The Society of Chemical Industry

"What A Chemist needs to know about Safety Assessment"

14/15 Belgrave Square, London

Thursday 7th June 2007

SAFETY TESTING OF GENOTOXIC IMPURITIES

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SAFETY TESTING OF GENOTOXIC IMPURITIES

- Genetic Toxicology - background
- Standard tests
- Test performance and SAR
- Current legislation and proposals
  Including Thresholds of Toxicological Concern (TTC)
- Tracing a genotoxic impurity

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"Milestones" in pharmaceutical Regulatory Genetic Toxicity testing

1973, 1975 Ames *et al.*	"Carcinogens are mutagens"
1980	CPMP Guidelines
1981	DHSS Guidelines
1983-6	OECD protocols
1984	EEC Guidelines
1985	2nd IPCS Collaborative Trial (Ashby *et al.*)
1989	Revised DoH Guidelines
1995	ICH Topic S2A
1997	ICH Topic S2B ICH Topic M3
2007	ICH S2 Revision: Step 2 expected October

Difference between genotoxic and non-genotoxic carcinogens realised

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Agents capable of causing direct or indirect damage to DNA

- Electrophilic species forming covalent adducts to DNA
  e.g. alkylating agents
  arylnitrenium ions
  diol epoxides of PAH
  etc
- UV and ionising radiations
- Reactive oxygen species
- Topoisomerase inhibitors
- Nucleoside analogues
- Protein synthesis inhibitors

Types of genetic damage known to be involved in carcinogenesis

Lesion	Detected by test system				Involvement in carcinogenesis	
	Ames	MLA	IVC	BM.MN	Oncogene activation	Suppressor inactivation
Base substitution	Yes	Yes			Yes	Yes
Small deletion	Yes	Yes		Yes	Yes	Yes
Large deletion		Yes	Yes	Yes		Yes
Chromosome translocation		Yes	Yes	Yes	Yes	Yes
Mitotic recombination		Yes			?	Yes
Chromosome loss				Yes		Yes

MLA mouse lymphoma *tk*
IVC in vitro chromosome aberrations
BM.MN rodent bone-marrow micronucleus

REGULATORY REQUIREMENTS FOR PHARMACEUTICALS

ICH Topics:

- **S2A:** Guidance on Specific aspects of Regulatory Tests for Pharmaceuticals
  (Adopted by CPMP September 1995; published in Federal Register April 1996)
- **S2B:** Genotoxicity: a Standard Battery for Testing of Pharmaceuticals
  (Adopted by CPMP September 1997; published in Federal Register November 1997)
- **M3:** Timing of Pre-Clinical Studies in Relation to Clinical Trials
  (Adopted by CPMP September 1996; published in Federal Register November 1997)

http://www.ich.org/cache/compo/276-254-1.html

ICH Topic S2B

3. The Standard Test Battery for Genotoxicity

i) A test for gene mutation in bacteria

ii) An *in vitro* test for cytogenetic evaluation of chromosomal damage with mammalian cells
OR
An *in vitro* mouse lymphoma TK assay

iii) An *in vivo* test for chromosomal damage using rodent haematopoietic cells

ICH Topic M3

7. Genotoxicity studies

Prior to first human exposure, in vitro tests for the evaluation of mutations and chromosome damage are generally needed. If an equivocal or positive finding occurs, additional testing should be performed.

The standard battery of tests for genotoxicity (Topic S2B) should be completed prior to the initiation of Phase II studies

Regulatory interpretation of genotoxicity data

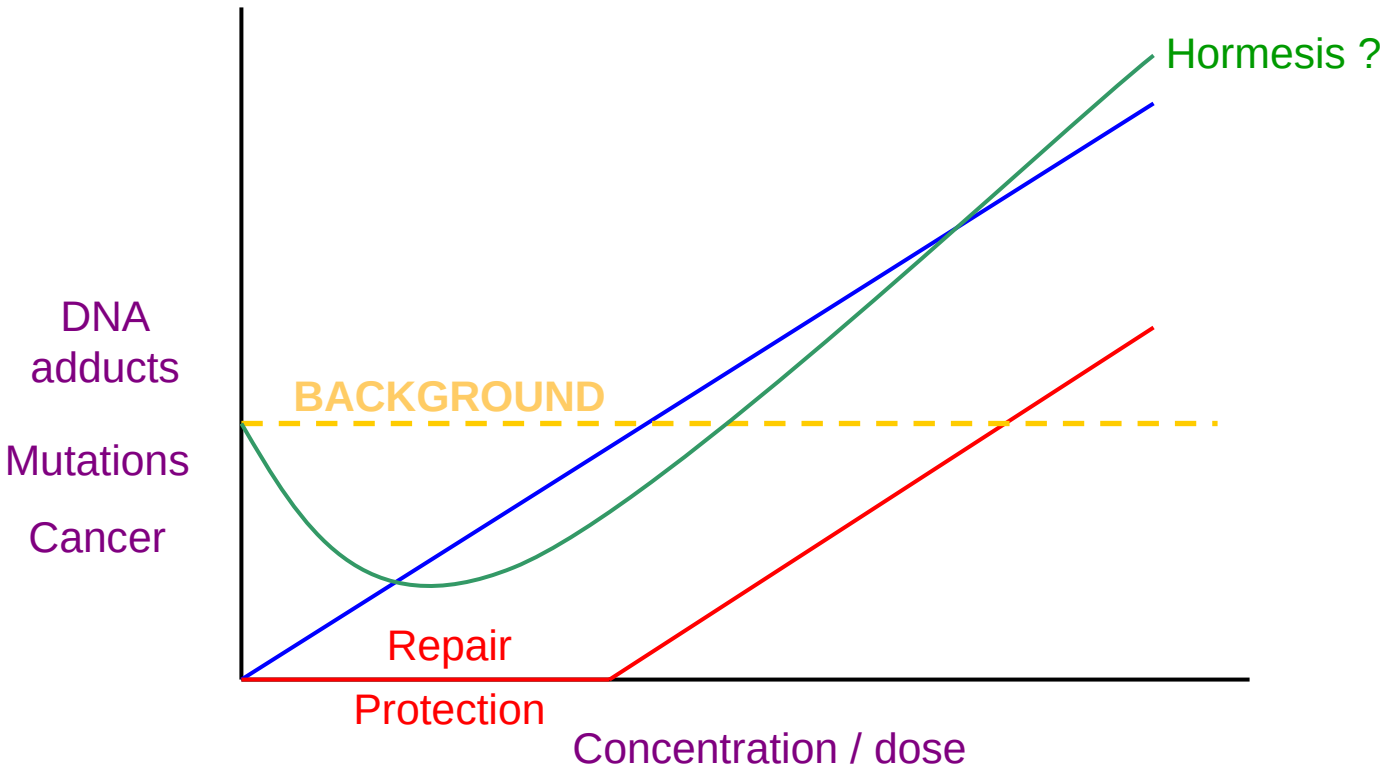
“Thresholds do not exist for DNA-binding chemicals”

- “According to current regulatory practice it is assumed that (in vivo) genotoxic compounds have the potential to damage DNA at any level of exposure and that such damage may lead/contribute to tumor development. Thus for genotoxic carcinogens it is prudent to assume that there is no discernible threshold and that any level of exposure carries a risk.”
  CHMP Guideline on the limits of genotoxic impurities (2006)

- Thresholds are accepted **IF** a mechanism showing lack of direct interaction with DNA can be demonstrated

- **Important to determine whether or not positive results are a consequence of DNA adduct formation**

Regulatory interpretation of genotoxicity data



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Endogenous levels of DNA adducts

		(~6 x10^9 nucleotides/cell)
❑ Oxidative damage	8-oxo-guanine,	1 in 10^5-10^6 nucleotides
❑ Alkylation	O^6-methylguanine,	1 in 10^6-10^7 nucleotides
❑ Lipid peroxidation	Etheno adducts,	1 in 10^7-10^8 nucleotides
❑ Smoking/pollution/diet	Bulky/PAH adducts,	1 in 10^7-10^8 nucleotides

(De Bont & van Larebeke. Mutagenesis 2004; 19: 169-185)

- ~ 20,000 DNA lesions per cell per day

  (Drablos et al. DNA Repair 2004; 3: 1389-1407)

STANDARD TESTS

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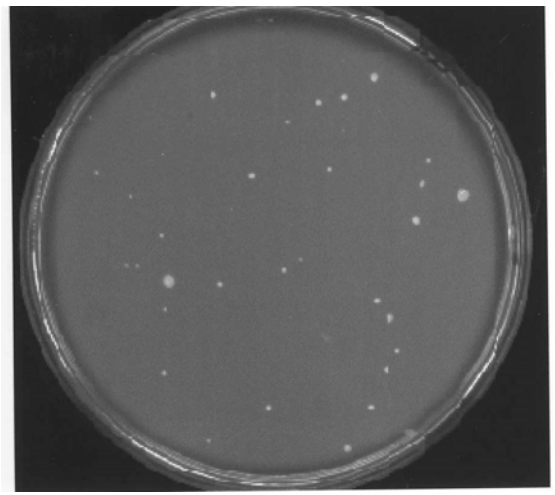
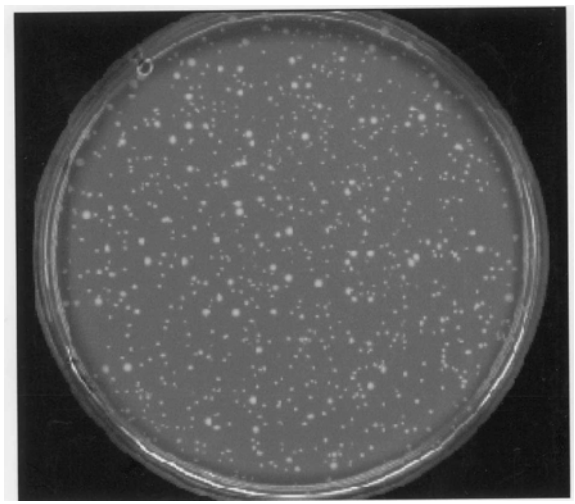
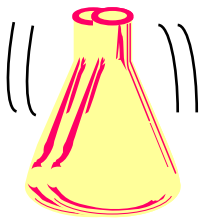
Ames test

- Uses strains of Salmonella typhimurium and Escherichia coli
- Strains have defined mutations making them require specific amino acids
  - S. typhimurium - histidine
  - E. coli - tryptophan
- Back mutation allows growth in medium without histidine or tryptophan

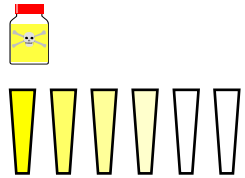




Ames test



Bacteria (2/5 strains) removed from freezer and incubated shaking (37°C) overnight

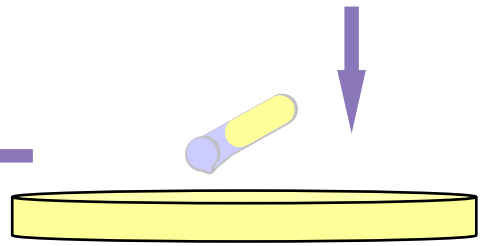


Serial dilution of test compound

&

+/-

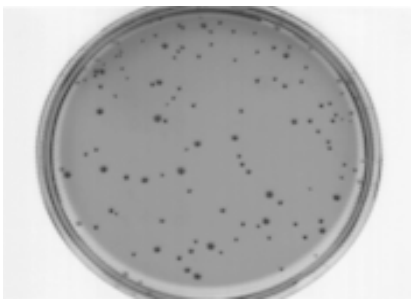
Metabolic activation system (Rat liver S9 fraction and co-factors)



Test compound, S9, top agar and bacteria mixed & poured onto agar plate (trace histidine/tryptophan). Three plates per concentration

INCUBATOR

Plates incubated at 37°C for 3 days



Mutant (revertant) colonies can grow without histidine. Number of colonies per plate counted

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Ames test

- Bacterial strains constructed to detect a range of mutagens
  - Salmonella strains are DNA repair-deficient (uvrB)
  - Increased cell wall permeability
  - Error-prone repair on pKM101 plasmid

Strain	Reversion event
TA1535 & TA100	Base-pair substitution
TA1537 & TA98	Frame-shift
E.coli *uvr*A pKM101	Base-pair substitution Excision-repair proficient (detects cross-linking agents)

Ames test

- OECD Guideline 471

- Highest level tested:
  - determined by solubility
  - determined by toxicity
  - 5000 µg/plate (free acid/base)

- Amount of compound required
  - 80 mg (2-strain screen)
  - 2 g (5-strain regulatory test)

- Strains used (AstraZeneca)
  - Screen TA98 & TA100
  - GLP TA1535, TA1537, TA98, TA100; E.coli *uvr*A pKM101

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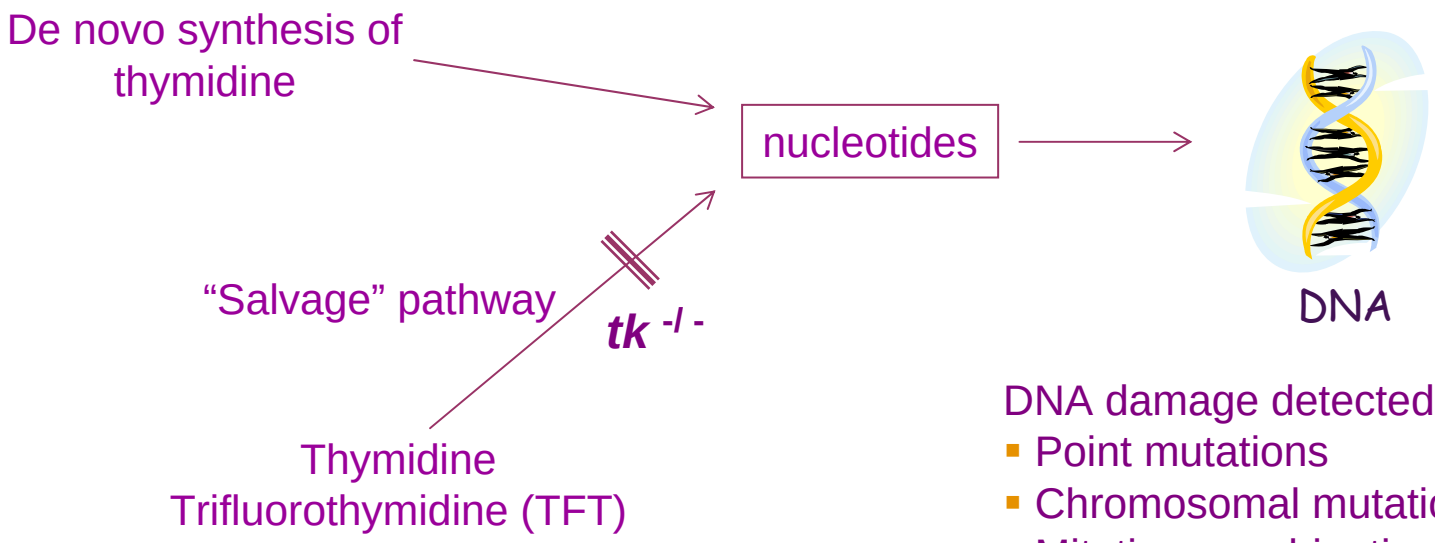
Mouse lymphoma TK assay

- Forward mutation of mouse lymphoma (*tk*$^{+/-}$) cells at the thymidine kinase locus



DNA damage detected:

- Point mutations
- Chromosomal mutations
- Mitotic recombination
- Large DNA deletions

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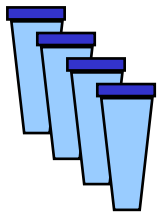
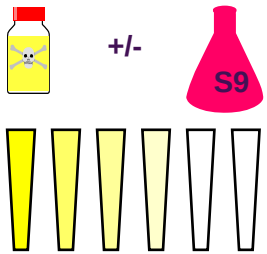
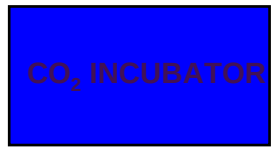
Mouse lymphoma TK assay



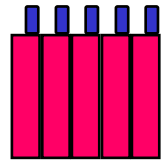
Cells dispensed at $1x10^7$/tube



Serial dilution of test compound prepared & cultures treated -/+ S9

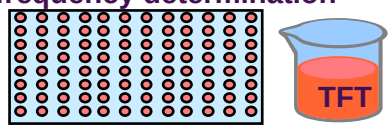


Cultures incubated at 37°C for 3 hours

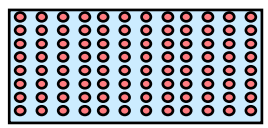


Cultures washed,diluted to $2x10^5$/mL& incubated 24 hr. Cultures counted, subbed to $1.5x10^5$/mL& incubated for another 24 hr

Cultures counted and plated into selective (with TFT) & non-selective medium

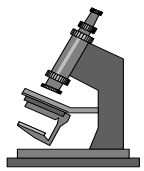


2000 cells/well for mutant frequency determination



1.6 cells/well for cloning efficiency determination

Incubated for 8 days. Wells containing viable clones scored



Incubated for 12 days. Wells containing small & large mutant clones scored

Mutant frequency calculated (number mutants per 10^{-6} viable cells)

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Mouse lymphoma TK assay

- OECD Guideline 476
- Highest concentration tested:
  - determined by solubility
  - determined by toxicity
  - 10 mmol/L or 5000 µg/mL (free base)
- Amount of compound required
  - 200 mg (non-GLP)
  - ~5 g (regulatory)

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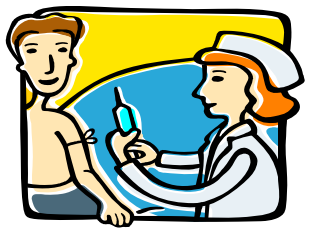
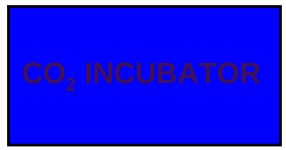
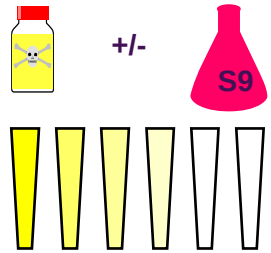
In vitro cytogenetics assay - lymphocytes



Whole blood cultures started in medium + PHA

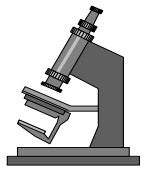


Cultures incubated at 37°C for 48 hours



Serial dilution of test compound prepared & cultures treated 3 hours+/-S9, 20 hours –S9

Add colcemid 18 hours after start of treatment
After 2 hours fix and stain with Giemsa



Score metaphases
a) Mitotic Index
b) Chromosome aberrations
c) Polyploid cells

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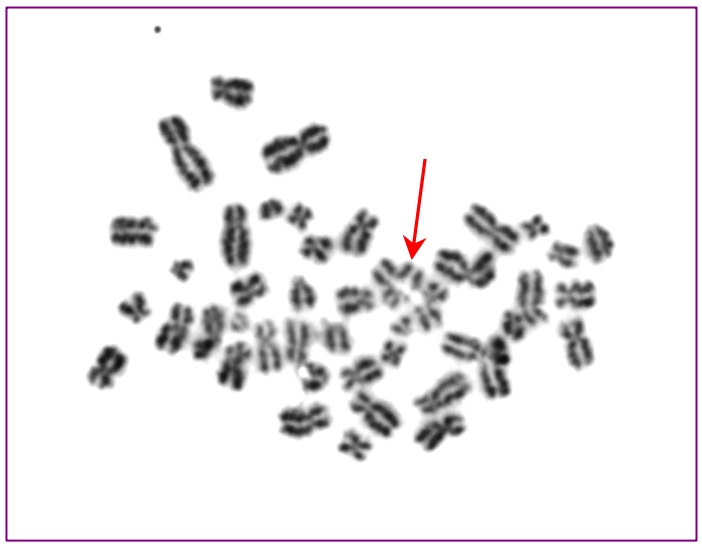
In vitro cytogenetics assay

- OECD Guideline 476
- Highest concentration tested:
  determined by solubility
  determined by toxicity
  10 mmol/L or 5000 µg/mL (free base)
- Detects: large scale DNA damage
  aneugens as increases in ploidy



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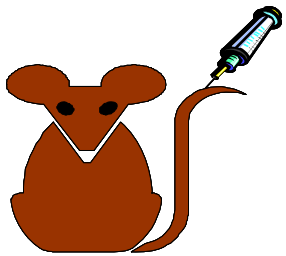
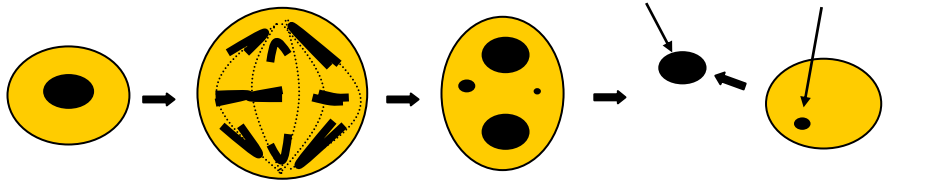
Rodent bone-marrow micronucleus test

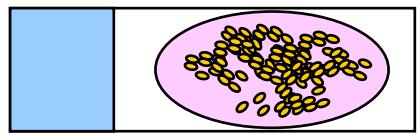




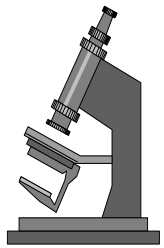
Rats/mice dosed with compound, three doses, seven animals / group. Animals sacrificed 24 or 48 hours later

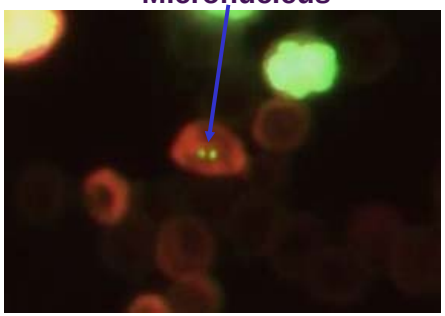
Micronuclei may be formed by loss of whole chromosome during division or by chromosome breakage. The erythrocyte's nucleus is extruded leaving any micronuclei behind

Femurs removed and bone marrow aspirated



Bone marrow cells spread onto slides. Slides fixed and stained (acridine orange)





2000 cells analysed per animal, number of micronucleated immature erythrocytes scored

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Rodent bone-marrow micronucleus test

- OECD Guideline 474
- Highest dose tested:
  2000 mg/kg (free base)
  MTD
- Amount of compound required
  Up to 20 g depending on MTD
- Detects: chromosome breakage
  chromosome loss (aneuploidy)

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TEST PERFORMANCE AND SAR

- First extensive validation reported from Ames laboratory (McCann et al 1975)
  - 90% (157/175) carcinogens positive (sensitivity)
  - 87% (94/108) non-carcinogens negative (specificity)
- Data for 241 chemicals from Sugimura's laboratory (Nagao et al 1978)
  - Sensitivity 85% (136/160)
  - Specificity 74% (60/81)
- European trial – 82 carcinogens, 7 non-carcinogens (Bartsch et al 1980)
  - Sensitivity 76%
  - Specificity 57%

Detection of rodent carcinogens by standard in vitro tests

	Ames	MLA	Ames + MLA
Sensitivity	318 / 541 **59%**	179 / 245 **73%**	389 / 436 **89%**
Specificity	130 / 176 **74%**	41 / 105 **39%**	34 / 105 **32%**

- Sensitivity = proportion of carcinogens giving positive results
- Specificity = proportion on non-carcinogens giving negative results

(Kirkland et al. Mutat Res 2005; 584: 1-256)

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New pharmaceutical compounds submitted to BfArM, 1990 – 1997

Muller and Kasper (2000) Mutat. Res., 464, 19-34

Marketed pharmaceuticals listed in the PDR (1999)

Snyder and Green (2001) Mutat. Res., 488, 151-169

	Number of positive tests			
Test system	**German pharmaceuticals 1990-1997**		**PDR review 1999**	
Bacterial mutation	23/298	8%	27/323	8%
In vitro cytogenetics	77/266	29%	55/222	25%
Mouse lymphoma *tk*	28/104	27%	24/96	25%
In vitro mammalian *hprt*	6/162	4%	2/91	2%
In vivo cytogenetics	19/283	7%	29/252	12%

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AstraZeneca (UK) data

- 5-strain Ames tests — 4 / 110 Positive **(4%)**
- 2-strain Ames tests — 65 / 185 Positive **(35%)**
- Mouse lymphoma TK tests — 25 / 132 Positive **(19%)**

(Data from Alderley Park, 2002 – 2005)

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AstraZeneca SAR predictivity for bacterial mutagenicity

- MCASE – learning set contains >4000 compounds
- DEREK - >80 rules for in vitro mutagenicity
- ANN – in-house dataset accumulating
- Predictivity of Ames test activity for 542 in-house structures
  - Negative predictions negative ~90%
  - Positive predictions positive ~75%

- Only ~5% of new Ames tests are positive
- 10-15% submissions return alerts
- Good filter to prioritise testing of discovery compounds
- Insufficient confidence to drop positive predictions
- Don’t expect better predictivity for other SAR

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Bacterial mutation test

- Ames test a good predictor of DNA reactivity
- SAR is accurate enough to focus testing

Mouse lymphoma TK assay or in vitro cytogenetics

- Has a higher incidence of positive results (20-25%)
- Detects agents inactive in bacterial systems
  - Topoisomerase inhibitors
  - Carbamates
  - Nucleoside analogues

IMPURITIES - CURRENT LEGISLATION AND PROPOSALS

- ICH Q3A (R) – Impurities in New Drug Substances (2002)
- ICH Q3B (R) – Impurities in New Drug Products (2006)
- CHMP Guideline on the Limits of Genotoxic Impurities (2006)
- Further guidance required
  - ICH Q3A/B do not provide sufficient confidence for genotoxic impurities
  - CHMP does not consider compounds in development
- PhRMA rationale (Müller *et al.* 2006)

CHMP Guideline on the Limits of Genotoxic Impurities

- Assumes no thresholds for DNA reactive agents
- Separates potential genotoxic impurities (PGI) into those with and without sufficient experimental evidence for threshold-related mechanisms e.g.
  - spindle disrupters
  - topoisomerase inhibitors
  - inhibitors of DNA synthesis
  - etc
- Impossible to define “safe” exposure to non-threshold genotoxins
- Pragmatic “**Threshold of Toxicological Concern**”

Threshold of Toxicological Concern (TTC) – origins

- 1958 Delaney Clause
  (amendment to 1954 US Federal Food, Drug and Cosmetic Act)
  No food additive can be deemed safe, or given approval, if found to cause cancer in animals or man
- Justification – experts unable to set absolutely safe levels for any carcinogen
- 1980's – improvements in analytical technologies showed quantifiable traces of numerous substances in food
- 1979 – US Court case involving Monsanto over leaching of a polymer from a drinks container allowed FDA to accept a negligible risk level of contamination

Threshold of Toxicological Concern (TTC) – origins

- Original work by Rulis followed by Gold, then Munro
  Low probability that 1ppb in diet will present a lifetime cancer risk >1 in 10^6
- FDA “Threshold of Regulation” for trace substances from food contact materials
  0.5ppb in 3kg food per day ⇛ 1.5 µg/day
- Carcinogenic contaminants justified without change to Delaney Clause
- TTC approach used in US1996 Food Quality Protection Act

CHMP Guideline – TTC

- Linear extrapolation of animal data for 730 carcinogens
- Daily exposure to ≤ **1.5 µg/day** for most carcinogens should not exceed a lifetime cancer risk of **1 in 10^6**
- High potency carcinogens include:
  - Aflatoxins
  - N-nitroso compounds
  - Azoxy compounds
  - **10-fold lower TTC**

Numbers and fractions of compounds in different structural groups that are estimated to give a risk greater than one in a million at different intake levels

Structural Group	0.15 mcg		1.5 mcg		3 mcg		6 mcg		15 mcg		30 mcg		60 mcg		150 mcg		Total
	n	*F*	*n*	*F*	*n*	*F*	*n*	*F*	*n*	*F*	*n*	*F*	*n*	*F*	*n*	*F*	
Aflatoxin-like compounds	**5**	**1**															**5**
Aromatic amines	5	0.03	51	0.31	71	0.44	82	0.51	106	0.65	126	0.78	138	0.85	153	0.94	162
Aromatic nitrates	2	0.06	8	0.24	12	0.36	12	0.36	15	0.45	21	0.64	24	0.73	30	0.91	33
Azo compounds	0	0	9	0.50	9	0.50	10	0.56	12	0.67	14	0.78	16	0.89	17	0.94	18
Azoxy compounds	**4**	**0.80**	**4**	**0.80**	**4**	**0.80**	**5**	**1**									**5**
Benzidine derivatives	2	0.14	6	0.43	8	0.57	9	0.64	10	0.71	12	0.86	12	0.86	13	0.93	14
Carbamates	0	0	8	0.40	8	0.40	10	0.50	15	0.75	17	0.85	18	0.9	19	0.95	20
Heavy metal containing compounds	1	0.14	4	0.57	4	0.57	5	0.71	6	0.86	7	1					7
Highly chlorinated compounds	5	0.09	23	0.43	27	0.50	30	0.56	35	0.65	42	0.78	43	0.8	51	0.94	54
Hydrazines	2	0.04	30	0.53	35	0.61	37	0.65	47	0.82	47	0.82	52	0.91	56	0.98	57
Miscellaneous ashby alerts	2	0.05	5	0.12	8	0.2	13	0.32	25	0.61	32	0.78	34	0.83	36	0.88	41
α-Nitro Furyl Compounds	1	0.03	16	0.47	24	0.71	31	0.91	33	0.97	34	1					34
N-Nitroso Compounds	**47**	**0.45**	**90**	**0.86**	**96**	**0.91**	**99**	**0.94**	**102**	**0.97**	**105**	**1**					**105**
Organophosphorus compounds	0	0	5	0.29	5	0.29	6	0.35	8	0.47	11	0.65	14	0.82	15	0.88	17
Steroids	5	0.45	9	0.82	9	0.82	10	0.91	10	0.91	11	1					11
Strained rings	1	0.07	9	0.60	11	0.73	11	0.73	12	0.8	13	0.87	14	0.93	15	1	15
Tetrahalogenated dibenzodioxins and dibenzofurans (2,3,7,8)	2	0.40	2	0.40	2	0.40	2	0.40	3	0.6	3	0.6	3	0.6	3	0.6	5
Vinyl containing compounds	2	0.05	13	0.33	16	0.40	22	0.55	28	0.7	34	0.85	38	0.95	40	1	40

Absolute numbers of compounds (*n*) in various structural groups in the database that would give estimated risks greater than 1 in 10^6 if the intake were at values given in the column heading (calculated for a 60 kg person and an intake of 3 kg of diet per day) along with the fraction (*F*) of all members of each structural group.

From: Kroes et al. Food & Chemical Toxicology 2004; 42: 65-83

CHMP Guideline – TTC

- Lifetime risk of 1 in 10^6 is conservative, since drugs have benefit
  - Therefore, TTC limit based on **1 in 10^5** I.e. ≤ **1.5 µg/day**
- Higher levels if justifiable e.g.
  - Acute drug treatment
  - Life-threatening disease
  - Lack of alternatives
- Proposed level of acceptable risk consistent with other regulations
  - WHO drinking water standards 1 in 10^5
  - USEPA drinking water standards 1 in 10^4 to 1 in 10^6
  - FAO/WHO flavouring substances 1 in 10^6
  - Q3C limit set for benzene, 20 µg/day 1 in 10^5

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Limits for drugs before MAA/NDA ?

- CHMP not clear “Higher limits may be justified under certain conditions such as short-term exposure periods”
- US PhRMA Genotoxicity Taskforce White paper
  Regulatory Toxicology and Pharmacology 2006; 44: 198-211

A rationale for determining, testing, and controlling specific impurities in pharmaceuticals that possess potential for genotoxicity

Lutz Müller [a,*], Robert J. Mauthe [b], Christopher M. Riley [c], Marta M. Andino [d], David De Antonis [d], Chris Beels [e], Joseph DeGeorge [f], Alfons G.M. De Knaep [g], Dean Ellison [f], Jane A. Fagerland [h], Rebecca Frank [i], Betsy Fritschel [j], Sheila Galloway [f], Ernie Harpur [k], Charles D.N. Humfrey [l], Alexander S. Jacks [i], Nirdosh Jagota [m], John Mackinnon [e], Ganapathy Mohan [k], Daniel K. Ness [n], Michael R. O’Donovan [l], Mark D. Smith [o], Gopi Vudathala [k], Larry Yotti [p]

Represented:
Hoffmann-LaRoche, Pfizer, ALZA, GSK, Merck, J&J, Abbott, Noranco, Sanofi, AstraZeneca, Wyeth, Lilly, BMS

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PhRMA Genotoxicity Taskforce proposal

- Staged TTC approach for allowable daily intakes of genotoxic inpurities during all phases of clinical development

	Duration of Exposure (months)				
	<1	1 to 3	3 to 6	6 to 12	>12
Allowable daily intake (µg)	120	40	20	10	1.5
Alternative maximum	0.5%	0.5%	0.5%	0.5%	n/a

- Known carcinogens should have compound-specific risk calculated
- Risk levels: up to 12 months 1 in 10^6 ; over 12 months 1 in 10^5

Relationship between Dose, Acceptable MDI and Concentration

Daily Dose of API (mg)	Concentration of Impurity (%) Maximum Daily Intake (MDI) and Duration of Exposure				
	< 4 weeks	1-3 months	3-6 months	6-12 months	> 12 months
	MDI =120 ug	MDI=40 mcg	MDI=20 mcg	MDI=10 mcg	MDI=1.5 mcg
3000.0	0.004		0.0007	0.0003	0.00005
1500.0	0.008			0.0007	0.00010
1200.0	0.010			0.0008	0.00013
1000.0	0.012			0.0010	0.00015
900.0	0.013				0.00017
500.0	0.024	0.0			0.00030
400.0	0.030	0.010			0.00038
300.0	0.040	0.013			0.0050
200.0	0.060	0.020			0.75
100.0	0.120	0.040	0.0		
90.0	0.133	0.044	0.022		
40.0	0.300	0.100	0.050		
30.0	0.400	0.133	0.067	0.033	
25.0	0.480	0.160	0.080	0.040	0.00600
20.0	0.600	0.200	0.100	0.050	0.00750
10.0	1.20	0.400	0.200	0.100	0.015
9.0	1.33	0.444	0.222	0.111	0.017
8.0		0.500	0.250	0.125	0.019
7.0			0.286	0.143	0.021
6.0			0.333	0.167	0.025
5.0			0.400	0.200	0.030
4.0	3		0.500	0.250	0.038
3.0	4.00			0.333	0.050
2.0	6.00			0.500	0.075
1.0	12.00			1.00	0.150
0.5	24.00				0.300
0.4	30.00	10.00			0.375
0.3	40.00	13.33			0.500
0.2	60.00	20.00	10.00		0.750
0.1	>100%	40.00	20.00		1.50

[Impurity] ≤100 ppm – analytical and process challenges

1 ppm

[Impurity] ≥0.5% - potential quality concerns

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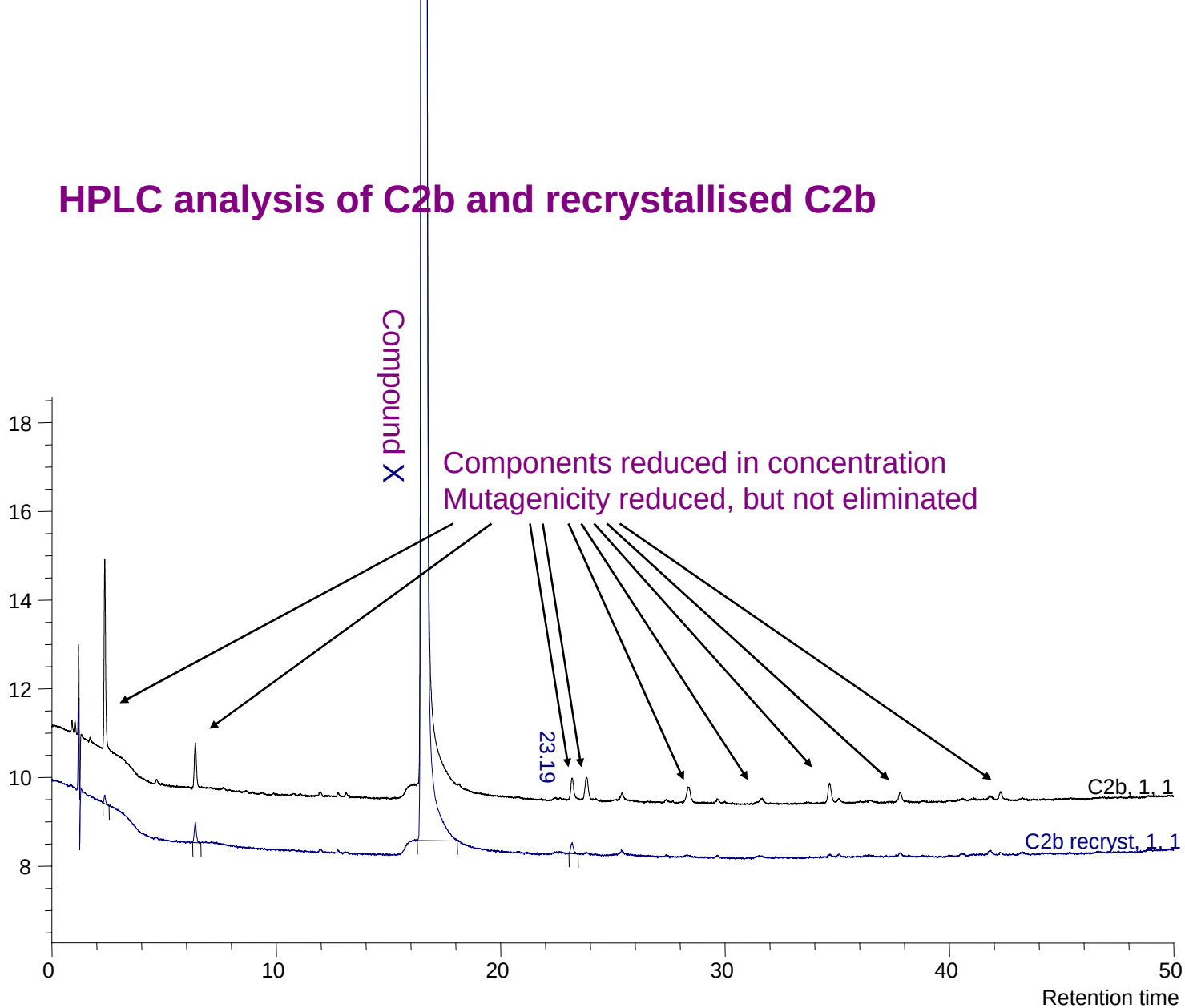
TRACING A GENOTOXIC IMPURITY

- Compound X - Discovery batches:
  - Some evidence of bacterial mutagenicity, possibly due to impurities
- Development batches:
  - C2a – no activity C2b – clearly active

Compound X µg/plate	Colonies/plate TA1535 +S9	
	Batch C2a	Batch C2b
Control	12	12
100	15	30
200	19	38
300	13	60
400	17	60
500	19	95
750	16	135
1000	22	130

HPLC analysis of C2b and recrystallised C2b



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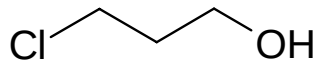
Compound X – Possible impurities

- Mutagenicity does not correspond to any drug-related material
- 4-Chlorobutanol known to be generated during synthesis
- 4-Chlorobutanol insufficiently potent to be responsible



µg/plate	Colonies/plate TA1535 +S9	
	Batch C2b	4-CB
Control	12	12
100	30	37
200	38	58
300	60	76
400	60	126
500	95	180
750	135	208
1000	130	297

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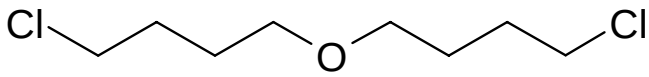
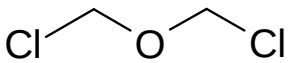
Compound X - Possible impurities

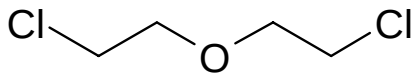
- bis(4-chlorobutyl) ether detectable in C2b



cf. bis(chloromethyl) ether
(IARC human carcinogen)



bis(2-chloroethyl) ether



- Sufficiently active to account for the mutagenicity of C2b ??

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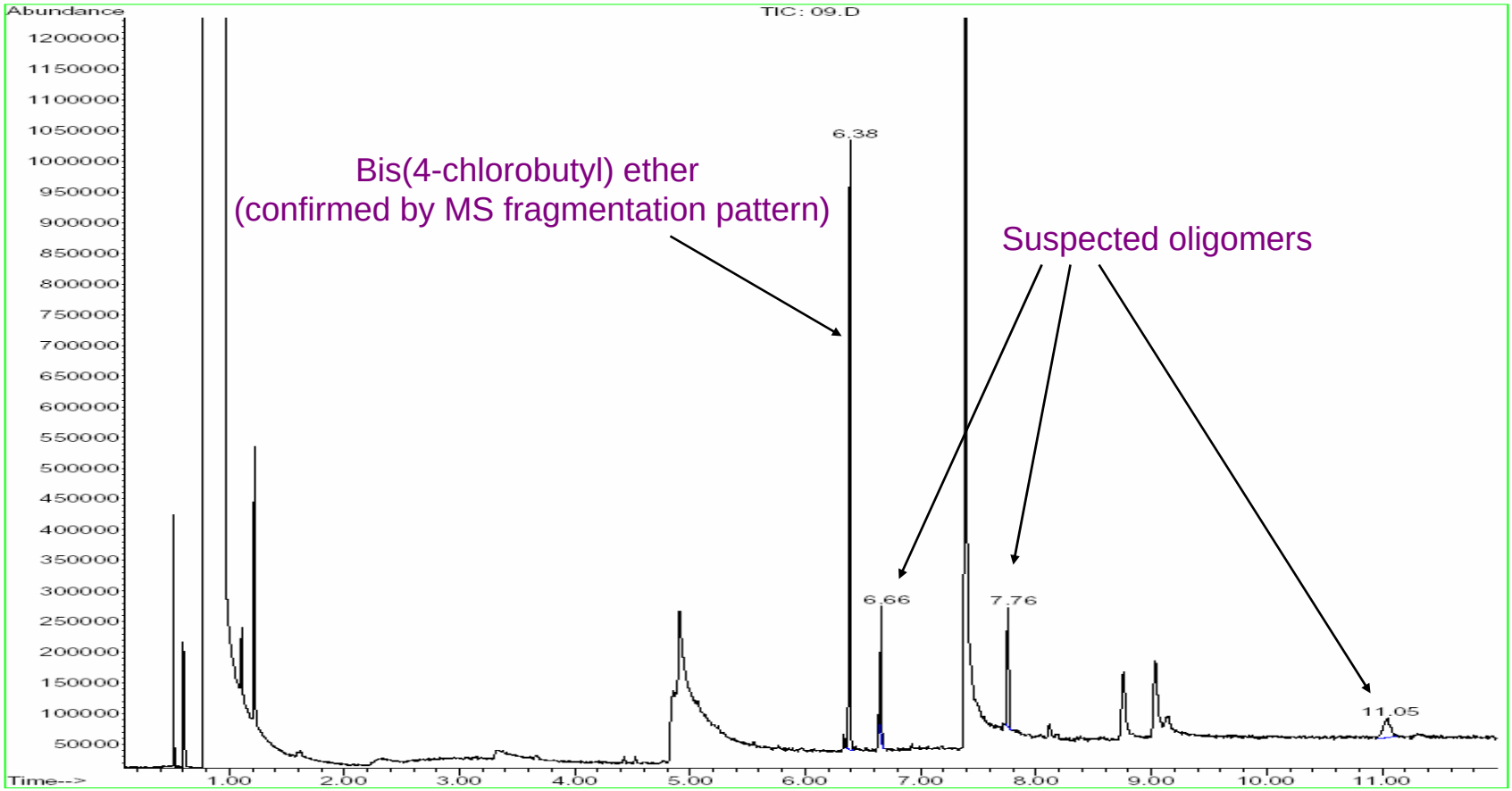
GC-MS analysis of C2b material



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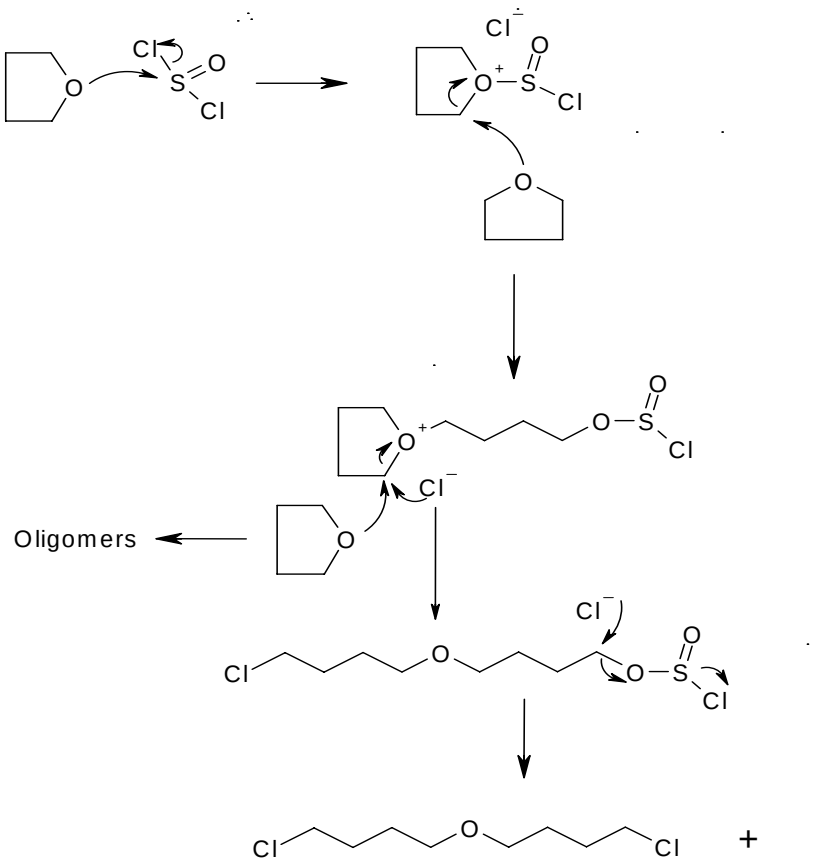
Mutagenicity of bis(4-chlorobutyl) ether

µg/plate	Colonies/plate TA1535 +S9	
	Batch C2b	Bis(4-chlorobutyl) ether
Control	12	15
1.0		25
3.3		31
10		99
33		276
100	30	528
200	38	
300	60	
330		1152
400	60	
500	95	
750	135	
1000	130	1723

Possible pathway to generate bis(4-chlorobutyl) ether

- Precursor intermedate has a step where THF is refluxed with $SOCl_2$



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Questions ?

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