In silico, in vitro, in vivo alternatives to the carcinogenicity bioassay

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Mechanistic findings at the basis of the science and regulation of mutagens and carcinogens

- **Miller**s' electrophilic reactivity theory of carcinogenesis
  (not including nongenotoxic carcinogens)

- **Chemical mutagenicity:**
  Malling's *in vitro* **S9** metabolic activation;
  Salmonella, **or Ames**' test for DNA-reactive chemicals

- **Ashby**'s theoretical model of carcinogenicity (compilation of **Structural Alerts**)

Mechanistic findings at the basis of the science and regulation of mutagens and carcinogens

- Because of the success **of Miller**s' electrophilic reactivity theory of carcinogenesis, and of **Ames**' test:

  major research efforts on the hypothesis **Mutation = Cancer**

- Later on, recognition of **nongenotoxic carcinogens**

Looking for further mutagenicity Short-Term Tests (STT) to predict carcinogenicity

- Hypothesis:
  Different **genetic endpoints** (gene mutation, chromosomal damage), and different **cells** (bacterial, mammalian) to cover the spectrum of cancer-relevant factors

- Development of **> 100 STTs** based on:

mutagenicity (e.g., gene mutation in mammalian cells, chromosomal aberrations, aneuploidy)

other **genotoxic** events (e.g., DNA damage)

Comparative carcinogenicity prediction exercise:

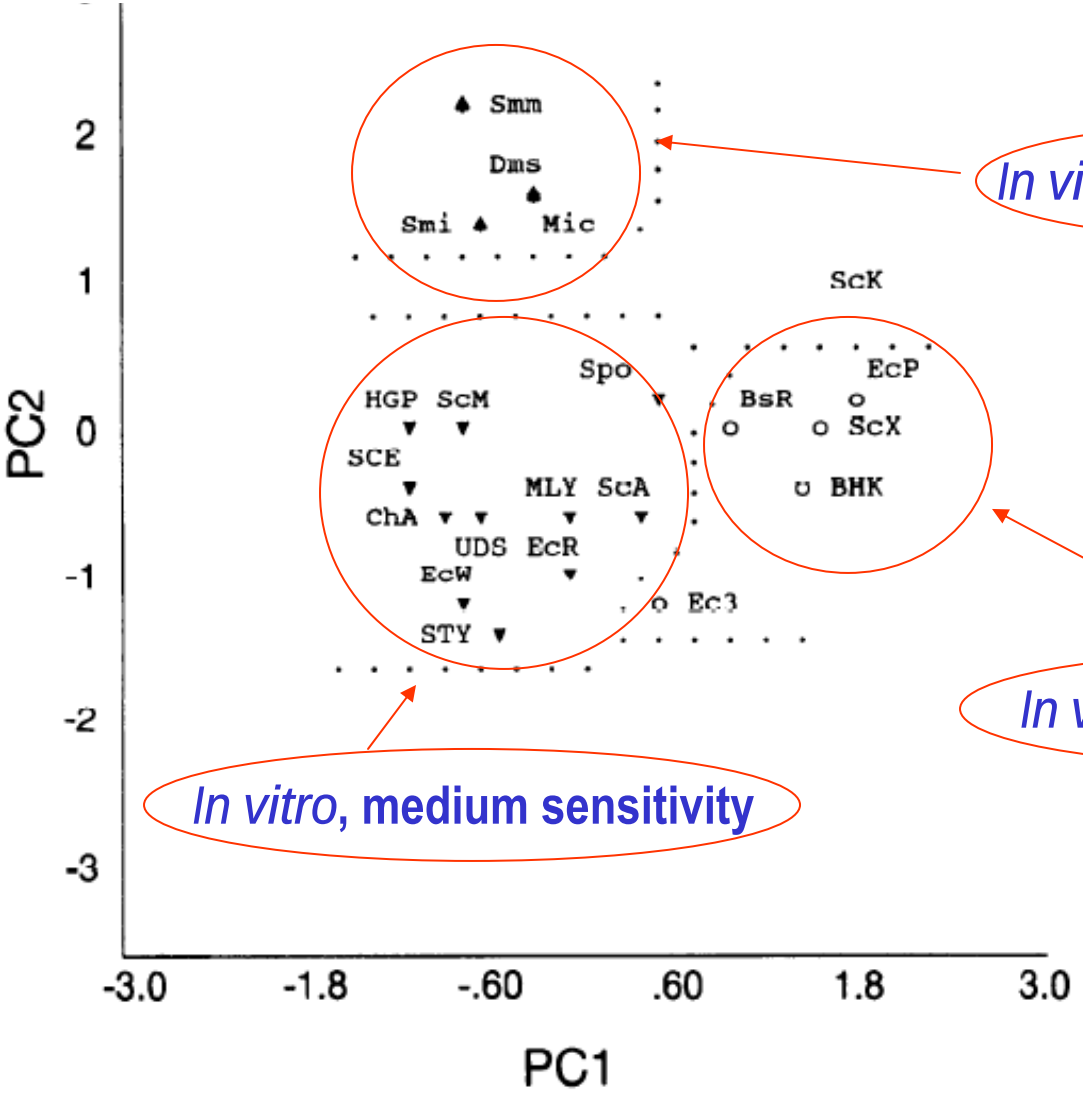
22 mutagenicity STTs x 42 chemicals

De Serres and Ashby, 1981, Evaluation of Short-Term Tests for Carcinogens,
Progr. Mutat.Res. Vol 1

Conclusion(s):

- Central role for *Salmonella*
- Continue with exploration of different **genetic endpoints** and different **cells**

Comparative carcinogenicity prediction: 22 mut. STTs x 42 chemicals



In vivo, low sensitivity

In vitro, **high sensitivity**

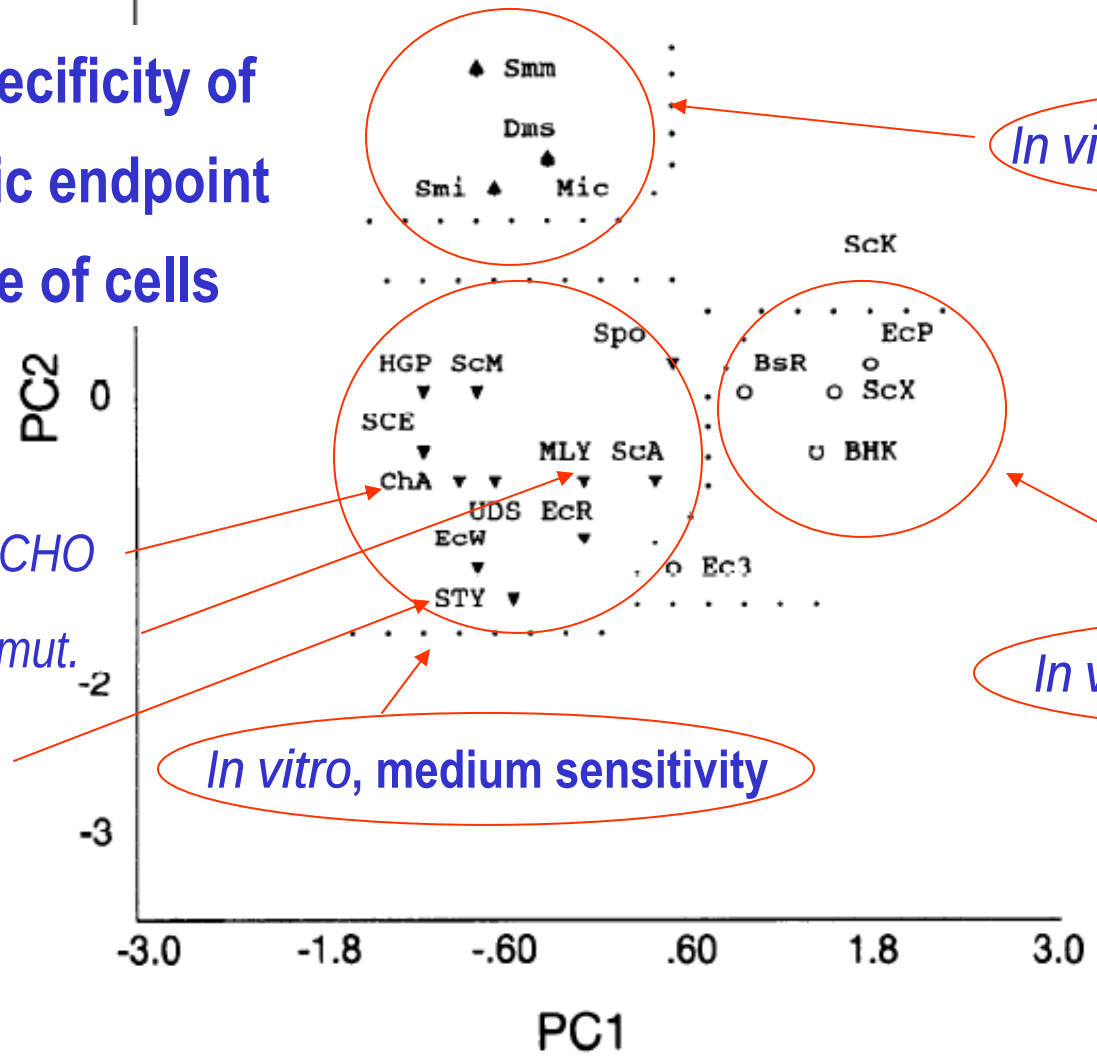
In vitro, **medium sensitivity**

Analysis: Benigni and Giuliani, 1985, Mutat.Res., 147: 139 - 151

Data from: De Serres and Ashby, 1981, Evaluation of Short-Term Tests for Carcinogens, Progr. Mutat.Res. Vol 1

Comparative carcinogenicity prediction: 22 mut. STTs x 42 chemicals

No specificity of genetic endpoint or type of cells



In vivo, low sensitivity

In vitro, **high sensitivity**

In vitro, **medium sensitivity**

Chrom. Aberr. CHO

Mouse lymph. mut.

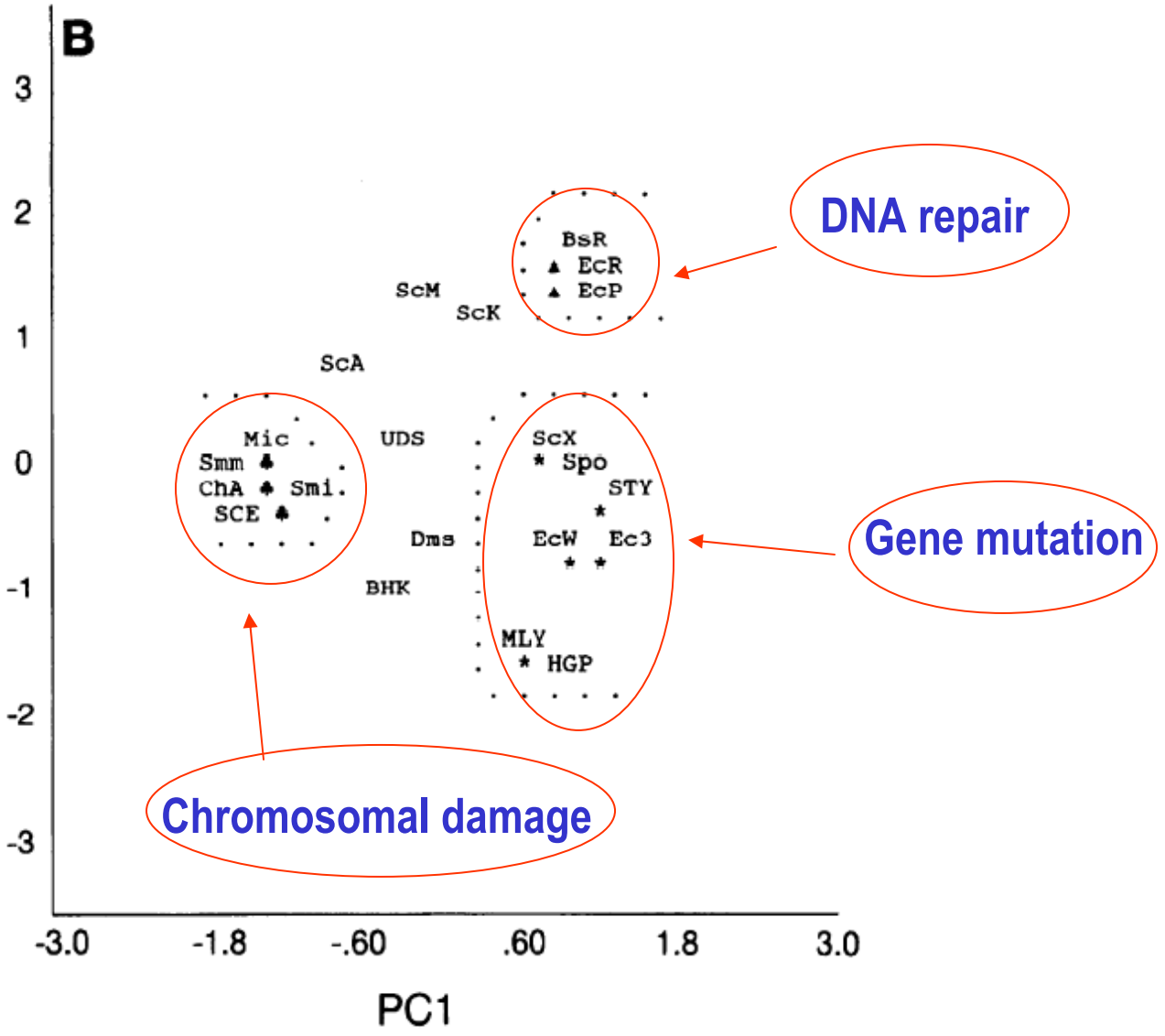
Salmonella

Analysis: Benigni and Giuliani, 1985, Mutat.Res., 147: 139 - 151

Data from: De Serres and Ashby, 1981, Evaluation of Short-Term Tests for Carcinogens, Progr. Mutat.Res. Vol 1

A questionnaire for biologists:

how 22 mutagenicity STTs will respond to 42 chemicals ?



Benigni and Giuliani, 1988, Mutat. Res. 205: 227; Benigni and Giuliani, 1994, Amer.J.Physiol., 266: R1697

Looking for further mutagenicity Short-Term Tests to predict carcinogenicity

- Looking for complements to *Salmonella (Ames)*:

  National Toxicology Progran evaluation of four *in vitro* STTs

  (n. chemicals = 114)

- Different **genetic endpoints** (gene mutation, chromosomal damage), and different **cells** (bacterial, mammalian)

  Salmonella typhimurium (Ames)

  Chromosomal Aberrations in CHO cells

  SCEs in CHO cells

  Mouse Lymphoma mutation

Tennant et al., 1987 Science 236: 933-941

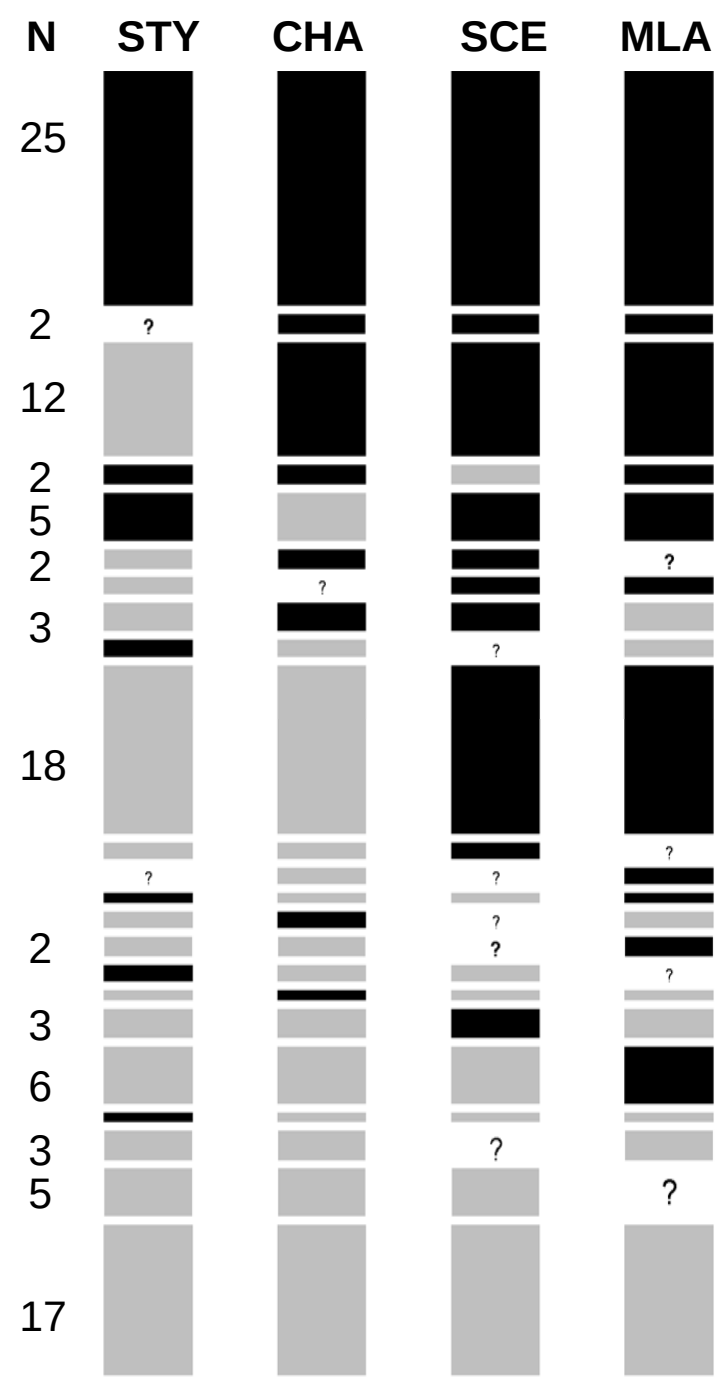
Zeiger et al., 1990 Environ. Mut. Mutagen. 16(18): 1-14

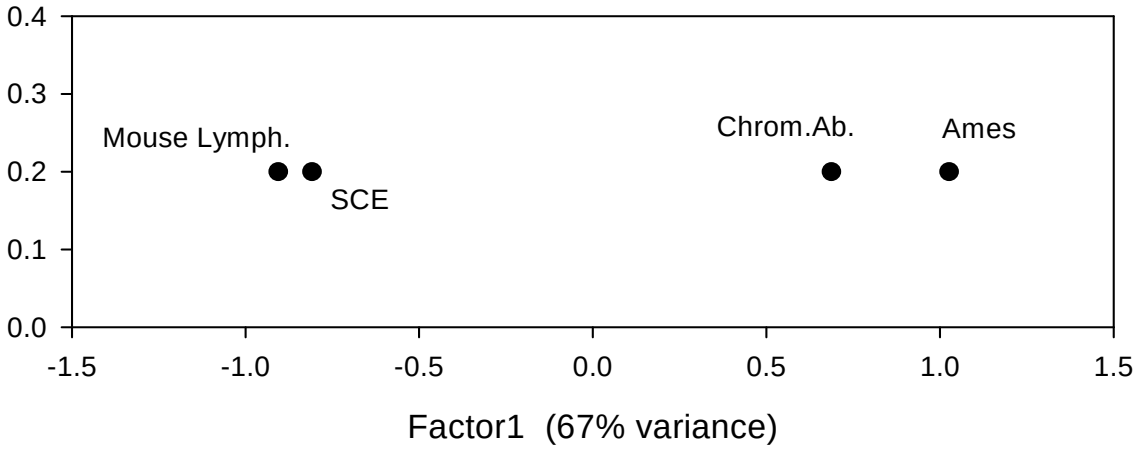
Relevance of STTs to rodent carcinogenicity

	Chi-square (p)
Ames	< 0.0001
Chrom. Ab (CHO)	0.011
Sce (CHO)	0.277
Mouse Lymphoma mut.	0.305
Batteries	
Ames + Chrom.Ab.	0.0004
Ames + Mouse Lymphoma	0.120

(our elaborations)

Genetic Toxicity Profiles: 114 NTP Chemicals



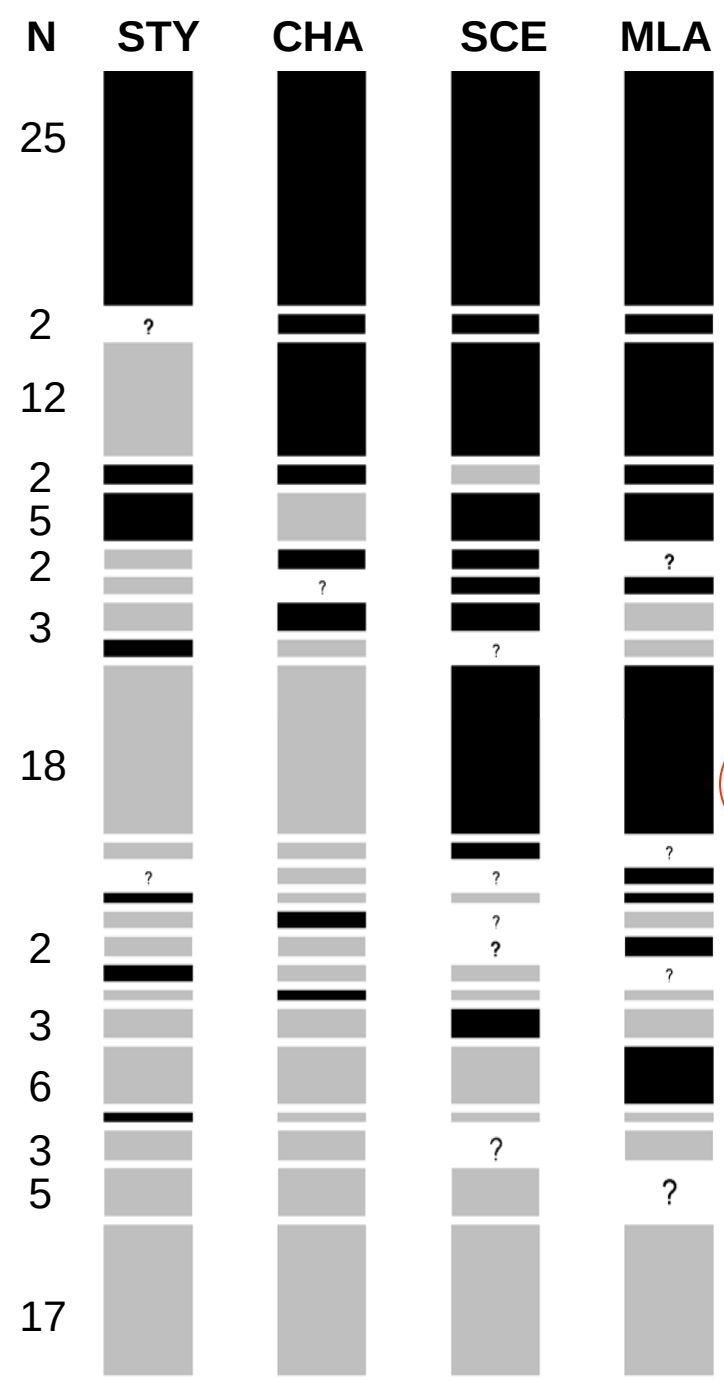


positive

negative

? equivocal

Benigni 1992 Mutagenesis 7: 335-341



Genetic Toxicity Profiles: 114 NTP Chemicals

Positive in Salmonella (and other tests): high correlation with carcinogenicity

Negative in Salmonella and positive in other tests: no correlation with carcinogenicity

positive

negative

? equivocal

Benigni 1992 Mutagenesis 7: 335-341

STTs to predict carcinogenicity

- **Mutagenicity = Carcinogenicity ?**

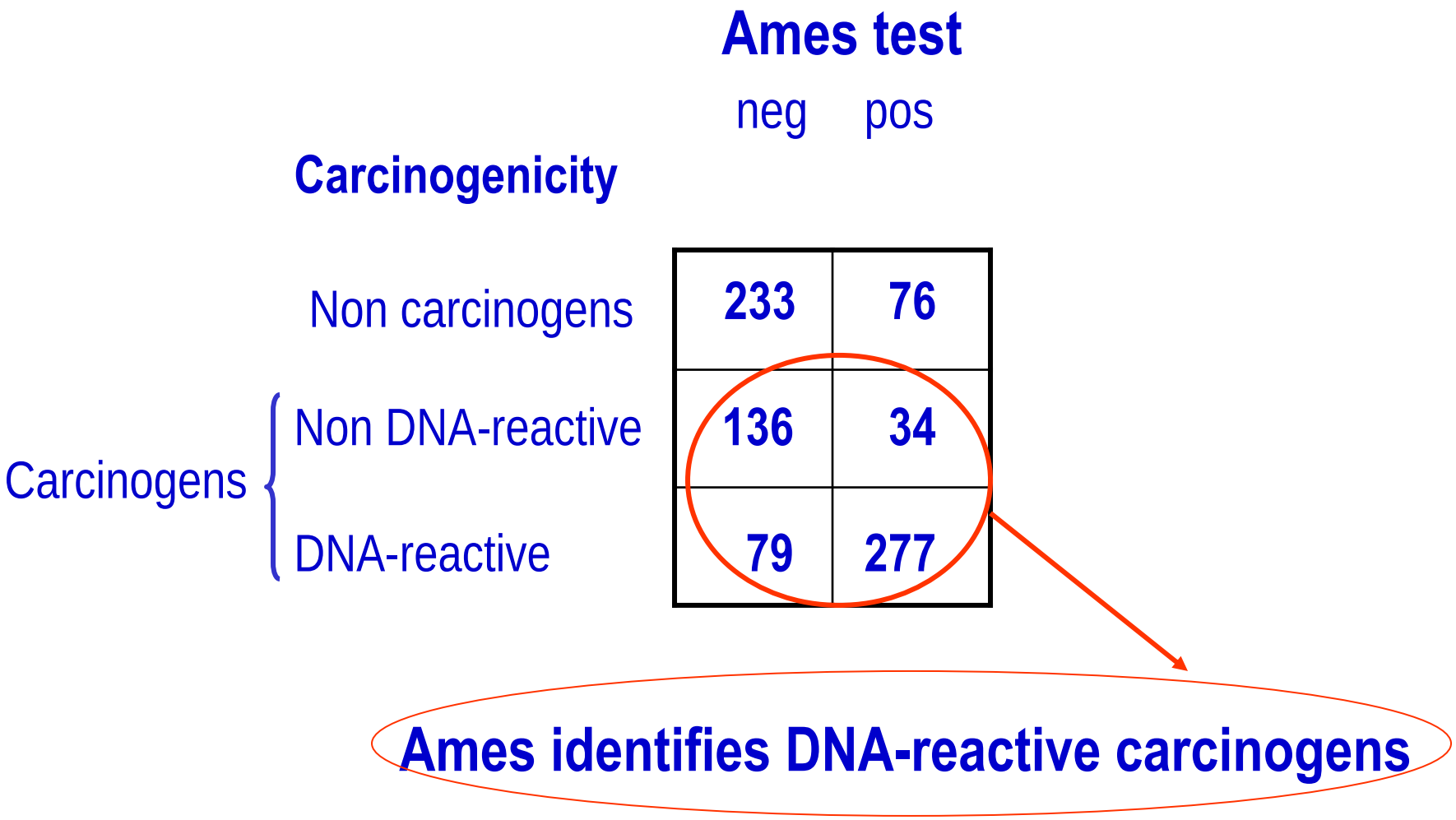
  Only within a limited area of the chemical space, i.e., **DNA-reactive** chemicals

- DNA-reactive chemicals induce **cancer, together with a wide spectrum of mutations**
- Most predictive mutagenicity-based assay: **Ames test**
- **Non DNA-reactive chemicals**, mutagenic in other *in vitro* assays (e.g., clastogenicity) but Ames-negative: no correlation with carcinogenicity

- No reliable *in vivo* **STT** (e.g., micronucleus) is available

Benigni R. et al., Exp.Opinion Drug Metab.Toxicol., 2010, 6: 1-11..
Zeiger E Regulat.Pharmacol.Toxicol. 1998;28:85-95.

Ames test *versus* rodent carcinogenicity



Results from 835 chemicals in ISSCAN v3a

http://www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7

Ames test *versus* rodent carcinogenicity

Carcinogenicity	Ames test: neg	Ames test: pos
Non carcinogens	233	76
Carcinogens — Non DNA-reactive	136	34
Carcinogens — DNA-reactive	79	277

Ames mutagen: 80% probability of being a carcinogen

Results from 835 chemicals in ISSCAN v3a
http://www.iss.it/ampp/dati/cont.php?id=233&lang=1&tipo=7

Prediction of carcinogenicity by the STTs

Mechanistic classes	*Ames*	*Chrom. Aberrations*
	Chi-square (p values)	
Direct Alkylating Agents		
All	0.0049	0.0001
Epoxides	0.0013	NS
Aliphatic halogens	NS	0.0055
Indirect Alkylating		
All	NS	NS
		too few data for most classes
Intercalating		
All	NS	---
Aminoaryl DNA-adducts forming		
Aromatic amines	0.0074	NS
Nitroarenes	0.0443	NS

Backing up the STTs with **Structure-Activity** concepts

Structure-activity relationship concepts:

application to different issues, through different approaches

Coarse-grain	**Structure Alerts** (mechanistic classes, category formation, priorities)
Fine-tuned	Quantitative Structure-Activity Relationships (**QSAR**) of congeneric classes
Hybrid (??)	non-local, or global QSARs

Toxtree: Rulebase for mutagens / carcinogens

Structure-based approach consisting of:

-New compilation of *Structure Alerts* (genotox and non-genotox)

-Three mechanistically-based *QSARs* for congeneric classes (aromatic amines, aldehydes)

Toxtree (version 1.6)

Open-source, freely available: *http://ecb.jrc.it/qsar/qsar-tools/index.php?c=TOXTREE*

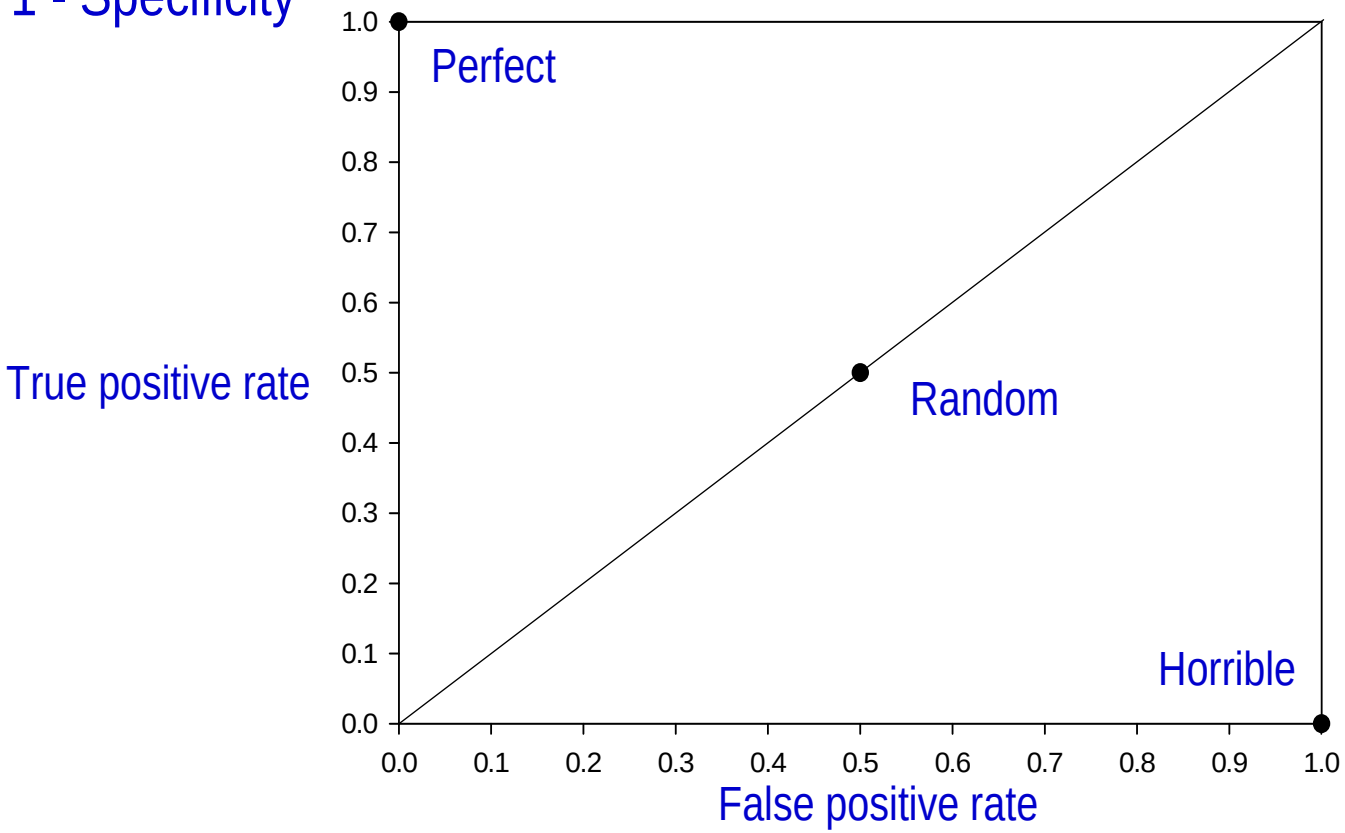
ROC graph: A simple, graphical way of comparing predictions with results

True positive rate = (Positives predicted as positive) / (Real positives)

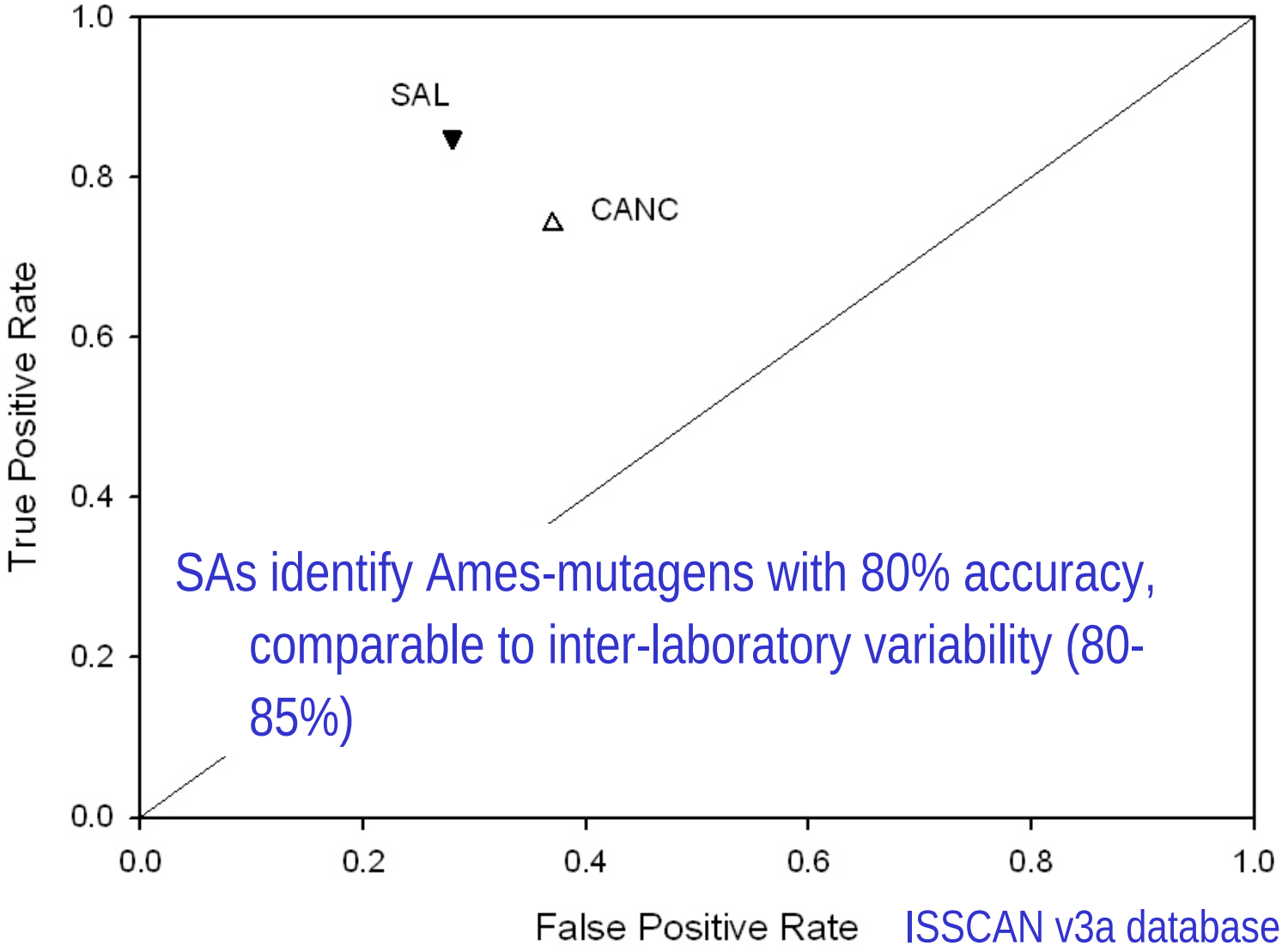
= Sensitivity

False Positive Rate = (Negatives predicted as positive) / (Real negatives)

= 1 - Specificity



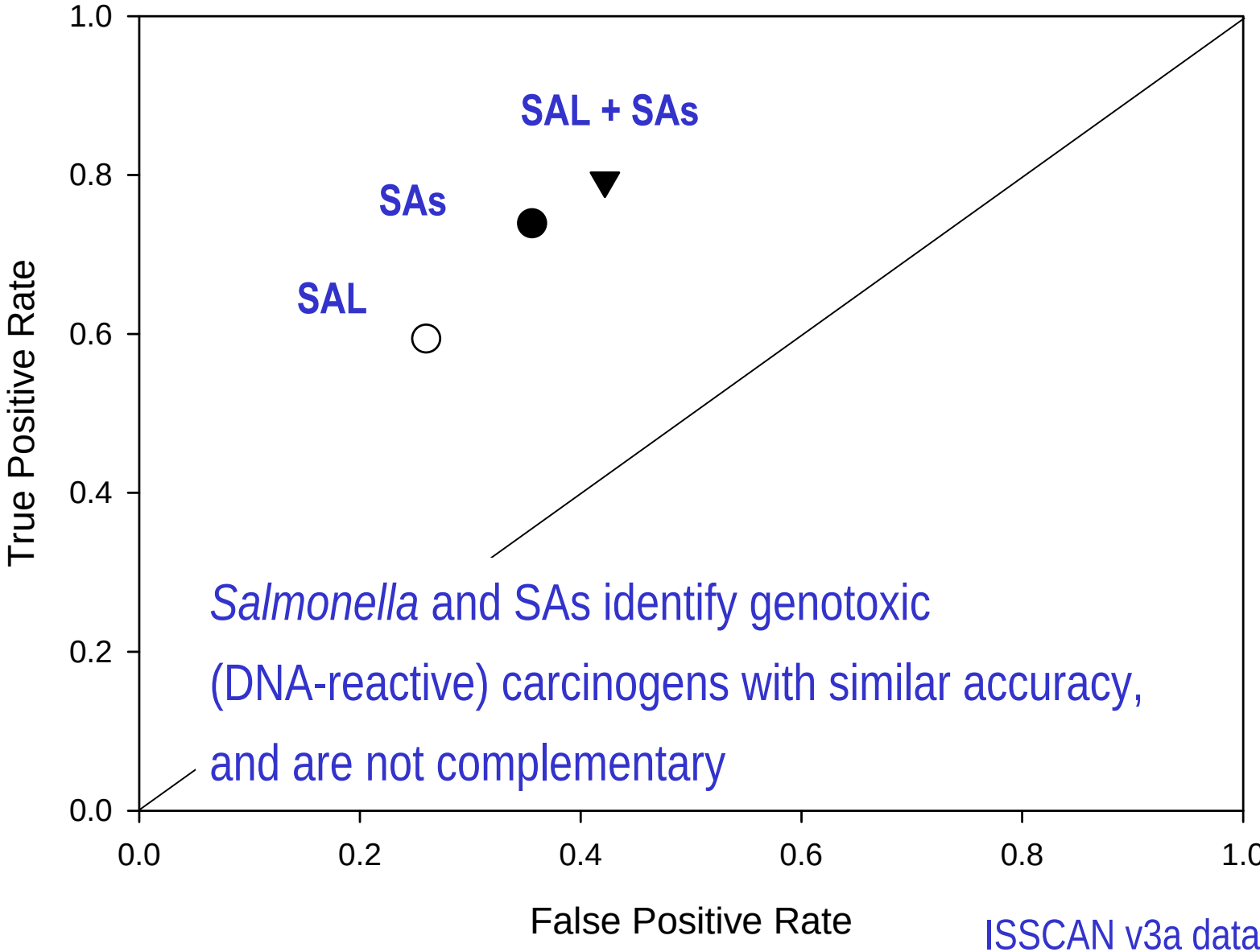
Toxtree SAs: agreement with Carcinogenicity and *Salmonella* (Ames)



SAs identify Ames-mutagens with 80% accuracy, comparable to inter-laboratory variability (80-85%)

ISSCAN v3a database

Carcinogenicity prediction: *Salmonella* (Ames) *versus* SAs



Salmonella and SAs identify genotoxic (DNA-reactive) carcinogens with similar accuracy, and are not complementary

ISSCAN v3a database

Which use for the Structural concepts (Alerts) ?

Success story: priority setting by human experts

Out of 400 chemicals tested by NCI/NTP:

- 2/3 selected as suspect carcinogens (n=267)

  68% carcinogenic (n=187)

- 1/3 selected on production/exposure considerations (n=133)

  20% carcinogenic (n=26); 6.8% positive in two species (n=9)

Fung, Barrett, and Huff (1995) Environ.Health Perspect. 103: 680-683

QSARs of Aromatic amines

Carcinogenic **activity** in rodents

Canc = - 1.16 HOMO + 1.76 LUMO – 2.86 L(R) + 2.65 B5(R) + 0.40 MR_3

+ 0.58 MR_5 + 0.54 MR_6 – 1.55 I(An) + 0.74 I(NO2) – 0.55 I(BiBr)

n = 66 (- =44; + = 73) Correct Classification = 87.9 %

Franke et al., 2001

Mutagenic **activity** in *Salmonella typhimurium* TA100 (+ S9)

Electronic ↓ Steric ↓

ActTA100 = 0.67 HOMO - 0.75 LUMO - 0.39 MR_2 – 0.38 MR_3 – 0.44 MR_6

- 0.62 Idist

n = 111 (- =47; + = 64) Correct Classification = 87. %

Benigni et al., 2007

European Chemicals Bureau - ISS Project

Assessment of local QSARs for congeneric classes:

- scientifically (mechanistically) interpretable
- good internal statistics

- **real external predictivity** tested
- applicability domain checked:
  - functional group
  - parameters range
  - chemical similarity

Conclusions on the local QSARs:

- scientifically interpretable, good internal statistics, but vary for their external predictivity
- **QSARs for potency: predictions 30 – 70 % correct**
- **QSARs for activity: predictions 70 – 100 % correct**
- Estimating intervals more reliable than estimating points
- **Internal validation measures do not correlate with external predictivity**

Benigni,R. and Bossa,C. (2008): Predictivity of QSAR. J.Chem.Inf.Model., 48:971-980

Which use for local QSARs ?

- Local QSARs for activity: 70 – 100 % correct external predictions

- Intra-Assay (inter-laboratory) agreement for the Ames test: 80 – 85%

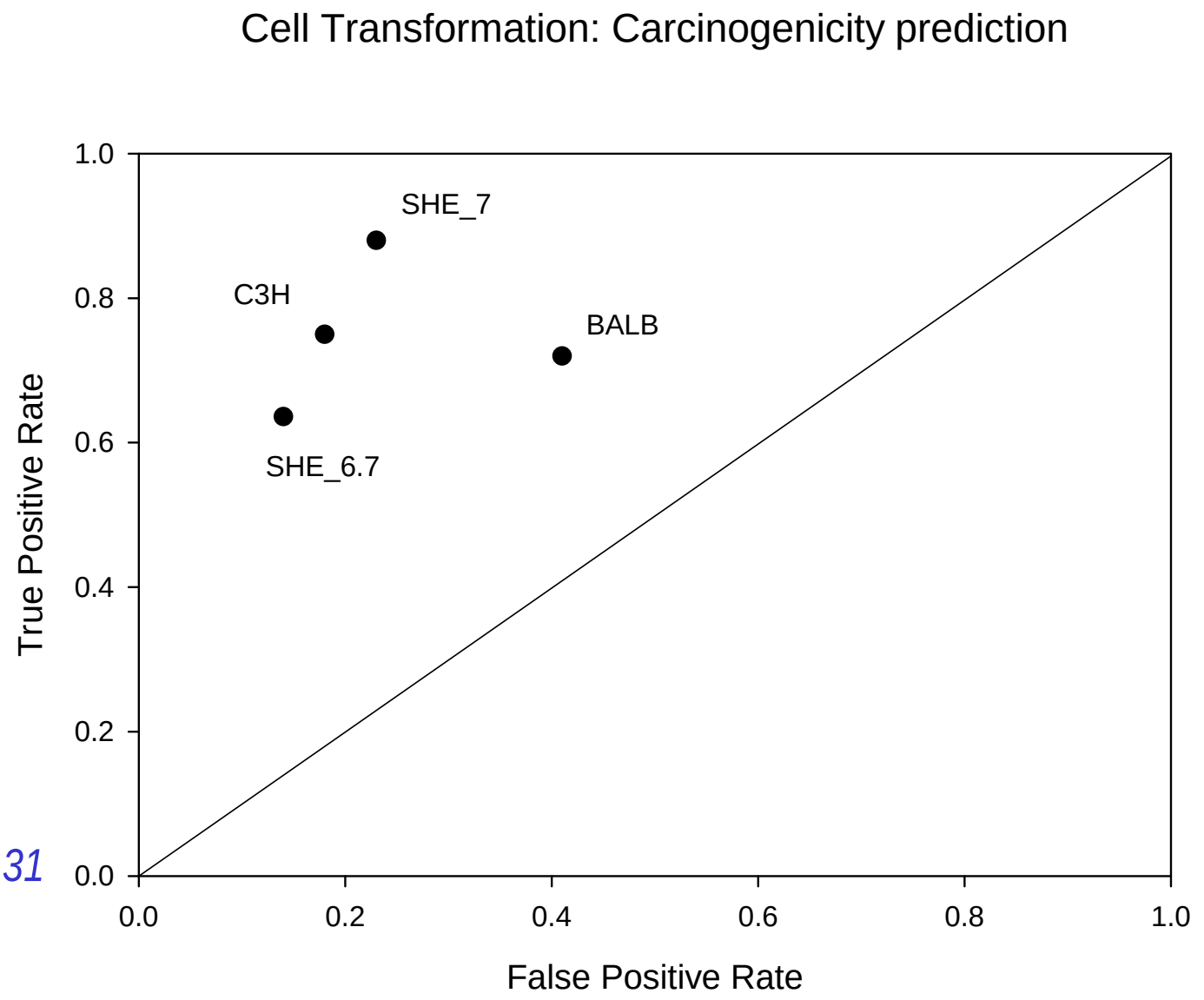
Piegorsch and Zeiger, 1990, in Statistical methods in Toxicology

Same range of uncertainty

Non-mutagenicity assays for predicting carcinogenicity ?

Cell transformation assays

in cultured cells, phenotypic alterations characteristic of tumorigenic cells



Data: *OECD Series on Testing and Assessment, 2007, vol.31*

Our elaboration

SHE pH≥7 Cell transformation *versus* rodent carcinogenicity

Carcinogenicity	SHE neg	SHE pos
Negatives	33	11
Non DNA-reactive	6	28
DNA-reactive	5	58

Carcinogens

Sensitive to DNA-reactive and non-DNA-reactive carcinogens

Chemicals n = 141, from OECD vol. 31

Great challenges:

overcoming the *in vitro* / *in vivo* gap

identifying “useful” steps in toxicity pathways

and more…

In vitro alternatives to carcinogenicity

The equation *mutation = cancer* is not of general validity

Out of > 100 STTs, only *Salmonella* and **cell transformation** (to be confirmed on larger database) predict chemical carcinogenicity

Present testing strategies to be revised ?

in vitro **alternatives**: state-of-the-art

• Toxicokinetic		
• **Acute toxicity**		*Reduction*
• **Skin irritation and corrosion**	*Replacement*	
• Skin sensitisation		
• **Eye irritation**		*Reduction*
• Acute systemic and local toxicity		
• **Genotoxicity**		*Reduction*
• **Carcinogenicity**		*Reduction*
• Repeated dose toxicity		
• Reproduction		
• Developmental toxicity		
• Ecotoxicity		

"Existing approaches incorporating replacement, reduction and refinement of animal testing: applicability in food and feed risk assessment" *The EFSA Journal* **(2009) 1052, 1-77**

Toxcast Phase 1

Pathway-based screening **approach**

Perturbations of biochemical / biological pathways supposedly related to toxicity

Extensive use of new omics technologies

Isolated, *in vitro* systems

309 unique chemicals

Mostly agrochemicals

- **76 in vivo bioassays**
  - ToxRefDB
    - Target organs (chronic), reproductive, developmental, **carcinogenicity**

  524 in vitro assays
  - 9 *in vitro* assay providers
    - 285 cell-based, 239 cell-free

- **can *in vivo* endpoints be predicted through *in vitro* ones ?**

Averting chance correlations
Cleaning Toxcast *in vitro* data

- **Statistical robustness:** 6 clusters of *in vitro* assays

(Cluster and Principal Component Analysis of response profiles)

- The clusters are **biologically meaningful**:

- Cluster 1: *Cell growth, cell adhesion and shape, etc…(immune-related)*
- Cluster 2*: Signaling (cell cycle, apoptosis…)*
- Cluster 3*: Nervous system factors*
- Cluster 4*: Metabolic factors*
- Cluster 5*: Cell growth, inflammation*
- Cluster 6*: Transcription factors, gene activation*

Prediction of Toxcast carcinogenicity through *in vitro*

- Discard erratic results
- Pick five representative assays from each cluster

Assays	Squared Canonical Correlation (sqcc)	
	Mouse	Rat
Cluster 1	0.02	0.03
Cluster 2	-	-
Cluster 3	-	-
Cluster 4	0.04	0.01
Cluster 5	0.01	-
Cluster 6	-	0.03

Prediction of Toxcast carcinogenicity through *in vitro*

- All representative assays (n=30) are used

Assays	sqcc	
	Mouse	Rat
30 representatives	0.04	0.09

- Toxcast *in vitro* assays are **poorly correlated** with animal carcinogenicity

Benigni, submitted

http://www.epa.gov/NCCT/toxcast/files/summit/ToxcastDataSummit_Poster_Benigni%20May 2009.ppt

Great challenges:

overcoming the *in vitro* / *in vivo* gap

identifying “useful” steps in toxicity pathways

and more…

- **Never take the scientific constructs at face value**
- **Always contrast theories with data**

The best laid schemes of mice and men go often askew
(R. Burns, 1785)

Acknowledgements

- Istituto Superiore di Sanita’

Alessandro Giuliani

Cecilia Bossa

Olga Tcheremenskaia

Structural Alerts: predictive ability

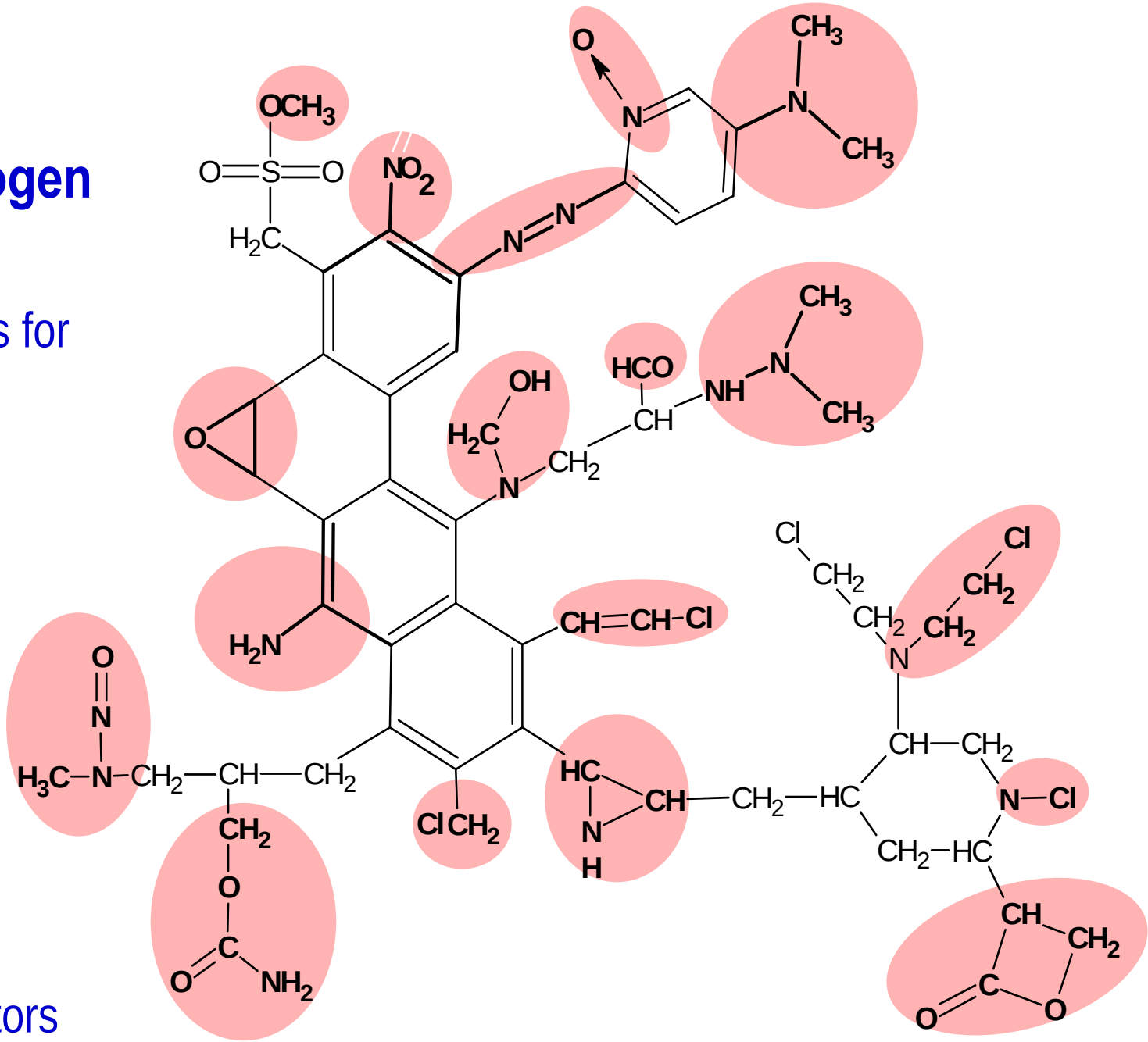
	N	Actives	%
Alkylating, direct acting			
SA_2: alkyl (C < 5) or benzyl ester of sulphonic or phosphonic acid	**13**	**12**	**92**
SA_5: S or N mustard	10	10	100
SA_7: epoxides and aziridines	28	20	71
SA_8: aliphatic halogens	75	51	68
SA_10: a,b-unsaturated carbonyls	55	37	67
SA_11: simple aldehyde	9	8	89
SA_12: quinones	**15**	**13**	**87**
Alkylating, indirect acting			
SA_13: hydrazine	**68**	**57**	**84**
SA_14: aliphatic azo and azoxy	8	8	100
SA_16: alkyl carbamate and thiocarbamate	8	7	88
SA_21: alkyl and aryl N-nitroso groups	**120**	**105**	**88**

Structural Alerts: predictive ability

	N	Actives	%
Intercalating and DNA-adducts forming, indirect acting			
SA_18: polycyclic aromatic hydrocarbons	14	11	79
SA_19: heterocyclic polycyclic aromatic hydrocarbons	**14**	**12**	**86**
Aminoaryl DNA-adducts forming, indirect acting			
SA_27: nitro-aromatic	88	63	72
SA_28: primary aromatic amine, hydroxyl amine and its derived esters	**107**	**87**	**81**
SA_28bis: aromatic mono- and dialkylamine	15	10	67
SA_28ter: aromatic N-acyl amine	17	13	76
SA_29: aromatic diazo	26	20	77
Nongenotoxic			
SA_20: (poly) halogenated cycloalkanes (nongenotoxic)	24	13	54
SA_17: thiocarbonyl (nongenotoxic)	18	14	78
SA_31a: halogenated benzene (nogenotoxic)	16	5	31
SA_31b: halogenated PAH (nogenotoxic)	10	8	80

Ashby's Poly-carcinogen

- Structural alerts for carcinogenicity;
- DNA-reactive functionalities



Some alerts accompanied by detoxifying (modulating) factors

Ashby (1995) Environ.Mutag. 7: 919-921

ECB Activities
- Biocides
- Classification & Labelling
- Computational Toxicology
- Existing Chemicals
- Export-Import
- New Chemicals
- REACH
- Testing Methods

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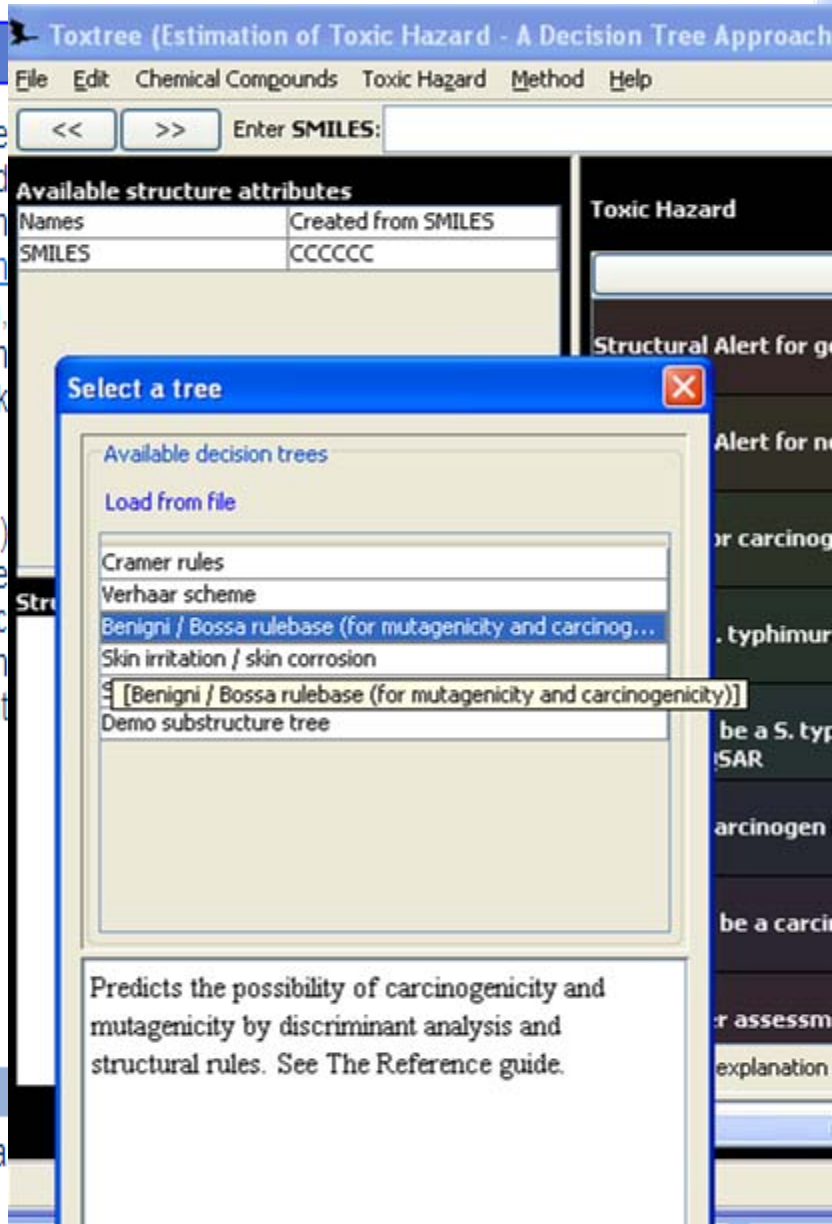
Toxtree

Toxtree is a flexible and user-friendly open-source application that places chemicals into categories and predicts various kinds of toxic effect by applying decision tree approaches, such as the Cramer classification scheme, the Verhaar scheme for aquatic modes of action, and a rulebase for skin irritation and corrosion based on rules developed by the German Federal Institute for Risk Assessment (BfR) and collaborators.

Toxtree was developed by IdeaConsult Ltd (Sofia, Bulgaria) under the terms of an ECB contract. The software is made freely available by ECB as a service to scientific researchers and anyone with an interest in the application of computer-based estimation methods in the assessment of chemical toxicity.

Toxtree (Version 1.20) - Download area

Following the original release of Toxtree (v1.00) in Ma functionalities was released in March 2007.



Non-local, "global" QSARs demonstrated problems in prediction exercises of external chemicals, e.g.,

NTP-1 carcinogenicity prediction exercise

Benigni,R. (1997): The first US National Toxicology Program exercise on the prediction of rodent carcinogenicity: definitive results. Mutat.Res., 387:35-45.

NTP-2 carcinogenicity prediction exercise

Benigni,R. and Zito,R. (2004): The second National Toxicology Program comparative exercise on the prediction of rodent carcinogenicity: definitive results. Mutat.Res.Revs., 566:49-63.

Predictive Toxicology Challenge 2000-2001

Helma,C. and Kramer,S. (2003): A survey of the Predictive Toxicology Challenge 2000-2001. Bioinformatics, 19:1179-1182.

Miscellaneous independent assessments

Benigni,R. and Bossa,C. (2008): Predictivity of QSAR. J.Chem.Inf.Model., 48:971-980.

Future

QSAR modeling:

- Identify further alerts for nongenotoxic carcinogens
- Build further local QSARs for congeneric classes

More work on *in vitro* methods

Integrate *in vitro* and QSAR –to overcome uncertainties on both sides-