In silico alternatives to the carcinogenicity bioassay: correlation with *in vivo*, *in vitro* results

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

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

- **Chemical mutagenicity:**
  Malling's *in vitro* metabolic activation (**S30, S9**);
  Salmonella, **or Ames**' test for DNA-reactive chemicals

- Structure-Activity (carcinogenicity) Relationships (**Ashby**'s **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

to cover the spectrum of cancer-relevant factors, use:

different **genetic endpoints** (gene mutation, chromosomal damage), different **cells** (bacterial, mammalian), and **animals** (ADME)

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

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

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

STTs to predict carcinogenicity: state-of-the-art

- **Mutagenicity = Carcinogenicity ?**

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

- DNA-reactive chemicals induce **cancer, and a wide spectrum of mutations**

- Most predictive mutagenicity-based assay: **Ames test**

- **Other *in vitro* assays (e.g., clastogenicity**), when Ames-negative : no correlation with carcinogenicity

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

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

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

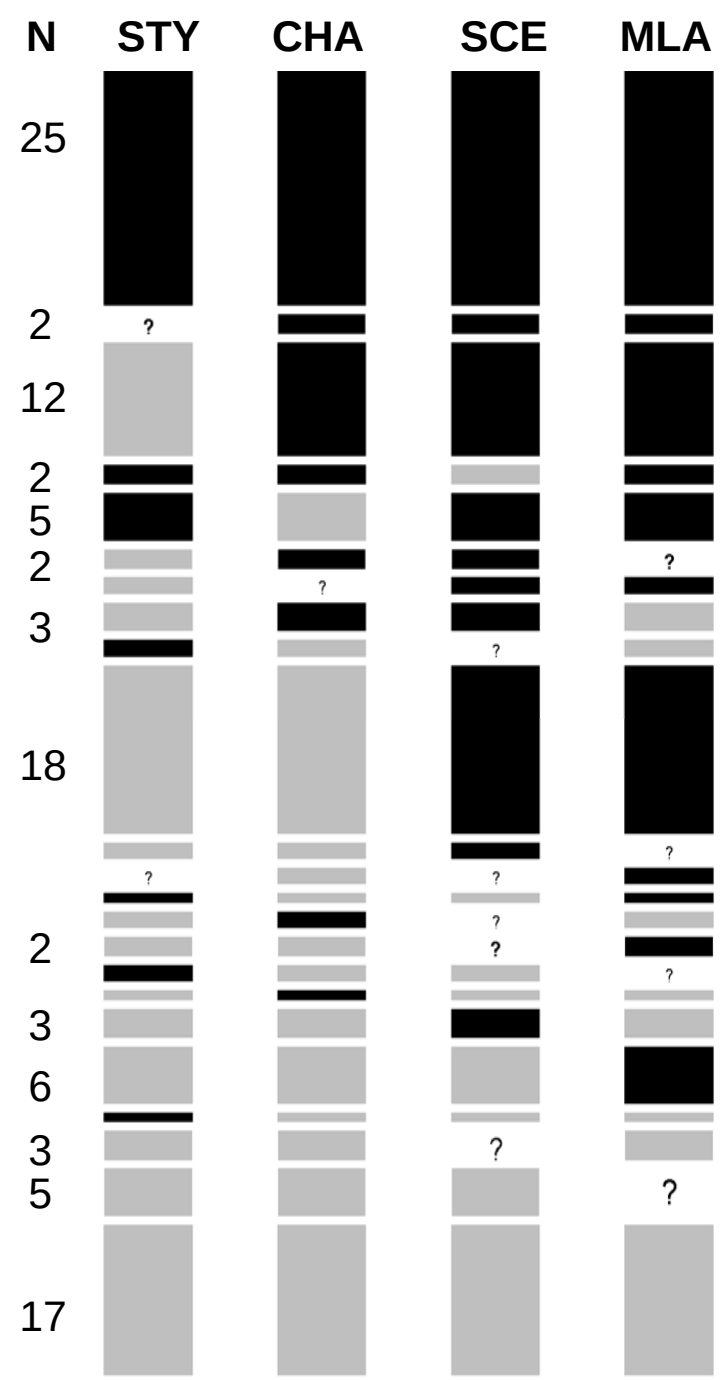
Zeiger et al., 1990 Environ. Mut. Mutagen. 16(18): 1-14

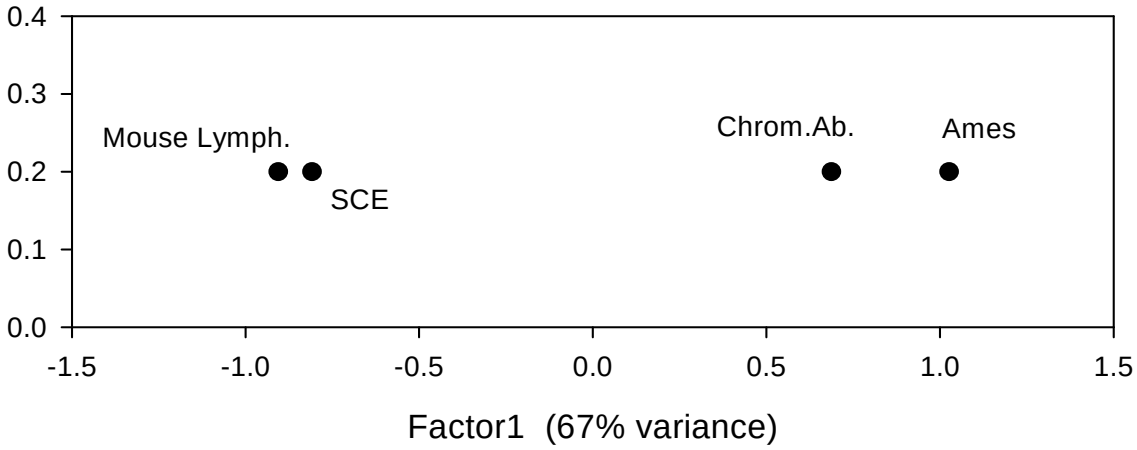
Relevance of STTs to rodent carcinogenicity

	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



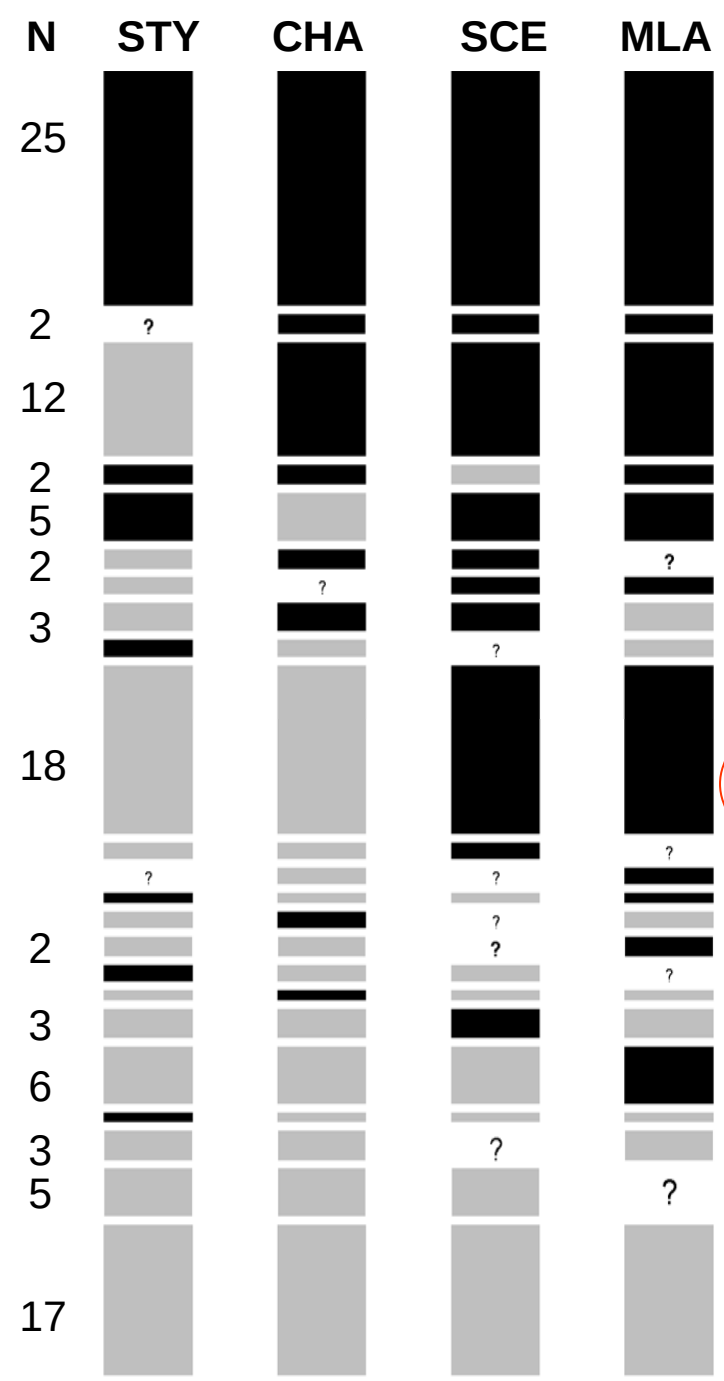


positive

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

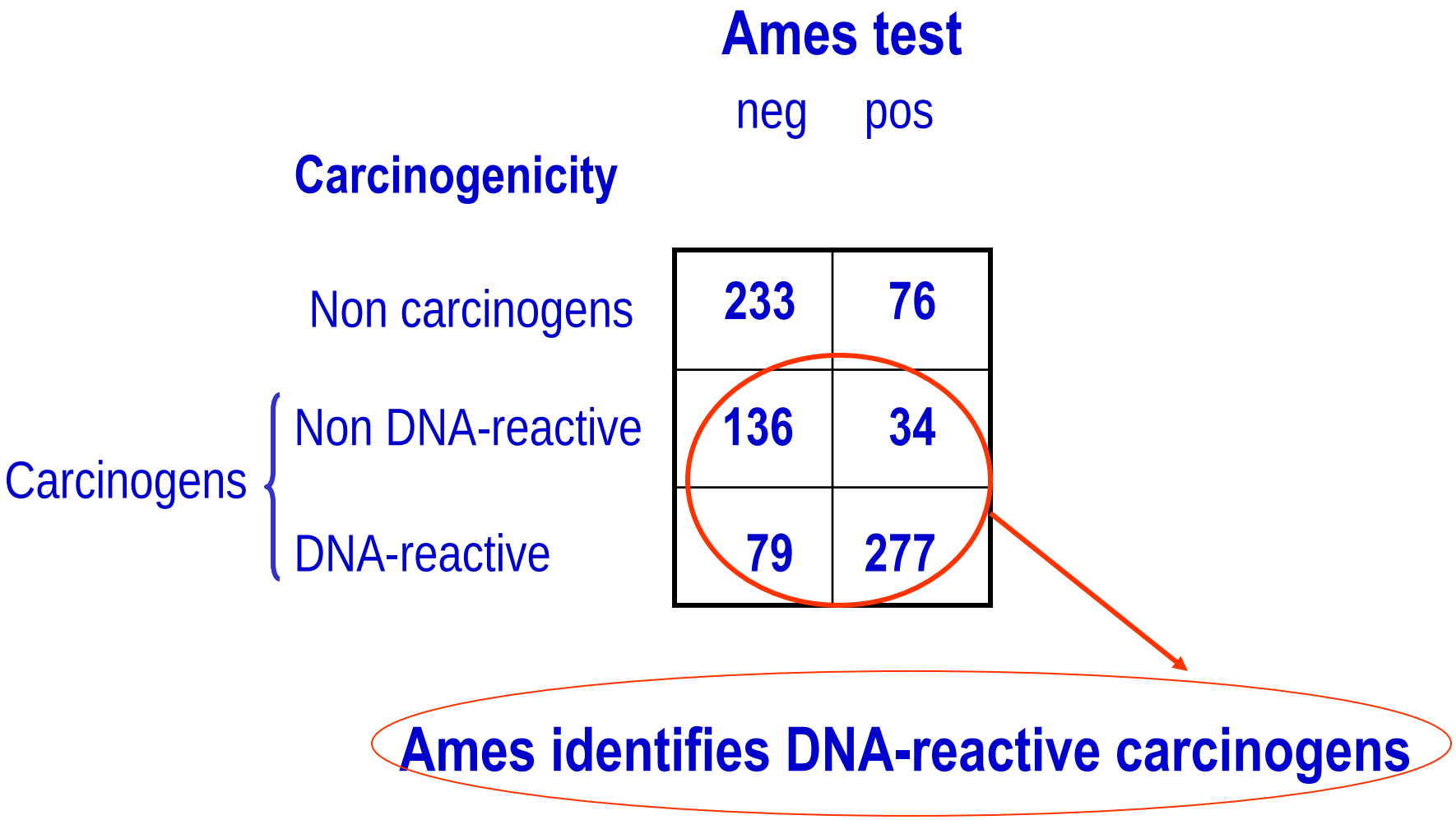
positive

negative

? equivocal

Benigni 1992 Mutagenesis 7: 335-341

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

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 (DNA-reactive) and non-genotox)

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

Expert system **Toxtree** (version 2.1.0)

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

Toxtree (Estimation of Toxic Hazard - A Decision Tree Approach) v2.1.0

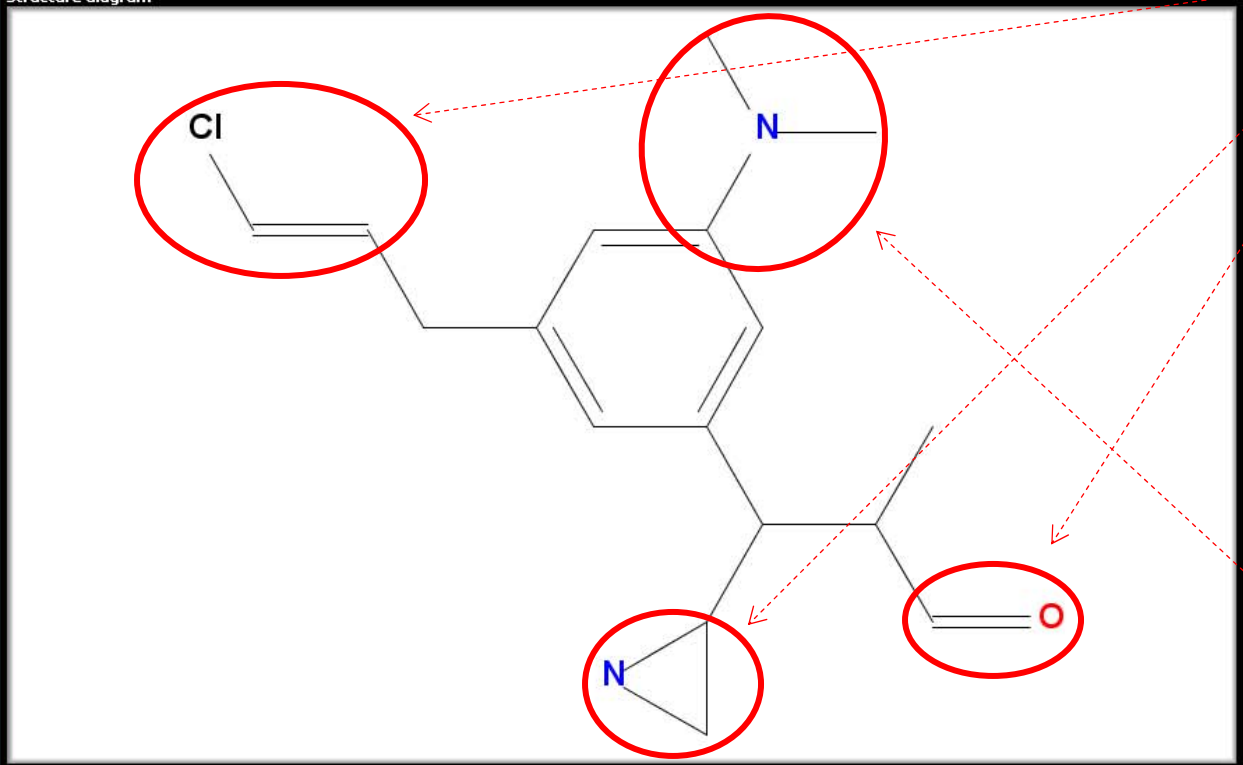
File Edit Chemical Compounds Toxic Hazard Method Help

<< >> Enter SMILES: Go!

Available structure attributes

B5STM1	2,0400
EHOMO	-8,7053
ELUMO	-0,0108
Error when applying the decision tree	YES
For a better assessment a QSAR calculation could be applied.	NO
LSTM1	2,8700
MR2	0,1000
MR3	NaN
MR5	NaN
MR6	0,1000
Negative for genotoxic carcinogenicity	NO
Negative for nongenotoxic carcinogenicity	YES
Potential S. typhimurium TA100 mutagen based on QSAR	NO
Potential carcinogen based on QSAR	NO
Proceed with QSAR6 and QSAR8?	YES
QSAR6,8 applicable?	YES
SA1	NO
SA10	NO
SA11	YES
SA12	NO
SA13	NO

Structure diagram



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Toxic Hazard by Benigni / Bossa rulebase (for mutagenicity and carcinogenicity)

Estimate

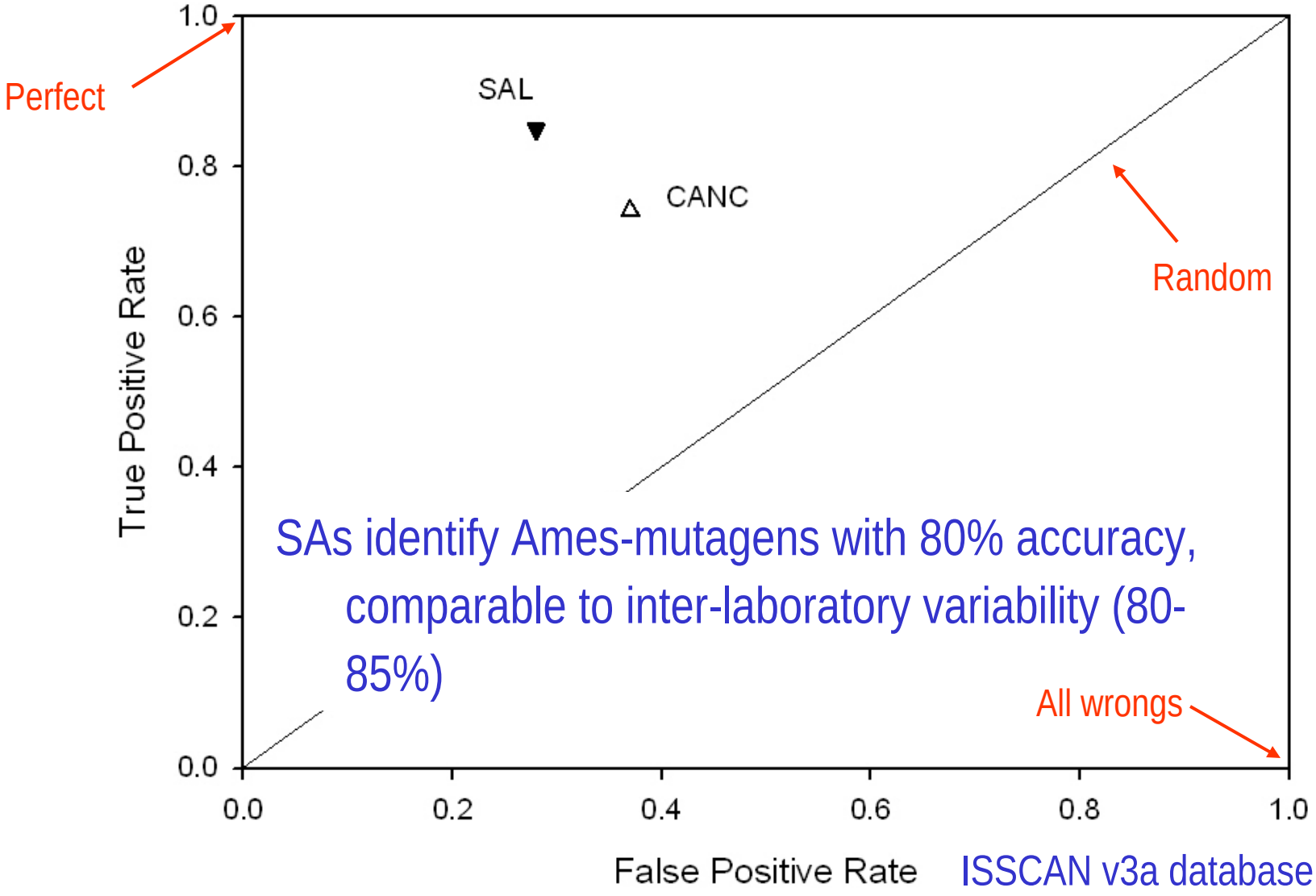
- Structural Alert for genotoxic carcinogenicity
- Structural Alert for nongenotoxic carcinogenicity
- Potential S. typhimurium TA100 mutagen based on QSAR
- Unlikely to be a S. typhimurium TA100 mutagen based on QSAR

Verbose explanation

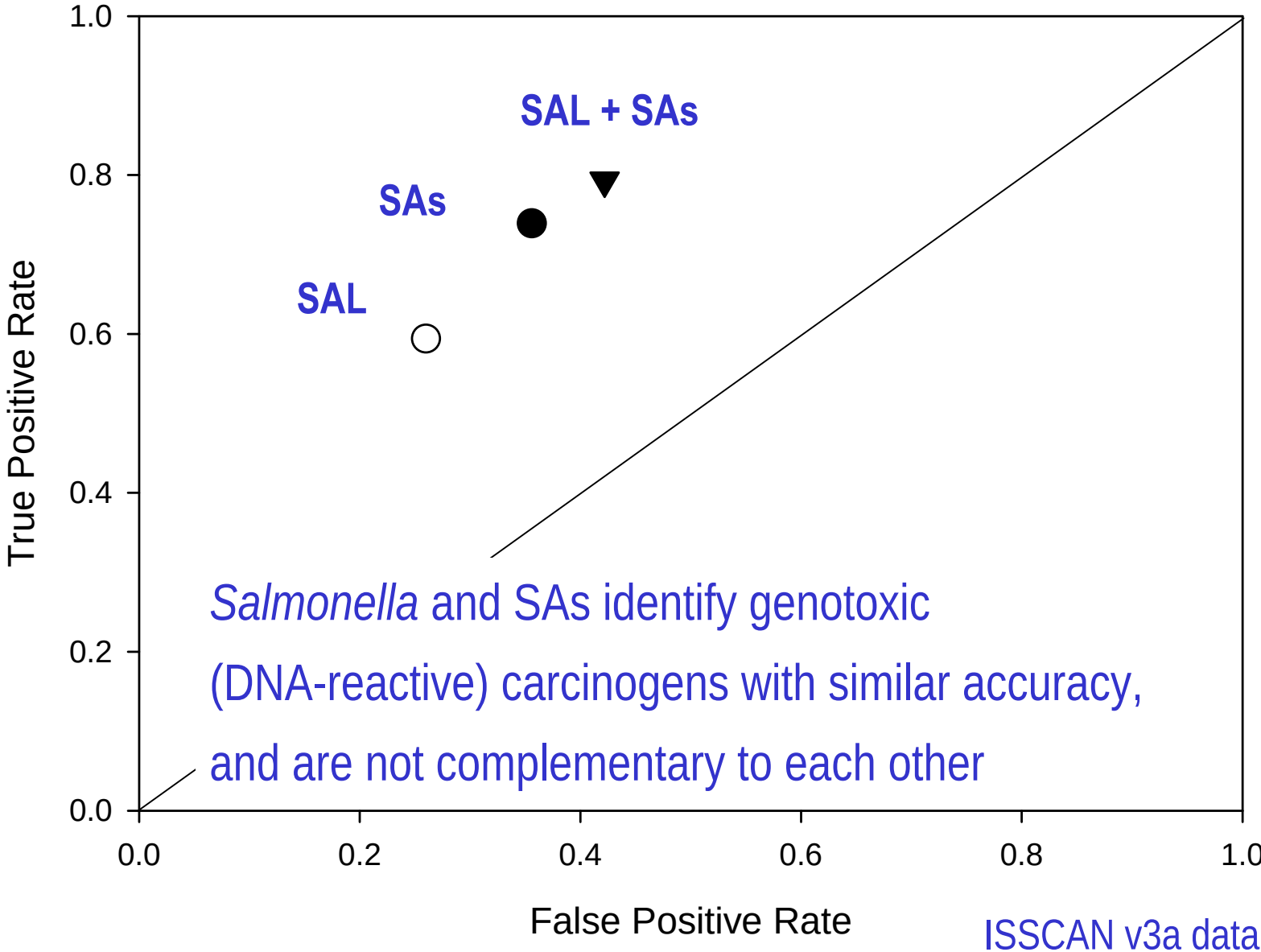
Benigni / Bossa rulebase (for mutagenicity and carcinogenicity)

- QSA1.Acyl halides No
- QSA2.Alkyl (C5) or benzyl ester of sulphonic or phosphonic acid No
- QSA3.N-methylol derivatives No
- QSA4.Monohaloalkene Yes
- QSA5.S or N mustard No
- QSA6.Propiolactones and propiosultones No
- QSA7.Epoxides and aziridines Yes
- QSA8.Aliphatic halogens No
- QSA9.Alkyl nitrite No
- QSA11.Simple aldehyde Yes
- QSA12.Quinones No
- QSA13.Hydrazine No
- QSA14.Aliphatic azo and azoxy No
- QSA15.Isocyanate and isothiocyanate groups No
- QSA16.Alkyl carbamate and thiocarbamate No
- QSA18.Polycyclic Aromatic Hydrocarbons No
- QSA19.Heterocyclic Polycyclic Aromatic Hydrocarbons No
- QSA21.Alkyl and aryl N-nitroso groups No
- QSA22.Azide and triazene groups No
- QSA23.Aliphatic N-nitro No
- QSA24.α,β unsaturated alkoxy No
- QSA25.Aromatic nitroso group No
- QSA26.Aromatic ring N-oxide No
- QSA27.Nitro aromatic No
- QSA28.Primary aromatic amine, hydroxyl amine and its derived esters (with restrictions) No
- QSA28bis.Aromatic mono- and dialkylamine Yes
- QSA28ter.Aromatic N-acyl amine No
- QSA29.Aromatic diazo No
- QSA30.Coumarins and Furocoumarins No
- QGenotoxic alert?.At least one alert for genotoxic carcinogenicity fired? Yes Class Structural Alert for genotoxic carcinogenicity
- QSA17.Thiocarbonyl (Nongenotoxic carcinogens) No

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



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



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

ISSCAN v3a database

Knowledge on DNA-reactivity (coded in SAs):

- Reliable enough to predict *Salmonella* results, and identify many carcinogens
- Identify human carcinogens
- Basis for successful priority setting in NTP bioassays (70% carcinogens among structurally suspect chemicals, only 10% among high exposure chemicals)
- Contribution to reduce DNA-reactive carcinogens among synthetic chemicals (pesticides, pharmaceuticals)

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

Local, mechanistically-based QSARs for congeners:

- 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 data 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%

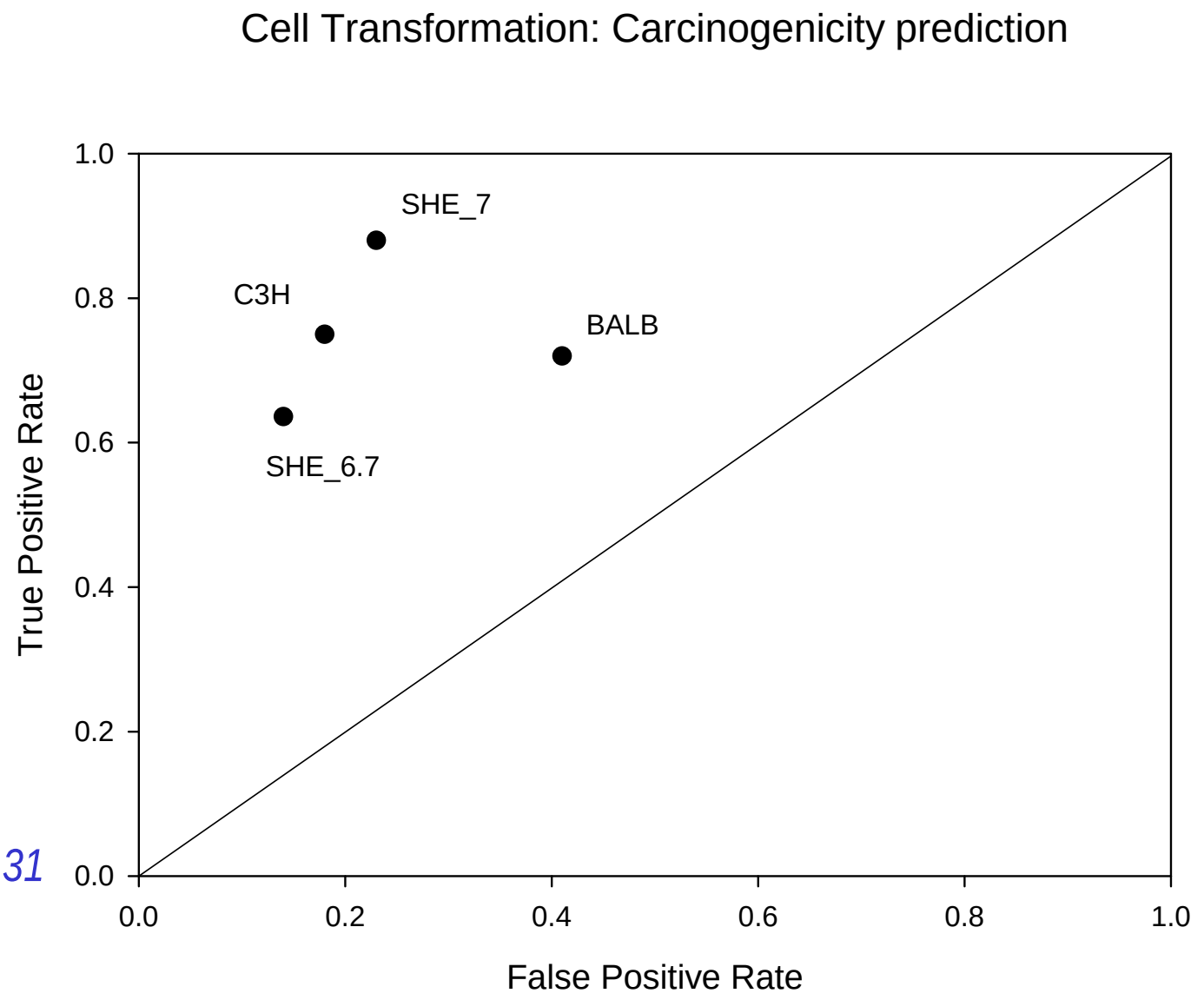
Piegorsch and Zeiger, 1990, in Statistical methods in Toxicology

Same range of uncertainty

Non-mutagenicity assays for non-DNA-reactive carcinogens ?

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

New testing strategy ?

In vitro (*Salmonella* and Cell transformation; sensitive to both DNA reactive and non DNA reactive carcinogens)

Structural Alerts to predict / rationalize experimental results

Presently available *in vivo* short-term tests generally not useful

Tiered strategy:

first Ames or SAs,

then SHE_7

Carcinogenicity

(initial sample)

-	+/-	+
32	2	87

SAs genotox

-	+/-	+
25	1	34

SAs nongenotox

-	+/-	+
25	1	28

SHE_7

-	+/-	+
18	-	5

Ames

-	+/-	+
25	1	44

SHE_7

-	+/-	+
19	-	10

Strong enrichment:

only ~8% undetected carcinogens

Refining structural considerations

Develop more **SAs for nongenotoxic carcinogens**

Whenever enough data, use / generate **QSARs for congeneric classes**

Gear QSARs to **increase sensitivity** (more robust negative predictions)
or specificity (more robust positive predictions)

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