbol 12-tetradecanoylphorbol 13-acetate,
12-tetradecanoylphorbol 13-acetate,
4beta-phorbol 12beta-myristate 13alpha-acetate

Structure:

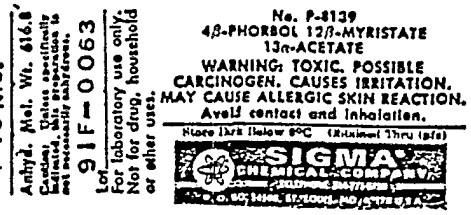


12-O-Tetradecanoylphorbol 13-Acetate (TPA)

Chemical abstracts service registry no.: 2o839-11-6

Data compilation:	Hecker and Schmidt (1974)
Physical and chemical properties	
Relative molecular mass:	618.8
Appearance:	white crystalline powder
Odor:	-
Congealing point (degrees centigrade):	72 (Hecker and Schmidt, 1974)
Boiling point (degrees centigrade):	-
Vapor pressure:	-
Density:	-
Solubility:	soluble in organic solvents, very insoluble in water
Stability:	sensitive to light and air
Acid:	instable
Alkali:	instable
Heat:	-
Light:	-
Oxygen:	instable (autoxidation)
Infrared spectrum:	(Hecker and Schmidt, 1974)
Absorption maxima:	232 and 333 nm (in ethanol) (Hecker and Schmidt, 1974)
Administrative regulations:	-
Source:	Sigma Chemie GmbH, D-8o28 Taufkirchen
Date of receipt at INBIFO:	1o.Jan.83
INBIFO substance no.:	S2866B
Shipment container:	colorless glass ampul in metal cylinder

Description on container:



Amount: 1o mg

Storage: minus 2o degrees centigrade

4.1.3 Cigarette (a)

Type (cigarette code): 2R1 (standard reference)

Source: Philip Morris, USA

Number of cigarettes: approx. 4x1oE5

Packaging: cartons with 2oo cigarettes,
1o packages with 2o cigarettes/package

Date of receipt at INBIFO: 44. KW 82

Storage

Main storage: walk-in cold room R911, 1 to 3 degrees centigrade, relative humidity uncontrolled

Laboratory storage: conditioning room R326, at least 1o days prior to smoking

storage in opened packages

temperature: 22 ± 1 degrees centigrade, relative humidity: 6o ± 3 o/o

Selection: no selection

(a) Actually used in this study is the smoke condensate of this cigarette. For the preparation of smoke condensate see 5.1 Condensates Preparation, Storage and Analyses

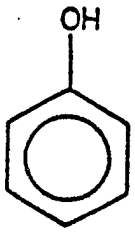
Specifications of cigarette:	see TABLE A
Physical properties and chemical composition of filler of cigarette:	see TABLE B
Composition of smoke components of cigarette:	see TABLE C

4.2 Test Substances

4.2.1 Phenol

Synonyms: carbolic acid, hydroxybenzene, monohydroxybenzene, oxybenzene, phenic acid, phenyl hydrate, phenyl hydroxide, phenylic acid, phenylic alcohol

Structure:



Phenol

Chemical Abstracts Registration Serial No.: 108-95-2

Data compilation: Merck Index 9 (1976)

Source: no. 77610, Fluka Feinchemicalien GmbH, D-7920 Neu-Ulm

TOTAL CIG. WEIGHT (mg/cig.)	FILTER AND PAPER WEIGHT (mg/cig.)	TOBACCO WEIGHT (a) (mg/cig.)	TOTAL CIG. LENGTH (mm)	DIAMETER (mm)	PUFF RESIS-TANCE (kPa)	COMPRESSI-BILITY (b) (mm)	STATIC BURNING TIME (min/4o mm)	PAPER POROSITY (ml/(cm2 x min))
1194	11o (c)	1o84	85	7.96	o.79	-	13.7	-

TABLE A

SPECIFICATIONS OF STANDARD REFERENCE CIGARETTE TYPE 2R1
specifications provided by the supplier, analyzed by PM, August 1977

(a) at tobacco moisture determined (see TABLE E)
(b) at 12 o/o moisture
(c) no filter with 2R1 cigarettes

TOTAL ALKALOIDS (o/o)	REDUCING SUGARS (o/o)	NITRATE (o/o)	AMMONIA (o/o)	NITRITE (o/o)	NITROGEN (o/o) KJELDAHL	PROTEIN	TOTAL
1.98	1o.5	o.89	o.13	-	-	-	2.17

TABLE B

PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF FILLER OF STANDARD REFERENCE CIGARETTE TYPE 2R1
specifications provided by the supplier, analyzed by PM, August 1977

CHLORIDE (o/o)	ASHES (o/o)	POTASSIUM (o/o)	CALCIUM (o/o)	MAGNESIUM (o/o)	HOT WATER SOLUBLES (o/o)	TOBACCO pH	MOISTURE TOBACCO (o/o)	EQUILIBRIUM (o/o)
0.66	14.8	-	-	-	59	5.5	-	12.6

TABLE B (continued)

PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF FILLER OF STANDARD REFERENCE CIGARETTE TYPE 2R1
specifications provided by the supplier, analyzed by PM, August 1977

PUFF NO.	SMOKE COMPONENTS (a)									
	TPM	WATER IN TPM	DPM	NICO-TINE	TAR	CO	NO	HCN	ALDE-HYDES	ISH
(1/cig.)	(mg/ cig.)	(mg/ cig.)	(mg/ cig.)	(mg/ cig.)	(mg/ cig.)	(mg/ cig.)	(mg/ cig.)	(ug/ cig.)	(mg/ cig.)	(o/o)
13.1	48.4	4.9	43.5	3.32	4o.2	25.4	o.39	453	3.13	71

TABLE C

SMOKE COMPONENTS OF STANDARD REFERENCE CIGARETTE TYPE 2R1
specifications provided by the supplier, analyzed by PM, August 1977

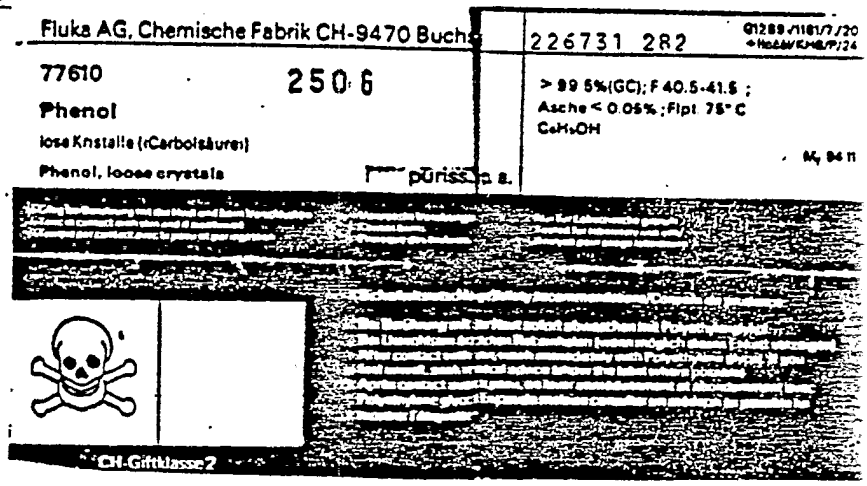
(a) given for completely smoked cigarettes, not corrected for minimum butt length (32 mm)

Date of receipt at INBIFO: 27.Jan.83

INBIFO substance no.: S2856B

Shipment container: plastic bottle

Description on container:



Amount: 25o g

Storage: at minus 2o degrees centigrade

Physical and chemical properties

Relative molecular mass:	94.1
Appearance:	colorless acicular crystals or white crystalline mass
Odor:	pungent
Congealing point (degrees centigrade):	41
Boiling point (degrees centigrade):	182
Vapor pressure:	181.32 Pa at 37 degrees centigrade
Density (a):	1.o6

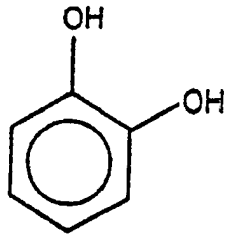
(a) density at 2o degrees referred to water at 4 degrees centigrade

Solubility:	very soluble: alcohol, chloroform, ether, glycerol, carbon disulfide, petrolatum, volatile and fixed oils, aqueous alkali hydroxides moderately soluble: water, benzol, insoluble: petrol ether
Stability	
Acid:	–
Alkali:	–
Heat:	–
Light:	instable (reddening)
Infrared spectrum:	–
Absorption maxima:	–
Chemical synthesis:	fusion of sodium benzenesulfonate with sodium hydroxide or heating of monochlorobenzene with aqueous sodium hydroxide under high pressure
Administrative regulations:	–

4.2.2 Catechol

Synonyms: brenzcatechin,
1.2-benzenediol,
1.2-dihydroxybenzene,
pyrocatechol

Structure:



Catechol

Chemical Abstracts Registration Serial No.: 120-80-9

Data compilation: Merck Index 9 (1980)

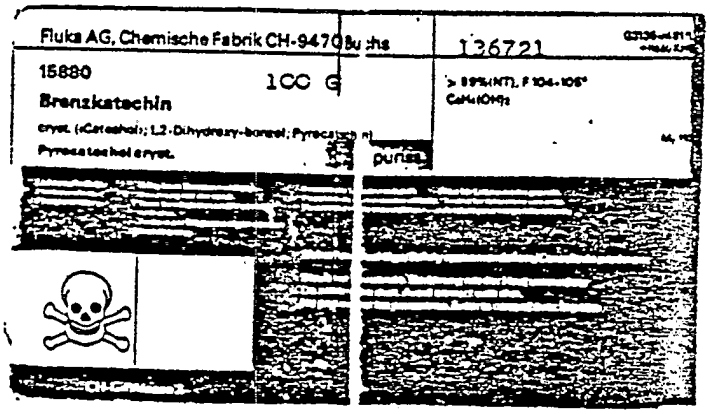
Source: no. 1588o,
Fluka Feinchemikalien GmbH,
D-7910 Neu-Ulm

Date of receipt at INBIFO: 14.Jan.83

INBIFO substance no.: S2857C

Shipment container: plastic bottle, gray

Description on container:



2062555060

Amount:	1oo g
Storage:	at 4 to 8 degrees centigrade, protected from light
Physical and chemical properties	
Relative molecular mass:	11o.11
Appearance:	colorless crystals
Odor:	pungent
Congealing point (degrees centigrade):	1o5
Boiling point (degrees centigrade):	245
Vapor pressure:	13.3 Pa at 37 degrees centigrade
Density:	1.344
Solubility:	soluble in water, alkaline and organic solutions
Purity:	.GT.99 o/o
Stability	
Acid:	-
Alkali:	instable
Heat:	-
Light:	instable (autoxidation)
Infrared spectrum:	-
Absorption maxima:	-
Administrative regulations:	

5 METHOD

5.1 Chronological Table

5.2 Condensate Preparation, Storage and Analyses

5.2.1 Preparation of mainstream whole smoke moist condensate by impaction trap (MWSC-I) (a)

Principle:	mechanical open-end smoking to a defined butt length in automatic negative pressure (vacuum pump) smoking machine, condensate collection in impaction trap
Time:	-
Sample material and quantity:	2R1 cigarettes (see 4 SUBSTANCES EXAMINED), 3o cigarettes/condensate batch, 3 batches prepared and pooled

Equipment

Smoking machine

Type:	automatic INBIFO smoking machine
Number of machines:	1
Machine no.:	oo25
Loading of cigarettes:	manually
Lighting of cigarettes:	automatically with an iodine spot lamp iodine spot lamp: Halogen-Bellaphot, 15 V, 15o W, gold-plated reflector, Osram, no. 64635, R. Schahl, D-8ooo München 1
Ejection of cigarettes:	automatically at butt length of 23 mm (automatic scanning with infrared photodiode)

(a) The terms "moist condensate" and "dry condensate" are in accordance with DIN 1o24o ("Maschinelles Abrauchen von Zigaretten, Bestimmung des feuchten und des trockenen Rauchkondensates").

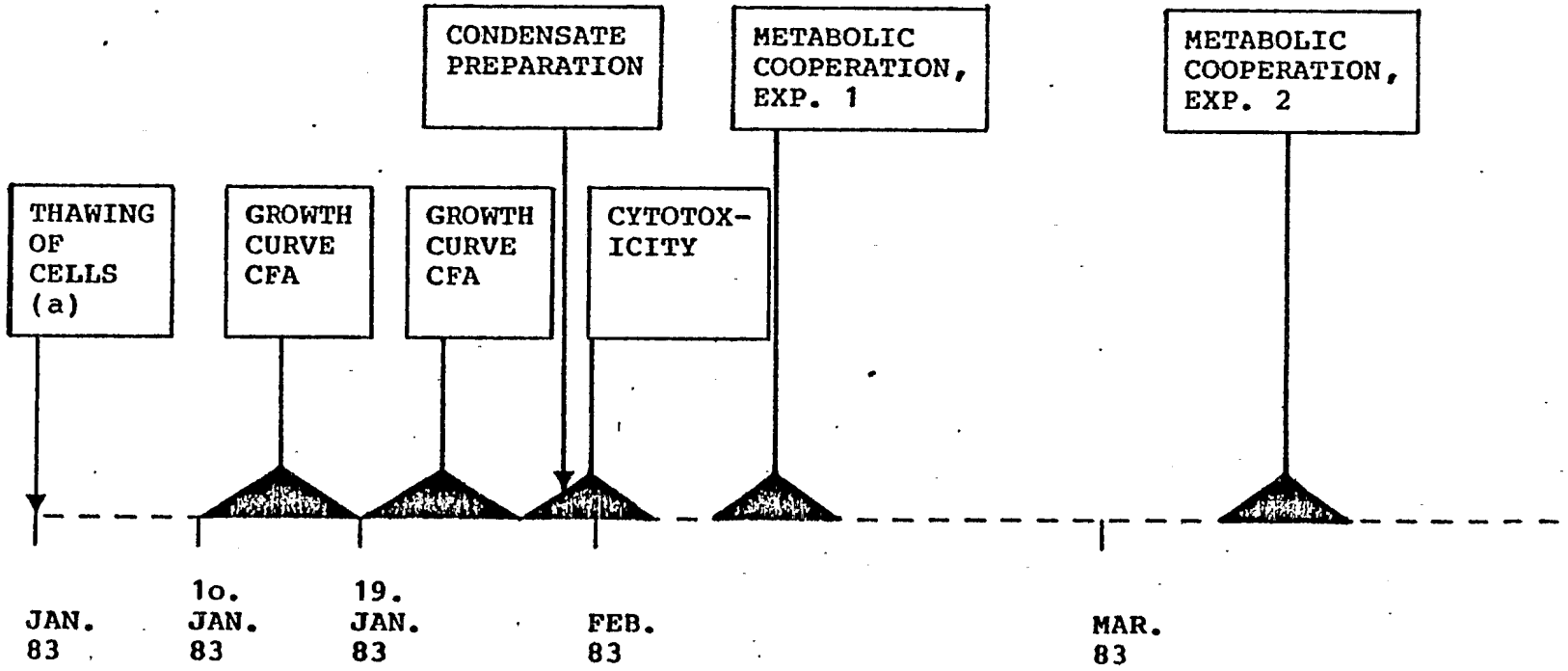


FIGURE B

CHRONOLOGICAL TABLE, WHOLE STUDY

Remarks: CFA : colony forming ability
EXP.: experimental part

(a) mutant cells: clone no. 4, cloned 29.Apr.8o, stored frozen
wild type cells: stored frozen
(see A -/3o65: Metabolic cooperation test, report in preparation)

Vacuum pump:	rotary valve pump, Medvak MP 1, Pfeiffer GmbH, D-633o Wetzlar
Flowmeter:	rotameter, L 4/16o, Rota, Dr. Henning KG, D-7867 Wehr/Baden
	soap-film flowmeter, Faust GmbH, D-5ooo Köln 9o
Impaction trap	
Type:	glass "Impaction trap for cigarette smoke condensate collection" according to Philip Morris (see FIGURE B), Faust GmbH, D-5ooo Köln 9o
Capillary:	length: 5 mm bore: o.4 mm
Mode of installation of the impaction trap insert:	distance of o.5 mm between capillary tip and wall of flask calibrated with o.5 mm thick teflon sheet spacer
Connection of impaction trap to smoking machine:	so that the impaction trap lies horizontally
Procedure	
Puffs/cigarette:	approx. 12
Puff frequency/cigarette:	1 puff/min
Puff duration:	approx. 2 s minus time for change of position
Puff volume:	35 ml
	parameter checked and regulated during condensation with rotameter or soap-film flowmeter
Scientific version:	14.Jan.81
Text version:	1.Feb.83

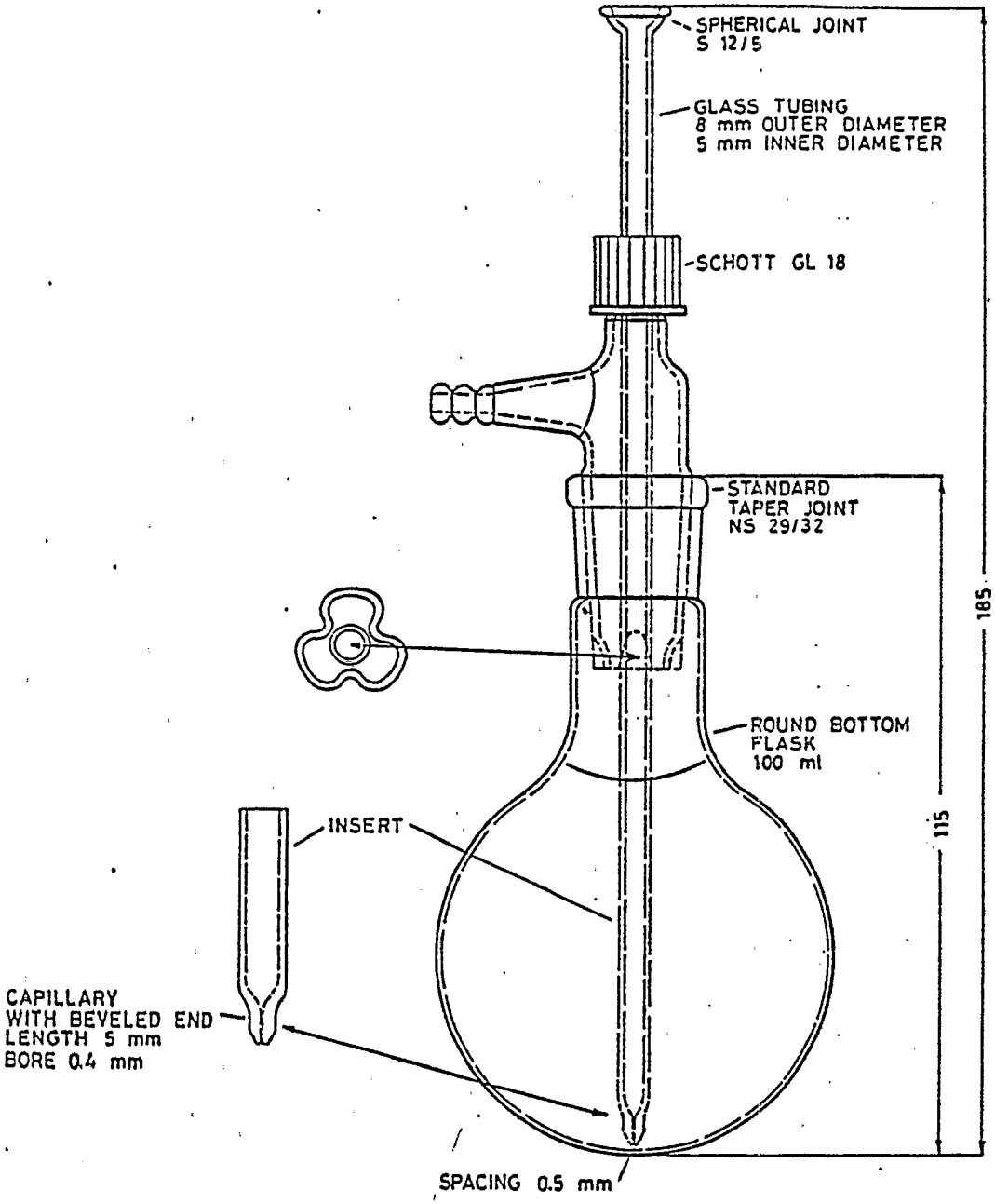


FIGURE C

IMPACTION TRAP

5.2.2 Suspension of condensate, storage and analyses

Principle:	suspension of MWSC-I in DMSO by sonication
Time:	immediately after MWSC-I preparation
Sample material and quantity:	total MWSC-I of 3 condensate batches, each prepared from 3o cigarettes with 1 smoking machine
Equipment:	sonication water bath: Sonorex RK 1oo, Bandelin KG, D-1ooo Berlin
	brown glass vials, 2 ml, no. 224981, screw caps, no. 24o2o6, Wheaton Scientific, via Zinsser, D-6ooo Frankfurt/Main
Chemicals and reagents:	DMSO, no. 295o, E. Merck, D-61oo Darmstadt 1
Procedure:	MWSC-I washed out of each impaction trap 6 times with approx. 3 ml portions of DMSO repeatedly after sonication (water bath) for approx. 3 min, washings transferred to a 1oo-ml volumetric flask and filled up to volume with DMSO
	amount of MWSC-I calculated from weight of impaction trap before and immediately after condensate preparation
	amount of dry condensate (a) calculated from MWSC-I and water concentration of suspension
	determination of water concentration: see 5.2.3

(a) The terms "moist condensate" and "dry condensate" are in accordance with DIN 1o24o ("Maschinelles Abrauchen von Zigaretten, Bestimmung des feuchten und des trockenen Rauchkondensates").

	determination of hydrogen-ion concentration: see 5.2.4
	determination of nicotine concentration: see 5.2.5
	dilution of MWSC-I suspension: with DMSO to 5 g dry condensate/l
	storage of MWSC-I suspension: fractionated in 1-ml aliquots, in the dark, in sterile brown glass vials, minus 75 degrees centigrade
	labeling of the vials: study no., batch no. (a), dry condensate concentration (g/l), date of condensate preparation
Scientific version:	15.Jan.81
Text version:	1.Feb.83

5.2.3 Determination of water concentration

Principle:	titration according to Karl Fischer
Time:	within 48 h after preparation of MWSC-I suspension
Sample material and quantity:	MWSC-I/DMSO suspension, 1 ml, 2 determinations/suspension
Results expressed in:	g/l
Equipment:	Karl Fischer Titrator E452, Deutsche Metrohm GmbH, D-7o24 Filderstadt

(a) Batch number consists of cigarette short code, type of condensate, consecutive number, date of condensate preparation, for example: 7A (I)/1o/o8128o (e. g.: code: 7A, condensate: I, batch no.: 1o, date: 8.Dec.8o)

Chemicals and reagents:	Karl Fischer solution, no. 9248, methanol, no. 6o12, DMSO, no. 295o, E. Merck, D-61oo Darmstadt 1
Procedure	
Titration:	1 ml DMSO mixed with 4 ml methanol in the reaction vessel of the titrator and titrated with Karl Fischer solution to determine the water content of DMSO. Afterwards 1 ml cigarette smoke condensate suspended in DMSO titrated in the same way
Computation:	titer of the Karl Fischer solution determined by titration of a mixture of 1 ml DMSO and 4 ml methanol with a known amount of water, e. g. 1o mg
Detection limit:	o.5 g H2O/l
Reproducibility (relative standard deviation):	2.8 o/o (1o g H2O/suspension, N = 8)
Scientific version: Text version:	2.Jun.8o 7.Jan.83

5.2.4 Determination of hydrogen-ion concentration

Principle:	electrochemical determination
Time:	on the day of condensate preparation
Sample material and quantity:	MWSC-I suspension, 2 ml
Results expressed in:	pH

Equipment:	pH meter: PW 9409, glass electrode: CA 1-S, Philips GmbH, D-3500 Kassel
Chemicals and reagents:	calibration buffer: pH 7.00, no. 9887, pH 4.00, no. 9884, E. Merck, D-6100 Darmstadt 1
Procedure:	determination at room temperature after calibration of pH meter with 2 standard buffers
Scientific version: Text version:	28.Feb.79 8.Jul.82

5.2.5 Determination of nicotine concentration

Principle:	gas chromatography after extraction with dichloromethane computer integration of peak areas
Time:	immediately after the preparation of the MWSC-I suspension
Sample material and quantity:	MWSC-I suspension (undiluted and diluted), 1 ml, 2 determinations/ suspension
Results expressed in:	g/l
Equipment:	gas chromatograph: HP 5710 A, detector: FID, automatic sampler: HP 7671 A, laboratory data system: HP 3351 A, Hewlett-Packard GmbH, D-6000 Frankfurt/Main recorder: Servogor 210, Metrawatt GmbH, D-8500 Nürnberg

centrifuge: model J-6B,
rotor: JS-4.2,
Beckman Instruments GmbH,
D-8000 München 40

Chemicals and reagents:

nicotine, no. 77635,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

quinoline, no. 802407,
dichloromethane, no. 822271,
DMSO, no. 2950,
sodium hydroxide, no. 5594,
sulfuric acid, no. 9074,
E. Merck,
D-6100 Darmstadt 1

nitrogen,
hydrogen,
air (synthetic),
Linde AG,
D-5000 Köln 50

Procedure

Extraction:

addition of 1 ml of the internal standard solution (0.5 mg quinoline/ml (0.1 mol/l) sulfuric acid), 1 ml sodium hydroxide (200 g/l) and 10 ml dichloromethane to 1 ml MWSC-I suspension, after agitation (5 min) and centrifugation (approx. 7.8x10E3 m/s2 (= 800 x g), 5 min, approx. 10 degrees centigrade), injection of 1 ul of the lower phase into the gas chromatograph

Gas chromatography

Column:

2 m x 1/8 inch outer diameter, stainless steel

Column packing:

100 g/kg Apiezon L and 100 g/kg potassium hydroxide on Chromosorb W-AW DMCS (a), 80 to 100 mesh

(a) AW: acid washed, DMCS: treated with dimethyldichlorosilane

Carrier gas and flow rate:	nitrogen, 3o ml/min
Oven temperature:	175 degrees centigrade
Injection port temperature:	2oo degrees centigrade
Detector temperature:	2oo degrees centigrade
Computation:	1 ml of a standard solution (1 mg nicotine and o.5 mg quinoline/ml (o.1 mol/l) sulfuric acid) diluted with 1 ml DMSO and extracted as described above determination of calibration factor
Detection limit:	o.o2 g/l
Recovery:	98.5 o/o
Reproducibility (relative standard deviation):	o.5 o/o (1 g nicotine/l (o.1 mol/l) sulfuric acid, N = 1o)

Scientific version: 2.Jun.8o
Text version: 17.Jan.83

5.3 Preparation of Reference and Test Substances

Principle:	suspension of reference and test substance in DMSO and dilution of the reference/test substance suspension with DMSO to the final concentration of the application solution (see TABLES D and E)
Time:	-
Sample material and quantity:	reference and test substance, i. e. phorbol, 5 mg, TPA, 5 mg, 2R1-MWSC-I, 5 mg/l, phenol, 1oo mg, catechol, 1oo mg

SUBSTANCE	AMOUNT (mg)	VOLUME, DMSO (ml)	STOCK SOLUTION (a) CONCENTRATION (g/l)
phorbol	5	1o	o.5
TPA	5	1o	o.5
2R1-MWSC-I	(b)	(b)	5
phenol	1oo	2o	5
catechol	1oo	2o	5

TABLE D

PREPARATION OF STOCK SOLUTIONS

(a) Stock solutions are fractionated in 1-ml aliquots and stored in dark glass vials at minus 75 degrees centigrade.
(b) see 5.2 Condensate Preparation, Storage and Analyses

SUBSTANCE	STOCK SOLUTION	DILUTED (a) STOCK SOLUTION	DOSE	APPLICATION SOLUTION (b), (A PLUS B)	
				A STOCK SOLUTION OR DILUTED STOCK SOLUTION	B DMSO
	(g/l)	(g/l)	(mg/l)	(ul)	(ul)
DMSO	-	-	-	-	3oo
phorbol	o.5	-	o.58	7o	23o
TPA	o.5	-	1	12o	18o
	o.5	-	o.1	12	288
	-	o.o5	o.o1	12	288
	-	o.oo5	o.oo1	12	288
MWSC-I	5	-	1o	12o	18o
	5	-	1	12	288
	-	o.5	o.1	12	288
	-	o.o5	o.o1	12	288
	-	o.oo5	o.oo1	12	288

TABLE E

PREPARATION OF APPLICATION SOLUTION (b)

(a) Stock solutions diluted with DMSO on the day of use.
(b) Application solutions are prepared on the day of use.
Application solution added to 59.7 ml 6-TG-selection-medium.

SUBSTANCE	STOCK SOLUTION	DILUTED (a) STOCK SOLUTION	DOSE	APPLICATION SOLUTION (b), (A PLUS B)	
				A STOCK SOLUTION OR DILUTED STOCK SOLUTION	B DMSO
	(g/l)	(g/l)	(mg/l)	(ul)	(ul)
phenol	5	-	1o	12o	18o
	5	-	1	12	288
	-	o.5	o.1	12	288
	-	o.o5	o.o1	12	288
catechol	5	-	1o	12o	18o
	5	-	1	12	288
	-	o.5	o.1	12	288
	-	o.o5	o.o1	12	288

TABLE E (continued)

PREPARATION OF APPLICATION SOLUTION (b)

(a) Stock solutions diluted with DMSO on the day of use.
(b) Application solutions are prepared on the day of use.
Application solution added to 59.7 ml 6-TG-selection-medium.

Results expressed in: -

Equipment:

brown glass bottles, 2 ml, no. 224981,
screw caps, no. 24o2o6,
Wheaton Scientific,
via Zinsser,
D-6ooo Frankfurt/Main

Chemicals and reagents

Chemicals:

DMSO, no. 295o,
E. Merck,
D-61oo Darmstadt 1

Dulbecco's Modification of Eagle's Medium (DMEM), no. 12-332-54,
fetal bovine serum, filter sterilized, mycoplasma and virus screened,
no. 29-1o1-54 (Lot no. (a)),
MEM 5o-fold amino acids (AA),
no. 16-o11-49,
MEM 1oo-fold non-essential amino acids (NEAA), no. 16-81o-49,
trypsin EDTA, no. 16-891-49,
phosphate-buffered saline (PBS),
no. 18-6o4-49,
sodium pyruvate, no. 16-82o-49,
Flow Laboratories GmbH,
D-53o9 Meckenheim

glutamine, no. 22942,
streptomycin, no. 355oo,
penicillin, no. 31749,
Serva Feinbiochemica GmbH und Co. KG,
D-69oo Heidelberg 1

6-thioguanine (6-TG), no. A 4882,
Sigma Chemie GmbH,
D-8o28 Taufkirchen

Reagents:

cell culture medium:
DMEM with
4 mmol/l glutamine,
1x1oE5 U pencillin/l,
1o mg streptomycin/l,
1o ml NEAA/1,
2o ml AA/1,
1 mmol/l sodium pyruvate and
3o g/l fetal bovine serum,
final pH: 7.4,
final osmolality: 33o mmol/kg

(a) will be given in report

	6-TG-selection medium: cell culture medium plus 1o mg/l 6-thioguanine
Procedure:	see TABLE D and TABLE E
Scientific version: Text version:	27.Jan.83 1.Feb.83

5.4 Routine Maintenance of V79 Cells

Principle:	maintenance of monolayer cell cultures in a subconfluent to confluent state by subculturing
Time	
Splitting:	2 times/week (see TABLES F and G)
Determination:	-
Sample material and quantity:	wild type and V79 mutant, approx. 1x1oE7 cells
Results expressed in:	-
Equipment:	centrifuge tubes: no. 2o7o, conical graduated, polypropylene, diameter: 3o mm, length: 115 mm, Falcon, Div. Becton and Dickinson GmbH, D-69oo Heidelberg-Wieblingen
	culture vials: tissue culture flask, growth area: 8o cm2, no. 147589, Nunc GmbH, D-62oo Wiesbaden
	micro vials: type "Eppendorf", polypropylene, no. 381o, Netheler und Hinz GmbH, D-2ooo Hamburg 65
	centrifuge: Digifuge GL, rotor: o335o, Heraeus-Christ GmbH, D-336o Osterode
	incubator: Cytoperm 8o8o/81oo124, carbon dioxide, humidity and temperature regulation, W. C. Heraeus GmbH, D-645o Hanau

DATE	ACTION	PASSAGE NO.
15.Feb.8o	received (a)	o
19.Feb.8o	splitted	1
24.Feb.8o	splitted	2
25.Feb.8o	splitted, frozen	3
17.Mar.8o	thawed	3
2o.Mar.8o	splitted	4
24.Mar.8o	splitted (b)	5
28.Mar.8o	splitted (b)	6
1.Apr.8o	splitted (b)	7
1o.Apr.8o	splitted	9
14.Apr.8o	splitted	1o
18.Apr.8o	splitted	11
22.Apr.8o	splitted	12
28.Apr.8o	splitted	13
2.May 8o	splitted	14
7.May 8o	splitted	15
12.May 8o	splitted	16
16.May 8o	splitted	17
2o.May 8o	splitted	18
23.May 8o	splitted, frozen	19
3.Jan.82	thawed	19
5.Jan.82	medium change	19

TABLE F

HISTORY OF CELLS, WILD TYPE

(a) Culture received from Dr. D. Wild, Zentrallaboratorium für Mutagenitätsprüfung der Deutschen Forschungsgemeinschaft, Freiburg. This passage was numbered o.
(b) cultures treated with amphotericin B

DATE	ACTION	PASSAGE NO.
29.Apr.8o	cloned (a)	o
7.May 8o	splitted	1
12.May 8o	splitted	2
16.May 8o	splitted	3
2o.May 8o	splitted, frozen	4
3.Jan.82	thawed	4
5.Jan.82	medium change	4

TABLE G

HISTORY OF CELLS, MUTANT

(a) Mutants were induced and cloned from V79 wild type passage no. 9. Detailed information about mutant induction and mutant cloning are given in the report A -/3o65: Metabolic cooperation test, report in preparation

inverted microscope D,
objectives: Ph2 Plan 16, Ph2 Plan 25,
microscope KM,
objective: Ph2 Plan 16,
hemocytometer chamber according to
Neubauer, no. 4229o3,
Carl Zeiss,
D-7o82 Oberkochen

hood: type Biogard Baker, no. B 6o-112,
Labotect,
D-34o6 Bovenden

Chemicals and reagents

Chemicals:

Dulbecco's Modification of Eagle's
Medium (DMEM), no. 12-332-54,
fetal bovine serum, filter steri-
lized, mycoplasma and virus screened,
no. 29-1o1-54 (Lot no. (a)),
MEM 5o-fold amino acids (AA),
no. 16-o11-49,
MEM 1oo-fold non-essential amino
acids (NEAA), no. 16-81o-49,
trypsin EDTA, no. 16-891-49,
phosphate-buffered saline (PBS),
no. 18-6o4-49,
sodium pyruvate, no. 16-82o-49,
Flow Laboratories GmbH,
D-53o9 Meckenheim

glutamine, no. 22942,
streptomycin, no. 355oo,
penicillin, no. 31749,
Serva Feinbiochemica GmbH und Co. KG,
D-69oo Heidelberg 1

sodium chloride, no. 64oo,
E. Merck,
D-61oo Darmstadt 1

Trypan Blue, no. 1B187,
Chroma Gesellschaft Schmidt und Co.,
D-7ooo Stuttgart-Untertürkheim

Reagents:

cell culture medium:
DMEM with
4 mmol/l glutamine,
1x1oE5 U pencillin/l,
1o mg streptomycin/l,

(a) will be given in the report

1o ml NEAA/l,
2o ml AA/l,
1 mmol/l sodium pyruvate and
3o g/l fetal bovine serum,
final pH: 7.4,
final osmolality: 33o mmol/kg

Trypan Blue solution:
5 g Trypan Blue/l saline

Procedure:

handling under sterile conditions!

medium taken off, monolayer washed 1 time with cold trypsin (5 ml), addition of 5 ml cold trypsin, incubation for 5 min in incubator, detachment of the cells from the flask bottom by mechanical shock, addition of 5 ml medium to stop trypsinization, cell suspension transferred into a centrifuge tube, flask washed 1 time with 5 ml medium, washing solution transferred into centrifuge tube, centrifugation at 49o5 m/s2 (= 5oo x g) for 5 min, supernatant discarded and pelleted cells resuspended in an appropriate volume (5 to 1o ml) medium

sample of the cell suspension mixed with equal volume of Trypan Blue solution in a micro vial, cells counted after 5 min of incubation at o degrees centigrade, unstained cells: viable, stained cells: dead, cells in 4-mm3 squares are counted. seeding of 3 to 5x1oE4 cells per culture vial

Calculation:

number of viable cells/ml =

$$\frac{\text{counted viable cells x 1oE4 x 2}}{4}$$

viability (o/o) =

$$\frac{\text{counted viable cells x 1oo}}{\text{counted viable and dead cells}}$$

Scientific version: 198o
Text version: 1.Feb.83

5.5 Metabolic Cooperation Assay (MC Assay)

Principle:	determination of the number of surviving colonies in a coculture of Chinese hamster V79 wild type and mutant (HPGRT-) cells in the presence of 6-thioguanine
Time	
Sampling:	-
Determination:	8 days after cell seeding (see TABLES H and I)
Sample material and quantity:	monocultures of V79 mutant cells and cocultures of V79 wild type and mutant cells: 1o cultures each per dose
Results expressed in:	recovery (o/o)
Equipment:	culture vials: tissue culture dish, diameter 6 cm, no. 3oo2, Falcon, Div. Becton and Dickinson GmbH, D-69oo Heidelberg-Wieblingen
	incubator: Cytoperm 8o8o/81oo124, carbon dioxide, humidity and temperature regulation, W. C. Heraeus GmbH, D-645o Hanau
	inverted microscope D, objectives: Ph2 Plan, 16, Ph2 Plan 25, microscope KM, objective: Ph2 Plan 16, hemocytometer chamber according to Neubauer, no. 4229o3, Carl Zeiss, D-7o82 Oberkochen
	hood: type Biogard Baker, no. 6o-112, Labotect, D-34o6 Bovenden

DAY	ACTION
1	seeding, medium change, start of exposure
2	exposure, proliferation
3	" , "
4	medium change, proliferation
5	proliferation
6	"
7	"
8	staining

TABLE H

CHRONOLOGICAL TABLE, MC AND CYTOTOXICITY ASSAYS

Remarks: start for cytotoxicity assay: 28.Jan.83
start for MC assay, 1st part: 8.Feb.83
start for MC assay, 2nd part: 7.Mar.83

GROUP	CULTURE NO. (a)	SUBSTANCE, CONCENTRATION (mg/l)	CELL TYPE SEEDED WILD TYPE	CELL TYPE SEEDED MUTANT
o-GR	o.1 to o.1o	-		
	o.11 to o.2o	-		
1-GR	1.1 to 1.1o	DMSO	+	+
	1.11 to 1.2o	"	-	+
2-GR	2.1 to 2.1o	phorbol, o.58	+	+
	2.11 to 2.2o	"	-	+
3-GR	3.1 to 3.1o	TPA, o.oo1	+	+
	3.11 to 3.2o	"	-	+
4-GR	4.1 to 4.1o	TPA, o.o1	+	+
	4.11 to 4.2o	"	-	+
5-GR	5.1 to 5.1o	TPA, o.1	+	+
	5.11 to 5.2o	"	-	+
6-GR	6.1 to 6.1o	TPA, 1	+	+
	6.11 to 6.2o	"	-	+
7-GR	7.1 to 7.1o	condensate, o.oo1	+	+
	7.11 to 7.2o	"	-	+
8-GR	8.1 to 8.1o	condensate, o.o1	+	+
	8.11 to 8.2o	"	-	+
9-GR	9.1 to 9.1o	condensate, o.1	+	+
	9.11 to 9.2o	"	-	+
1o-GR	1o.1 to 1o.1o	condensate, 1	+	+
	1o.11 to 1o.2o	"	-	+

TABLE I

GROUPS AND DOSES, MC ASSAY

(a) no. .1 to .5 and .11 to .15: assay 1
no. .6 to .1o and .16 to .2o: assay 2

GROUP	CULTURE NO. (a)	SUBSTANCE, CONCENTRATION (mg/l)	CELL TYPE SEEDED	
			WILD TYPE	MUTANT
11-GR	11.1 to 11.1o	phenol, o.o1	+	+
	11.11 to 11.2o	"	-	+
12-GR	12.1 to 12.1o	phenol, o.1	+	+
	12.11 to 12.2o	"	-	+
13-GR	13.1 to 13.1o	phenol, 1	+	+
	13.11 to 13.2o	"	-	+
14-GR	14.1 to 14.1o	phenol, 1o	+	+
	14.11 to 14.2o	"	-	+
15-GR	15.1 to 15.1o	catechol, o.o1	+	+
	15.11 to 15.2o	"	-	+
16-GR	16.1 to 16.1o	catechol, o.1	+	+
	16.11 to 16.2o	"	-	+
17-GR	17.1 to 17.1o	catechol, 1	+	+
	17.11 to 17.2o	"	-	+
18-GR	18.1 to 18.1o	catechol, 1o	+	+
	18.11 to 18.2o	"	-	+

TABLE I (continued)

GROUPS AND DOSES, MC ASSAY

(a) no. .1 to .5 and .11 to .15: assay 1
no. .6 to .1o and .16 to .2o: assay 2

Chemicals and reagents

Chemicals:

Dulbecco's Modification of Eagle's Medium (DMEM), no. 12-332-54,
fetal bovine serum, filter sterilized, mycoplasma and virus screened, no. 29-1o1-54 (Lot no. (a)),
MEM 5o-fold amino acid (AA), no. 16-o11-49,
MEM 1oo-fold non-essential amino acids (NEAA), no. 16-81o-49,
trypsin EDTA, no. 16-891-49,
phosphate-buffered saline (PBS), no. 18-6o4-49,
sodium pyruvate, no. 16-82o-49,
hypoxanthine aminopterine thymidine (HAT) 5o-fold concentrate, no. 73-157-49,
Flow Laboratories GmbH,
D-53o9 Meckenheim

glutamine, no. 22942,
streptomycin, no. 355oo,
penicillin, no. 31749,
Serva Feinbiochemica GmbH und Co. KG,
D-69oo Heidelberg 1

6-thioguanine (6-TG), no. A 4882,
Sigma Chemie GmbH,
D-8o28 Taufkirchen

DMSO, no. 295o,
methanol, no. 6oo9,
Giemsa, no. 92o4,
E. Merck,
D-61oo Darmstadt 1

Reagents:

cell culture medium:
DMEM with
4 mmol/l glutamine,
1x1oE5 U pencillin/l,
1o mg streptomycin/l,
1o ml NEAA/1,
2o ml AA/1,
1 mmol/l sodium pyruvate and
3o g/l fetal bovine serum,
final pH: 7.4,
final osmolality: 33o mmol/kg

6-TG-selection medium:
cell culture medium plus 1o mg/l 6-thioguanine

(a) will be given in the report

hypoxanthine aminopterine thymidine (HAT) medium:
cell culture medium plus 2o ml/l HAT 5o-fold concentrate

fixative:
37 ml formaldehyde/l PBS

Procedure:

seeding of cells in culture medium:
5 ml medium/dish, monoculture mutant: 1x1oE2 mutant cells/dish, coculture wild type and mutant, 4x1oE5 wild type plus 1x1oE2 mutant cells/dish

medium change and start of treatment:
3 h after cell seeding microscopic control of cell attachment

aspiration of culture medium and addition of 6-TG-selection medium plus test substance

expression period:
on day 4 aspiration of 6-TG-selection medium plus test substance and addition of fresh 6-TG-selection medium

staining:
on day 8 aspiration of medium,
fixation of colonies with methanol, 3 ml/dish for 5 min,
staining of colonies with Giemsa, 1 ml/dish for 2 min,
rinsing of colonies: 1 time with bi-distilled water, 3 times with tap water

evaluation:
counting of colonies visually

recovery (o/o) = CC/CM x 1oo

CC: number of colonies coculture
CM: number of colonies monoculture mutant

Scientific version: - (a)
Text version: 1.Feb.83

(a) will be given in the report

5.6 Cytotoxicity Assay

Principle:	determination of the colony forming ability of treated cells as compared to the colony forming ability of untreated cells
Time	
Sampling:	-
Determination:	8 days after cell seeding (see TABLE J)
Sample material and quantity:	monocultures of V79 wild type and V79 mutant cells respectively, 3 cultures each per dose, 1x1oE2 cells per culture
Results expressed in:	survival (o/o)
Equipment:	culture vials: tissue culture dish, diameter 6 cm, no. 3oo2, Falcon, Div. Becton and Dickinson GmbH, D-69oo Heidelberg-Wieblingen incubator: Cytoperm 8o8o/81oo124, carbon dioxide, humidity and temperature regulation, W. C. Heraeus GmbH, D-645o Hanau inverted microscope D, objectives: Ph2 Plan, 16, Ph2 Plan 25, Carl Zeiss, D-7o82 Oberkochen hood: type Biogard Baker, no. B 6o-112, Labotect, D-34o6 Bovenden

GROUP	CULTURE NO.	SUBSTANCE, CONCENTRATION (mg/l)	CELL TYPE	
			WILD TYPE	MUTANT
o-GR	o.21 to o.23	-	+	-
	o.24 to o.26	-	-	+
1-GR	1.21 to 1.23	DMSO	+	-
	1.24 to 1.26	"	-	+
2-GR	2.21 to 2.33	phorbol	+	-
	2.24 to 2.26	"	-	+
3-GR	3.21 to 3.23	TPA, o.oo1	+	-
	3.24 to 3.26	"	-	+
4-GR	4.21 to 4.23	TPA, o.o1	+	-
	4.24 to 4.26	"	-	+
5-GR	5.21 to 5.23	TPA, o.1	+	-
	5.24 to 5.26	"	-	+
6-GR	6.21 to 6.23	TPA, 1	+	-
	6.24 to 6.26	"	-	+
7-GR	7.21 to 7.23	condensate, o.oo1	+	-
	7.24 to 7.26	"	-	+
8-GR	8.21 to 8.23	condensate, o.o1	+	-
	8.24 to 8.26	"	-	+
9-GR	9.21 to 9.23	condensate, o.1	+	-
	9.24 to 9.26	"	-	+
1o.1-GR	1o.1.21 to 1o.1.23	condensate, 1	+	-
	1o.1.24 to 1o.1.26	"	-	+
1o.2-GR	1o.2.21 to 1o.2.23	condensate, 1o	+	-
	1o.2.24 to 1o.2.26	"	-	+

TABLE J

GROUPS AND DOSES, CYTOTOXICITY ASSAY

GROUP	CULTURE NO.	SUBSTANCE, CONCENTRATION (mg/l)	CELL TYPE	
			WILD TYPE	MUTANT
11-GR	11.21 to 11.23	phenol, o.o1	+	-
	11.24 to 11.26	"	-	+
12-GR	12.21 to 12.23	phenol, o.1	+	-
	12.24 to 12.26	"	-	+
13-GR	13.21 to 13.23	phenol, 1	+	-
	13.24 to 13.26	"	-	+
14-GR	14.21 to 14.23	phenol, 1o	+	-
	14.24 to 14.26	"	-	+
15-GR	15.21 to 15.23	catechol, o.o1	+	-
	15.24 to 15.26	"	-	+
16-GR	16.21 to 16.23	catechol, o.1	+	-
	16.24 to 16.26	"	-	+
17-GR	17.21 to 17.23	catechol, 1	+	-
	17.24 to 17.26	"	-	+
18-GR	18.21 to 18.23	catechol, 1o	+	-
	18.24 to 18.26	"	-	+

TABLE J (continued)

GROUPS AND DOSES, CYTOTOXICITY ASSAY

Chemicals and reagents

Chemicals:

Dulbecco's Modification of Eagle's Medium (DMEM), no. 12-332-54,
fetal bovine serum, filter sterilized, mycoplasma and virus screened, no. 29-1o1-54 (Lot no. (a)),
MEM 5o-fold amino acid (AA), no. 16-o11-49,
MEM 1oo-fold non-essential amino acids (NEAA), no. 16-81o-49,
trypsin EDTA, no. 16-891-49,
phosphate-buffered saline (PBS), no. 18-6o4-49,
sodium pyruvate, no. 16-82o-49,
Flow Laboratories GmbH,
D-53o9 Meckenheim

glutamine, no. 22942,
streptomycin, no. 355oo,
penicillin, no. 31749,
Serva Feinbiochemica GmbH und Co. KG,
D-69oo Heidelberg 1

DMSO, no. 295o,
methanol, no. 6oo9,
Giemsa, no. 92o4,
E. Merck,
D-61oo Darmstadt

Reagents:

cell culture medium:
DMEM with
4 mmol/l glutamine,
1x1oE5 U pencillin/l,
1o mg streptomycin/l,
1o ml NEAA/l,
2o ml AA/l,
1 mmol/l sodium pyruvate and
3o g/l fetal bovine serum,
final pH: 7.4,
final osmolality: 33o mmol/kg

Trypan Blue solution:
5 g Trypan Blue/l saline

(a) will be given in the report

Procedure:

seeding of cells in culture medium:
5 ml medium/dish, monoculture mutant:
1x1oE2 mutant cells/dish, monoculture
wild type 1x1oE2 wild type cells/dish

medium change and start of treatment:
3 h after cell seeding microscopic
control of cell attachment

aspiration of culture medium and
addition of medium plus test substance

expression period:
on day 4 aspiration of medium plus
test substance and addition of fresh
medium

staining:
on day 8 aspiration of medium,
fixation of colonies with methanol,
3 ml/dish for 5 min,
staining of colonies with Giemsa,
1 ml/dish for 2 min,
rinsing of colonies 1 time with
bidistilled water, 3 times with tap
water

evaluation:
counting of colonies visually

survival (o/o) = CT/CU x 1oo

CT: number of colonies, treated
culture
CU: number of colonies, untreated
culture

Scientific version: - (a)
Text version: 1.Feb.83

(a) will be given in the report

5.7 Determination of Population Doubling Time, Growth Curve

Principle:	determination of the number of cells on 1o subsequent days
Time	
Sampling:	-
Determination:	on 1o subsequent days
Sample material and quantity:	wild type and V79 mutant cells, dependent on the day of determination
Results expressed in:	h
Equipment:	centrifuge tubes: no. 2o7o, conical graduated, polypropylene, diameter: 3o mm, length: 115 mm, Falcon, Div. Becton and Dickinson GmbH, D-69oo Heidelberg-Wieblingen
	culture vials: tissue culture flask, growth area: 25 cm2, no. 163371, Nunc GmbH, D-62oo Wiesbaden
	micro vials: type "Eppendorf", polypropylene, no. 381o, Netheler und Hinz GmbH, D-2ooo Hamburg 65
	centrifuge: Digifuge GL, rotor: o335o, Heraeus-Christ GmbH, D-336o Osterode
	incubator: Cytoperm 8o8o/81oo124, carbon dioxide, humidity and temperature regulation, W. C. Heraeus GmbH, D-645o Hanau

inverted microscope D,
objectives: Ph2 Plan 16, Ph2 Plan 25,
microscope KM,
objective: Ph2 Plan 16,
hemocytometer chamber according to
Neubauer, no. 4229o3,
Carl Zeiss,
D-7o82 Oberkochen

hood: type Biogard Baker, no. B 6o-112,
Labotect,
D-34o6 Bovenden

Chemicals and reagents

Chemicals:

Dulbecco's Modification of Eagle's
Medium (DMEM), no. 12-332-54,
fetal bovine serum, filter steri-
lized, mycoplasma and virus screened,
no. 29-1o1-54 (Lot no. (a)),
MEM 5o-fold amino acids (AA),
no. 16-o11-49,
MEM 1oo-fold non-essential amino
acids (NEAA), no. 16-81o-49,
trypsin EDTA, no. 16-891-49,
phosphate-buffered saline (PBS),
no. 18-6o4-49,
sodium pyruvate, no. 16-82o-49,
Flow Laboratories GmbH,
D-53o9 Meckenheim

glutamine, no. 22942,
streptomycin, no. 355oo,
penicillin, no. 31749,
Serva Feinbiochemica GmbH und Co. KG,
D-69oo Heidelberg 1

sodium chloride, no. 64oo,
E. Merck,
D-61oo Darmstadt 1

Trypan Blue, no. 1B187,
Chroma Gesellschaft Schmidt und Co.,
D-7ooo Stuttgart-Untertürkheim

Reagents:

cell culture medium:
DMEM with
4 mmol/l glutamine,
1x1oE5 U pencillin/l,
1o mg streptomycin/l,

(a) will be given in the report

1o ml NEAA/l,
2o ml AA/l,
1 mmol/l sodium pyruvate and
3o g/l fetal bovine serum,
final pH: 7.4,
final osmolality: 33o mmol/kg

Trypan Blue solution:
5 g Trypan Blue/l saline

Procedure:

handling under sterile conditions

day 1:
2o culture flasks each for wild type and mutant cells filled with 5 ml medium, seeding of 3x1oE4 wild type and mutant cells respectively per flask

incubation at 37 degrees centigrade

days 2 to 11:
splitting and determination of the number of cells of 2 wild type and mutant cultures respectively

(1) splitting:
medium taken off, monolayer washed 1 time with cold trypsin (3 ml), addition of 3 ml cold trypsin, incubation for 5 min at 37 degrees centigrade, detachment of the cells from the flask bottom by mechanical shock, addition of 3 ml medium to stop trypsinization, cell suspension transferred into a centrifuge tube, flask washed 1 time with 5 ml medium, washing solution transferred into centrifuge tube, centrifugation at 49o5 m/s2 (= 5oo x g) for 5 min, supernatant discarded and pelleted cells resuspended in an appropriate volume (5 to 1o ml) medium

(2) determination of the number of cells:
sample of the cell suspension mixed with equal volume of Trypan Blue solution in a micro vial, cells counted after 5 min of incubation at o degrees centigrade,

unstained cells: viable,
stained cells: dead,
cells in 4-mm3 squares are counted.

days 4 and 8:
aspiration of medium and addition of 5 ml fresh medium

Calculation:

number of viable cells/ml =

$$\frac{\text{counted viable cells} \times 1oE4 \times 2}{4}$$

number of viable cells/culture vial = number of cells/ml x volume of cell suspension

population doubling time (T)

$$T = \frac{t}{n}$$

t : time between 2 determinations of the number of cells
n : number of cell division in t, n = 3.32 (log N2 - log N1)
N1: number of cells at the 1st determination of the number of cells
N2: number of cells at the relative to the 1st determination subsequent determination of the number of cells

Scientific version:
Text version:

- (a)
1.Feb.83

(a) will be given in the report

6 STORAGE OF MATERIALS AND RECORDS

Remains of substances examined, protocols and records are stored in our archives for at least 5 years, they can be claimed by the client. Stained colonies are stored at least 3 months after the delivery of the report.

7 REPORTING

Report language: English

Report concept: The report will contain the following parts:

1 SUMMARY
2 RESPONSIBILITY
3 INTRODUCTION
4 SUBSTANCES EXAMINED
5 METHOD
6 STORAGE OF MATERIALS AND RECORDS
7 RESULTS AND DISCUSSION
8 REFERENCES

8 REFERENCES

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9 FORMS

TITLE	TEXT VERSION	IDENTIFICATION (a)
Zellsplit	1. KW 83	A
Metabolic cooperation/Zytotoxizität, Monokultur	2. KW 83	B
Metabolic cooperation, Mischkultur	2. KW 83	C
Colony counting	2. KW 83	D

TABLE K

TITLE, TEXT VERSION AND IDENTIFICATION OF FORMS

(a) arbitrary identification for this proposal

ZELLSPLIT

A /

Zelltyp: Medium: Durchführende(r) (ZN):

KULTUR NR. DATUM	NEUE PASSAGE	AUFGENOMMEN IN ..... ml	ZELLEN GEZÄHLT LEBEND/TOT	ZELLZAHL, LEBEND $\bar{x}$/ml	$\bar{x}$/gesamt	VIABILITY (o/o)	VERDÜN-NUNG	ZELL-MENGE (ml)	ZELL-ZAHL IM KOLBEN	ANZAHL DER KOLBEN

BEMERKUNGEN:

FORM A

DATUM/ZN

METABOLIC COOPERATION/ZYTOTOXIZITÄT (a), MONOKULTUR	A /

Zelltyp:

Zellernte

- Kulturgefässe
  - Art
  - Anzahl
  - Volumen (ml)
- Zellzahl
  - gezählt, lebend/tot
  - lebend/ml
  - lebend gesamt

Zellaussaat

- Kulturgefässe
  - Art
  - Anzahl
- benötigte Zellzahl/Kulturgefäss
- benötigte Zellzahl gesamt
- Verdünnungen (ml)
  - (1) +
  - (2) +
  - (3) +
  - (4) +
- Zellzahl/ml nach Verdünnung
- benötigte Zellsuspension (ml)/ Kulturgefäss
- benötigte Zellsuspension gesamt (ml)
- angesetzte Zellsuspension (ml)

Bemerkungen

QA 13. JAN. 1993

FORM B

(a) Nichtzutreffendes durchstreichen

DATUM/SN

METABOLIC COOPERATION, MISCHKULTUR	A /

Zellaussaat

Kulturgefässe
Art

Anzahl

PARAMETER	WILDTYP	MUTANTE
benötigte Zellzahl/Kulturgefäss		
benötigte Zellzahl gesamt		
Zellzahl/ml nach Verdünnung		
benötigte Zellsuspension (ml)/ Kulturgefäss		
benötigte Zellsuspension gesamt (ml)		
angesetzte Zellsuspension gesamt (ml)		
eingegebene Zellsuspension (ml)/ Kulturgefäss		

Bemerkungen

QA 13. JAN. 1983

FORM C

DATUM/ZI

0042555104

COLONY COUNTING

A /

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

GROUP:

CULTURE NO.	NUMBER OF COLONIES

M

SE

RSD (o/o)

Bemerkungen

QA 13. JAN. 1983

2062555105

FORM D

END OF PROPOSAL

DATUM/ZN

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
adrenaline	not reported	o	Umeda et al., 198o
aldrin	2.5 to 2o	+ (at doses .GT.5 mg/l)	Kurata et al., 1982
anthralin	o.1	o	Kinsella, 1982
	o.o5	+	Trosko et al., 1982
	o.o5 to o.5	(+)	Umeda et al., 198o
benzo(a)anthracene	1	o	Newbold and Amos, 1981
benzo(a)pyrene	1	o	"
benzo(e)pyrene	1	o	"
alpha-benzene hexachloride	5 to 4o	(+)	Kurata et al., 1982
gamma-benzene hexachloride	2.5 to 4o	(+)	"
beta-benzene hexachloride	8o to 64o	-	"

TABLE

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
caffeine	not reported	o	Umeda et al., 1980
catechol	0.0005 to 0.5 .GT.1	(+) ++	INBIFO Dr. J.C. Charles, personal communication
chaetoglobosin A	0.01 to 1	+ (a)	Umeda et al., 1980
chenodeoxycholic acid	0.5 to 5	(+)	Noda et al., 1981
cholic acid	20 to 200	o	"
cigarette smoke condensate			
neutral fraction, 2R1-CSC	?	++	Dr. J.C. Charles, personal communication
basic fraction, 2R1-CSC	?	++	"
2R1-MWSC	0.001 to 1	(+)	INBIFO, 1983
concanavalin A	10 to 100	++ (a)	Umeda et al., 1980

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
danthron	not reported	o	Umeda et al., 198o
DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane)	2 to 1o	++ (a)	Kurata et al., 1982
o,p'-DDT(1,1,1-trichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl)ethane)	1.25 to 2o	+ (at doses .GT.5 mg/l)	"
dehydrocholic acid	1o to 1oo	o	Noda et al., 1981
deoxycholic acid	5 to 5o	o	"
dieldrin	2.5 to 2o	+	Kurata et al., 1982
diethylstilboestrol	5	o	Kinsella, 1982
7,12-dimethylbenzo(a)-anthracene	1 o.5 to 5	o o	Newbold and Amos, 1981 Noda et al., 1981

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
2,4-dinitrofluorobenzene	1.86 to 93x1oE-3	+ (a)	Warren et al., 1982
dispase	not reported	o	Umeda et al., 198o
disodium EDTA	"	o	"
elastinal	"	o	"
endrin	2.5 to 2o	+ (at doses .GT.5 mg/l)	Kurata et al., 1982
estradiol	o.5 to 5	o	Umeda et al., 1982
griseofulvin	5	o	Kinsella, 1982
delta-haemolysin	1, 2	o	"
heptachlor	2.5 to 2o	(+)	Kurata et al., 1982
hydroquinone	.GT.1	++	Dr. J.C. Charles, personal communication
iodoacetic acid	o.1	o	Kinsella, 1982
kepone	1 to 4	+ (a)	Tsushimoto et al., 1982 a

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
lithocholic acid	2 to 2o	++	Noda et al., 1981
	5 to 5o	++ (a)	Umeda et al., 198o
methoxychlor (1,1,1-trichloro-2,2-bis(p-metaoxyphenyl)ethane)	1.25 to 2o	+ .GT.5 mg/l	Kurata et al., 1982
n-methyl-n-nitro-n-nitrosoguanidine	not reported	o	Noda et al., 1981
4-o-methylphorbol-12-myristate-13-acetate	not reported	o	Yotti et al., 1979
	o.6x1oE-3	o	Newbold and Amos, 1981
mezerein	o.7x1oE-3	(+)	Newbold and Amos, 1981
mineral dusts:			
ball-milled amosite	o.o1 to 5	o	Chamberlain, 1982
min-U-sil silica	5 to 5o	o	"
UICC amosite	o.o1 to 5	o	"
mirex	1 to 15	+ (at doses .GT.12mg/l)	Tsushimoto et al., 1982 a

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
4-nitroquinoline 1-oxide	not reported	o	Noda et al., 1981
oleic acid	o.1, 1	o	Kinsella, 1982
	3.o to 1o	+ (a)	Trosko et al., 1982
pepstatin	not reported	o	Umeda et al., 198o
phenol	?	o	Dr. J.C. Charles, personal communication
	o.oo5 to 5	o	INBIFO, 1983
phenylhydroquinone	not reported	o	Noda et al., 1981
o-phenylphenol	not reported	o	"
phorbol	not reported	o	Yotti et al., 1979
	o.4x1oE-3	o	Newbold and Amos, 1981
	o.58	o	INBIFO, 1983
4 alpha phorbol-12,13-didecanoate	o.7x1oE-3	o	Newbold and Amos, 1981
4 alpha phorbol-12,13-didecanoate	not reported	o	Yotti et al., 1979
phorbol-13,2o-diacetate	o.5x1oE-3	o	Newbold and Amos, 1981

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
phorbol-12,13-diacetate	not reported	o	Yotti et al., 1979
phorbol-12,13-dibutyrate	not reported	+	"
phorbol-12,13-didecanoate	"	++	"
phorbol-12-myristate-13-acetate (TPA)	"	+++	"
	o.oo1 to 1x1oE-3	+++ (a) up to 1 ug/l	Umeda et al., 198o
	o.6x1oE-3	+++	Newbold and Amos, 1981
	o.1	+++	Kinsella, 1982
	o.oo4	++	Trosko et al., 1982
	o.1	+++	Trosko et al., 198o
	o.o1	+++	Chamberlain, 1982
	o.oo1 to 1	+++	INBIFO, 1983
phytohemaglutinin-P	o.o1 to o.1 (b)	o	Noda et al., 1981
polybrominated biphenyl	2.5 to 1o	+ (at doses .GT.5 mg/l)	Trosko et al., 1981
2,4,5,2',4',5'-hexa-bromobiphenyl	1 to 1o	++ (a)	Tsushimoto et al., 1982 b

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
3,4,5,3',4',5'-hexa-bromobiphenyl	1 to 1o	o	Tsushimoto et al., 1982 b
2,3,4,5, 2',4',5'-hepta-bromobiphenyl	1 to 1o	+ (a)	"
2,3,4,5,2',3',4',5'-octa-bromobiphenyl	1 to 1o	+ (a)	"
2,4,5,3',4',5'-hexa-bromobiphenyl	1 to 1o	(+)	"
2,4,5,3',4'-penta-bromobiphenyl	1 to 1o	(+)	"
3,4,5,3',5'-penta-bromobiphenyl	1 to 1o	o	"
polymixin B	not reported	o	Umeda et al., 1981
putrescine	1oo to 5oo	o	Noda et al., 1981
saccharin	1oo to 5oo	(+) (at doses .GT.2oo mg/l)	Trosko et al., 198o
	25o to 2ooo	(+)	Umeda et al., 198o
silica see mineral dusts	-	-	-

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: stronqly positive response

(a) dose-dependent response

SUBSTANCE	CONCENTRATION TESTED (mg/l)	IMC	REFERENCE
sodium deoxycholate	1o to 5oo	o	Umeda et al., 198o
sodium phenobarbital	1o to 5oo	o	"
sodium o-phenylphenolate	1.8 to 18x1oE3	o	Noda et al., 1981
spermidine	not reported	o	"
spermine	not reported	o	"
stilboestrol diproprionate	o.1, 1	o	Kinsella, 1982
TDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane)	1.25 to 1o	+ (at doses .GT.5 mg/l)	Kurata et al., 1982
TPA see phorbol-12-myristate-13-acetate			
urea	5oo to 5ooo	+	Umeda et al., 198o
ursodeoxycholic acid	5 to 5o	(+)	Noda et al., 1981

TABLE (continued)

SUBSTANCES TESTED IN THE METABOLIC COOPERATION ASSAY

remarks: IMC: inhibition of metabolic cooperation
o : no IMC
(+): questionable positive response
+ : weakly positive response (= IMC)
++ : positive response
+++: strongly positive response

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