UMONS
Université de Mons

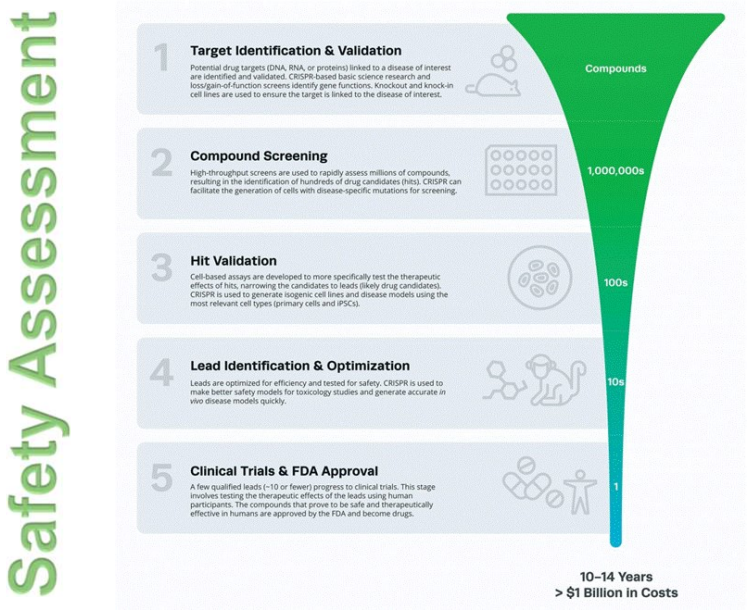
Biological Function of the Usual Suspects in Metabolomics : Examples of Fumarate and Succinate

UCL - Seminars - November 21st 2022

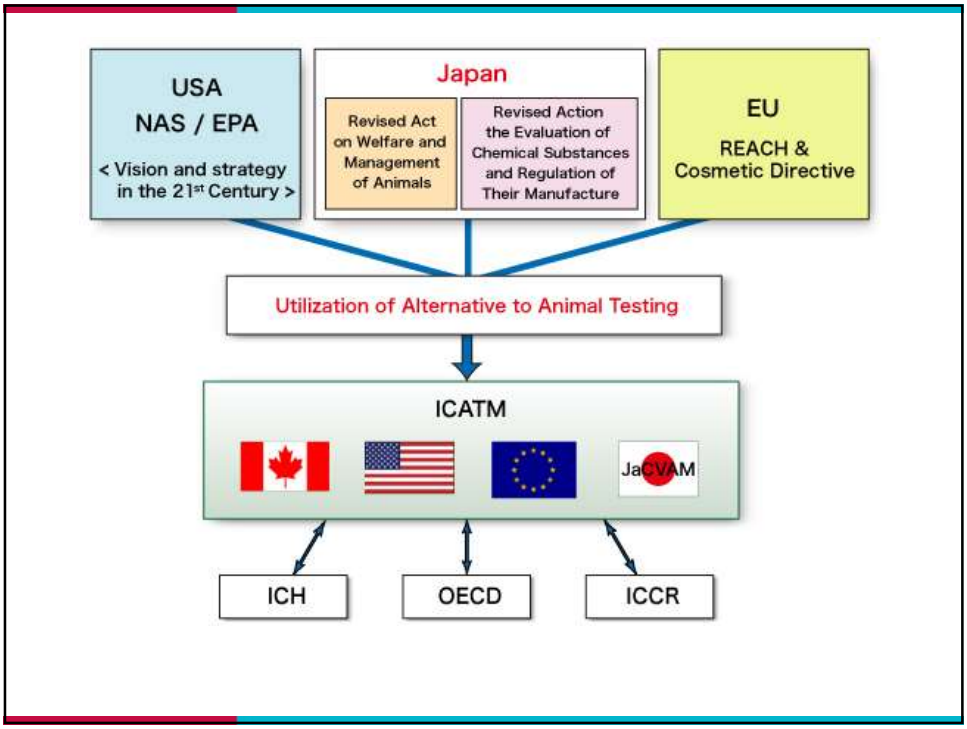
Jean-Marie Colet

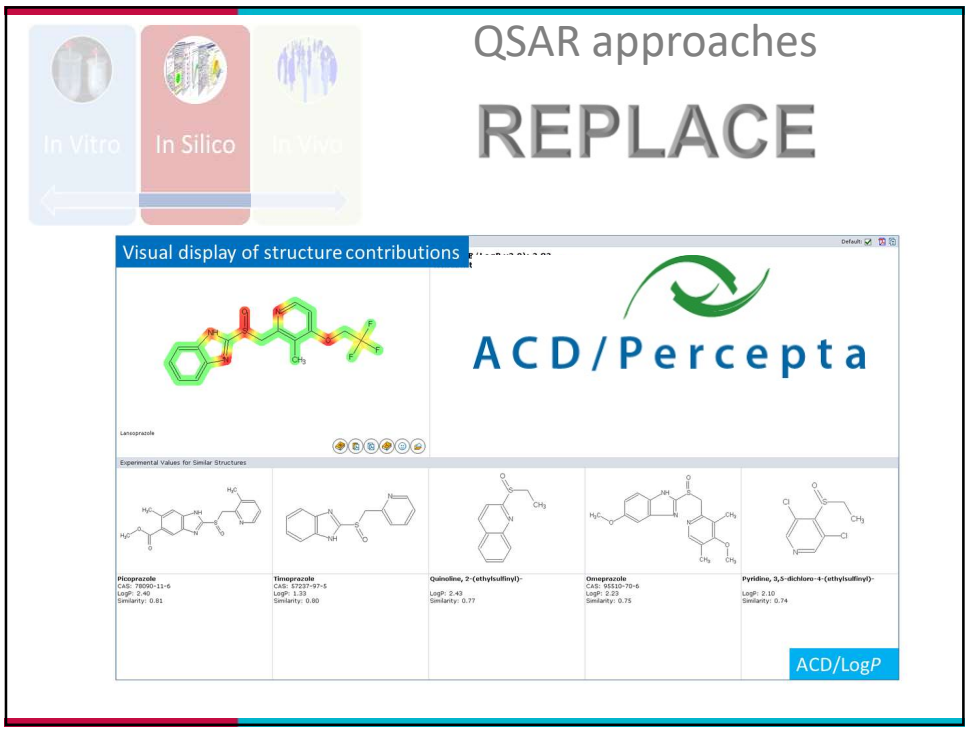
Faculté
de Médecine
et de Pharmacie

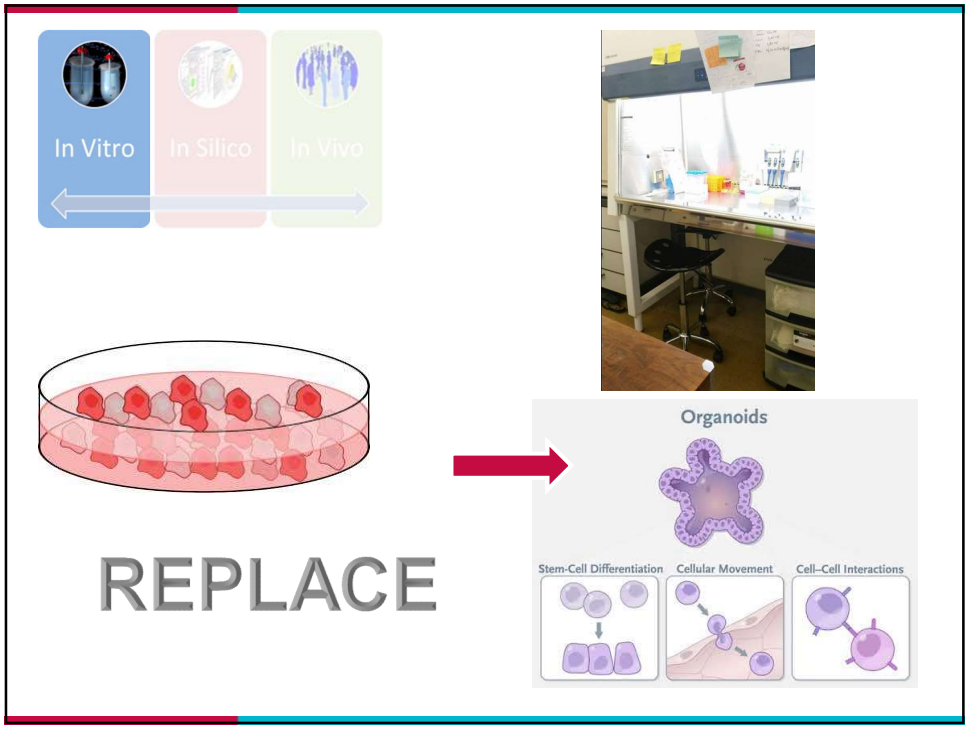
Jean-marie.colet@umons.ac.be

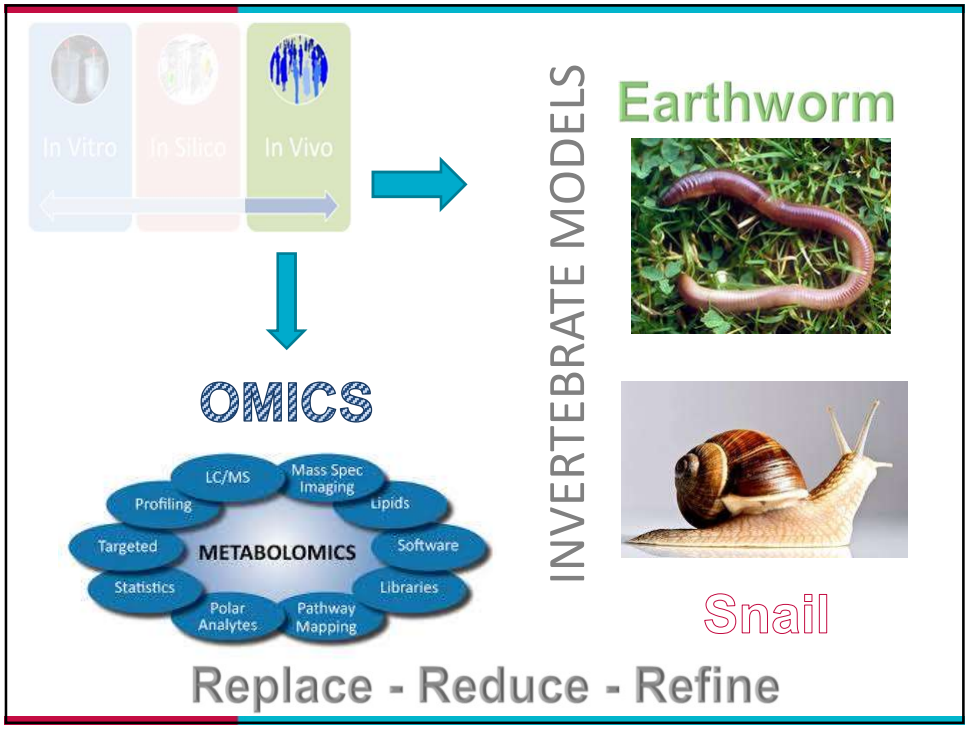



ANIMAL TESTING
3R
Replace
Replace animal studies with other methods
Reduce
As many trials as required, as few as possible
Refine
Minimize stress of study animals


USA
NAS / EPA
< Vision and strategy in the 21st Century >
Japan
Revised Act on Welfare and Management of Animals
Revised Action the Evaluation of Chemical Substances and Regulation of Their Manufacture
EU
REACH & Cosmetic Directive
Utilization of Alternative to Animal Testing
ICATM
JaCVAM
ICH
OECD
ICCR


In Vitro
In Silico
In Vivo
QSAR approaches
REPLACE
Visual display of structure contributions
ACD/Percepta
ACD/LogP


In Vitro
In Silico
In Vivo
REPLACE
Organoids
Stem-Cell Differentiation
Cellular Movement
Cell–Cell Interactions


In Vitro
In Silico
In Vivo
OMICS
LC/MS
Mass Spec Imaging
Profiling
Lipids
Targeted
METABOLOMICS
Software
Statistics
Libraries
Polar Analytes
Pathway Mapping
INVERTEBRATE MODELS
Earthworm
Snail
Replace - Reduce - Refine


BIO profiling
UMONS
Université de Mons
LIÈGE université
ULB
UNIVERSITÉ LIBRE DE BRUXELLES

BIOPROFILING IN A FEW WORDS …

- Financial support from the European Union and Wallonia under the ERDF-ESF 2014-2020 program
- Interuniversity project
- 3,4M€ for equipment acquisition
- Located at UMONS (Mons, Belgium)
- Open to all actors in the biotechnology sector

BIO profiling

Two complementary pl

Meta-Vision
- Small molecules
- NMR based

MS-Quanta
- Macromolecules
- MS-based

WWW.BIOPROFILING.EU

BIO profiling

Meta-Vision

Equipment

NMR Spectrometer
600 MHz Bruker AVANCE™

BIO profiling

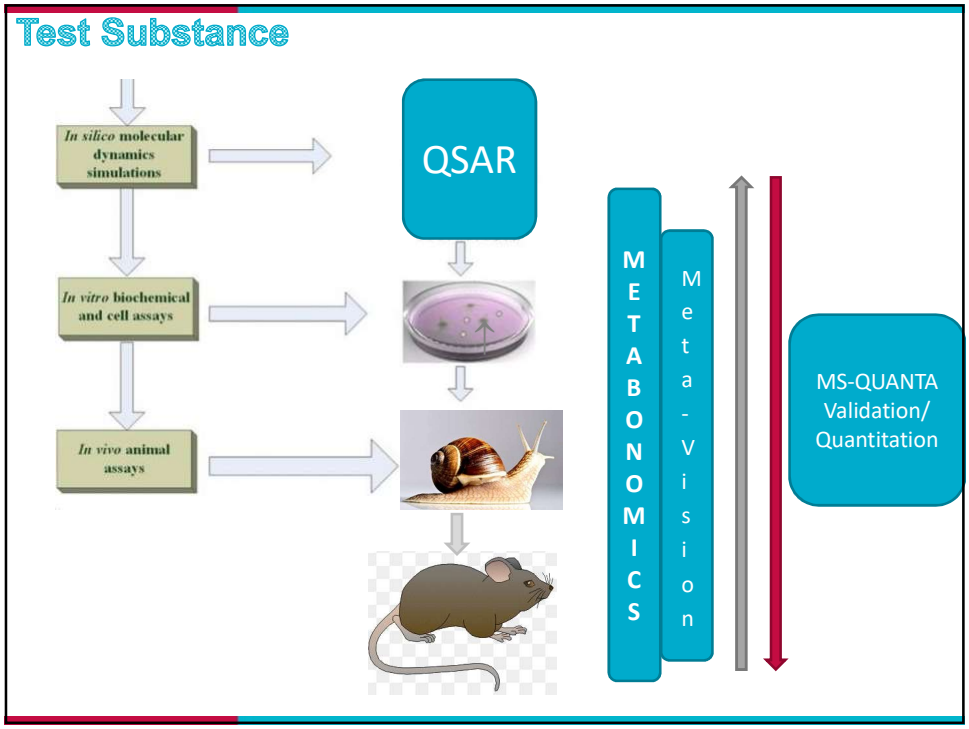
Université de Mons

MS-Quanta

1. **High Resolution mass Spectrometers :**
   Exploratory quantitative analysis in DDA and DIA mode
   Relative quantitative profile of more than 2000 targets
   Triple TOF 3600+ (SCIEX) Q Extractive (Thermo Scientific)
   Triple TOF 6600 (Sciex) Q Extractive Plus (Thermo Scientific)
2. **High sensitivity and high speed mass spectrometers :**
   SRM/MRM/PRM analysis
   Simultaneous quantification (multiplex mode) > 50 targets
   Qtrap 6500+ (Sciex) Xevo TQ-S (Waters)
3. **High resolution mass spectrometers in mass or space :**
   FT-ICR SolarX XR (Bruker)
   MALDI-TOF/TOF Rapiflex (Bruker)

BIO profiling UMONS ULB LIEGE université

Université de Mons

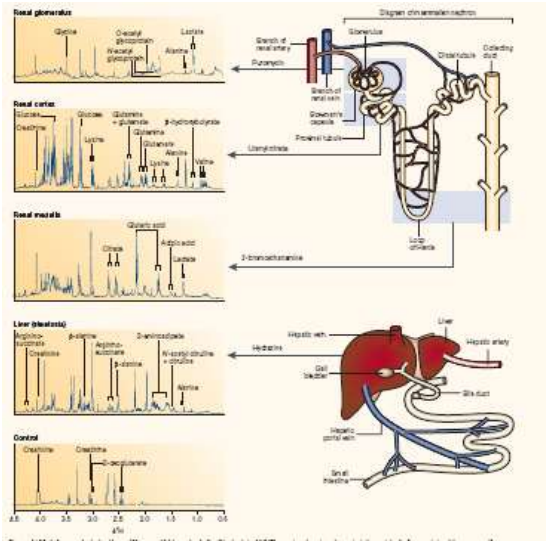


The COMET Initiative

Consortium on Metabonomics in Toxicology

How to apply MBX in preclinical drug development programs ?

M
E
T
A
B
O
N
O
M
I
C
S



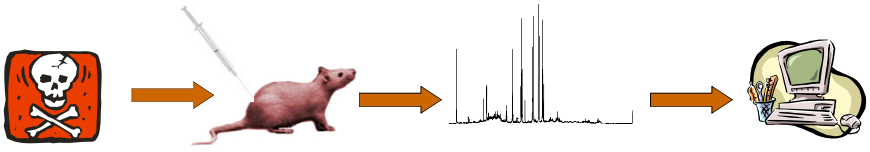
Nicholson JK Nat rev Drug Disc 1(2):153-161 (2002)

COMET

Consortium on Metabonomic in Toxicology

Imperial College-London
BMS Lilly Novo Pfizer Pharmacia Roche

2001-2004



147 prototoxicants

Acute dosing

^{1}H-NMR analysis

Database
+
Chemometrics models
for toxicity prediction

16

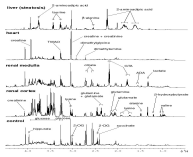
Experimental Protocol

Metabolism cages on refrigerated racks

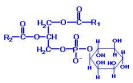
Urine

Blood

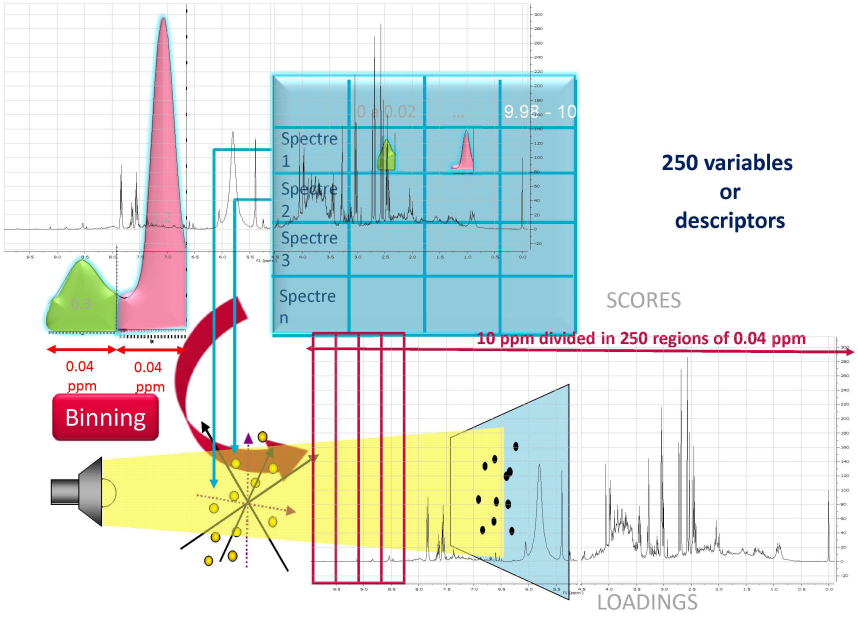
^{1}H NMR



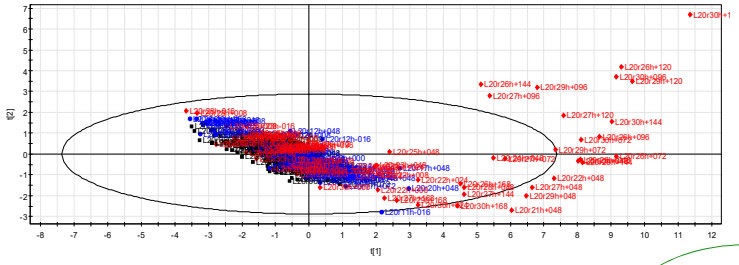
Pattern recognition



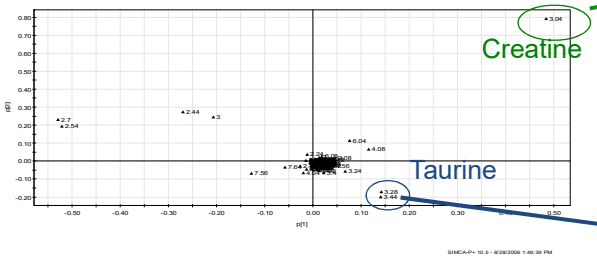
Potential biomarkers



Multivariate MBX data analysis



Scores Plot



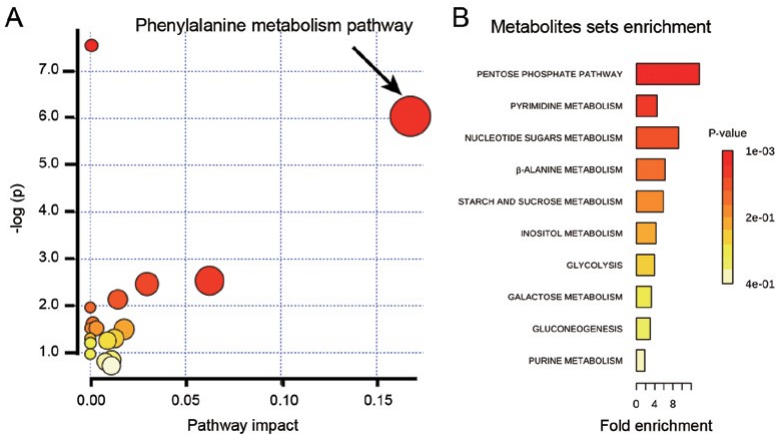
3.04 ppm

Loadings Plot

3.28 & 3.44 ppm

MetaboAnalyst :

From metabolites VIP to Biochemical pathways



COMET: Control Model

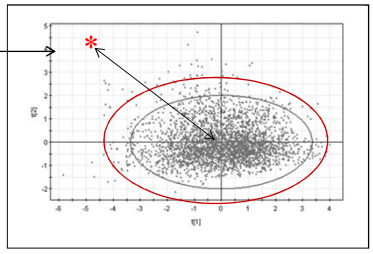
Prediction and Classification of Drug Toxicity Using Probabilistic Modeling of Temporal Metabolic Data: The Consortium on Metabonomic Toxicology Screening Approach
T. Ebbels, H. Keun, O. Beckonert, M. Bollard, J. Lindon, E. Holmes, and Jeremy K. Nicholson
Journal of Proteome Research/2007

Identifying abnormal urine samples

- 4521 "normal" urine samples
- PCA-based approach
- Determine the distance to the model (DmodXPS+) as well as the probability of belonging to the model (PmodXPS+):

Test sample

- Normal
- Marginal: 95%<x< 99%
- Abnormal: <95%



COMET: CLOUDS Model (Classification Of Unknowns by Density Superposition)

Prediction and Classification of Drug Toxicity Using Probabilistic Modeling of Temporal Metabolic Data: The Consortium on Metabonomic Toxicology Screening Approach
T. Ebbels, H. Keun, O. Beckonert, M. Bollard, J. Lindon, E. Holmes, and Jeremy K. Nicholson
Journal of Proteome Research/2007

Identifying toxicity type

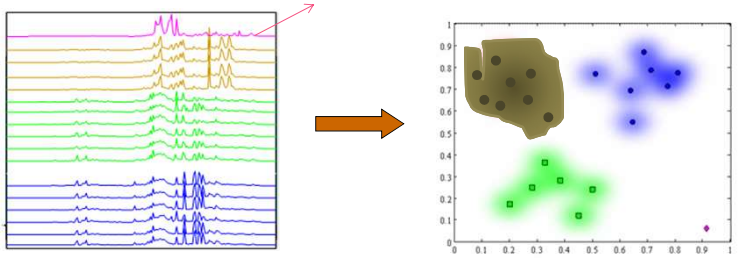
- PNN-based model (Probabilistic Neural Network – Specht 1990)

Test sample

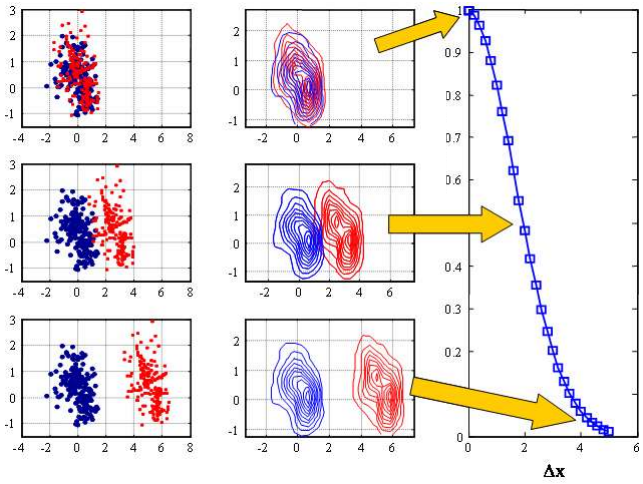
Cardiac toxicity

Renal toxicity

Liver toxicity



Clouds approach



Classification based on similarity

The « Usual Suspects »

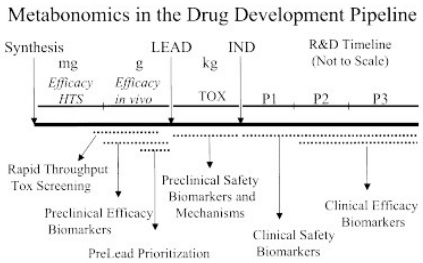


TABLE 2
The "Usual Suspects"[a]

2-oxoglutarate
acetate
citrate
creatine
creatinine
glucose
hippurate
lactate
succinate
taurine
trimethyl amine/trimethyl amine oxide (TMA/TMAO)

Metabonomics in toxicology: a review.
Robertson DG.
Toxicol Sci. 2005 Jun;85(2):809-22

The « Usual Suspects »

- Whatever the pathological case or the experimental model, at very early stages of the pathological process, a series of recurrent metabolites seem to be systematically recruited by the impacted cells to maintain the homeostasis essential to their survival

- Among those " **usual suspects** " :
  - Some osmoprotectors (betaine and taurine)
  - Some antioxidants (glycine, cysteine, glutamate/glutamine as precursors of glutathione)
  - Others, such as **Krebs cycle intermediates**, indicators of cellular energy imbalance

The « Usual Suspects »

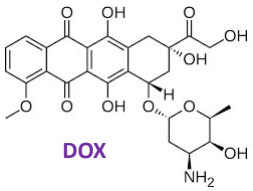
But several questions remain unanswered :

- ✓ Do those intermediates only reflect cell energy status / metabolic switch ?

  OR

- ✓ Do they contribute more actively to cell survival ?

- Research interest of our group in drug-induced cardiotoxicity (i.e. chemotherapies such as **Doxorubicin**)



DOX

- To identify very early during treatment the onset of cardiac adverse events (better patient care and offer alternative solution to minimize any risk of complication)

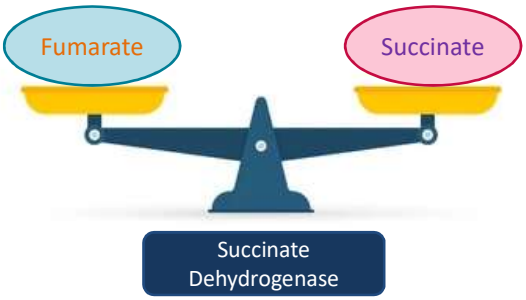
- Metabolomic study on cultured cardiomyoblasts (H9C9 cell line) and cardiomyocytes (AC16 cll line) exposed to DOX

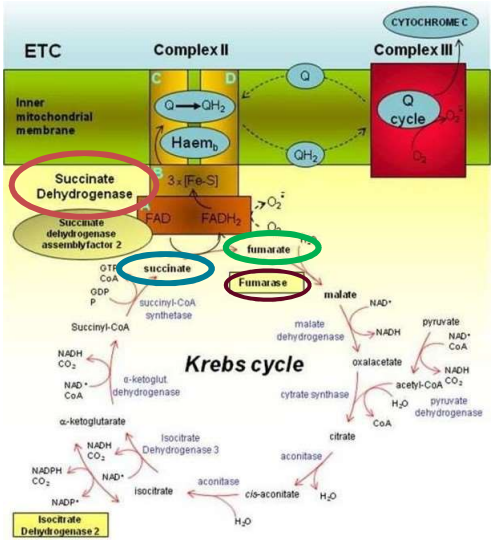
- Results : Very soon after exposure, the intra- and extra-cellular levels of the "usual suspects" were indeed altered

- Confirming the observations mentioned previously in the literature in other pathological contexts

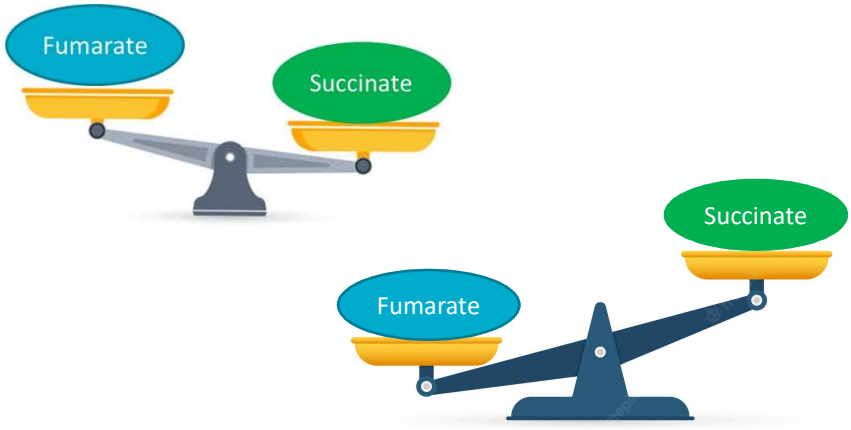
➢ Among these suspects, two caught our attention:

- ✓ **Fumarate** and **Succinate**
- ✓ **2** Krebs cycle intermediates
- ✓ Interconverted by the succinate dehydrogenase



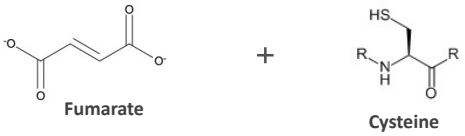


Energy imbalance ?

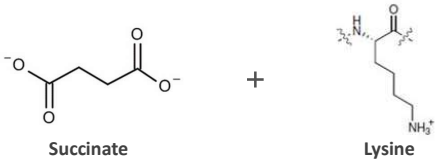


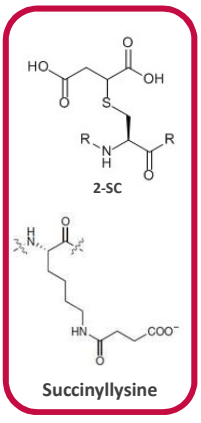
Succinylation versus Succination

Succination



Succinylation

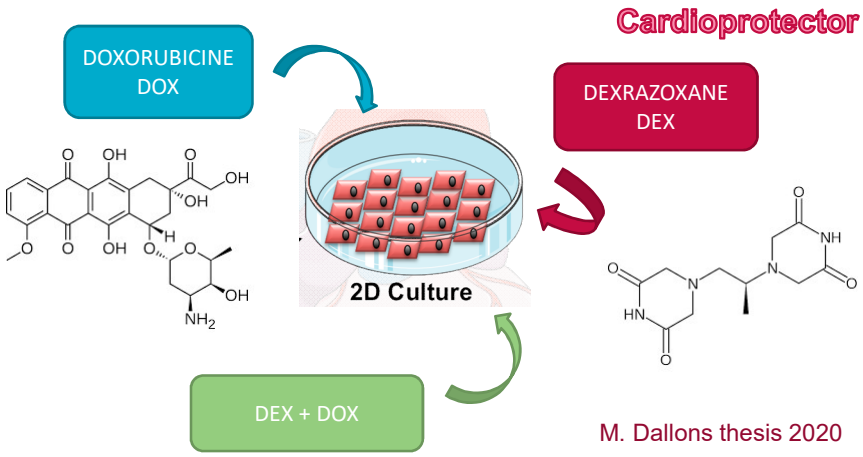




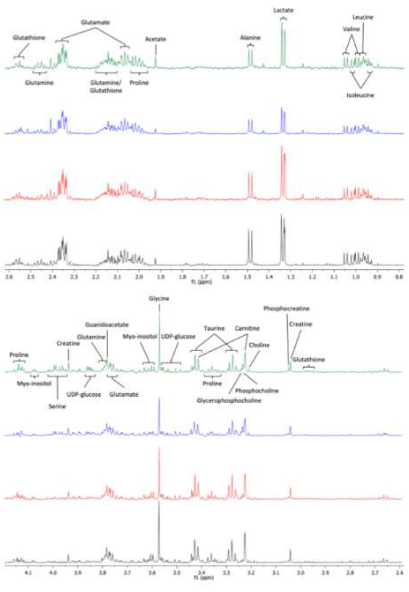
32

Succinate & Succinylation

- H9C2 cardiomyoblasts and AC16 cardiomyocytes were exposed to DOX and/or to DEX



M. Dallons thesis 2020

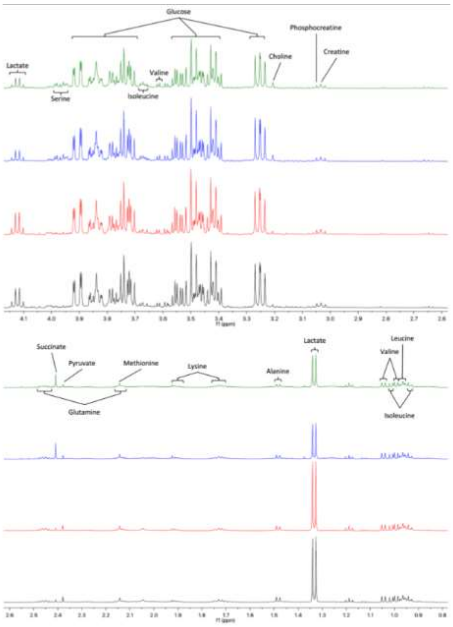


Cardiac cells exposed for 4h or **<u>24h</u>** to :

- Black = Control
- Red = DOX
- Blue = DEX
- Green = DEX + DOX

Intracellular extracts (polar phase)

Dallons M, Schepkens C, Dupuis A, Tagliatti V and Colet J-M
New Insights About Doxorubicin-Induced Toxicity to Cardiomyoblast-Derived H9C2 Cells and Dexrazoxane Cytoprotective Effect: Contribution of In Vitro ^{1}H-NMR Metabonomics.
Front. Pharmacol. 11:79 (2020)



Cardiac cells exposed for 4h or **24h** to :

- Black = Control
- Red = DOX
- Blue = DEX
- Green = DEX + DOX

Cell culture medium

Dallons M, Schepkens C, Dupuis A, Tagliatti V and Colet J-M
New Insights About Doxorubicin-Induced Toxicity to Cardiomyoblast-Derived H9C2 Cells and Dexrazoxane Cytoprotective Effect: Contribution of In Vitro ^{1}H-NMR Metabonomics.
Front. Pharmacol. 11:79 (2020)

C

	CTR	DOX	DEX	DEX+DOX
Alanine	100	107	103	102
Creatine	100	89	100	101
Glucose	100	97	104	105
Glutamine	100	101	102	106
Isoleucine	100	105	97	99
Lactate	100	94	100	97
Leucine	100	100	99	100
Lysine	100	112	98	100
Phosphocreatine	100	90	92	93
Proline	100	113	95	109
Pyruvate	100	100	128	130
Succinate	100	96	97	109
Valine	100	105	98	98

D

	CTR	DOX	DEX	DEX+DOX
Glucose	100	96	97	97
Glutamine	100	124	96	87
Isoleucine	100	105	103	103
Lactate	100	105	60	65
Leucine	100	102	92	92
Pyruvate	100	84	74	77
Serine	100	138	514	514
Succinate	100	104	550	523
Valine	100	104	110	110

FIGURE 6 | Heatmaps constructed from relative means of discriminant metabolites normalized integrals. **(A)** H9C2 intracellular compartment after 4 hours of DOX/DEX exposure. **(B)** H9C2 intracellular compartment after 24 hours of DOX/DEX exposure. **(C)** H9C2 culture media after 4 hours of DOX/DEX exposure. **(D)** H9C2 culture media after 24 hours of DOX/DEX exposure.

Dallons M, Schepkens C, Dupuis A, Tagliatti V and Colet J-M
New Insights About Doxorubicin-Induced Toxicity to Cardiomyoblast-Derived H9C2 Cells and Dexrazoxane Cytoprotective Effect: Contribution of In Vitro ^{1}H-NMR Metabonomics.
Front. Pharmacol. 11:79 (2020)

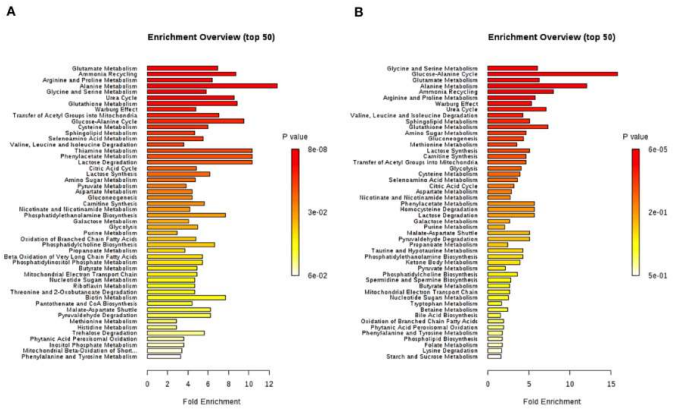
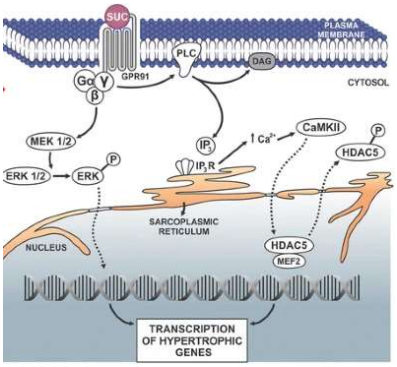


FIGURE 7 | Metabolite set enrichment analysis performed on ^{1}H-NMR metabonomic data, using MetaboAnalyst 4.0 online software. **(A)** 4 hours H9C2 cells exposure to DOX/DEX. **(B)** 24 hours H9C2 cells exposure to DOX/DEX.

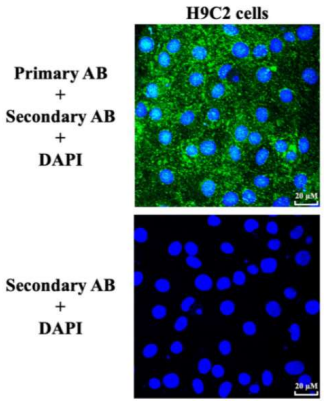
Does succinate play a biological role in cardiac protection ?



- Possible role of secreted succinate via an autocrine/paracrine effect by binding to the extracellular part of its **GPR91 receptor** anchored in the plasma membrane of cardiac cells
- Once activated GPR91 receptor initiates a signaling pathway **promoting cell survival**

M. de Castro Fonseca et al.
GPR91: expanding the frontiers of Krebs cycle intermediates
Cell Communication and Signaling volume

Immunofluorescent detection of GRP91 on cardiac cells



Indirect immunofluorescence staining of the GPR91 receptor on H9C2.

Cells were labeled with 4′,6-diamidino-2-phenylindole (DAPI) and rabbit anti-GPR91 antibody (AB) revealed with fluorescent Alexa Fluor 488 secondary AB

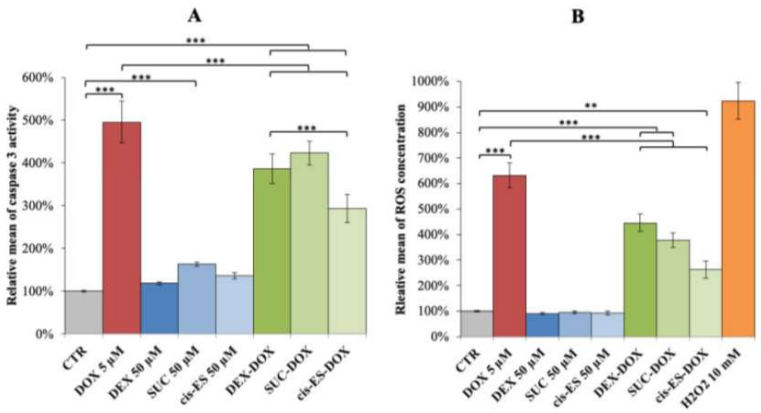
A control without primary AB was made for both cell lines

M. Dallons, E. Alpan, C. Schepkens, V. Tagliatti, J.-M. Colet
GPR91 Receptor Mediates Protection against Doxorubicin-Induced Cardiotoxicity without Altering Its Anticancer Efficacy. An In Vitro Study on H9C2 Cardiomyoblasts and Breast Cancer-Derived MCF-7 Cells
Cells . ;9(10):2177. (2020)

Succinate & Succinylation

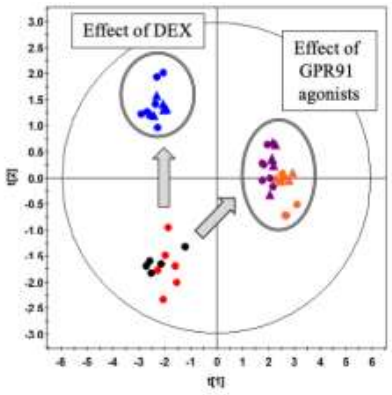
- Addition of either succinate or cis-epoxysuccinate (cis-ES) in the extracellular fluid
- 2 agonists of the GPR91 receptor
- Comparison with DEX protective effect against DOX

Succinate



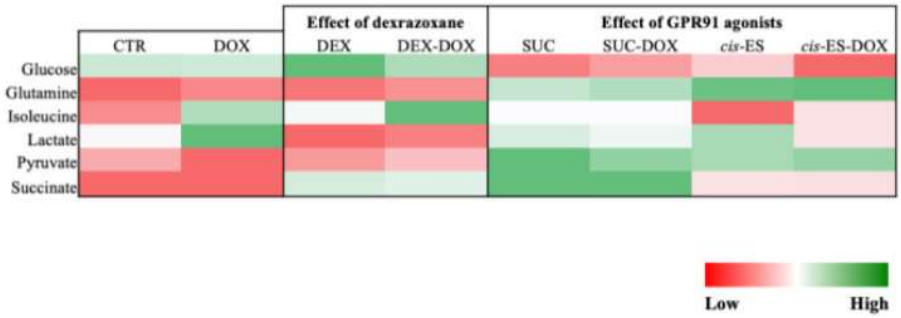
M. Dallons, E. Alpan, C. Schepkens, V. Tagliatti, J.-M. Colet
GPR91 Receptor Mediates Protection against Doxorubicin-Induced Cardiotoxicity without Altering Its Anticancer Efficacy. An In Vitro Study on H9C2 Cardiomyoblasts and Breast Cancer-Derived MCF-7 Cells
Cells . ;9(10):2177. (2020)

MBX evaluation of GRP91 agonists



M. Dallons, E. Alpan, C. Schepkens, V. Tagliatti, J.-M. Colet
GPR91 Receptor Mediates Protection against Doxorubicin-Induced Cardiotoxicity without Altering Its Anticancer Efficacy. An In Vitro Study on H9C2 Cardiomyoblasts and Breast Cancer-Derived MCF-7 Cells
Cells . ;9(10):2177. (2020)

Succinate



M. Dallons, E. Alpan, C. Schepkens, V. Tagliatti, J.-M. Colet
GPR91 Receptor Mediates Protection against Doxorubicin-Induced Cardiotoxicity without Altering Its Anticancer Efficacy. An In Vitro Study on H9C2 Cardiomyoblasts and Breast Cancer-Derived MCF-7 Cells
Cells . ;9(10):2177. (2020)

Succinate & Succinylation

- Addition of extracellular succinate or cis-epoxysuccinate (cis-ES)
- 2 agonists of the GPR91 receptor
- Significant reduction in cell stressox and apoptosis induced by DOX
- Protective effect of succinate similar to DEX
- Protective effect of cis-ES higher on oxidative stress and apoptosis

Succinate & Succinylation

- Moreover, our metabolomics study also highlighted several metabolic pathways involved in the cardioprotective effects of the two GPR91 agonists: stimulation of aerobic metabolism with glucose as the main fuel, redox balance and phospholipid synthesis

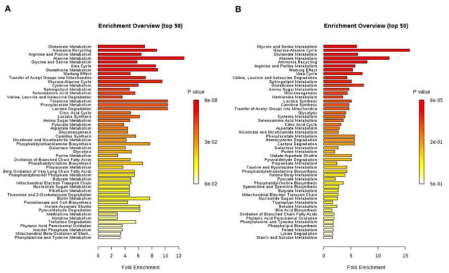
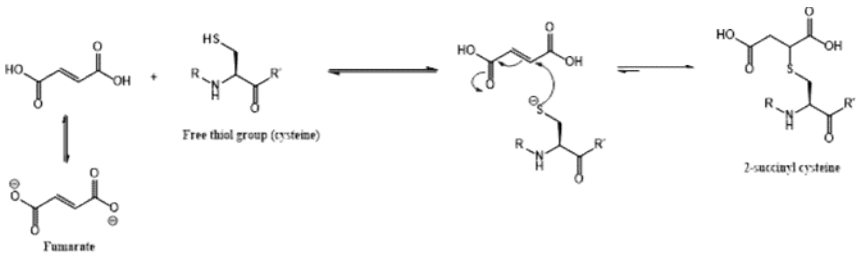


FIGURE 7 | Metabolite set enrichment analysis performed on ^{1}H-NMR metabonomic data, using MetaboAnalyst 4.0 online software. **(A)** 4 hours H9C2 cells exposure to DOX/DEX. **(B)** 24 hours H9C2 cells exposure to DOX/DEX.

Enrichment

Fumarate - Succination

- **Succination** = Spontaneous binding of fumarate to cellular targets ?
- Michael addition of free thiol group (from cysteine) to fumarate metabolite
- Thioether bond very stable ⇔ physiologically irreversible
- Regulation of the metabolism independently of any enzymatic activity



Fumarate - Succination

- **Fumarate** accumulates during hypoxic episodes and acts as a competitive inhibitor of prolyl hydroxylase
- Fumarate = oncometabolite (by stabilizing HIF)
- Enzyme inhibitor (i.e., glyceraldehyde-3-phosphate dehydrogenase, a glycolytic enzyme)

- Succination of key proteins often occurs during a germline mutation of the fumarase gene (also called fumarate hydratase), an enzyme converting fumarate in malate in the Krebs cycle
- This mutation is for instance present in the case of renal cancers and in the Leiomyomatosis disease

Fumarate - Succination

- In excess, fumarate intensively undergoes an addition reaction of cysteine residues of target proteins, significantly impacting different metabolic pathways

- Succination is favored by the inhibition of the activity of the Krebs cycle and respiratory chain, 2 events commonly observed in drug cardiotoxicity

Fumarate - Succination

- Finally, as the level of cellular 2-succinyl cysteine (2-SC) can be considered as a biomarker of succination and its potentially adverse consequences, a rabbit polyclonal antibody directed against 2-SC has been marketed by the company Discovery® Antibodies

- It is currently the only test to assess cellular levels of 2-SC. However, its effectiveness is questioned in the literature due to issues related to its non-specificity which calls for the development of a more robust detection method for succination.

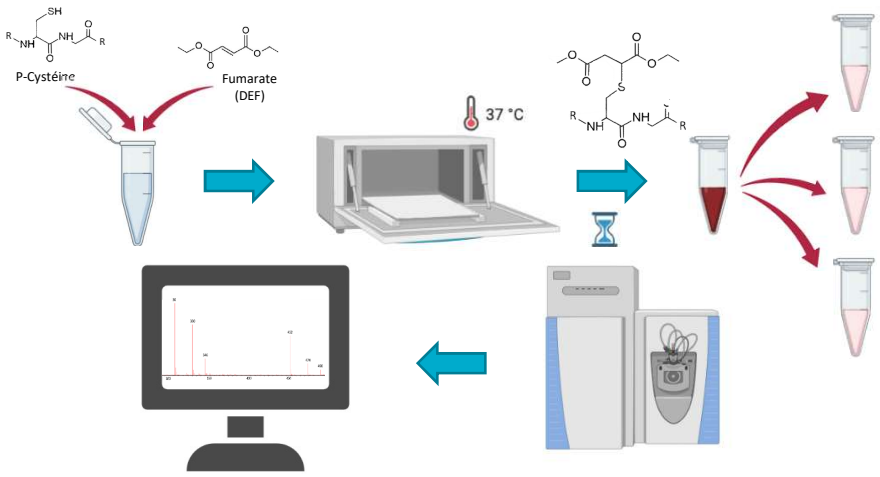
Fumarate - Succination

- In this context, our group is currently working on two goals :

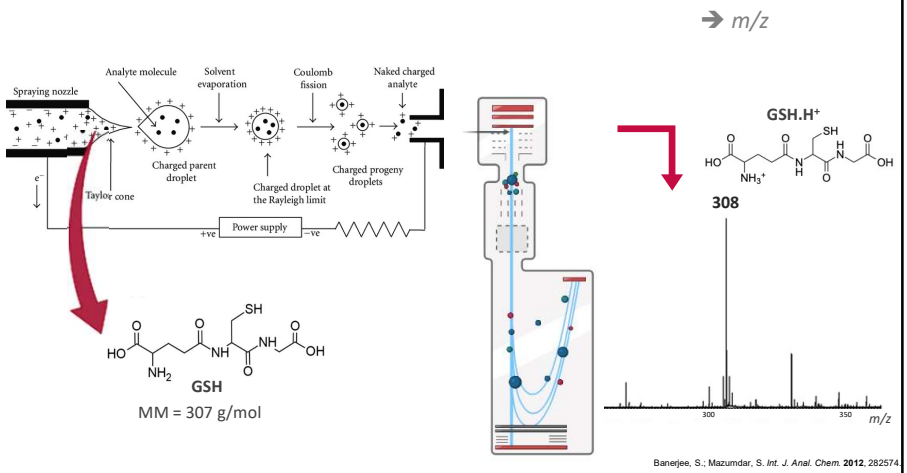
1. To develop a new MS-based protein succination detection methodology (addition of Di-Ethyl-Fumarate DEF)

2. Evaluate the possible succination reaction of SUMO-1 (Small Ubiquitin-like Modifier -1) and its impact in cardiotoxicity

Fumarate – Succination - Cysteine

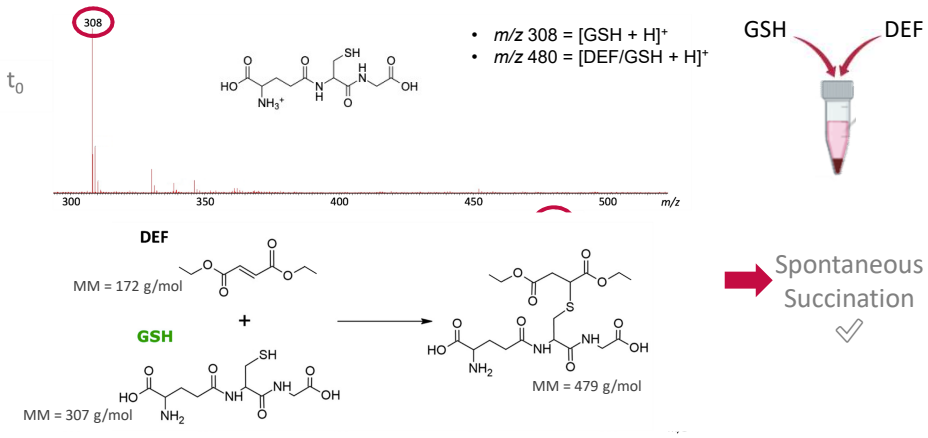


Fumarate – Succination - Glutathione



Banerjee, S.; Mazumdar, S. *Int. J. Anal. Chem.* **2012**, 282574.

Fumarate – Succination - Glutathione



Fumarate – Succination – SUMO-1

- Myocardial infarction is a prevalent and life-threatening cardiovascular disease
- Treatments :
  - ✓ Mainly restore coronary reperfusion
  - ✓ Few target post-translational modifications of critical proteins
- Small ubiquitin-like modifier (SUMO) proteins form a new type of protein post-translational modifications (PTM), known as SUMOylation
- SUMOylation ⇔ deSUMOylation dynamically balanced in the maintenance of various biological processes including cell division, DNA repair, epigenetic transcriptional regulation, and cellular metabolism

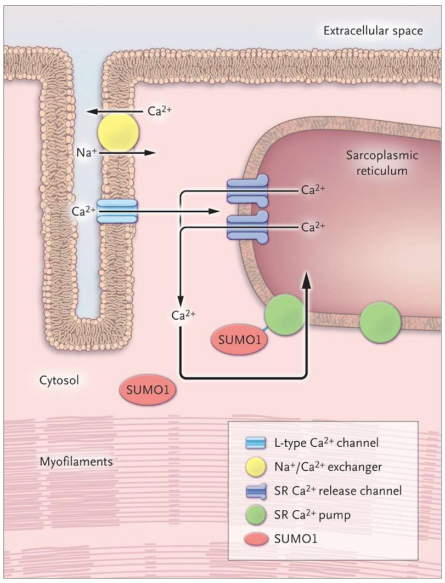
Fumarate – Succination – SUMO-1

- Importantly, SUMOylation plays a critical role in the regulation of cardiac functions
- Imbalance of SUMOylation – deSUMOylation associated with cardiovascular diseases, especially in heart failure and myocardial infarction

⇒ **SUMOylation as a key therapeutic target for treating cardiovascular diseases**

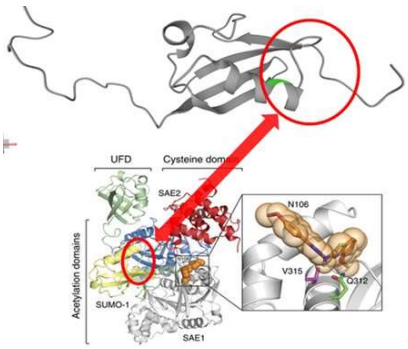
Fumarate – Succination – SUMO-1

- SUMOylation of SERCa2A is a crucial process to ensure the homeostatic calcium exchange in cardiomyocytes
- It is suspected that succination of SUMO-1 or SUMO-E1 (Ligase) can disturb the SUMOylation reaction



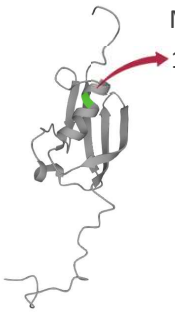
Fumarate – Succination – SUMO-1

- we hypothesize that **succination** could induce **3D conformational changes of SUMO-1** and, thereby, impair the process of cardiac contraction



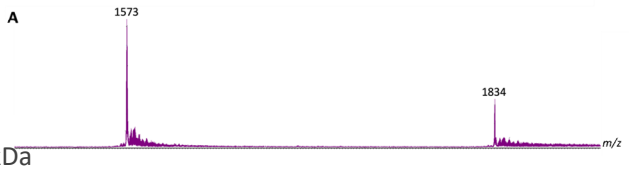
Fumarate – Succination – SUMO-1

SUMO1

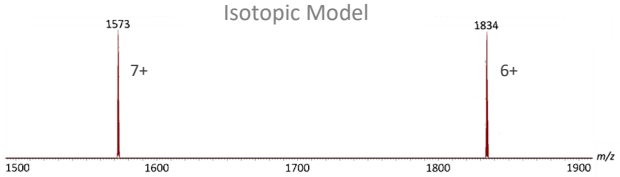


MW = 11kDa

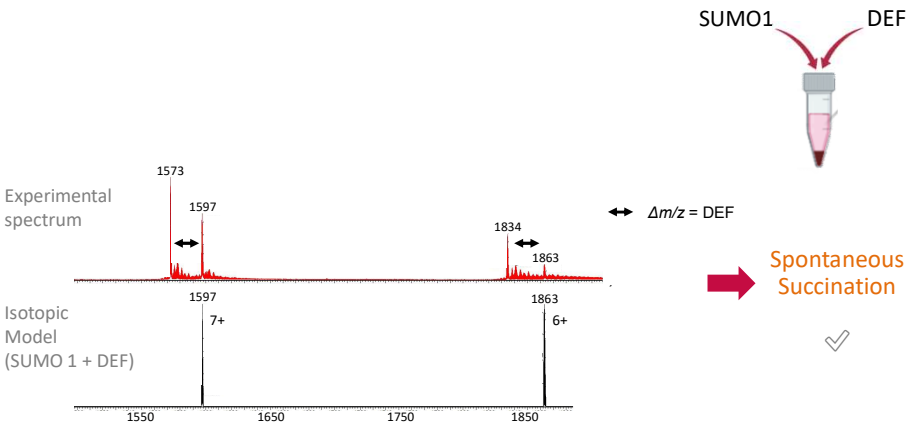
1 single Cysteine



Isotopic Model



Fumarate – Succination – SUMO-1



ARC 2023-2027 funding

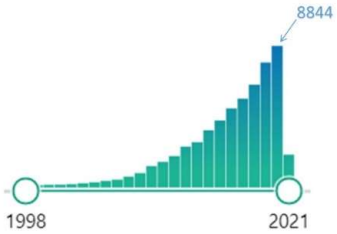
May the post-translational process of succination be involved in cardiac arrhythmia?

A joint biological and chemical investigation of the impact of proteins succination on their structure and activity.

Conclusions

Metabonomics from 1998 till now …

- ✓ Analytical progress
- ✓ Softwares development
- ✓ Robust and coherent metabolic patterns of diseases
- ✓ Identification of biomarkers



Yearly publications including « Metabol(n)omics » as key words (source : Pubmed)

- 🔎 Biological roles of metabolites
- 🔎 Deciphering cell communication
- 🔎 Better understanding organ crosstalk

Thank you for your attention

Adaptative versus toxic phases

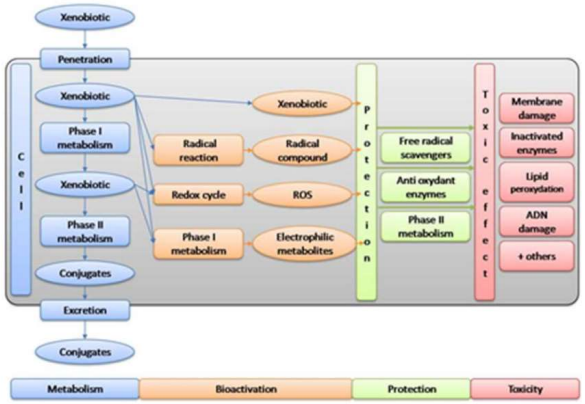
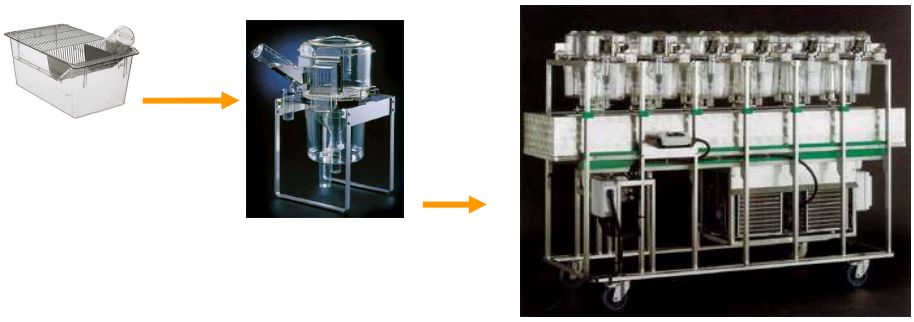


Schéma général des voies principales de biotransformation chez l'homme

Tiré de Monod, G., 1997. L'induction du cytochrome P450(1A1). pp. 33-52. in Lagadic, L., T. Caquet, J.-C. Amiard & F. Ramade eds. , 1997. Paris, Masson, 419 pp.

- Animals placed in metabolic cages for urine collection
- Acclimatation (at least 2 days) to minimize stress
- Water and food ad libitum or controlled if necessary



Avoid bacterial contamination !