In silico, *in vitro*, and *in vivo* methods as alternatives to the carcinogenicity bioassay for epigenetic chemicals

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Testing strategy for carcinogenicity pre-screening in REACH

- **Pivotal role of genotoxicity short-term tests**
- Bioassay may be required in the case of high exposure plus genotoxicity evidence

Testing strategy for carcinogenicity pre-screening in REACH

- Pivotal role of genotoxicity short-term tests
- Bioassay may be required in the case of high exposure plus genotoxicity evidence
- **Nongenotoxic carcinogens**

  are negative in genotoxicity tests, and go undetected

Nongenotoxic carcinogens: an overlooked issue

- theory on nongenotoxic carcinogenicity is much less developed than that on the genotoxic mechanisms

- nongenotoxic carcinogens are often considered to represent a lesser risk for human health

however

- nongenotoxic carcinogens are **a remarkable proportion of recognized human carcinogens** (17 - 27% of IARC Group 1)

Hernandez et al., 2009, Mutat.Res., 682: 94-109.

Human Carcinogens *(IARC Group 1)* negative in genotox assays

Dimethylarsinic acid
Monosodium methane arsenate
Beryllium
Beryllium sulfate tetrahydrate
Chromium carbonyl
Cyclosporin
Estrogens, non-steroidal
Chlorotrianise
Estrogen/progesterone therapy
Estradiol
Estrogens, steroidal
Ethinyl estradiol
Ethanol
Gallium arsenide
Nickel sulfate hexahydrate
Nickel (II) oxide
Nickelocene
2,3,7,8-Tetrachlorodibenzo-para-dioxin

Hernandez et al., 2009, Mutat.Res., 682: 94-109.

…Alternative approaches to the identification of nongenotoxic carcinogens ?

Backing up the STTs with Structure-Activity concepts:

more **SAs for nongenotoxic carcinogens**

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- **Analysis of mechanistic knowledge**

e.g.,

- Woo,Y.T. and Lai,D.Y. (2010). In: Cancer Risk Assessment: Chemical Carcinogenesis, Hazard Evaluation, and Risk Quantification, ed. G.Hsu, et al, pp. 517-556. Wiley, New York.
- Woo,Y.T. (2003) In: Quantitative Structure-Activity Relationship (QSAR) models of mutagens and carcinogens., ed. R.Benigni, pp. 41-80. CRC Press, Boca Raton.

Combined with

- **Data Mining of unflagged carcinogens –with mechanistic explanation-**

more **SAs for nongenotoxic carcinogens:** results

-**18** new **SAs** not contained in the previous rulebase

mechanistic classes of:

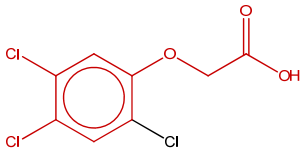
a) **Peroxisome Proliferators** (including DNA methylating agents, and chemicals affecting Gap Junction);
b) inducers of **Hormonal Imbalance**;
c) inducers of **Oxidative Stress**;
d) **Aryl Hydrocarbon Receptor** agonist / antagonist chemicals

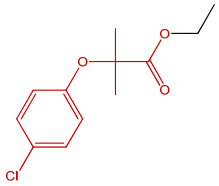
- **2** new **SAs** for genotoxic carcinogenicity coded as well

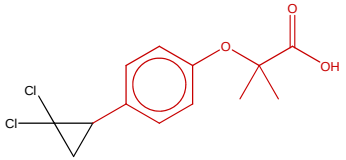
- implemented into **Toolbox 2.3,** to be implemented into **Toxtree**

more **SAs for nongenotoxic carcinogens:** examples

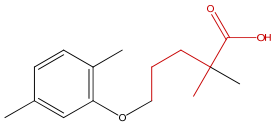
substituted phenoxyacid

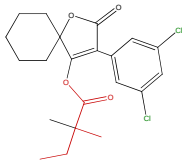




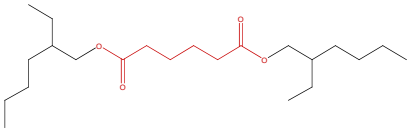


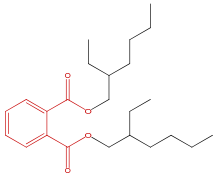
substituted n-alkylcarboxylic acids



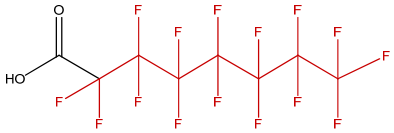


phthalate (or butyl) diesters and monoesters

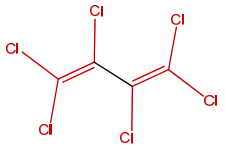




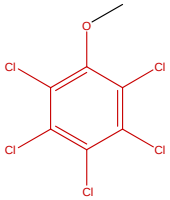
perfluorooctanoic acid (PFOA)



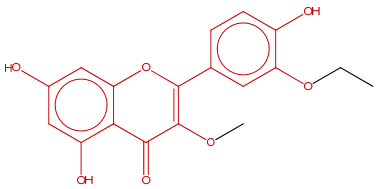
tri (or tetra) chloroethylene



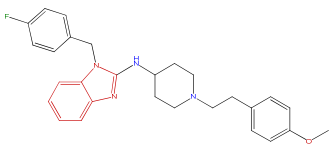
pentachlorophenol



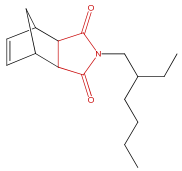
quercetin-type flavonoids



imidazole, benzimidazole



dicarboximide



Carcinogenicity prediction:

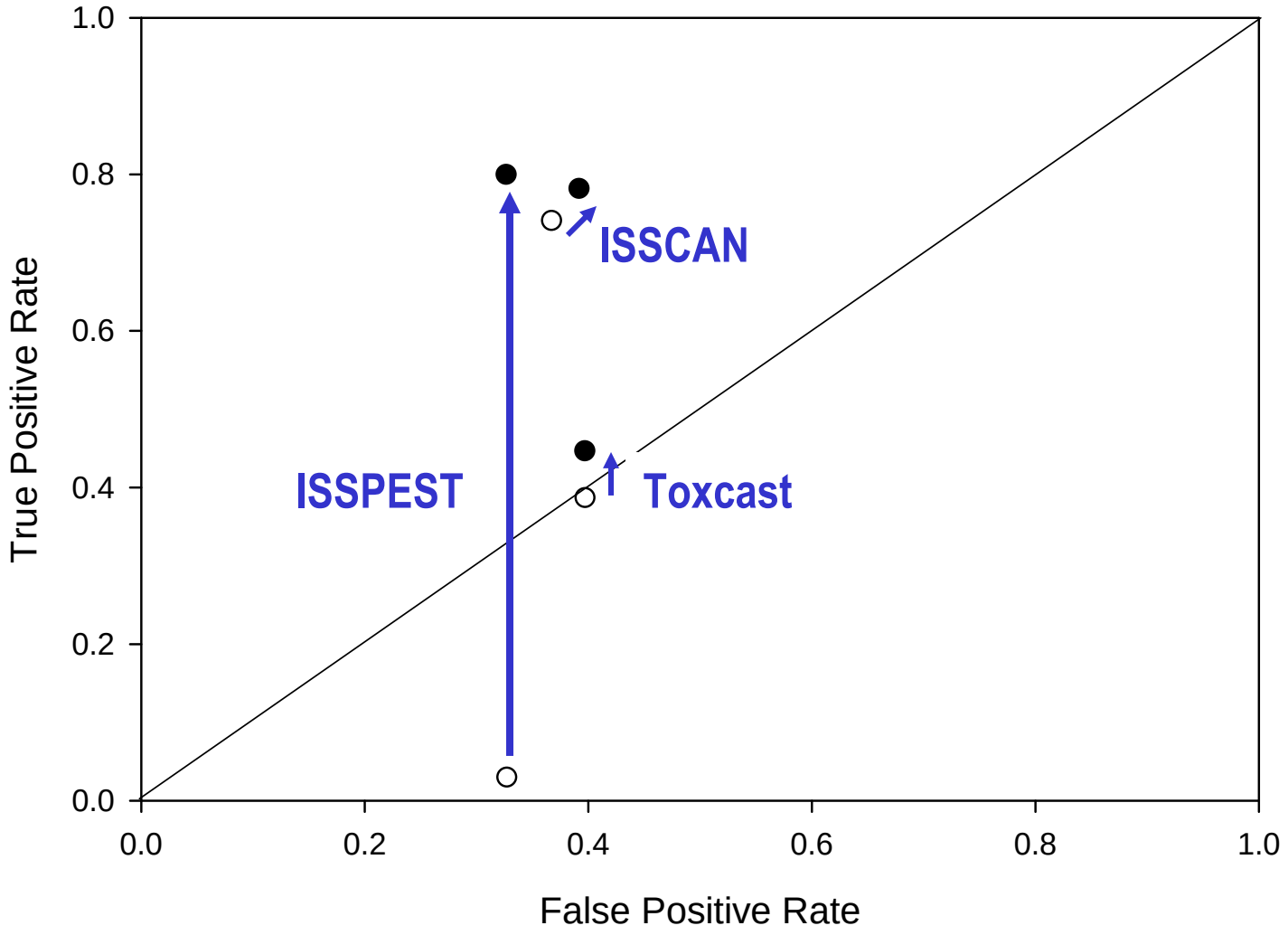
Old (mainly genotoxic) *versus* expanded SAs rulebase

-Carcinogenicity databases:

-ISSCAN

-Toxcast phase 1

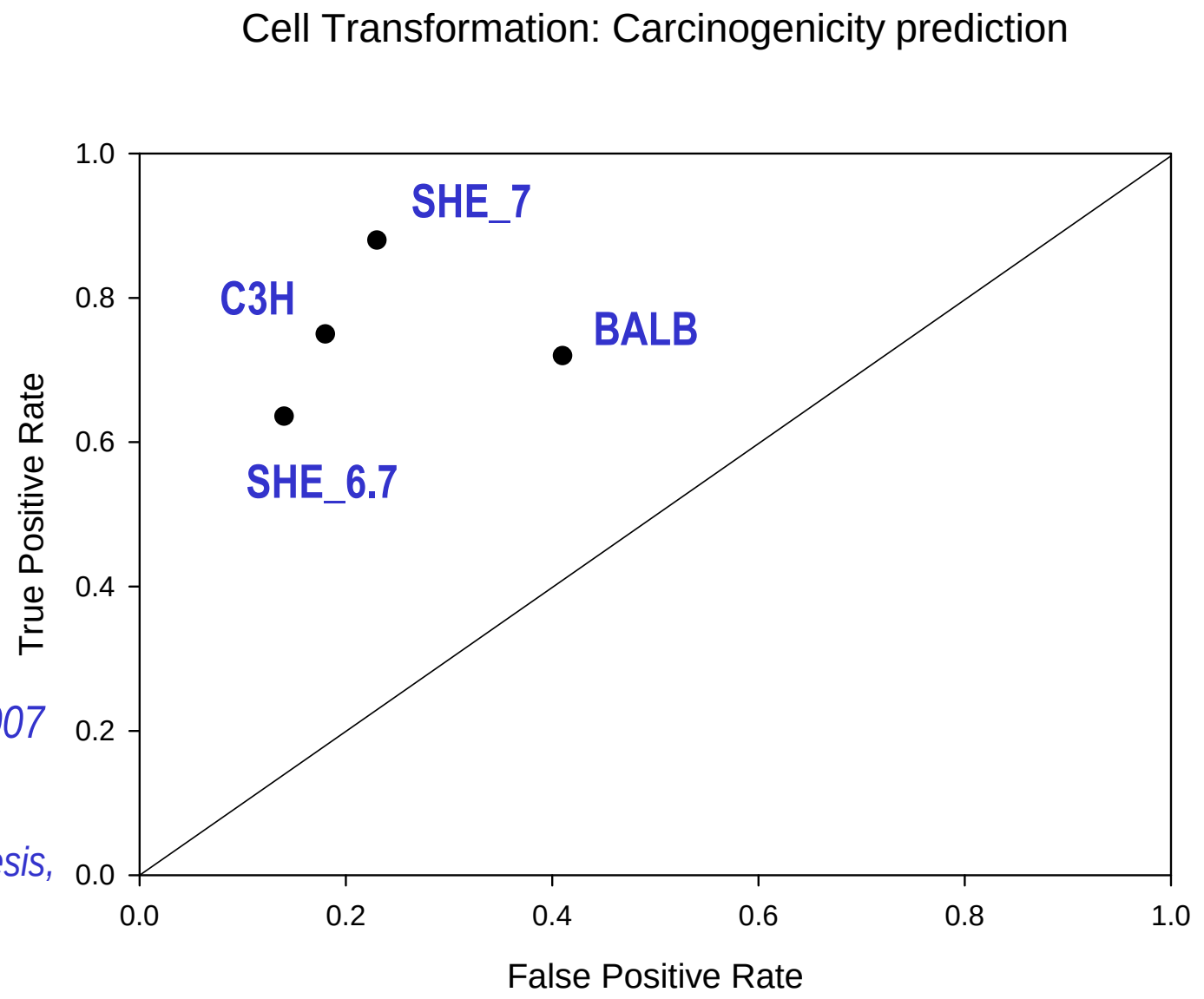
-ISSPEST pesticides (PAN)



Non-mutagenicity ***in vitro*** **assays** for non-DNA-reactive carcinogens ?

Cell transformation assays

in cultured cells, phenotypic alterations characteristic of tumorigenic cells



Data: *OECD vol.31 2007*

no inorganics

Benigni et al., Mutagenesis, 2011, 26: 455-460

ISSCTA: a database of Cell Transformation Assays results

to be included into the **OECD (Q)SAR Toolbox** and the **ISSTOX** database

n = 370 (including inorganics and organics)

Systems

primary normal diploid cells: Syrian Hamster Embryo cells assay (pH 7)
Syrian Hamster Embryo cells assay (pH 6.7)

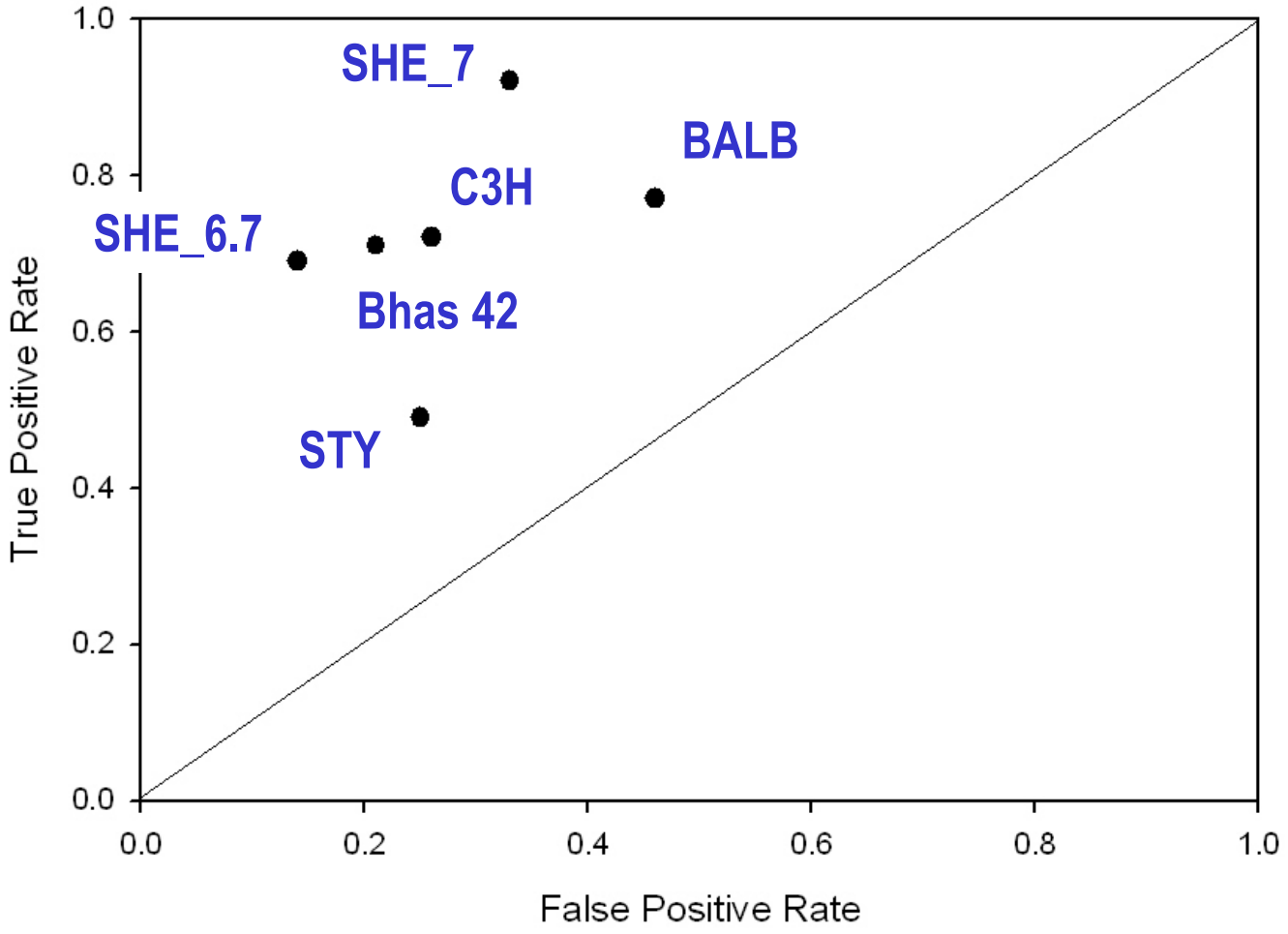
immortalized aneuploid mouse cells: BALB/c 3T3
C3H10T1/2
Bhas 42

Data: *OECD vol.31, 2007*

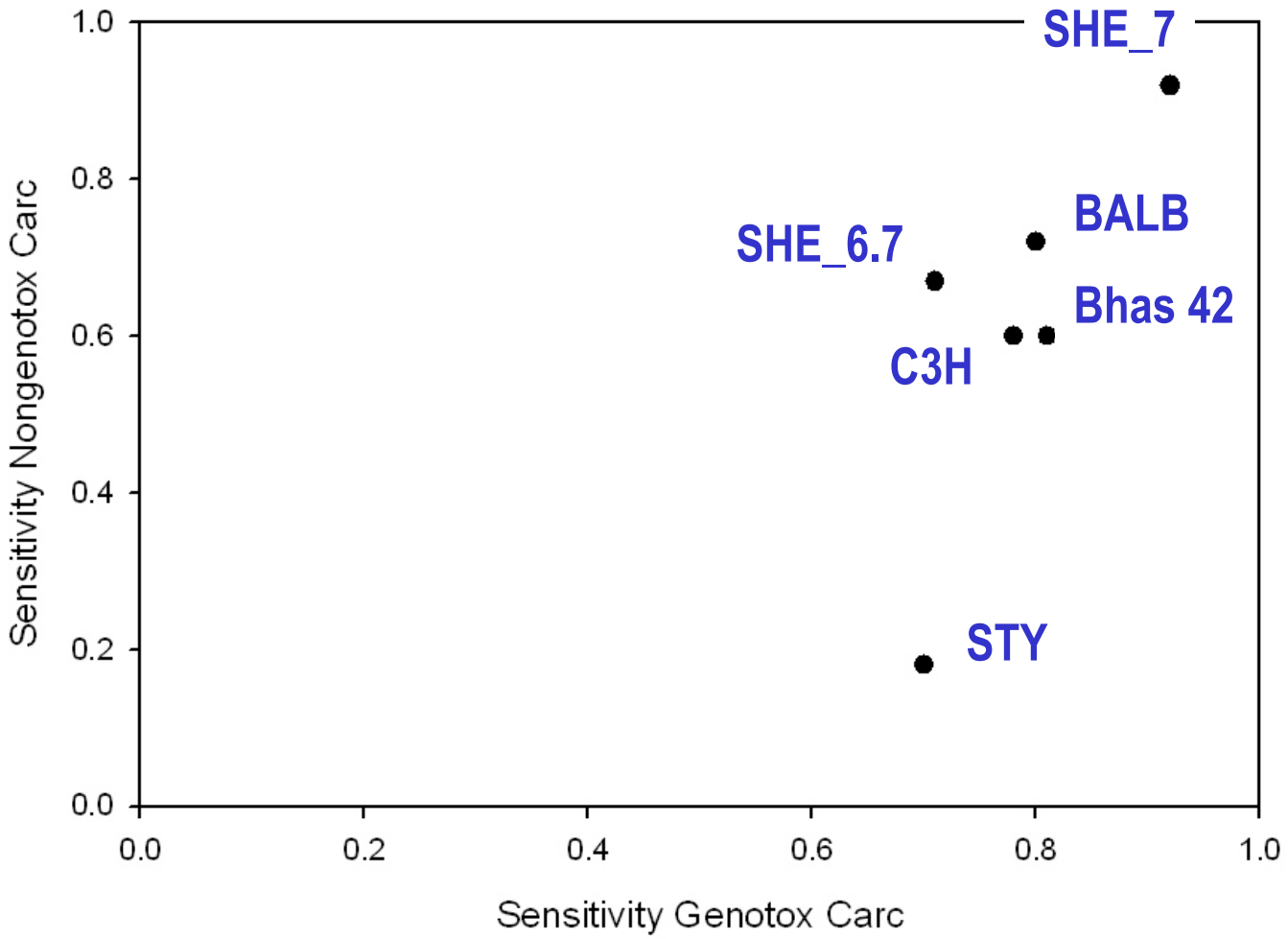
Sakai et al., 2010 Mutat.Res. 702:100-122

Cell Transformation Assays: carcinogenicity prediction

A new analysis on ISSCTA, including inorganics and Bhas 42

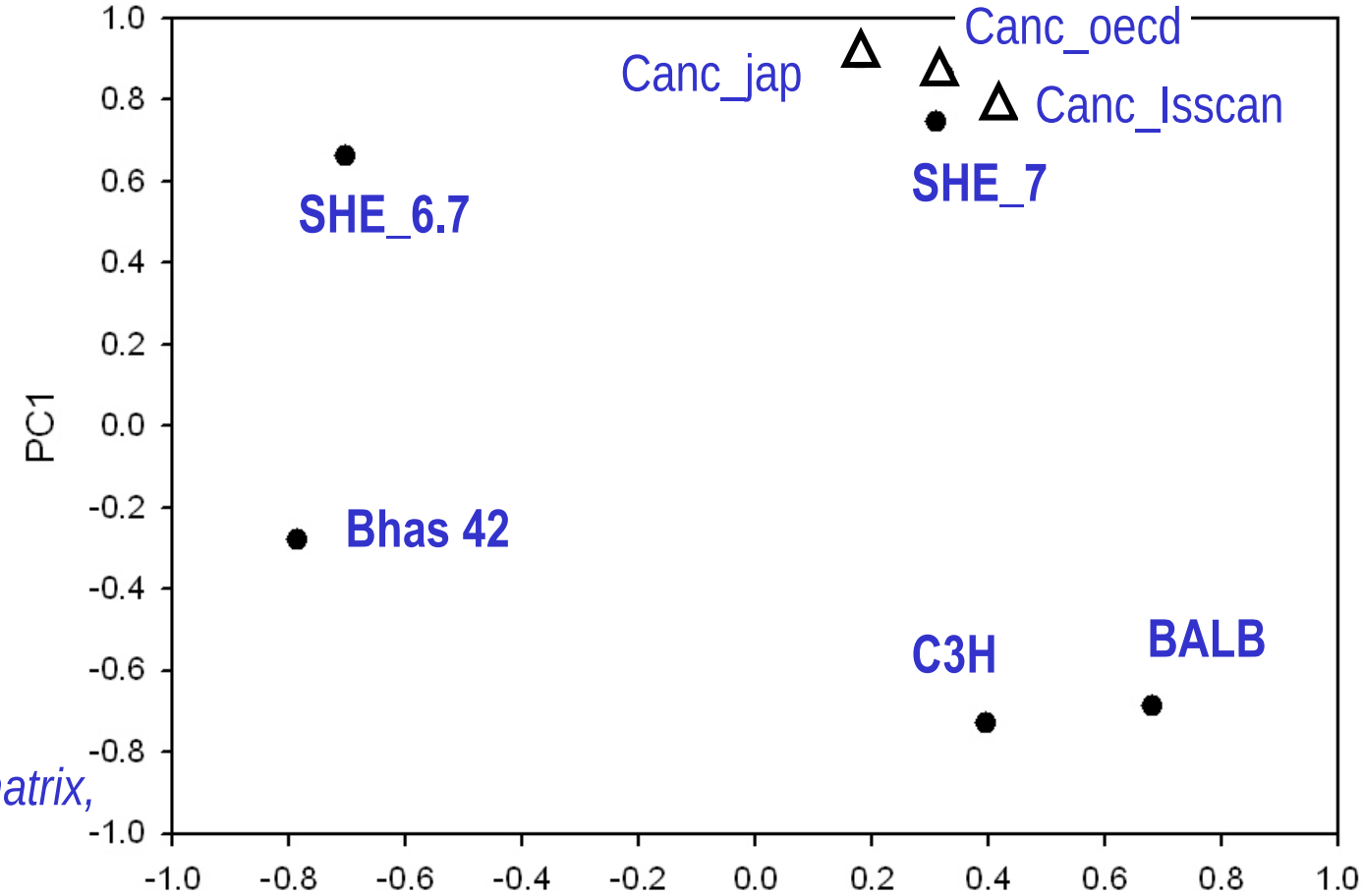


CTAs prediction of genotoxic and nongenotoxic carcinogens



CTAs and Rodent Bioassay:

global similarity between responses to the chemicals



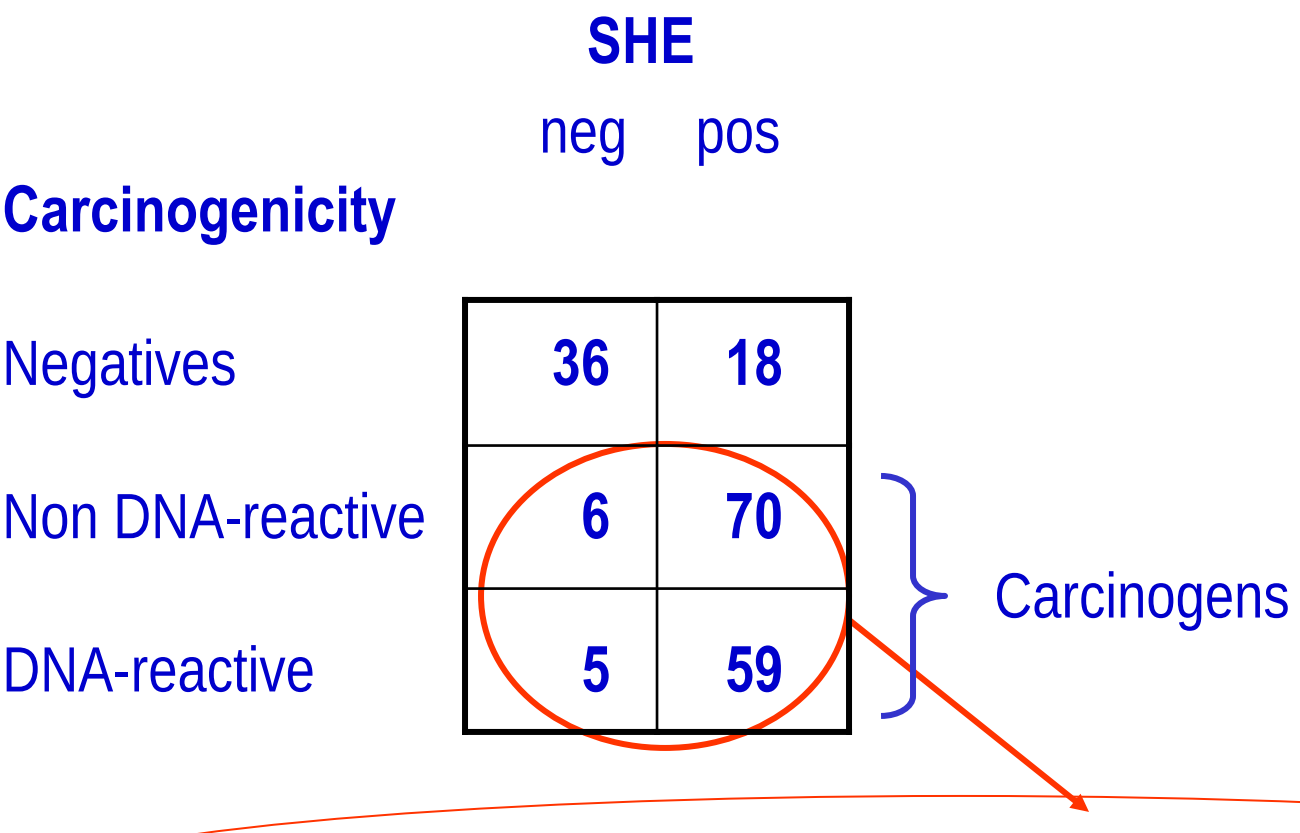
Procedure:

Tests similarity matrix, then

Principal Component Analysis

(PC1 + PC2: 0.80 Exp. Var.)

SHE pH≥7 Cell transformation *versus* rodent carcinogenicity



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

Chemicals n = 194, from OECD vol. 31

Efficient, alternative testing strategy with tools available today?

in vitro **assays** {*Salmonella* and Cell transformation}
sensitive to both DNA-reactive and non DNA-reactive carcinogens

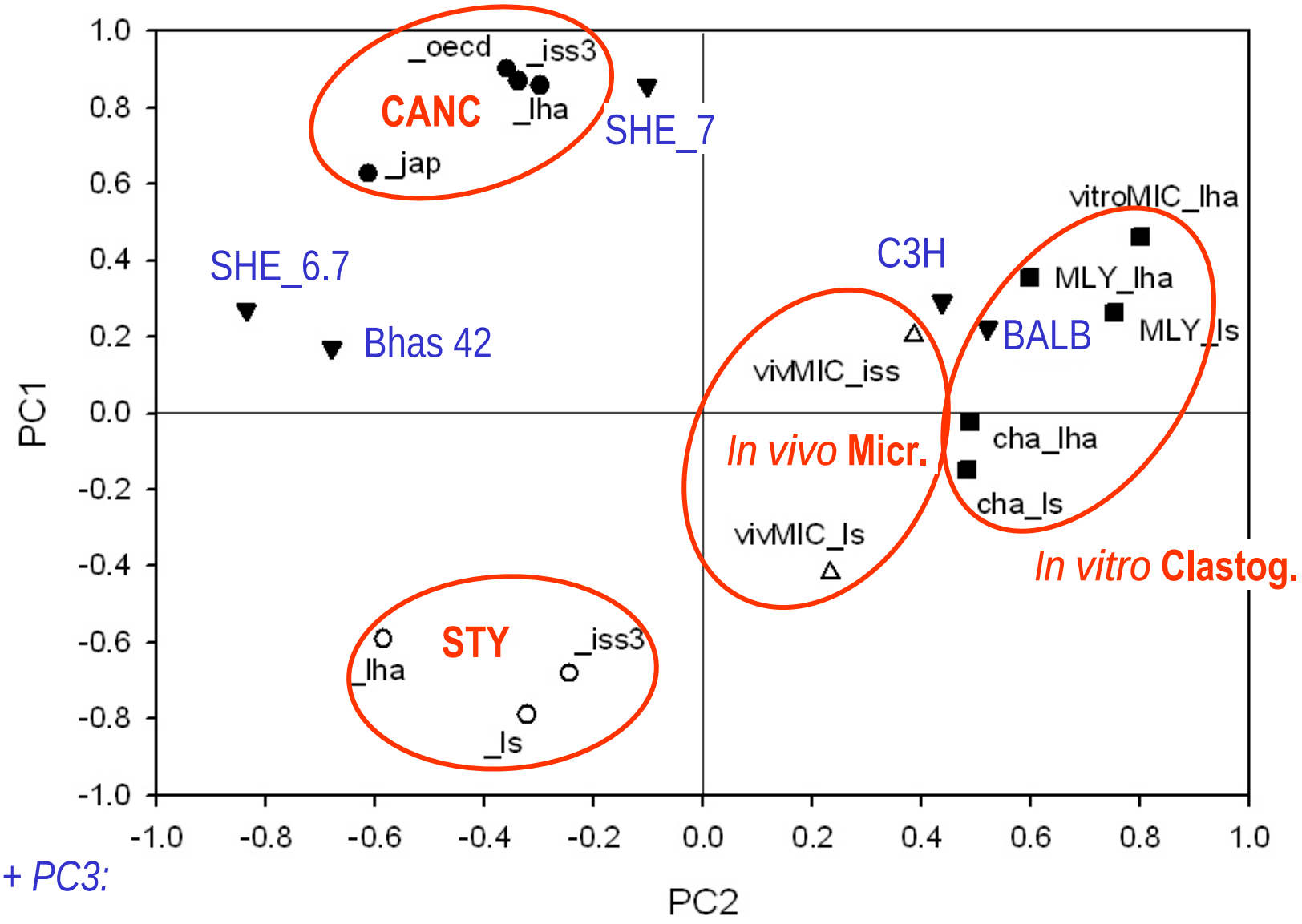
Structural Alerts to predict / rationalize experimental results

A tiered approach to carcinogens identification

Tiered Approach A			Tiered Approach B		
	Noncarc	Carc		Noncarc	Carc
Initial Sample	36	86	Initial Sample	52	130
	After Tier 1			After Tier 1a	
STY Negative	27	41	SAgeno Negative	32	66
				After Tier 1b	
			SA nongeno Negative	27	43
	After Tier 2			After Tier 2	
SHE Negative	17	8	SHE Negative	17	5
% initial sample	47 %	9%		33%	4%

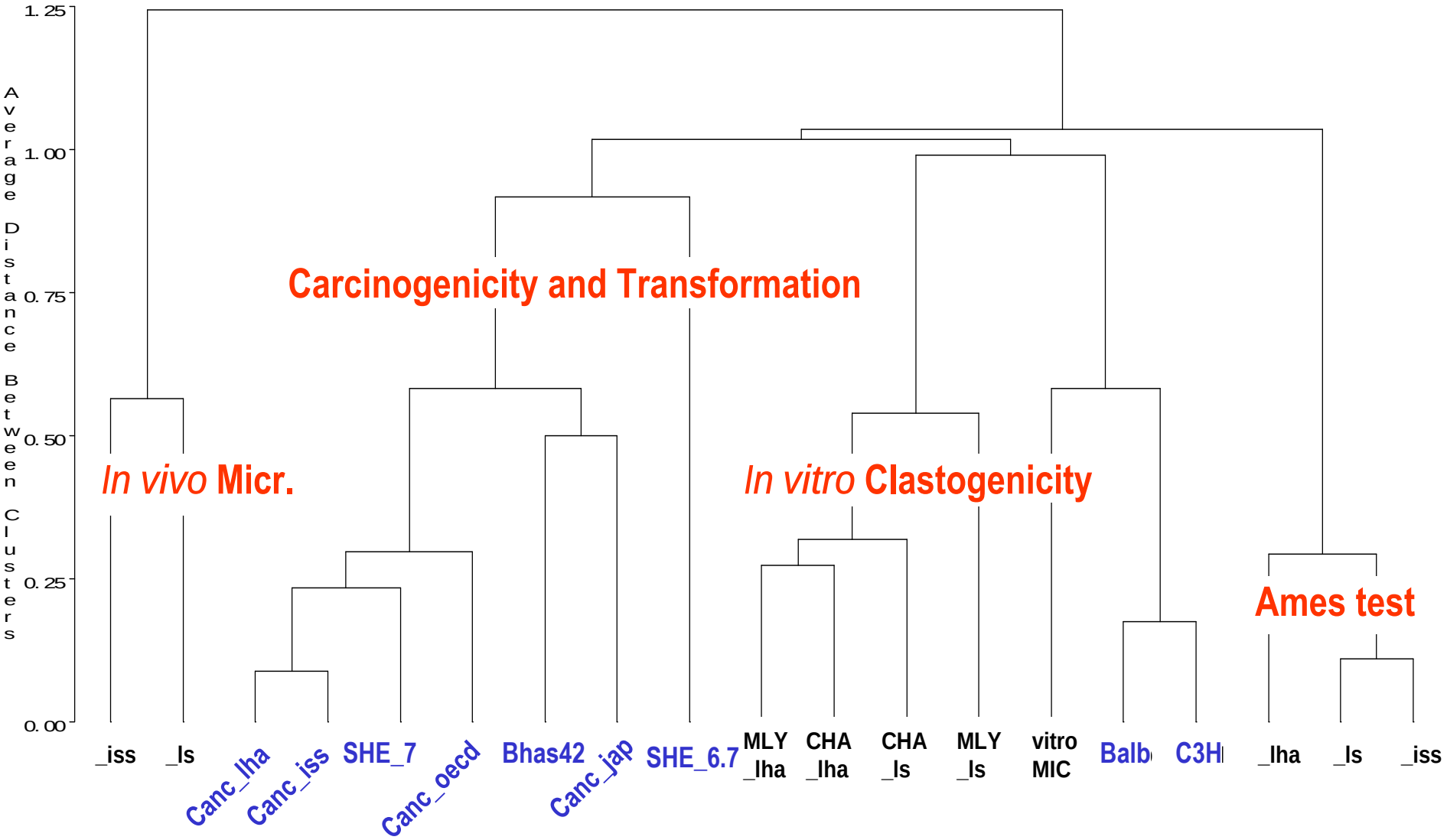
only ~ 5-10% undetected carcinogens

Meta-"omics" of toxicological endpoints



(PC1 + PC2 + PC3: 0.74 Exp. Var.)

Panels of chemicals (databases) as mechanistic probes


Average Distance Between Clusters
1.25
1.00
0.75
0.50
0.25
0.00
Carcinogenicity and Transformation
In vivo Micr.
In vitro Clastogenicity
Ames test
_iss
_ls
Canc_lha
Canc_iss
SHE_7
Canc_oecd
Bhas42
Canc_jap
SHE_6.7
MLY _lha
CHA _lha
CHA _ls
MLY _ls
vitro MIC
Balb
C3H
_lha
_ls
_iss

Do “nongenotoxic carcinogens" lack genotoxic activity (and viceversa)?

Cyproterone acetate and **tamoxifen**:

liver carcinogens that had been thought to act by nongenotoxic mechanisms. Later studies indicated that are genotoxic and have tumor-initiating activity.

Steroidal estrogens: nongenotoxic, but also DNA-adducts

Inducers of **Oxidative stress**: also DNA damage

DNA-reactive chemicals can interact with proteins as well

Animal studies demonstrate that **tumor promoters** can cause cancer in the absence of an initiating agent

Do “nongenotoxic carcinogens" lack genotoxic activity (and viceversa)? **An issue for risk assessment**

…classification of carcinogens into genotoxic and non-genotoxic or into initiating or promoting agents may not only be unhelpful but even an impediment to risk assessment.

Once we can accumulate enough relevant mechanistic information about individual chemicals, it will be more reasonable to use this information directly for risk estimation, without expending efforts in classifying them… *Yamasaki 1995*

Classification systems based on labeling chemicals as genotoxic or nongenotoxic and on presumed mechanisms of action for each class lead to ambiguous reconstructions of the carcinogenic process….

… the existence or absence of threshold dose-responses cannot be determined from current knowledge of carcinogenic mechanisms.

The prudent policy for protecting public health is the one that considers the dose-response of all potential contributing effects of each specific chemical *(in each specific stage of carcinogenesis*)… *Melnick et al. 1996*

…some (final) considerations

- Most **reductionist approaches** are weak predictors of reality

(e.g., chromosomal aberrations and other STTs,
DNA-hypomethylation in tissues,
omics in pharmacology)

- Two **success stories**

- **Ames test** maps a large family of DNA- (and protein-) reactive carcinogens
- **Cell Transformation** as model for cell-cell and cell-stroma interplay in cancer tissues (not a single-cell condition)

- Balance of details and generality as **challenge** in describing **complex systems**

In a fluctuating (stochastic) environment

is governed by the three fundamental laws of conservation:

i) ***mass***,
ii) ***energy*** &
iii) ***momentum***

Courtesy of Kumar Selvarajoo

Resulting in Navier–Stokes equations

$$\rho\left(\frac{\partial \mathbf{v}}{\partial t} + \mathbf{v}.\nabla\mathbf{v}\right) = -\nabla p + \nabla.\mathrm{T} + f$$

v : flow velocity, ρ : fluid density, p : pressure, T : stress tensor, f : body forces/volume

…some (final) considerations

• The **present strategies** for carcinogenicity pre-screening **do not defend** adequately **human health,** and need **to be updated**

• **Simpler and more efficient alternative strategies available**

• **Reliable ground for refinements**:

-Expand SAs for nongenotoxic carcinogens to decrease reliance on experiments (mechanistic knowledge plus data mining)

-Improve specificity

- ……

Freely-available tools generated at the ISS

Structural Alerts for - canc / *in vitro* mut
- *in vivo* micronucleus

Toxtree

http://ihcp.jrc.ec.europa.eu/our_labs/computational_toxicology/qsar_tools/toxtree

OECD (Q)SAR Toolbox

http://www.oecd.org/document/54/0,3746,en_2649_37465_42923638_1_1_1_37465,00.html

Curated toxicological databases

ISSTOX

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

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