Overview of Animal Tumors with Questionable Relevance to Human Risk Assessment

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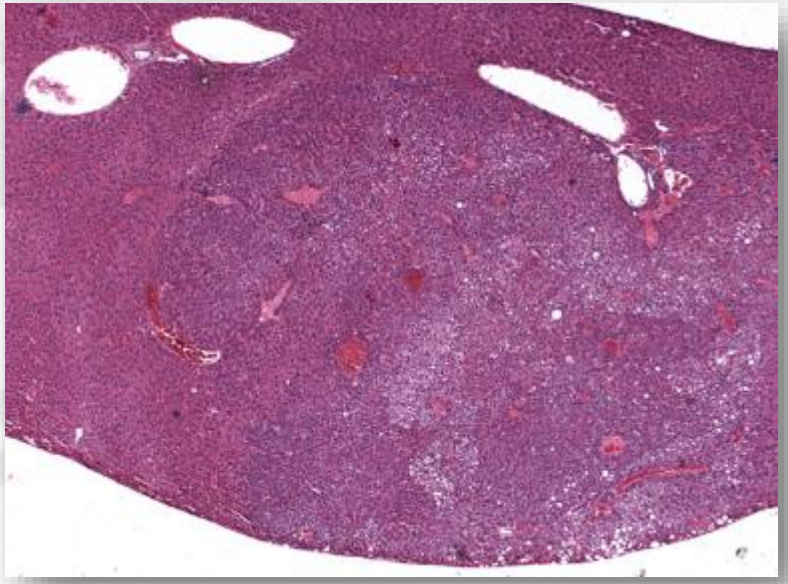
Introduction

- Study evaluation
- Distribution of tumors by chance
- Prestep lesions
- Value of Statistics
- Value of control data
- Data interpretation
- Considerations on MOA
- Mechanistical approaches

Problems by diagnostic terms/criteria

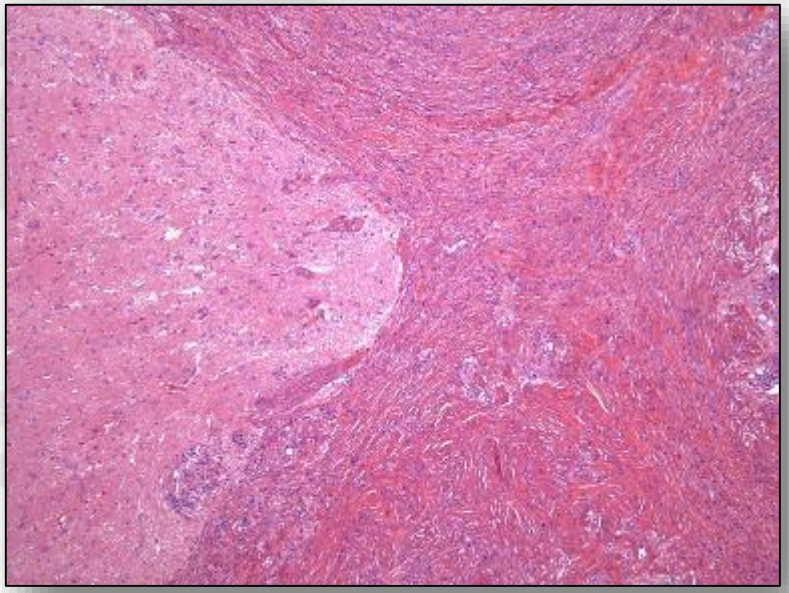
- **Examples:**
  liver: nodular hyperplasia



Total examined	M	F	M	F	M	F	M	F
	52	52	52	52	52	52	52	52
Original Diagnoses								
Hepatocellular adenoma	12	1	7	4	12	7	27	22
Nodular hyperplasia	9	3	9	4	18	8	22	6
Diagnoses after Peer Review								
Hepatocellular adenoma	14	4	12	8	18	12	34	24

Single-High-Dose Cases

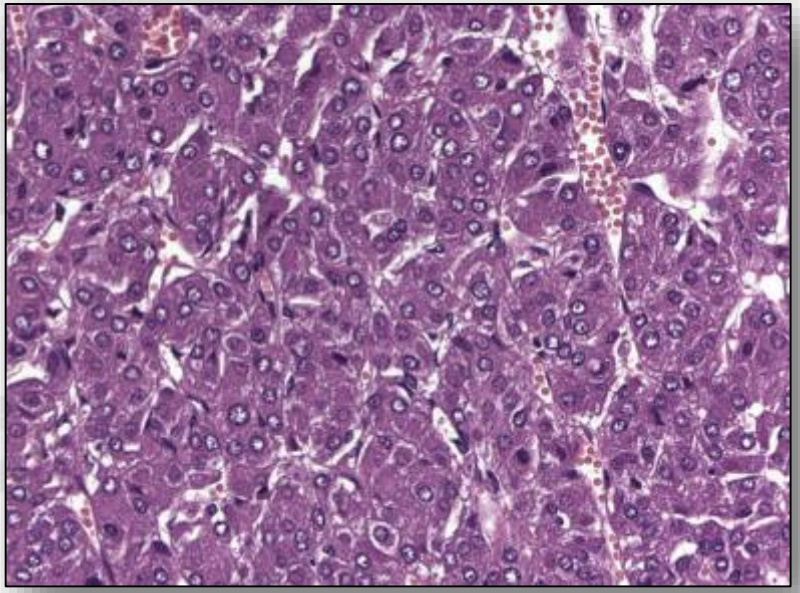
- **Example: Meningiosarcoma (malignant meningioma)**
- **In males: 1 high dose case P > 0.001**
- **True?**



Male	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3805					
Meningioma	2	0.05	0.06	0.31	0.00	2.04
Meningial sarcoma	1	0.03	0.03	0.20	0.00	1.43

Single-High-Dose Cases

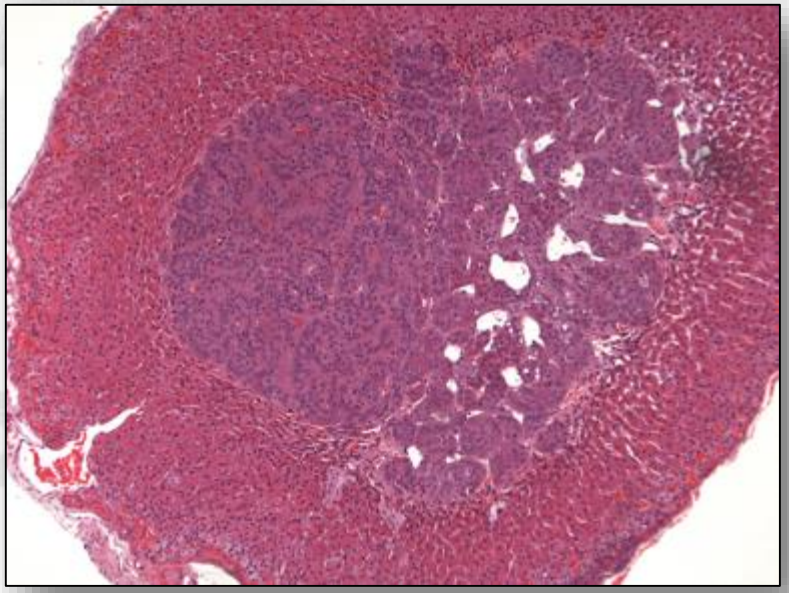
- **Example: Aesthesioneuroblastoma in nasal cavity**
- **In males: 1 high dose case P > 0.001**
- **True?**



- **Not any information in control animals**
- **Only described as induced tumor**

By-chance distribution

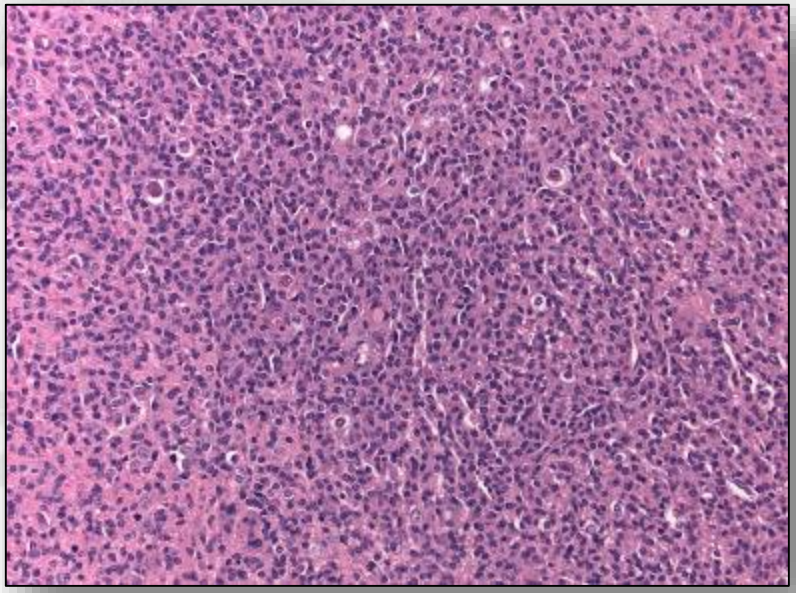
- **Example: Pheochromocytoma, B6C3F1 mouse**
- **Induced?**



Groups	1		2		3		4	
Total examined	M	F	M	F	M	F	M	F
	52	52	52	52	52	52	52	52
Pheochromocytoma, b.	1	0	0	0	0	0	0	1
Pheochromocytoma, m.	0	0	0	0	0	0	0	1
Hyperplasia, medulla	2	5	5	1	6	3	5	1

By-chance distribution

- **Example:**
  3 astrocytomas in rat females, high dose
  3 oligodendrogliomas in rat males, low dose
- **Why should be the high dose tumors test item-related?**



Female	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3777					
Astrocytoma	7	0.19	0.21	0.55	0.00	2.00

Single cases exceeding control data incidences

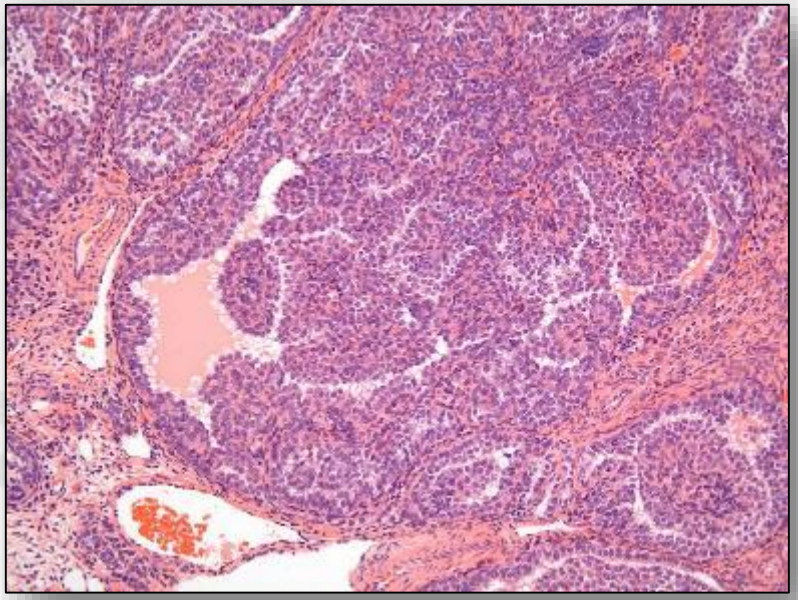
- **Example: hepatocellular carcinoma in Wistar Rats: 0, 0, 0, 0, and 2 (groups 1, 2, 3, 4 and 5)**

Male	Total n	Total %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3973					
Adenoma, hepatocellular	79	1.99	1.95	1.96	0	8.00
Carcinoma, hepatocellular	18	0.45	0.45	0.78	0	2.80

Study ID	1				2				3				4				5			
Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Group	3		4		3		4		3		4		3		4		3		4	
Adenoma	2	1	2	0	0	0	1	2	0	0	0	0	0	0	0	0	0	1	1	0
%	4.0	2.0	4.1	0	0	0	6.7	9.1	0	0	0	0	0	0	0	0	0	2.0	2.0	0
Carcinoma	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Relevance of pre-neoplastic lesions

- **Example: increased granulosa cell tumors (benign) in high dose females**
- **And Precursors?**
- **Sex stromal cord hyperplasia vs granulosa cell hyperplasia.**



Female	Total n	Total n %	Mean %	STDEV %	MIN %	MAX %
Numbers of rats examined	3766					
Theca cell hyperplasia	3	0.08	0.08	0.34	0.00	1.49
Granulosa cell hyperplasia	79	2.10	2.51	6.09	0.00	28.00
Granulosa-theca cell hyperplasia	11	0.29	0.38	2.46	0.00	17.86
Interstitial cell hyperplasia	314	8.34	10.16	23.58	0.00	95.83
Sertoli cell hyperplasia	300	7.97	9.42	19.17	0.00	68.00
Luteal hyperplasia	1	0.03	0.04	0.27	0.00	2.00
Sex cord stromal hyperplasia	163	4.33	4.11	14.55	0.00	88.00

Statistics related issues

- **rare neoplasm: if in an assay involving one or two hundred animals there may be no such neoplasm, or at most one or two such neoplasms in animals of one sex and strain**
- **if only one or two animals have a particular type of neoplasms in a standard assay, a statistically significant result is completely impossible. This holds true even if one or two such neoplasms occur in the top dose group and there are none elsewhere in the study.**

Statistics related issues

- **Common neoplasm: "…if it occurs spontaneously in five or ten or more animals in most experiments performed with animals of one strain"**

- **Peto, R.; Pike, M.C.; Day, N.E.; Gray, R.G.; Lee, P.N.; Parish, S.; Peto, J.; Richards, S.; Wahrendorf, J.: Guidelines for simple, sensitive significance tests for carcinogenic effects in long-term animal experiments. International Agency for Research on Cancer Monographs (suppl. 2), 311-426 (1980).**

Statistics related issues

- only if the number of neoplastic lesions exceeded 5% in at least one sex/dose group the statistical calculation was considered to give evidence of a significant trend

- Gart, J.J.; Krewski, D.; Lee, P.N.; Tarone, R.E.; Wahrendorf, J.: Statistical Method in Cancer Research. Vol III – The Design and Analysis of Long-term Animal Experiments. IARC Scientific Publications No. 79. IARC, Lyon (1986).

Statistics related issues

- **Only one-tailed p-values of $p<0.025$ for rare neoplasm's and p-values of $p<0.05$ for common neoplasms are considered to be statistically significant .**

- **Lin, K.K.; Rahman, M.A.: Overall false positive rates in tests for linear trend in tumor incidence in animal carcinogenicity studies of new drugs. J Biopharm Statistics, 8: 1-15 (1998).**

Relevance of control data

Regulatory Forum

Toxicologic Pathology, 37: 679-693, 2009

ISSN: 0192-6233 print / 1533-1601 online
DOI: 10.1177/0192623309336154

Best Practices for Use of Historical Control Data of Proliferative Rodent Lesions

Control data: recomendations

- Concurrent control group is most relevant.
- Used as a tool for to detect an abnormal control group.
- Useful in interpretation of rare lesions.
- Study design bears variables.
- Necropsy and trimming affect control data.
- Data from own lab are more useful than from other labs.
- Peer Reviewed control data are considered of greater value.
- Published data may be used with care.
- 2-7 years collection are recommended but greater time span may be useful.

Mechanistic Studies as Interdisciplinary Approach

Example: Lung AB adenoma in Mice (Agrochemical)

Issue:

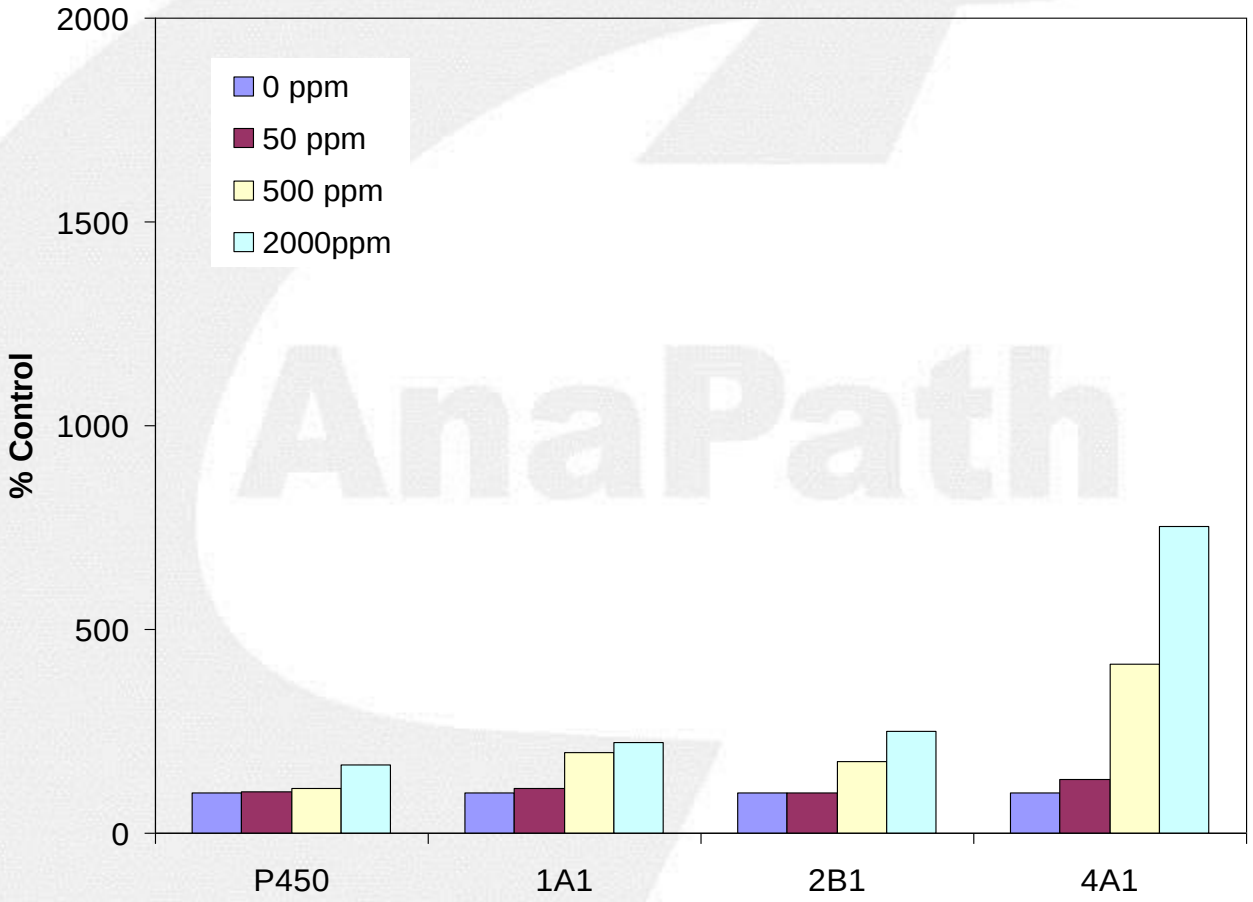
- **Lung tumors in mice**
- **Not mutagenic in Ames Test**
- **Not carcinogenic in rats**

Hypothesis:

- **Epigenetic non-genotoxic mode of action**
- **Liver enzyme induction**

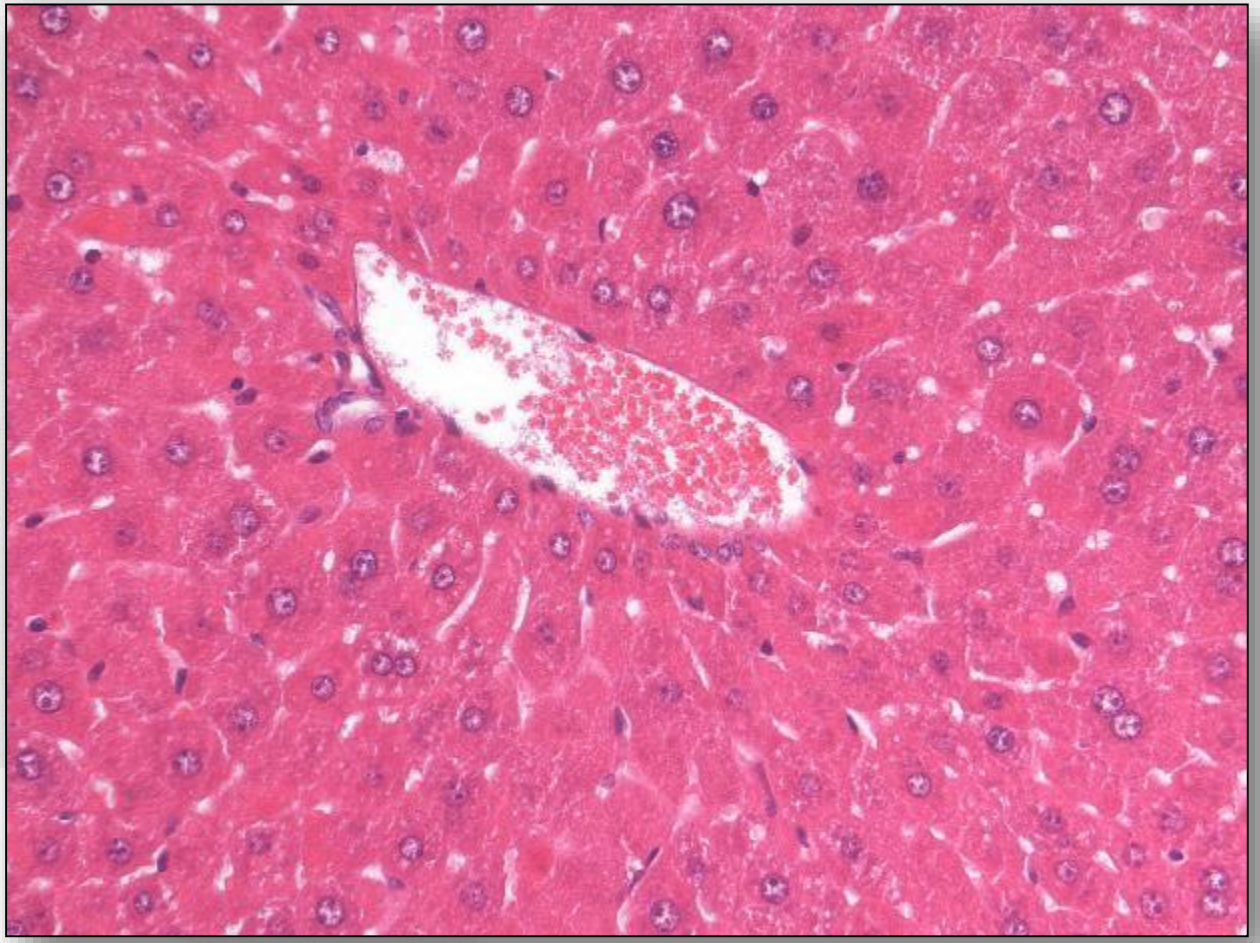
Weber K, Sagelsdorff P: Mechanistical Considerations on Induced Pathological Lesions, JSTP, 2011

Liver enzyme induction

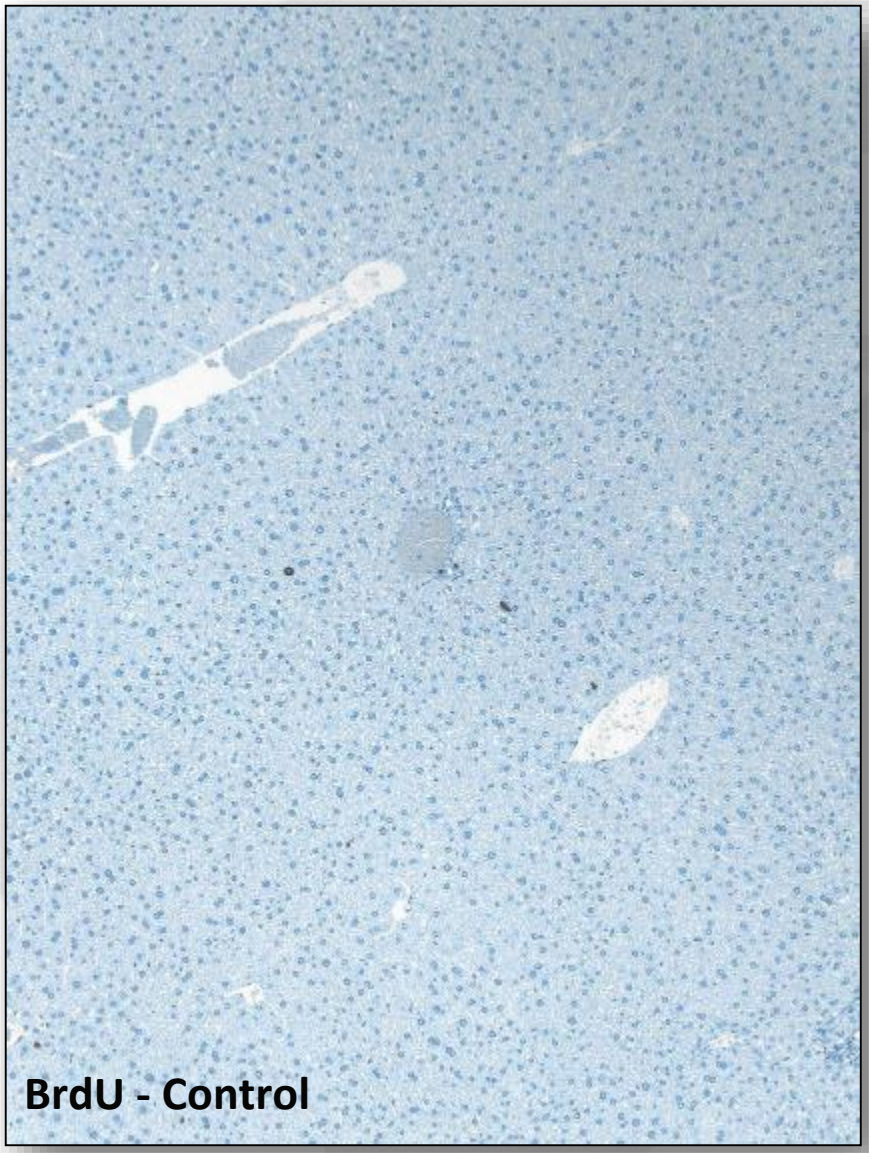


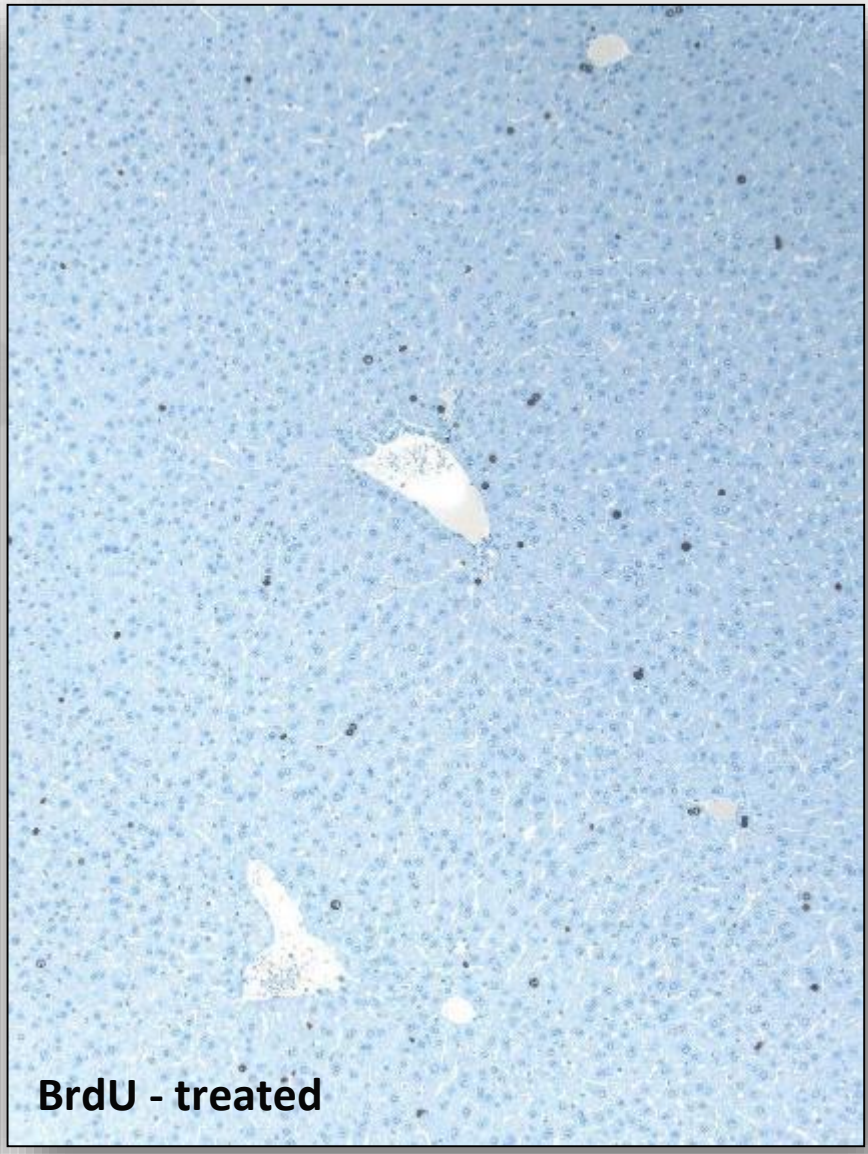
Partial confirmation of hypothesis

- **Liver enzyme induction**
- **Moderate peroxisome proliferator in mouse liver**
- **Weak peroxisome proliferation in mouse lung**

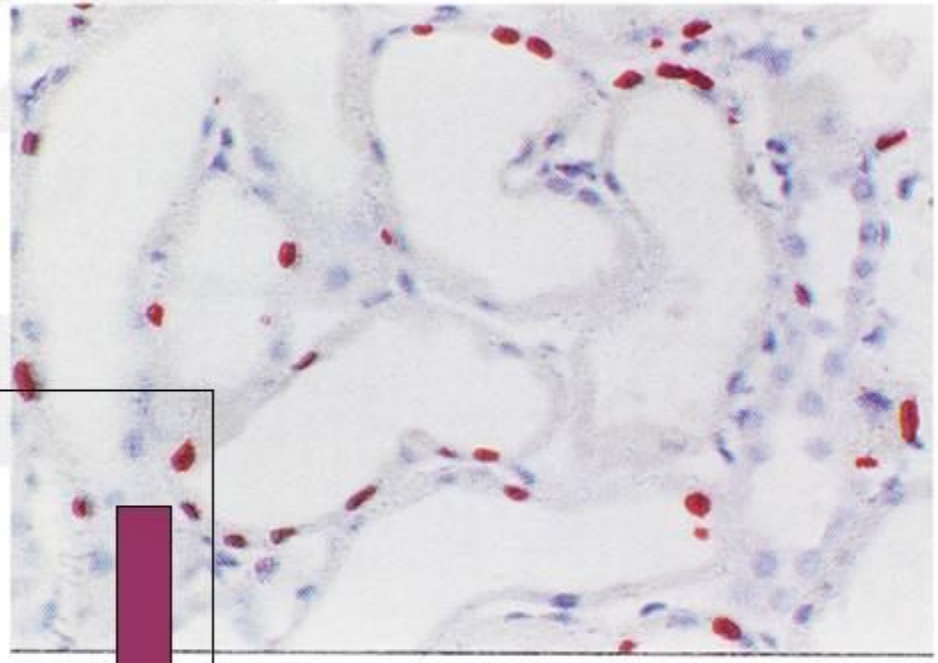


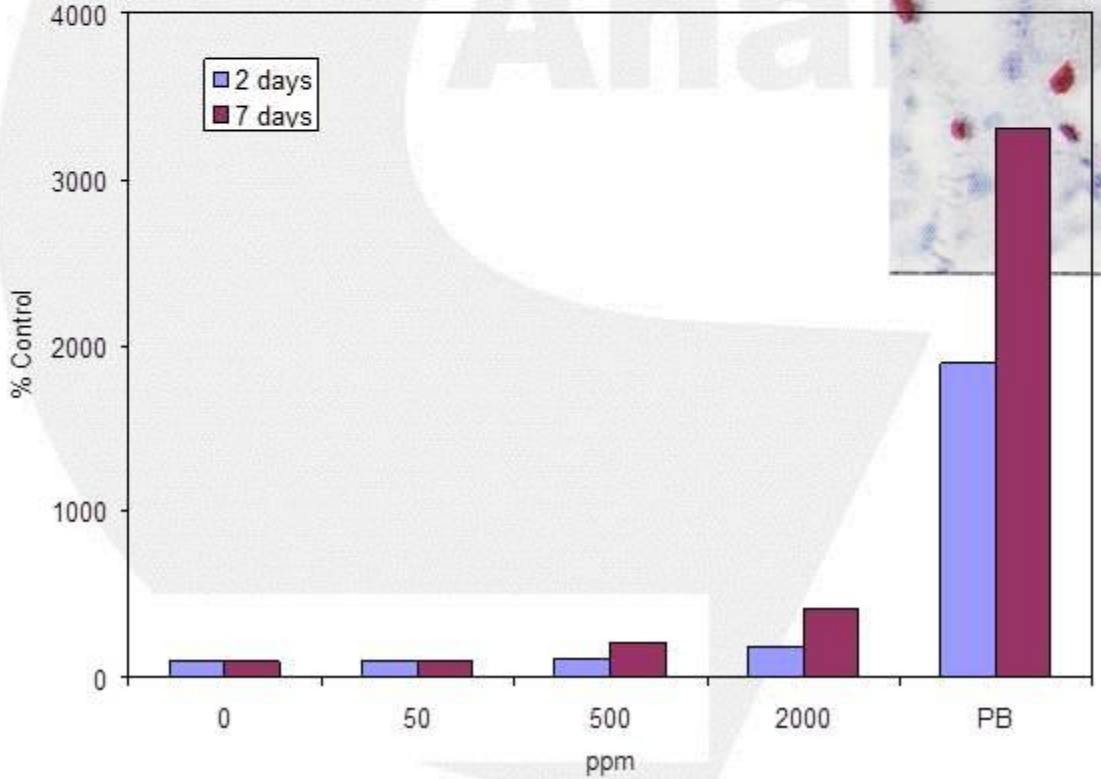
Proliferation study with weak results





Cell proliferation in lungs?





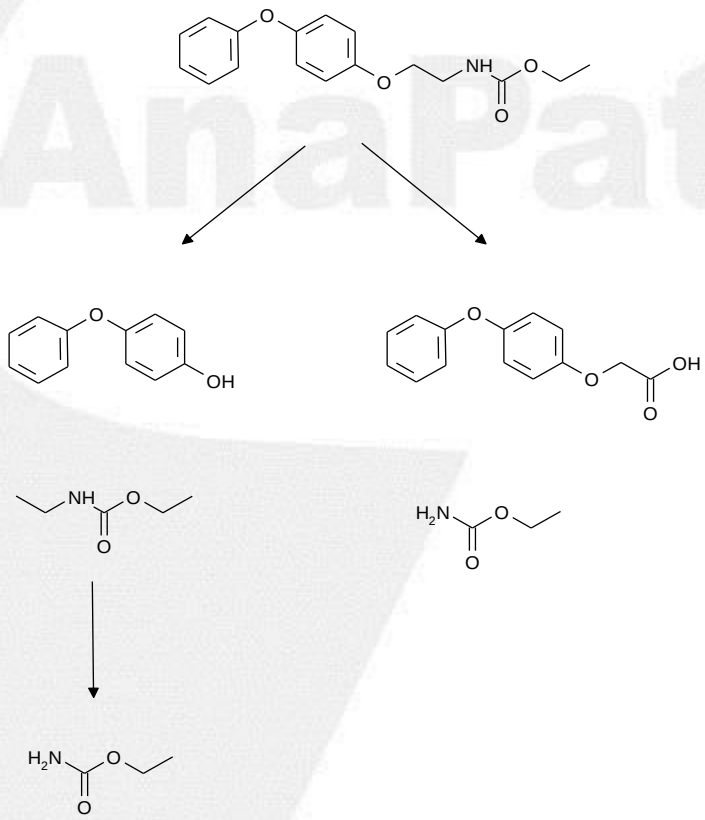
Summary on proliferation studies

- **Weak stimulation of cell proliferation in mouse liver**
- **No stimulation of cell proliferation in mouse lung**

- **Stimulation of cell proliferation did not explain tumor formation in mouse lung by a non-genotoxic mode of action.**

Next hypothesis: genotoxicity

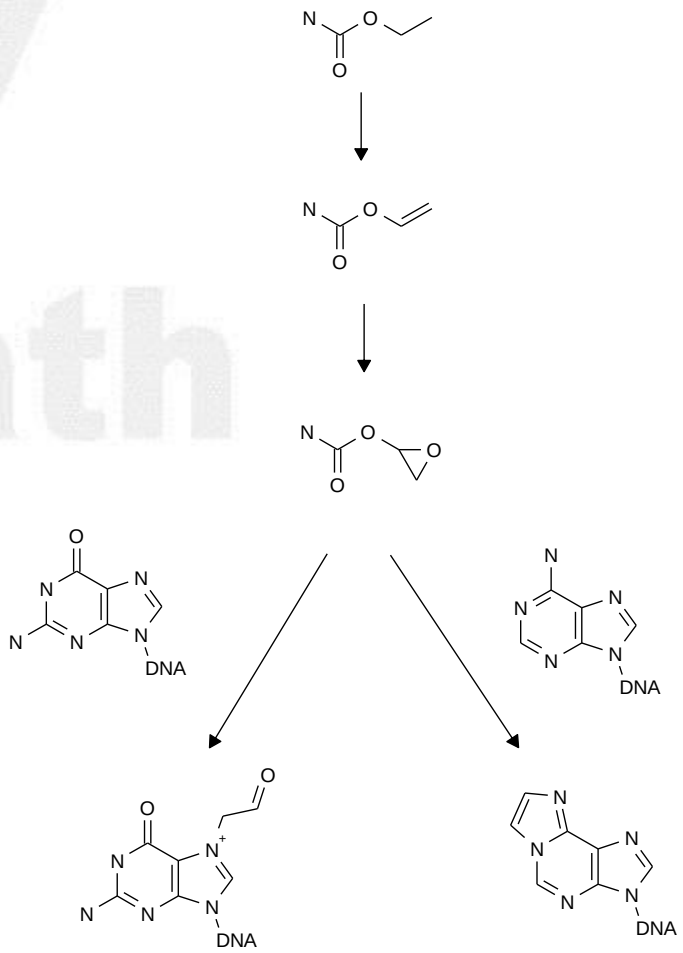
- **DNA Binding Assay**
  Mouse specific metabolism to urethane



Urethane

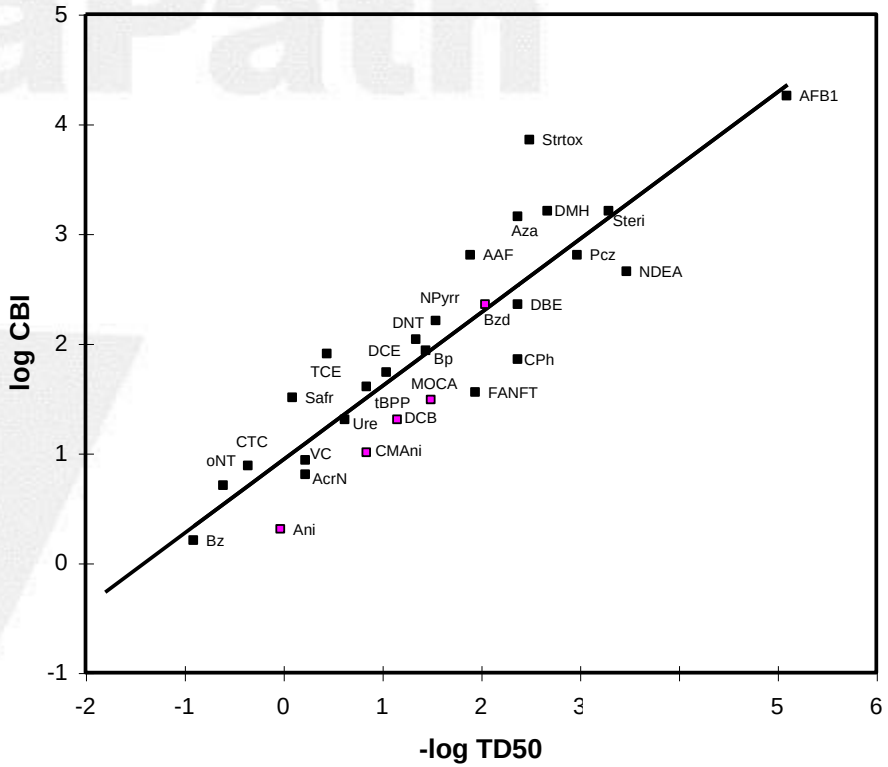
Carcinogenic to rodents (liver, lung)

- **Not mutagenic in Ames-Test**
- **Oxidation to vinylcarbamate**
- **Formation of DNA adducts (mainly oxoethyl-guanine, less ethenoadenine)**

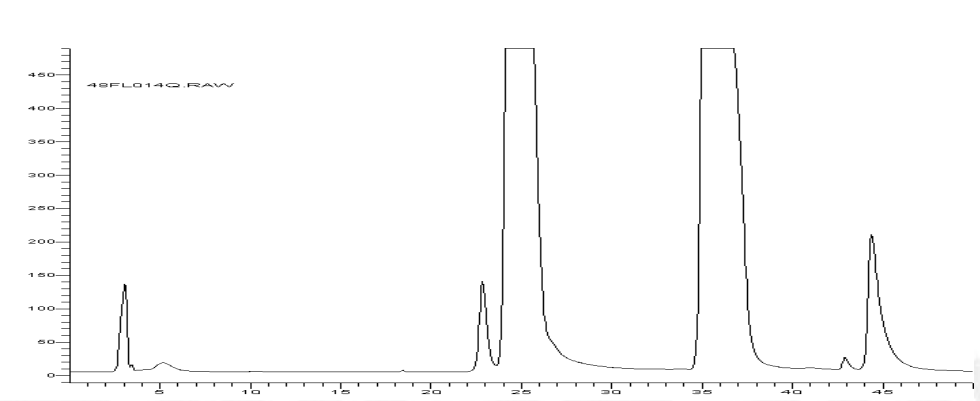


DNA binding assay

- Single (oral) treatment with radiolabelled compound
- Isolation of (liver) DNA and hydrolysis of DNA (to purines)
- HPLC Separation of normal DNA constituents from adduct (Purines from 7OEG)
- Calculation of CBI (Covalent Bound Radioactivity)



DNA binding assay



Results in mice

- Liver:
  7OEG Index: 0.5-0.8[a]
  EA Index: 0.07-0.1[a]

- Lung:
  7OEG Index: 0.7[a]

Results in rats

- Liver:
  7OEG Index: not detectable (LoD < 0.07[a])
  EA Index: not detectable (LoD < 0.07[a])
- Lung: 7OEG Index: not detectable (LoD < 0.07[a])

[a] µmol adduct per mol DNA nucleotide / mmol chemical applied per kg b.w.

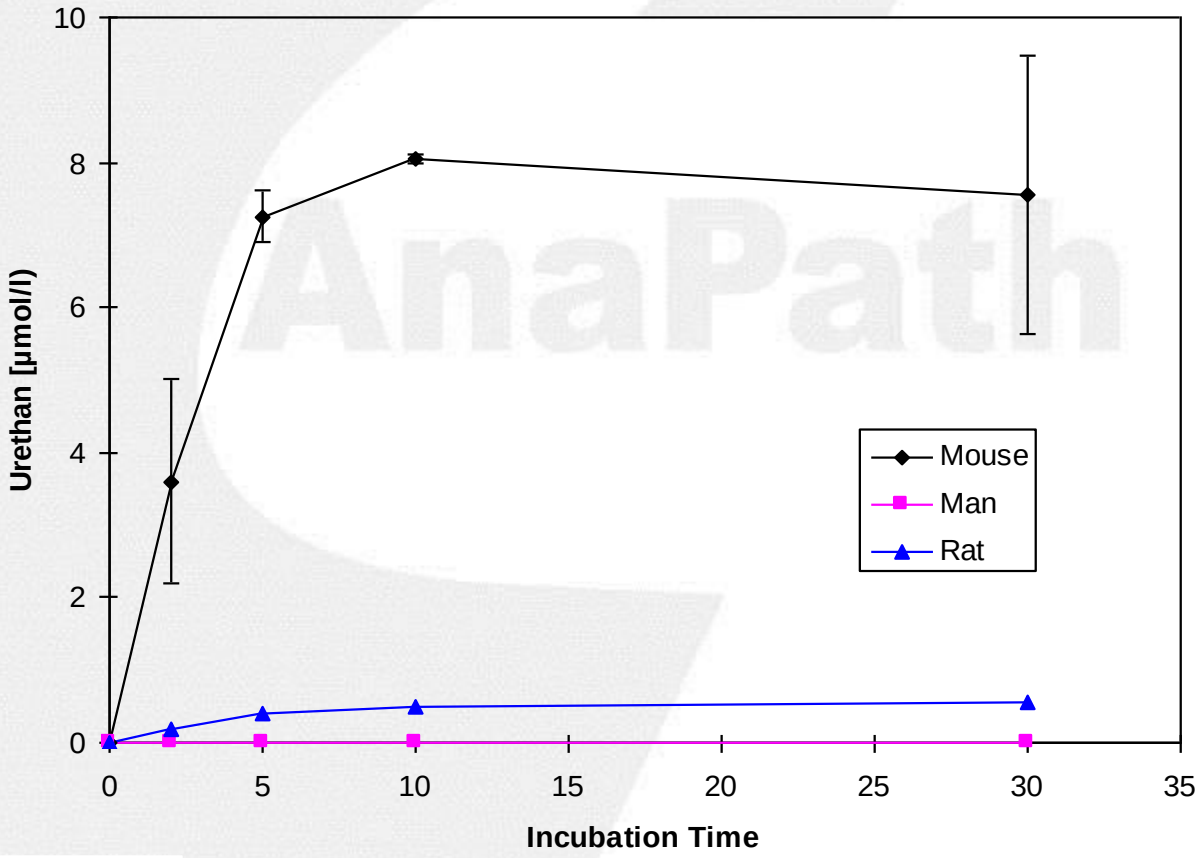
Next hyopthesis: Species-specific effect?

- Moderate/week peroxisome proliferator in mouse
- Weak/no stimulation of cell proliferation in mouse
- DNA adduct formation in mouse

→

- Incubation of microsomes with test item
- Extraction of urethane
- GC/MS analysis of urethane in extracts

In vitro metabolism: liberation of urethane



Urethane: results and summary

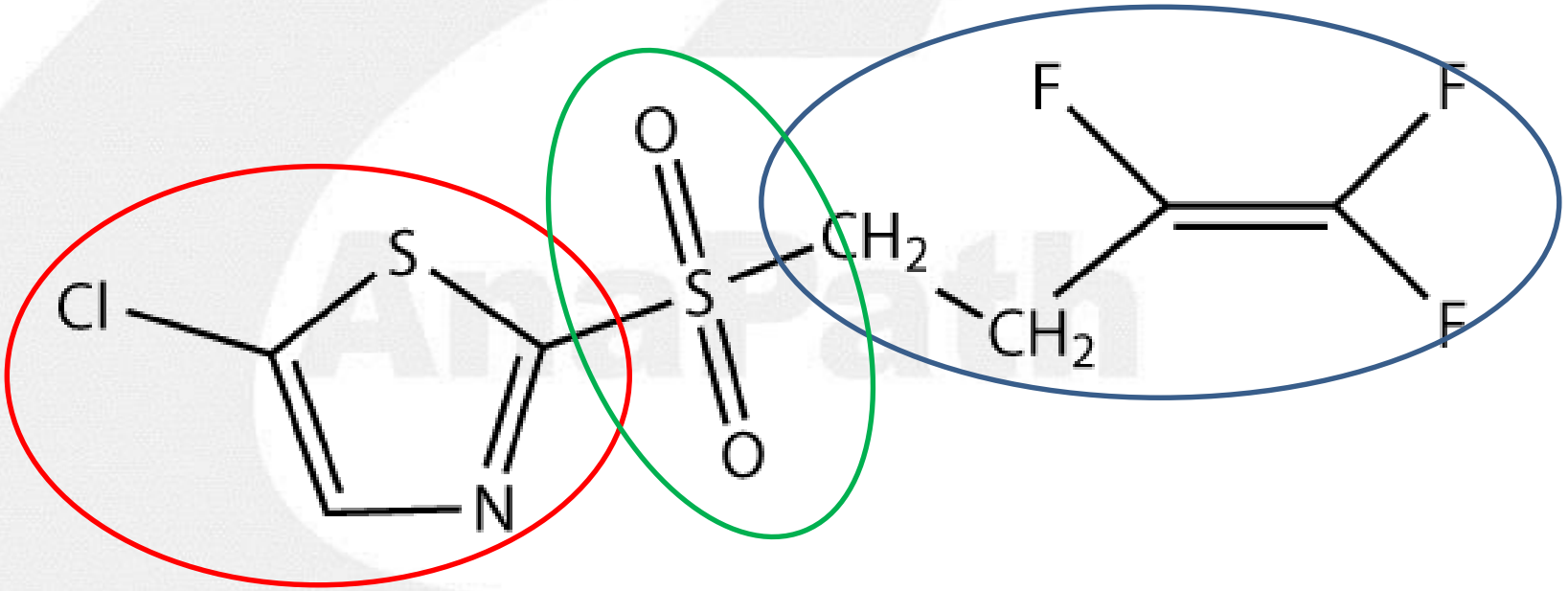
Release of urethane from parental compound

- **Mouse: 200 pmol/min/mg**
- **Rat: 10 pmol/min/mg**
- **Man: <0.5 pmol/min/mg**

- **Human microsomes release 400 times less**
- **Urethane than the susceptible mice**
- **20 times less Urethane the non-susceptible rat**

Man is not susceptible to tumor formation by this test item

Another Example: Fluensulfone – AB-adenoma



5-Chloro-2-(3,4,4-trifluoro-but-3-ene-1-sulfonyl)-thiazole

Detailed information

Toxicol Sci. 2012 Jul;128(1):284-94. doi: 10.1093/toxsci/kfs127. Epub 2012 Apr 5.

Relationship of metabolism and cell proliferation to the mode of action of fluensulfone-induced mouse lung tumors: analysis of their human relevance using the IPCS framework.

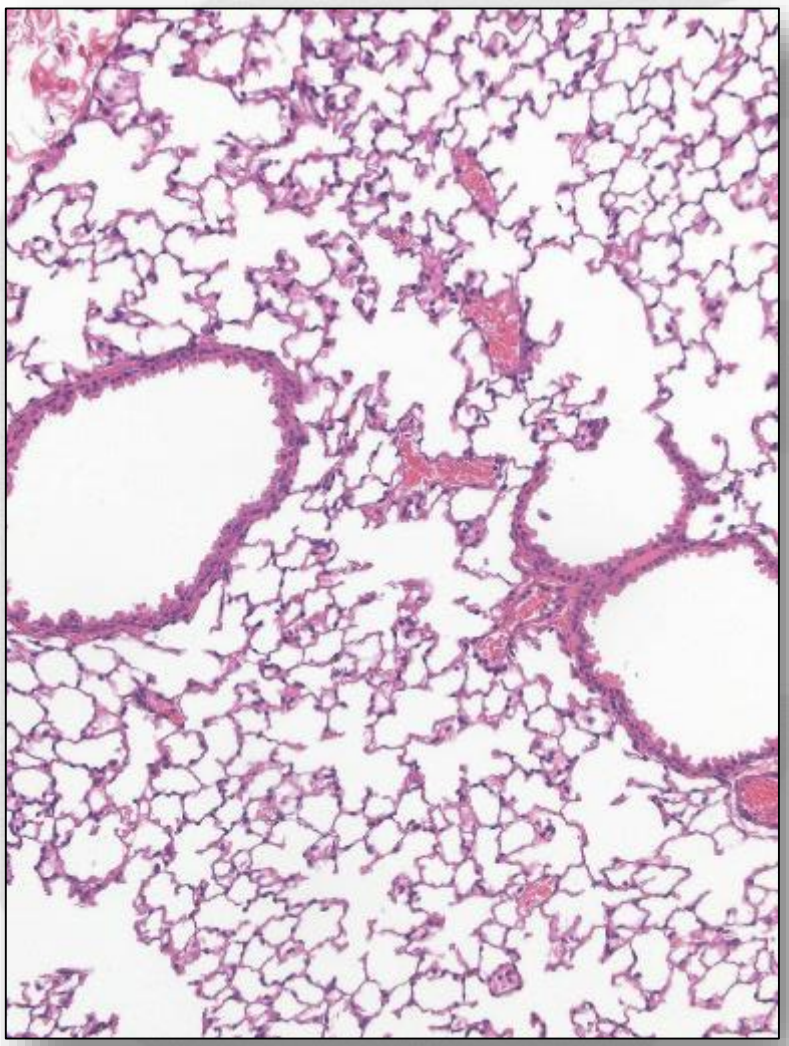
Strupp C, Banas DA, Cohen SM, Gordon EB, Jaeger M, Weber K.

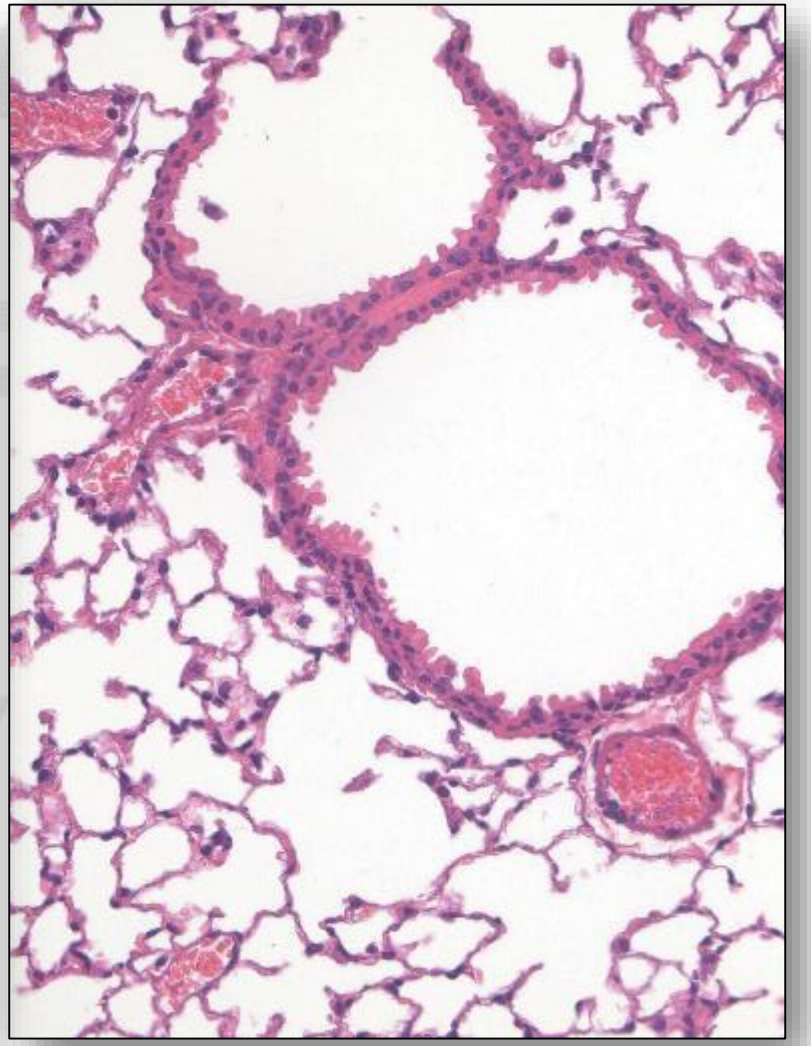
Source
Makhteshim Agan Holding B.V., Schaffhausen Branch, 8200 Schaffhausen, Switzerland. christian.strupp@ma-europe.com

Design: 78-week mouse study

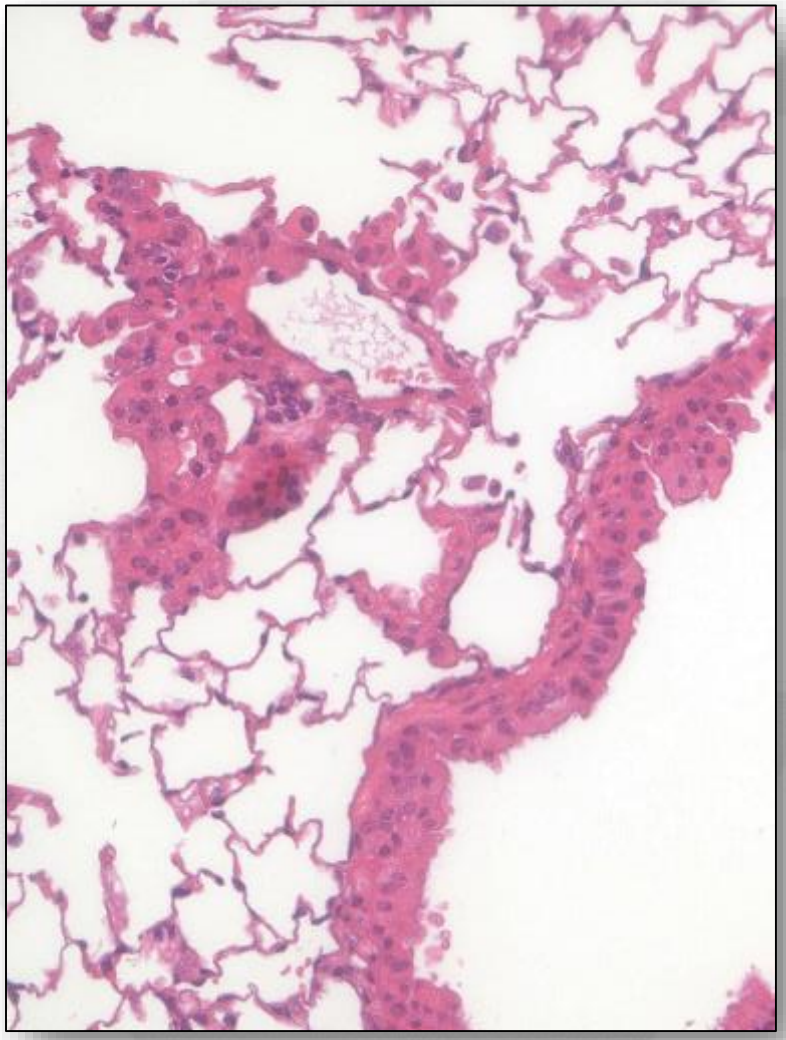
Allocation	Control	30 mg/kg	200 mg/kg	1200 mg/kg
Males	50	50	50	50
Females	50	50	50	50

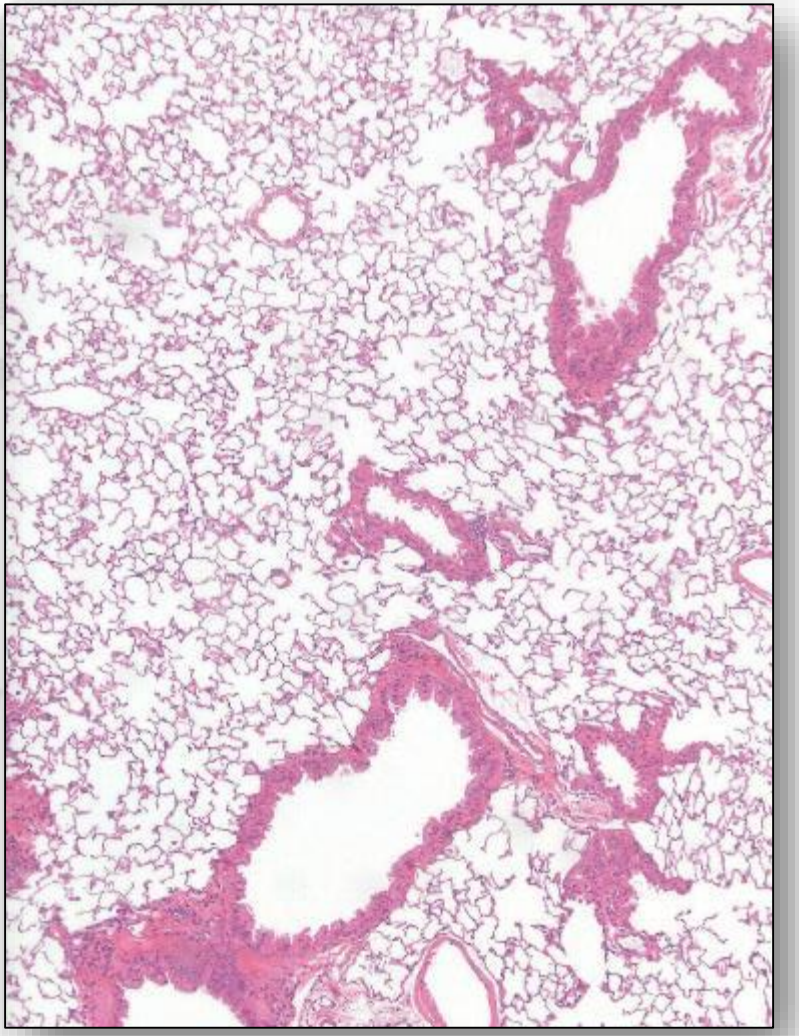
Control mice: lungs



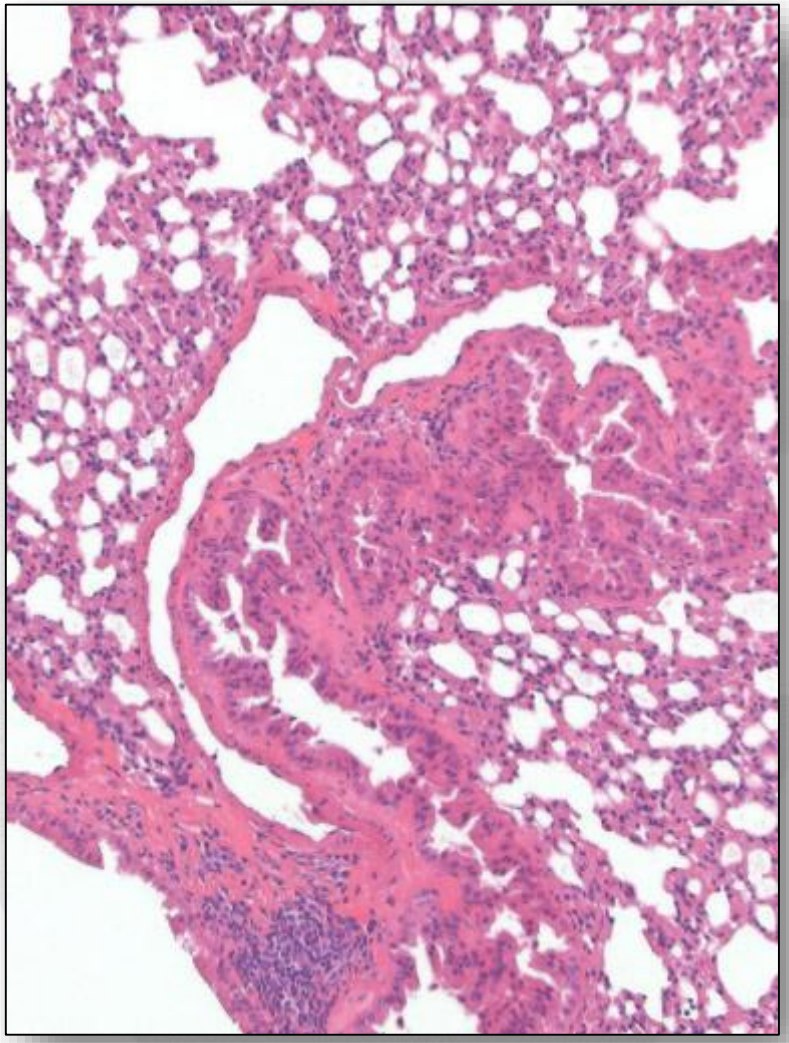


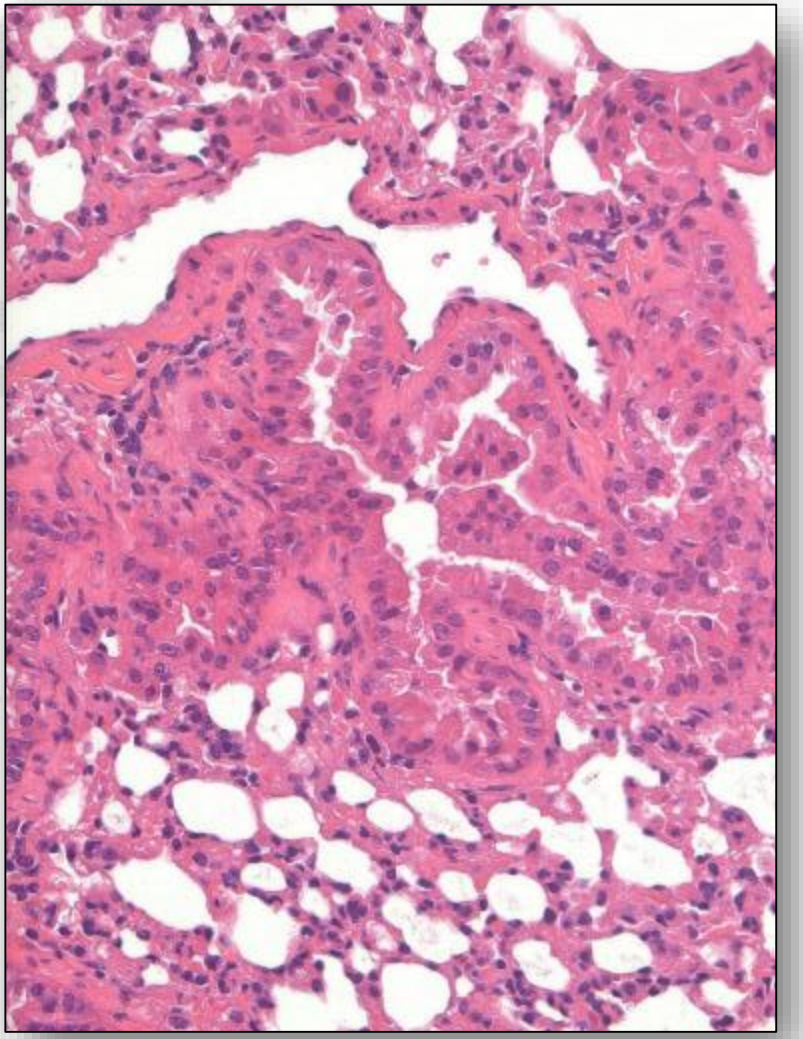
High dose mouse: lungs



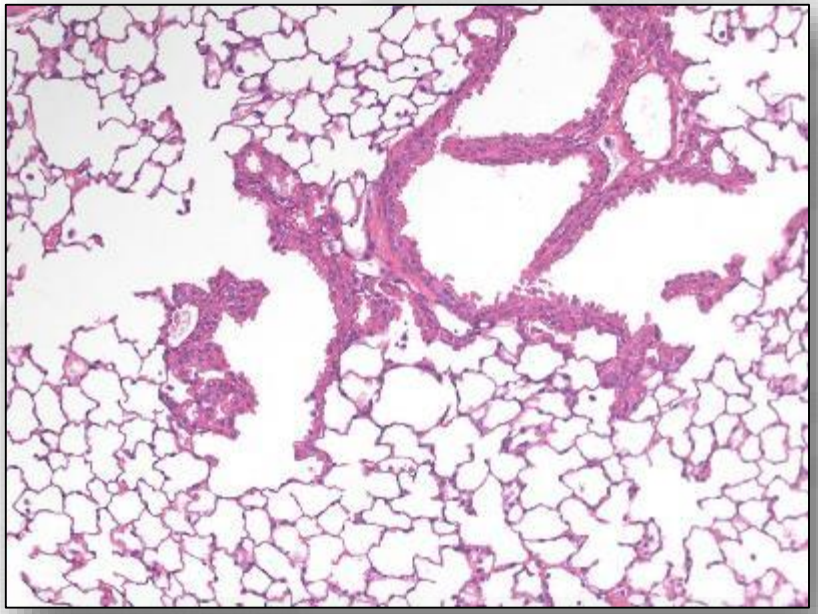


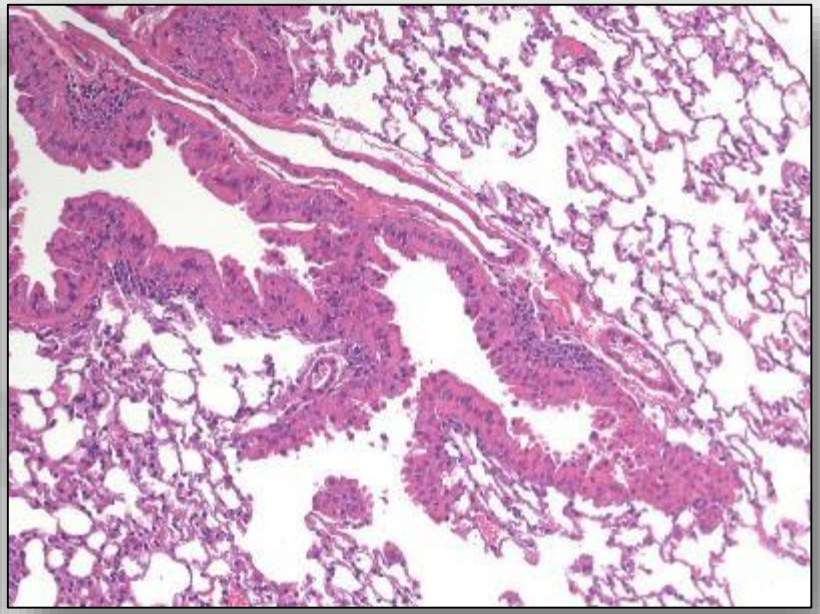
High dose mouse: lungs

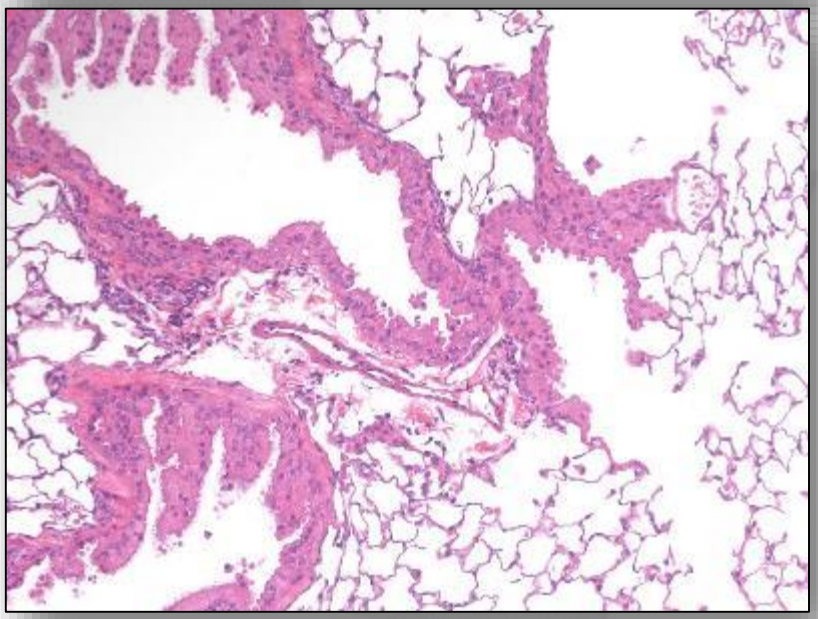


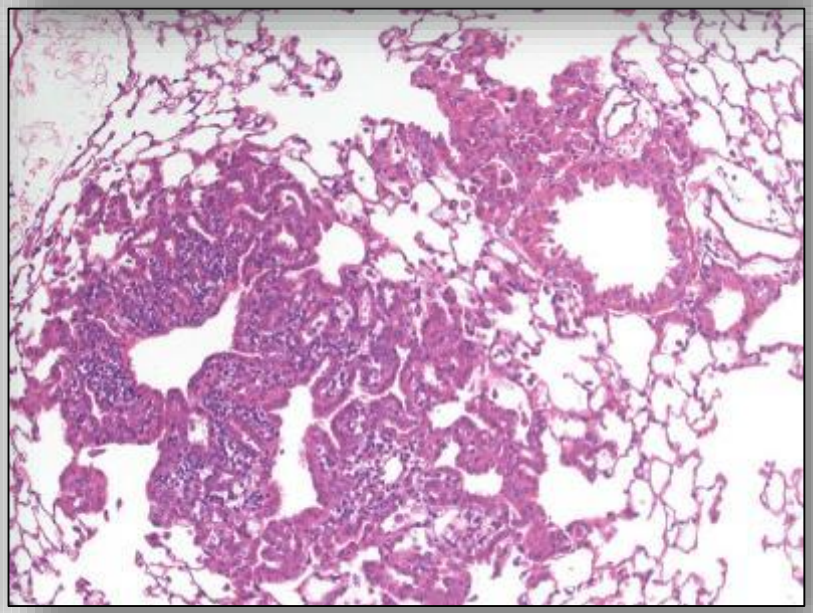


High dose mouse: lungs - spectrum

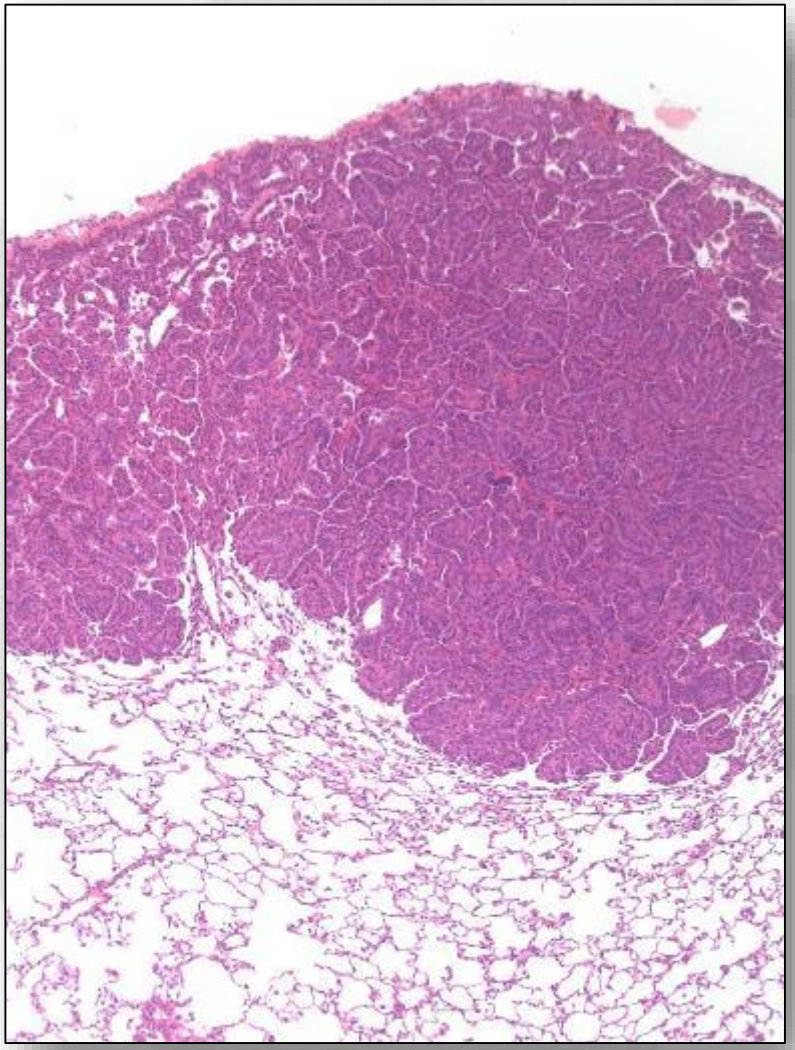


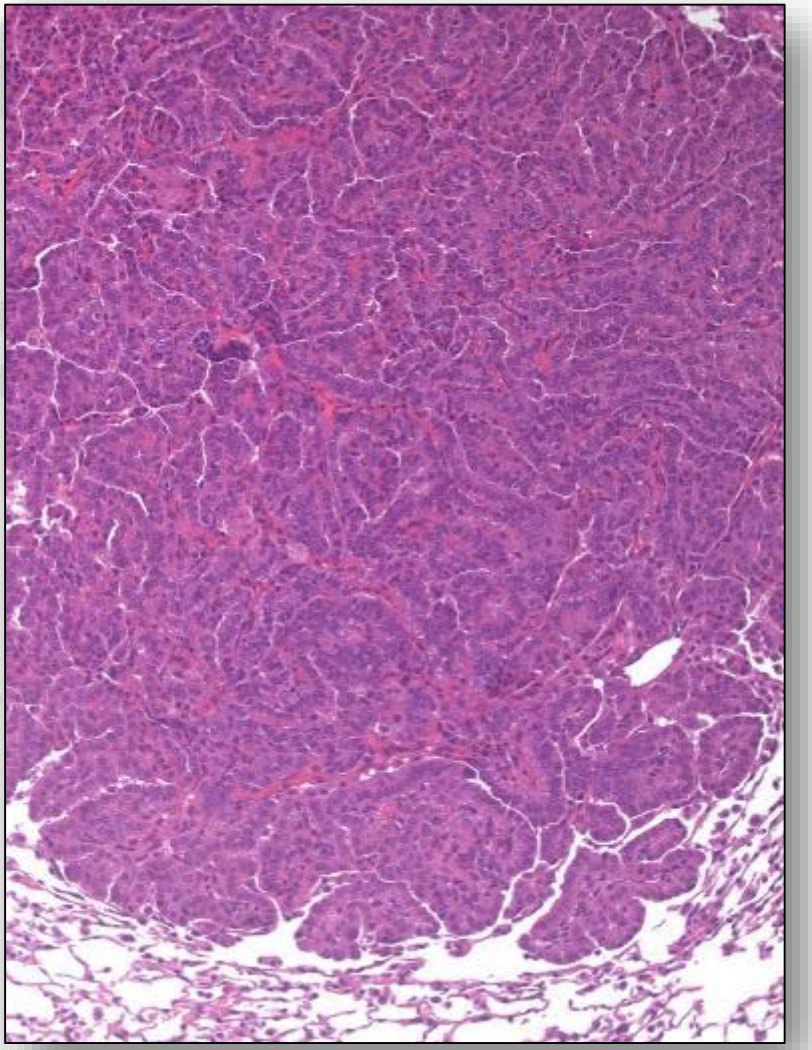




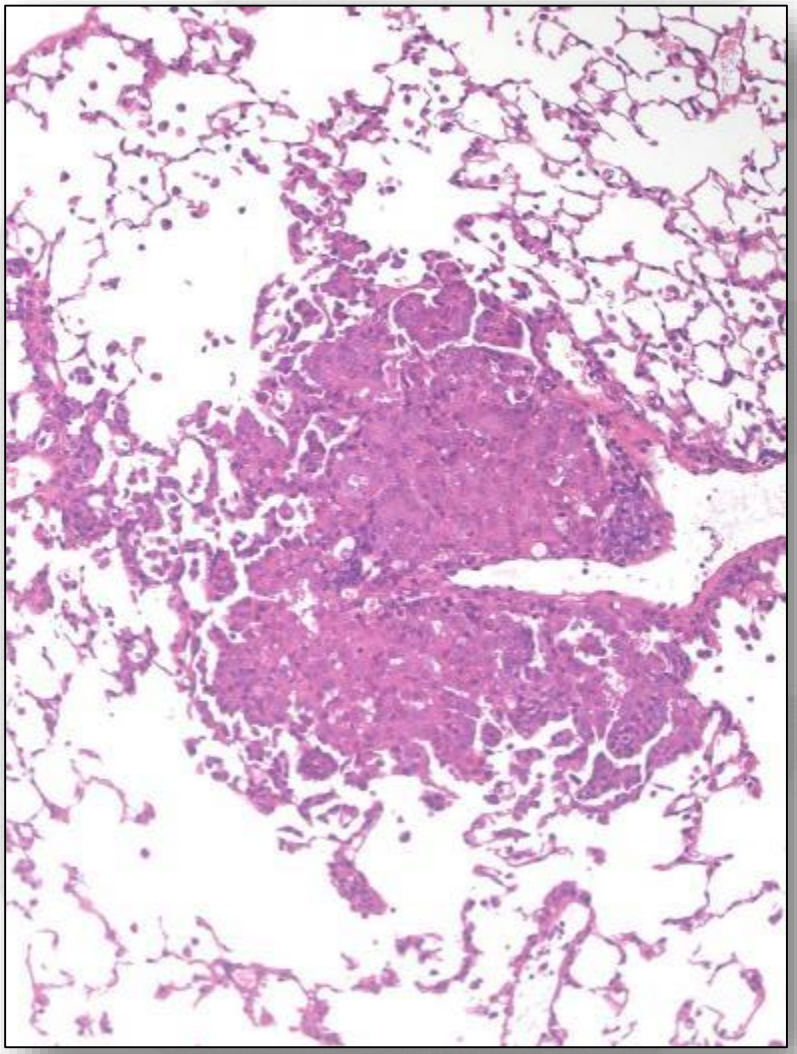


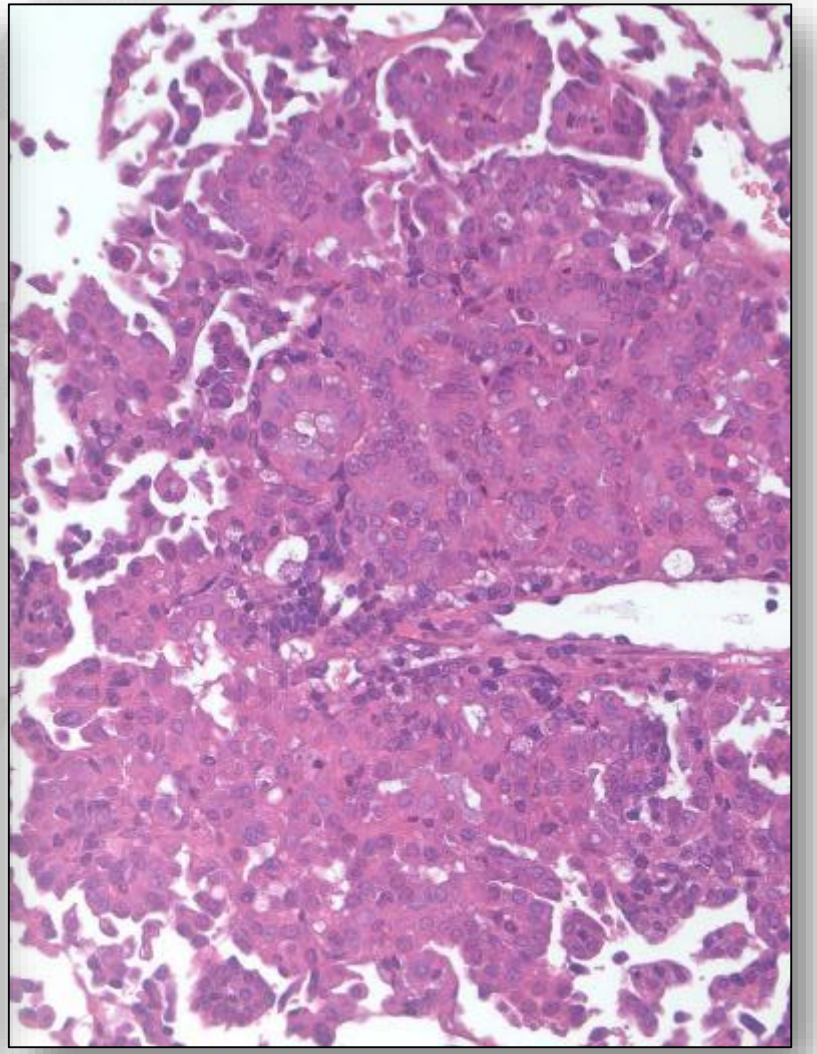
High dose mouse: lungs – AB adenoma





High dose mouse: lungs – exceptional case





Incidence on lung lesions in mice

	Males				Females			
Fluensulfone [mg/kg diet]	0	30	200	1200	0	30	200	1200
Animals examined	50	50	50	50	50	50	50	50
Bronchiolar hyperplasia	1	-	24**	31**	5	7	43**	48**
Mean grade (0-5)	1.0	-	1.3	1.6	1.0	1.0	1.8	2.6
Alv/bronch. adenoma	7	9	5	12	2	4	14**	9*
Alv/bronch. carcinoma	8	3	3	4	2	1	1	4
Pooled alv/bronc. carc. & alv/bronc. adenoma	15	12	8	16	4	5	15	13

Fisher exact test (one-sided): $p<0.05$*, $p<0.01$**

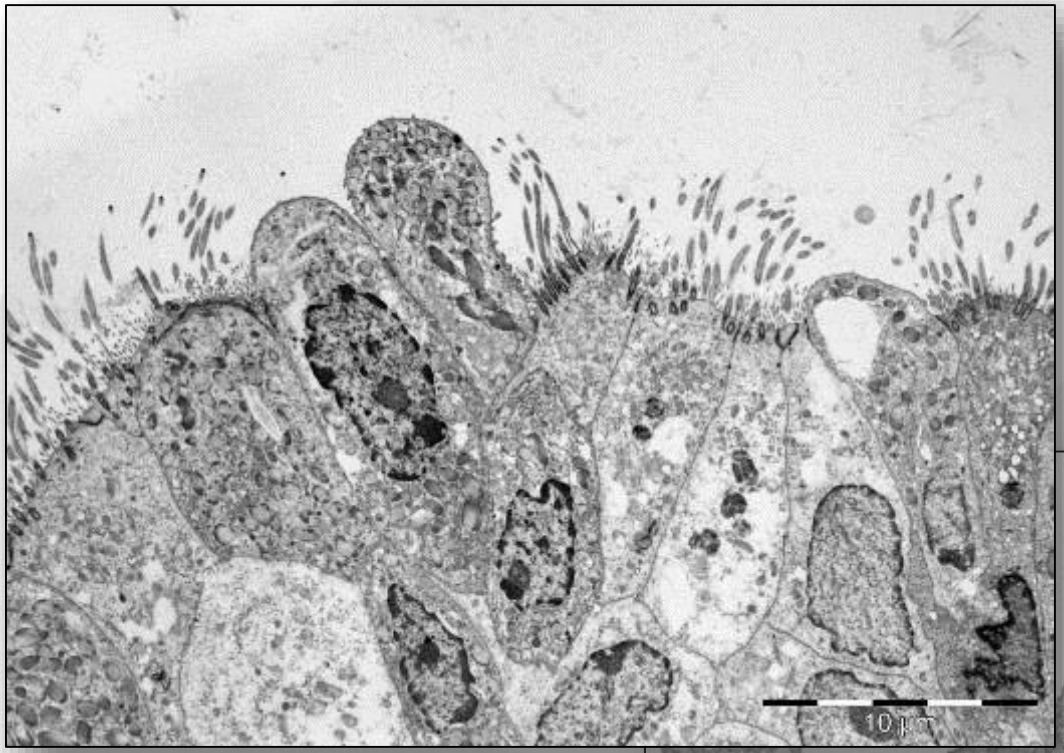
MOA: First step

- basophilic and multifocal hypertrophic epithelium
- mainly non-ciliated cells involved
- possible Clara cell origin
- electron microscopy by re-fixation and contrasting of previously formaldehyde-fixed material (Dept. Anatomy, University of Bern, Switzerland)

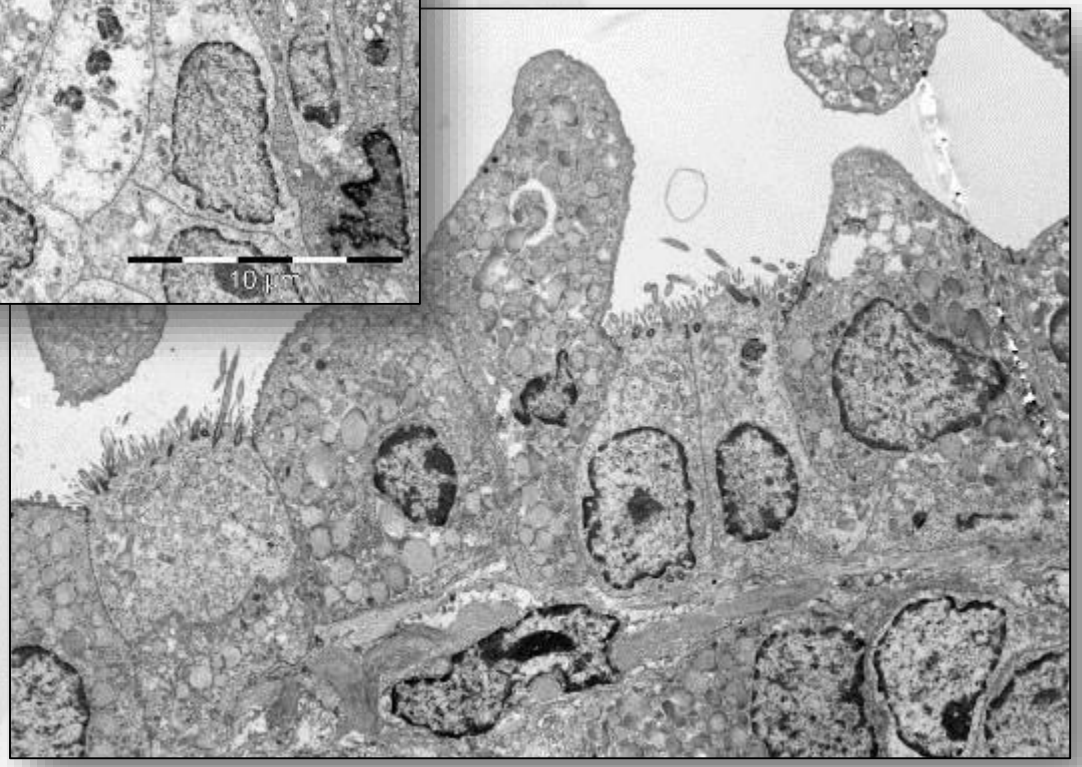
Results:

- increased number of Clara cells
- ciliated cells visible, however very low in number related to Clara cells
- Clara cell layers with pseudo-stratification indicating hyperplastia

Normal bronchiolar epithelium

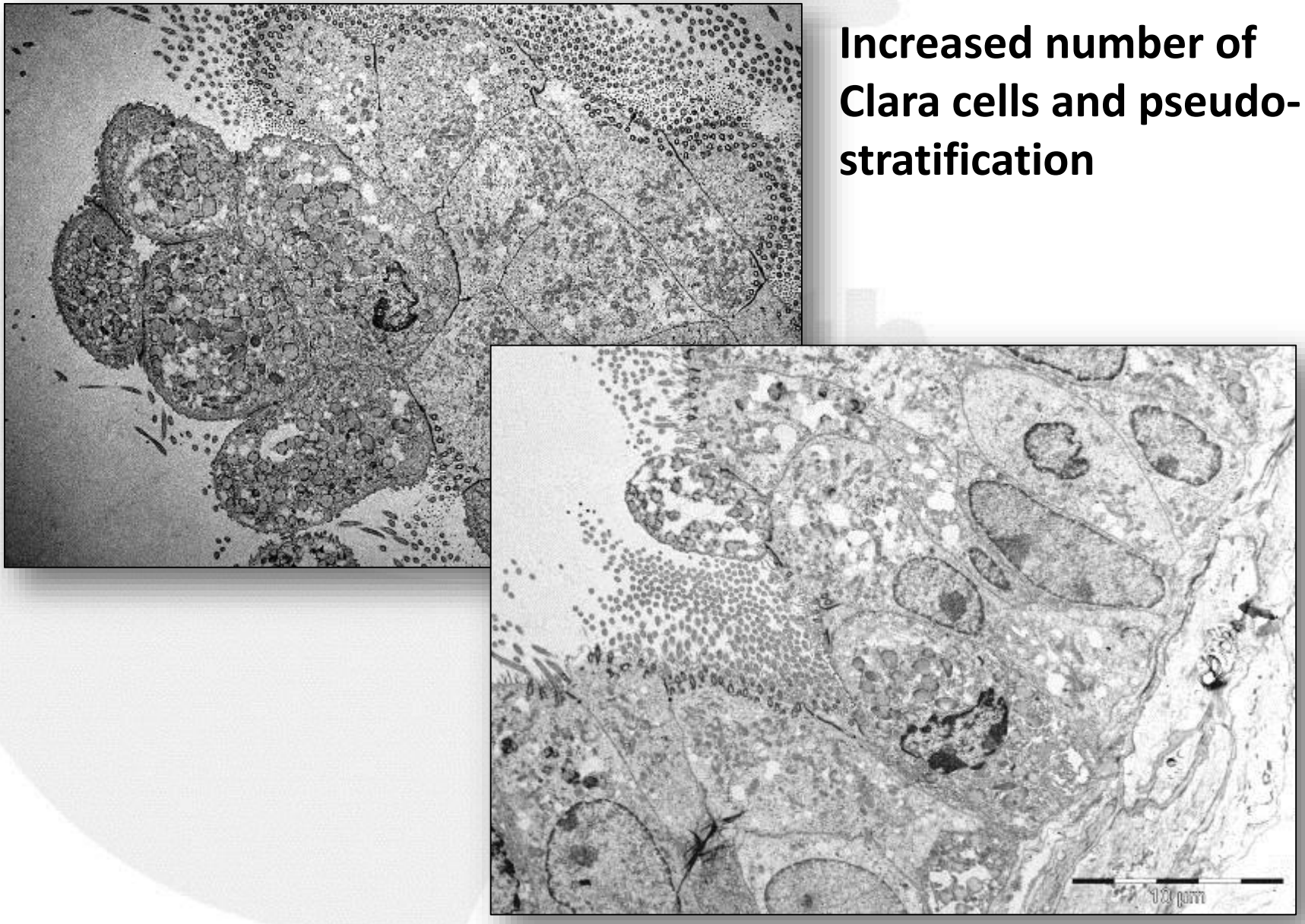


Normal appearance of single layered epithelium



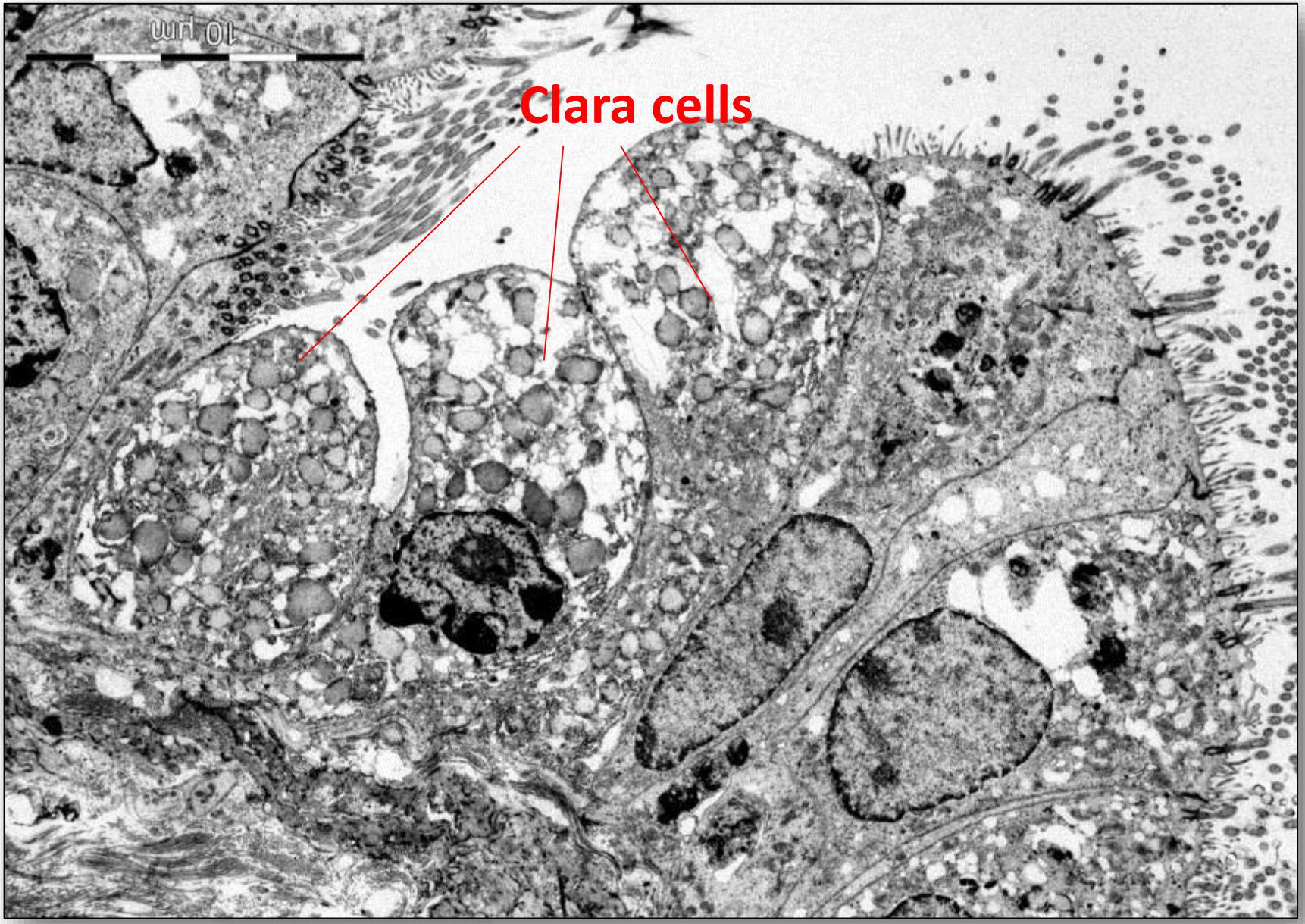
- **Ciliated cells**
- **Clara cells are non-ciliated containing vesicles**

Hyperplastic bronchiolar epithelium



Increased number of Clara cells and pseudo-stratification

Hyperplastic bronchiolar epithelium



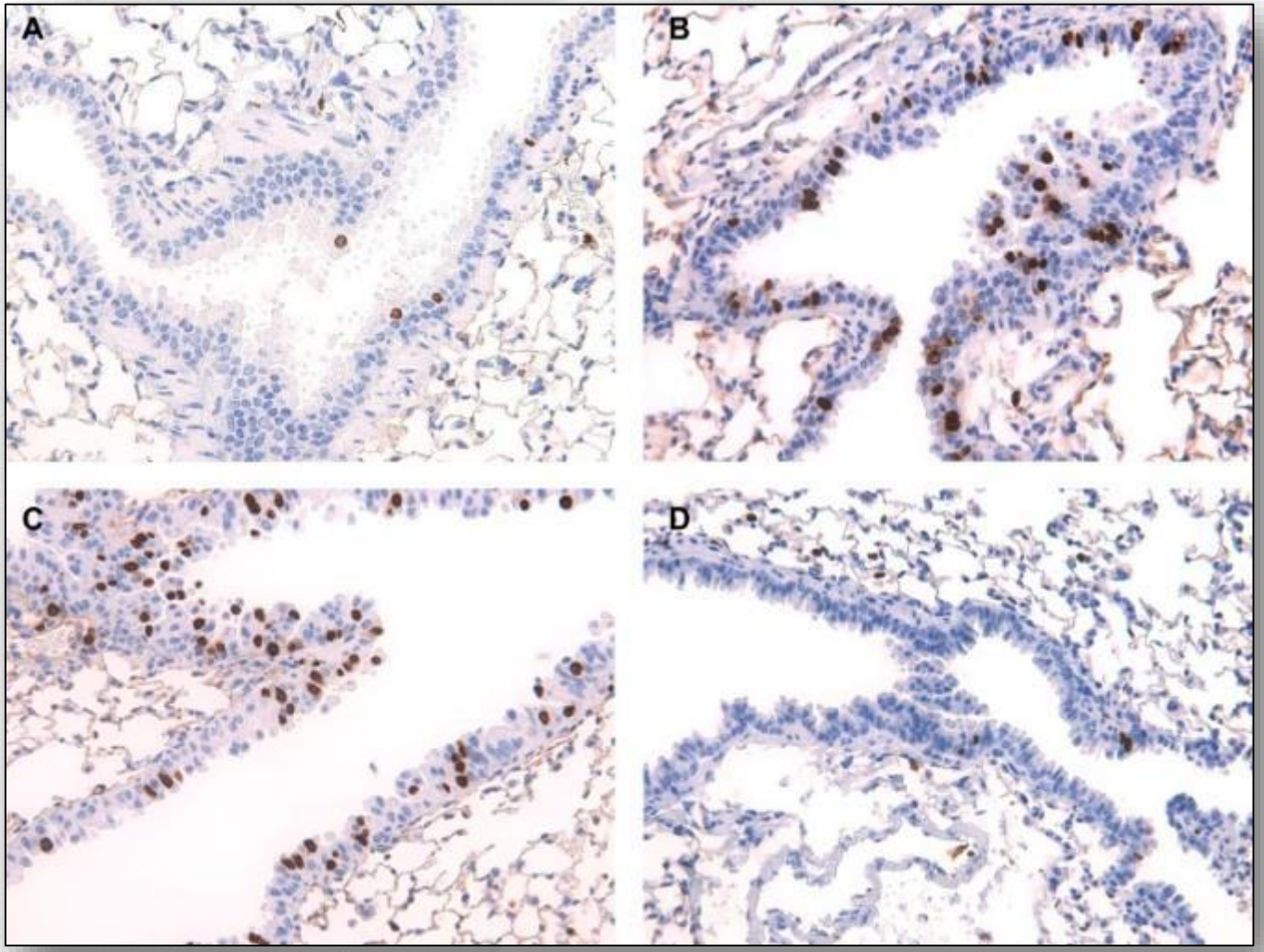
MOA: Cell proliferation study in mice

Group	Control	Fluensulfone 1200 mg/kg	Isoniazid 1305 mg/kg
Number of mice 3 days	5	5	5
7 days	5	5	5

MOA: Cell proliferation study in mice

- 2 and 14 h before sacrifice, the animals were injected ip with 100 ml of a 10 mg/ml aqueous BrdU
- Lungs (and a piece of small intestine as a labeling control)
- Histopathological evaluation and immunohistochemistry
- Number of BrdU-positive cells within all positive staining bronchiolar epithelial cells counted manually (distinction between Clara cells and other bronchial epithelial was not possible by light microscopy, therefore, all visible bronchioles on a slide were evaluated to reach at least 500 cells.
- Final counted number of all cells and positive cells was calculated for a ratio with 1000 cells in the denominator

MOA: 4x increase of cell proliferation after 3 days (recovery after 7 days)

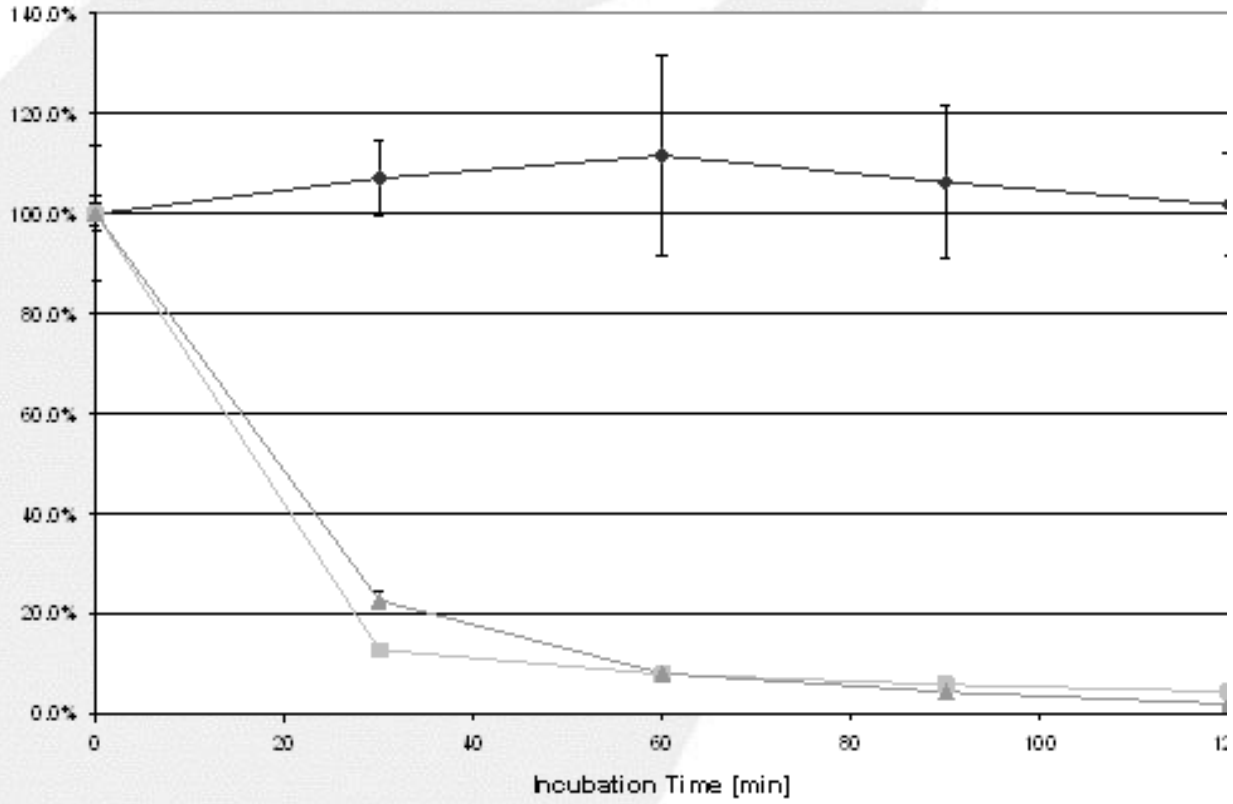


A) Control, (B) Fluensulfone (day 3), (C) Isoniazid (day 3), (D) Fluensulfone (day 7).

MOA: comparative metabolism-results

- **Metabolic capacity of the microsomes of both species confirmed by activity in converting chlorzoxazone to hydroxyl-chlorzoxazone**
- **In presence of inhibitors of CYP2E1 and Cyp 2f2:**
  CYP 2E1 - no important role
  Cyp 2f2-inhibitor partly inhibited the metabolism
- **After 120 minutes, fluensulfone was almost completely metabolized in mouse lung microsomes whereas in the human lung microsomes there was no indicator for metabolic transformation**

MOA: species-specific metabolism



Time-dependent metabolic conversion (%) of fluensulfone in human and mouse lung microsomes

Key events

Key Event	Mice	Humans
Metabolic activation by Cyp 2f2	Yes	No (known lack of Cyp 2f2 in human Clara cells and fewer Clara cells in humans)
Increased Clara cell proliferation	Yes	Unlikely
Bronchio-alveolar hyperplasia and neoplasia	Yes	Unlikely

Summary

- Pathologists as a source of misinterpretation
- PR and PWG
- Fortuities occur often
- Stars and statistics
- Species specificity
- Metabolism
- MOA vs mechanistical studies