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Proteomics in mechanistic toxicology: history, concepts, achievements, caveats and potential

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Abstract

Toxicoproteomics can be defined as the application of proteomic approaches to the understanding of toxicology problems, and this review deals with the various types of applications that have been described in the literature. Toxicoproteomics has been applied to very different classes of toxicants, from drugs and natural products to metals, or from industrial chemicals to nanoparticles and nanofibers. It has also been applied to address questions at different levels, from the search of the primary molecular targets of toxicants to the deciphering of the molecular responses of cells and tissues to toxicants. Although restricted to mammalian cells and tissues, this paper reviews these two levels of investigation and the different application areas of toxicoproteomics, leading to the discussion of the advantages and drawbacks of the most popular proteomic platforms. Some of the pending questions in toxicoproteomics are also critically addressed, such as the specificity, validation and result hierarchization issues. The question of shared mechanisms, which are encountered in many toxicoproteomic papers dealing with different toxicants, is also discussed. Finally the future of toxicoproteomics is briefly outlined.

1. Introduction: history of proteomics in toxicology

One of the major challenges for toxicology is to find how toxicants exert their effects on living organisms. Toxicology needs to provide knowledge of the molecular targets of the toxicants, and also on how an action on a (or a few) target(s) propagate in living cells to give rise to adverse effects. With regard to the complexity of living cells, this appears as a daunting task with almost infinite possibilities of direct and indirect effects. Progressive accumulation of biochemical and physiological knowledge at both the organism and cellular levels has allowed the toxicologists to decipher many mechanisms of toxicity. However, the price to pay in this strategy is that toxicology often follows well-known and well-paved avenues and has difficulties in unraveling new mechanisms or even to put into perspective different putative mechanisms. It is therefore not surprising that with its wide scope and low degree of preconception hypotheses, proteomics would appear as a seducing approach in toxicology. Historically speaking, the first attempts to apply a wide-scope protein screen to toxicology started soon after the first publications of the initial technique of proteomics, namely two-dimensional electrophoresis. Those publications were essentially descriptive (e.g. in [1-6]), because protein identification from 2D gels was an ordeal at that time. Only comigration with a purified know protein, blotting [7], or immunoprecipitation prior to analysis by 2D gels [8], was feasible at that time. Nevertheless, these authors had identified cytochromes P450 [8] and also Hsp proteins [2] as induced by toxicants. With the development of protein identification methods, first by Edman sequencing then by mass spectrometry, it became possible to identify almost every protein spot present on a 2D gels, and modern toxicoproteomics came to birth twenty years ago [9-11] (Figure 1).

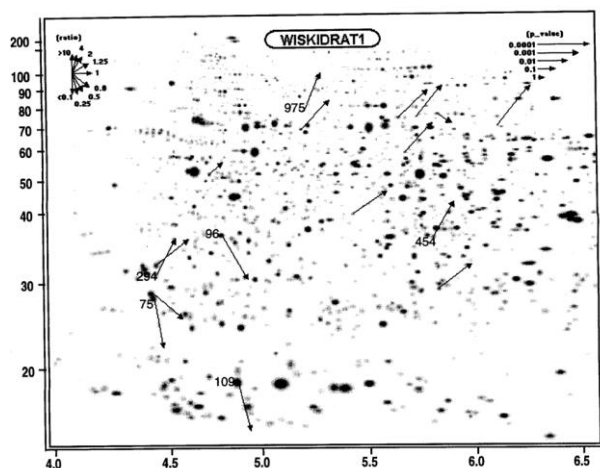


Figure 1: Historical illustration of the interest of proteomics in toxicology

This figure is extracted from one of the oldest toxicoproteomic papers [11], and illustrates a classical approach of expression proteomics. The synthetic image represents a 2D gel profile of rat kidney proteins, and the few proteins which amount is altered are indicated with arrows. The direction of the arrow indicates the direction and intensity of the change (decrease or increase), while its length indicates the statistical significance of the change. Among the six proteins showing the most significant changes, three were identified with the technical means available at that time (Edman microsequencing). Spot 75 was identified as Calbindin D, spot 96 as Regucalcin, and spot 109 as Major Urinary Protein.

Since this start toxicoproteomics has flourished in almost all possible directions and for all possible biological systems, from bacteria to plants going through all classes of animals. It would be overwhelming to review all these various aspects of toxicoproteomics, so that choices were made in preparing this paper. In order to select the cited references, Medline was searched with the keywords "toxicoproteomics" and "toxicity + proteomics". Only the papers dealing with mammalian cells and tissues were then kept for inclusion in this review, without any further selection on the type of toxicant or on the proteomic platform used. So despite their obvious interest, nothing will be found here about the responses of plants or bacteria to metals or to organic pollutants, nor will be anything mentioned about the field of ecotoxicology (reviewed for example in [12]) nor about biological fluids.

Even in this limited frame, toxicoproteomics has attracted a strong interest, and has been regularly reviewed (e.g. [13-27], to quote just a few). However, several of these reviews have either focused into specialized topics in toxicoproteomics (e.g. on polyaromatic hydrocarbons [28]), or have diluted proteomics with the other omics in the field of toxicology [29-31]. It is thus felt that a review encompassing the various types of proteomics in the field of human-oriented toxicology can be of interest. In this context, two major types of proteomic studies in the field of toxicology can be distinguished: those aiming at studying the molecular responses in a biological system exposed to a toxicant and those aiming at finding directly the molecular targets of a toxicant.

2. Proteomics for studying biological responses to toxicants

The rationale of this approach is that examination of the biological responses will provide valuable insights into the molecular mechanisms at play when a cell or an organism is exposed to a given toxicant (Figure 2). Thus, the general implementations of this approach are to submit the cell or organism either to a moderately toxic (sub-lethal) dose of the chemical of interest or to a highly toxic (lethal) dose but to examine the target cell or tissue at early time points, where the cellular death has not increased too much. In both cases, the rationale is to use a high enough dose to induce a significant cellular response that can be easily observed by proteomics, keeping at the same time the cell mortality low enough not to get the proteomic analysis polluted by events that are strictly related to cell death and will not give interesting insights into the specific molecular mechanisms involved in toxic injury.

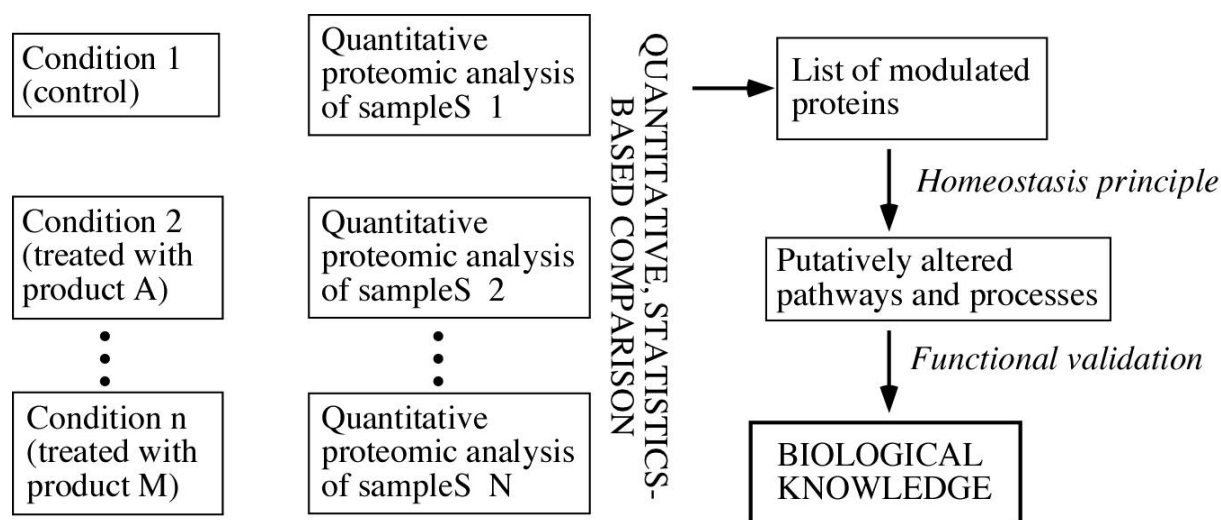


Figure 2: Scheme of principle of expression proteomics

This figure describes the major steps involved in an expression proteomic experiment. The uppercase S at the end of sampleS stresses the importance of biological replicates for each condition. It should also be kept in mind that all expression proteomic strategies rely on quantitative differences, so that the quality of quantification and especially the variance of the quantification is crucial in such approaches. The steps downstream the proteomic process per se are also highlighted, e.g. the homeostasis hypothesis, which implies that what changes is important in the biological process studied, and the necessity of functional validation to derive solid biological knowledge from the proteomic results

The proteomes of the control and treated samples are then compared to determine which proteins are modulated (underexpressed or overexpressed) in the treated samples compared to the control ones. This statement implies that the proteomic setup used to perform this task must be quantitative. The number of biological replicates required to obtain reliable data also implies that the precision of the quantification must be kept over large series of samples. These features have a strong impact on the type of proteomic technology that is used to perform such studies.

The responses that emerge from this type of toxicoproteomic analyses are a mixture of direct and indirect responses. The direct responses concern the target(s) of the toxicant. They can show up either in a decrease of the target protein(s), e.g. when the toxicant chemically modifies it and drive the protein(s) into degradation, e.g. by the proteasome pathway, or in an increase of the target proteins, by which the cell reacts to the toxicant by an increased synthesis of the target protein(s) to overcome the molecular inhibition caused by the toxicant.

These direct responses however, are usually diluted by much wider indirect responses by which the cell/tissue tries to restore its homeostasis that has been compromised by the toxicant. These indirect responses are also of interest, as (i) they provide clues to the molecular pathways that are disturbed by the toxicant, even if the primary targets are not identified, and (ii) also give clues to molecular pathways that the cells upregulate/downregulate to survive, providing testable hypotheses on how helping the cells to survive (or oppositely increase their death rate) by chemically modulating these pathways.

This frame of indirect toxicoproteomics is clearly the most widely used up to now, and it has been applied for all major types of toxicants.

2.1. Toxicoproteomics of responses to drugs

Adverse reactions are a major cause of drug withdrawal, either at the non-clinical stage or even worse at the clinical stage [32]. It is therefore of great importance to understand these toxic effects for several reasons:

(i) it could enable the researchers to devise new leads keeping the efficacy and decreasing the toxic side effects

(ii) according to the "fail early principle" the faster the toxic effect can be detected and even better predicted, the better it is in the drug development process.

As the toxic effects can arise through many different molecular mechanisms, it is therefore not surprising that the wide scope of proteomics has been used to detect and understand the involved mechanisms. The studies have focused on two types of drugs. First, the drugs that has been withdrawn from the market despite their efficacy because of strong adverse reactions (e.g. troglitazone) or which have shown important and potentially severe side effects (e.g. acetaminophen, cyclosporine, statins). Second, the anticancer drugs, for which toxicity is a concern for normal cells but is wanted for the cancer cells. In this respect, decoding the often more-complex-than-anticipated toxic mechanisms is of great importance to understand how to devise more efficient anticancer drugs with less resistances.

Examples of such toxicoproteomic studies on drugs are given in Table 1

Table 1: Toxicoproteomic studies on drugs

Chemical	In vivo/in vitro	Organ/cell type	Proteomic setup*	reference
acetaminophenol	In vivo (mouse)	liver	2D gels/MALDI	[33]
aminoacridines	In vitro (human)	various	2D gels/LCMS	[34]
andrographolide	In vitro (human)	hepatocytes	Silac	[35]
aubipy	In vitro (human)	Ovary tumor cells	2D gels/MALDI	[36]
aza cytidine	In vitro (human)	Pancreas tumor cells	2D gels/MALDI	[37]
aza cytidine	In vitro (human)	myeloma	TMT	[38]
bortezomib	In vitro (human)	myeloma	iTRAQ	[39]
cisplatin	In vitro (human)	Hela	Silac	[40]
cisplatin	In vitro (rat)	hepatocytes	Shotgun label free	[41]
cisplatin	In vitro (human)	Ovary mitochondria	Shotgun spectral count	[42]

cyclosporine	In vivo (rat)	kidney	2D gels/Edman	[10]
cyclosporine	In vitro (human)	Renal cell line	Silac	[43]
doxorubicin	In vitro (rat)	Isolated heart	2D gels/MALDI	[44]
dexamethasone	In vitro (mouse)	osteoblast	Silac	[45]
dopamine	In vitro (rat)	Neuron like	DIGE/MALDI	[46]
fluorouracil	In vitro (human)	Colon cancer cells	Silac	[47]
fluoxetine	In vitro (rat)	Primary neurons	2D gels/MALDI	[48]
flutamide	In vivo (rat)	testis	2D gels/MALDI	[49]
fluvastatin	In vivo (rat)	liver	2D gels/MALDI	[50]
levo tetrahydro-palmatine	In vitro (human)	hepatocytes	Shotgun spectral counting	[51]
lovastatin	In vivo (rat)	liver	2D gels/MALDI	[52]
lovastatin	In vitro (mouse)	ESC-derived cardiomyocytes	2D gels/MALDI	[53]
pregabalin	In vitro	glioma	2D gels/MALDI	[54]
proteasome inhibitor I	In vitro (rat)	Neuron like	DIGE/MALDI	[55]
simvastatin	In vitro (rat)	hepatocytes	Shotgun label free	[56]
tacrolimus	In vitro (human)	Renal cell line	Silac	[43]
terpyridine platinum	In vitro (rat)	glioma	2D gels/MALDI	[57]
tolbutamide	in vitro (rat)	hepatocytes	Shotgun label free	[58]
troglitazone	In vitro (human)	hepatoma	2D gels/LCMS	[59]
troglitazone	In vivo (mouse)	Liver mitochondria	iTRAQ	[60]
warfarin	In vitro (mouse)	ESC-derived cardiomyocytes	2D gels/MALDI	[53]

*the proteomic setups are defined as follows :

2D gels : non multiplexed 2D gels, where proteins are detected by Coomassie staining, silver staining, fluorescence or radioactivity

DIGE : multiplexed 2D gels with several fluorophores (one different per sample)

MALDI : protein identification by MALDI-MS (peptide mass fingerprinting) with or without an added MS/MS stage

LCMS : protein identification by nanoLC-MS/MS techniques

Shotgun spectral counting : label-free and 2D gels-free proteomics in which the proteins are empirically quantified through the spectral counting of their independent peptides appearing in the MS/MS analysis

Shotgun label-free : label-free and 2D gels-free proteomics in which the proteins are empirically quantified by the measurement of the ion current corresponding to their peptides.

The data compiled in Table 1 deal with studies that investigate mostly the effect of a single drug, sometimes two [43, 53]. However, some studies investigate the toxicity of a combination of drugs, either of the same therapeutic class (e.g. non steroidal anti-inflammatory drugs on rat cardiomyocytes using a 2D gel approach [61]) or of different classes but with the same target organ, such as the nephrotoxicity study carried out in [62] using 2D DIGE or the hepatotoxicity studies carried out either using 2D gels [63, 64], or 2D DIGE [65].

Series of studies addressing the same topic with different experimental designs (e.g. [63-65]) are very important on a general point of view, as they allow assessing the relevance and specificity of the proteomic findings while removing the experimenter and technical setup variables. Such studies indeed allowed demonstrating that different drugs induce different responses. Conversely, comparison of studies made on similar drugs with the same proteomic setup and the same tissues, e.g. [50, 52] show similar cellular responses.

However, when taken globally, some pathways and some proteins are found rather often, highlighting potential roles in response to injury or in detoxification processes. For example, calreticulin is found as a responsive protein in 7 studies made on different toxicants [33, 36, 38, 40, 53, 54, 63]. Mitochondrial proteins (albeit different ones) are almost always found, chaperones of the grp family are found in 10 studies [33, 39, 43, 46, 48, 55, 57, 59, 63, 65], and proteasome subunits in 10 studies [34, 36, 37, 39, 45, 53-55, 57, 63], just to quote a few. It is noteworthy that P450 cytochromes are only found in a few studies using shotgun-type proteomics, [41, 56, 58], as they are not visualized by 2D electrophoresis. However, they are not seen in most of the shotgun-type proteomic studies either.

A few additional studies can be related to this topic of drugs toxicity, namely the studies that investigate either the interference of natural products with drug effects (e.g. with doxorubicin in [66] and in [67]) or the studies that investigate resistance to drugs, especially resistance to anticancer drugs (e.g. [68-76]). This subject is of old interest for the proteomic community, as can be seen from reviews that are older than a decade, e.g. [77], and research papers that are of course even older [78, 79].

2.2. Toxicoproteomics of responses to natural products

Owing to the fact that many drugs derive from natural products, the boundary between toxicoproteomics of drugs and of natural products is fuzzy and somewhat arbitrary. Despite the fact that the financial incentive is much less for this area of research than for drugs, an important number of toxicoproteomic papers has been published in this area, and a selection of the mis displayed in table 2.

Table 2 : Toxicoproteomics of natural products

chemical	In vivo/in vitro	Organ/cell type	Proteomic setup	reference
azaspiracid	In vitro (human)	neuroblastoma	silac	[80]
bilirubin	In vitro (human)	neuroblastoma	2D gels/MALDI	[81]
butyrate	In vitro (human)	Colon cancer cells	2D gels/MALDI	[82]
celastrol	In vitro (human)	HeLa	2D gels/MALDI	[83]
celastrol	In vitro (human)	lymphoblast	silac	[84]
deoxynivalenol	In vitro (human)	lymphocytes	2D gels/MALDI	[85]
digoxin	in vitro (human)	HUVEC	2D gels/LCMS	[86]
isoline	In vivo (mouse)	liver	2D gels/LCMS	[87]
palytoxin	In vitro (human)	Mammary tumor cells	2D gels/LCMS	[88]
phalloidin	In vivo (mouse)	liver	2D gels/MALDI	[89]
polyphyllin	In vitro (human)	Various cell lines	2D gels/MALDI	[90]
resveratrol	In vitro (human)	Colon cancer cells	Shotgun label free	[91]
staurosporine	In vitro (human)	neuroblastoma	2D gels/MALDI	[92]
tanshinone	In vitro (human)	Cervix carcinoma	2D gels/MALDI	[93]
thapsigargin	In vitro (human)	neuroblastoma	2D gels/LCMS	[94]
tubeimoside	In vitro (human)	Hela	2D gels/MALDI	[95]
usnic acid	In vivo (rat)	liver	2D gels/MALDI	[96]

When the data that are compiled in this table are cross-analyzed, the emerging keyword is ER stress, where the key players are the ER chaperones. ER stress is mentioned in 8 of the 17 entries of Table 2 ([82, 84, 90, 92-96]). The second keyword is mitochondria, mitochondrial proteins being found modulated in 7 of the 17 entries of Table 2 ([80, 82-84, 87, 89, 94]). This suggests that the mitochondrial response and the ER stress response are core cellular responses to which many different toxic mechanisms converge, and which may represent, together with oxidative stress and central metabolism [97], a type of core response to stress and to cellular injury.

2.3. Toxicoproteomics of responses to industrial chemicals

Another large class of toxicants that has been submitted to toxicoproteomic studies is represented by industrial chemicals, which can induce various toxic effects upon exposure. Various classes have been investigated, with of course a more intense focus on chemical classes that are known to represent a major public health concern, such as aromatic hydrocarbons or endocrine disruptors. These studies are summarized on table 3

Table 3: Toxicoproteomic studies on industrial chemicals

chemical	In vivo/in vitro	Organ/cell type	Proteomic setu	reference
acrolein	In vitro (rat)	Lung epithelium	2D gels/MALDI	[98]
benzene	In vitro (human)	Lung carcinoma	DIGE/MALDI	[99]
benzopyrene	In vitro (human)	lymphoblast	2D gels/MALDI	[100]
benzopyrene	In vitro (pig)	urothelium	2D gels/MALDI	[101]
bisphenol A	In vivo (rat)	Mammary gland	2D gels/MALDI	[102]
bisphenol A	In vitro (mouse)	Sertoli cell line	2D gels/MALDI	[103]
bromate	In vivo (rat)	kidney	DIGE/LCMS	[104]
Butylidene phthalide	In vitro (human)	Prostate cancer cells	2D gels/LCMS	[105]
CCl4	In vivo (rat)	Liver mitochondria	2D gels/MALDI	[106]
chlorobenzenes	In vitro (human)	Lung carcinoma	DIGE/MALDI	[107]
decadienal	In vitro (human)	Bronchial cells	2D gels/LCMS	[108]
diazinon	In vitro (mouse)	neuroblastoma	2D gels/MALDI	[109]
dibutylphthalate	In vivo (rat)	Fetal testis	2D gels/MALDI	[110]
dibutylphthalate	In vivo (rat)	Fetal testis	2D gels/MALDI	[111]
diethylmaleate	In vivo (mouse)	Airway epithelium	DIGE/LCMS	[112]
dimethoxyhexane	In vivo (rat)	testis	2D gels/MALDI	[113]
dinitrobenzene	In vivo (rat)	testis	2D gels/MALDI	[114]
dioxin (TCDD)	In vitro (human)	hepatoma	2D gels/MALDI	[115]
dioxin (TCDD)	In vitro (rat)	hepatoma	Shotgun (ICPL)	[116]
dioxin (TCDD)	In vitro (mouse)	Leydig+sertoli cell lines	2D gels/Edman	[117]
fuel (alkanes)	In vivo (mouse)	lung	2D gels/MALDI	[118]
Glyphosate	In vivo (mouse)	skin	2D gels/LCMS	[119]
hydrazine	In vivo (rat)	liver	DIGE/MALDI	[120]

Naphthalene	In vivo (mouse)	Airway epithelium	DIGE/LCMS	[112]
nonylphenol	In vitro (human)	Colon carcinoma	2D gels/MALDI	[121]
Perfluorooctanoic acid	In vitro (human)	Liver cells	DIGE/MALDI	[122]
Polychloro biphenyls	In vivo (rat)	brain	DIGE/MALDI	[123]
Polychloro biphenyls	In vivo (rat)	brain	Shotgun (label free)	[124]
Polychloro biphenyls	In vitro (rat)	neurons	Shotgun (spectral counting)	[125]
thioacetamide	In vivo (rat)	liver	2D gels/MALDI	[126]
toluene	In vitro (human)	Lung carcinoma	DIGE/MALDI	[99]

It must be emphasized that some topics, such as the toxicity of polycyclic aromatic hydrocarbons, have raised an important interest that is reflected in the proteomic community, e.g. by a recent review on this specific topic [28]. This review goes beyond the two papers that have been compiled in Table 3 and addresses topics such as the use of reactive metabolites of benzopyrenes and/or chemoprevention of the carcinogenic effects of polycyclic aromatic hydrocarbons by natural products.

As in Table 1, Table 3 compiles studies that investigate the effect of one chemical, or sometimes of a few related ones [99, 107, 125]. However, very few studies are available where several different chemicals have been investigated in parallel. One exception is a study on various polycyclic aromatic hydrocarbons and related products such as coal tar [127]. Conversely, some studies introduce an extra focus and apply proteomic technologies either to investigate a small subset of proteins such as the proteasome subunits [128] or to target proteins bearing a given post-translational modification such as tyrosine phosphorylation [129].

From Table 3, it can be easily seen that toxicoproteomic studies are focused on a few classes of chemicals (e.g. aromatic hydrocarbons) showing long-term effects. For these chemicals, where there are no obvious short-term effects in in vitro or on animal models, deciphering early mechanisms of action through omics technologies should enable the prediction a long-term outcome and would be of great value for effective and fast toxicological assessment. However, it must be recalled that this goal has not been reached yet.

As previously discussed, some proteins/subsets of proteins are found at a high frequency in this area of toxicoproteomics of industrial products, illustrating their central role in response to toxic injury. HSP proteins (mostly hsp70 and hsp27) are found in 14 papers out of 30 ([99, 101-103, 105, 107, 112, 113, 116, 117, 120, 122, 123, 127]). Mitochondrial proteins, most often matricial enzymes, are also found in 14 papers out of 30 ([98, 99, 106, 107, 113, 114, 116-118, 120, 122, 123, 125, 126]). More surprisingly, the single protein enolase is found in 13 papers out of 30 ([98-101, 104, 105, 107, 109, 112, 116, 118, 125, 127]). Peroxiredoxins are also found, frequently, in 10 papers out of 30 ([98, 99, 102, 107, 110-112, 116, 119, 121]) and proteasome subunits in also 10 papers out of 30 ([99- 101, 105, 107, 110, 111, 114, 125, 128]).

Furthermore, as the toxicoproteomic studies on industrial chemicals are carried out

on a limited range of chemicals, it is possible (and tempting) to compare various studies carried out on the same chemical in different papers (e.g. [100] vs [101] on benzopyrene, [102] vs [103] on bisphenol A, [110] vs [111] on dibutylphthalate, and [116] vs. [117] on dioxin). These comparisons show that proteomic studies carried out on the same chemical by different groups usually share a moderate overlap in the lists of modulated proteins. However, such comparisons should be handled with care, as the variation of the identified proteins depends on several factors. The first is of course biological variability, i.e. the fact that different cells may have a different response. The second factor is a purely technical one, i.e. the depth at which the proteome is investigated. Even keeping with studies that use the same proteomic setup, e.g. 2D gels, it is obvious that a 2D gel showing 2000 spots will show protein variations that will not appear on gels showing only 500-600 spots. In addition to these two obvious factors, a third subtler one must be taken into account, and relates to the way by which significant changes are detected in proteomic experiments. Significant changes are detected by statistical tests, so that the coefficient of variation observed for each protein in each condition plays a key role in defining the significance of a variation. Thus, if for any biological/technical reason the CV observed on protein X is higher in study B than in study A, then the same low-fold difference may be found significant (and thus reported) in study A, while it may be found not significant (and thus not reported) in study B.

2.4. Toxicoproteomics of responses to metals and metalloids

Apart from the few metal complexes that are used as drugs (mostly platinum complexes and gold complexes), toxicology of metals, metalloids and their organic complexes is a branch of occupational toxicology. This does not prevent toxicoproteomics to participate to the general study of inorganics toxicology, as can be shown in the studies mentioned in Table 4. However, a review recently published on toxicoproteomics for the investigation of metal toxicity [130] clearly indicates that in this specific area, toxicoproteomics on plants, microorganisms and metazoans of ecotoxicological interest dominates the field.

Table 4 : Toxicoproteomic studies on metals and metalloids

chemical	In vivo/in vitro	Organ/cell type	Proteomic setup	reference
arsenic trioxide	In vitro (human)	myeloma	2D gels/MALDI	[131]
arsenite	In vitro (human)	myeloma	SILAC	[132]
arsenite	In vitro (human)	keratinocytes	2D gels/MALDI	[133]
diphenylarsinic acid	In vitro (human)	Hepatoma cell line	2D gels/MALDI	[134]
calcium oxalate	In vitro (dog)	Kidney cell line	2D gels/MALDI	[135]
calcium oxalate	In vitro (human)	Monocyte cell line	2D gels/LCMS	[136]
calcium oxalate	In vitro (human)	Macrophage cell line	2D gels/LCMS	[137]

cadmium	In vitro (human)	HeLa	2D gels/MALDI	[138]
cadmium	In vitro (human)	Lung cell line	2D gels/MALDI	[139]
cadmium	In vitro (rat)	Leydig cell line	2D gels/MALDI	[140]
chromium	In vivo (mouse)	liver	2D gels/MALDI	[141]
chromium	In vitro (rat)	Lung cell line	2D gels/MALDI	[142]
chromium	In vivo (mouse)	skin	2D gels/MALDI	[143]
chromium	In vitro (rat)	Osteoblast cell line	2D gels/LCMS	[144]
chromium	In vitro (human)	Monocyte cell line	2D gels/LCMS	[144]
chromium	In vitro (human)	Keratinocyte cell line	2D gels/MALDI	[145]
copper	In vitro (human)	Embryonic carcinoma cells	2D gels/MALDI	[146]
iron	In vitro (human)	Hepatoma cell line	2D gels/MALDI	[147]
methyl mercury	In vitro (human)	Hepatoma cell line	SILAC	[148]
manganese	In vivo (rat)	Brain mitochondria	2D gels/MALDI	[149]
neodymium	In vitro (human)	Keratinocyte cell line	2D gels/MALDI	[145]
nickel	In vitro (human)	Keratinocyte cell line	2D gels/Edman	[150]
lead	In vivo (mouse)	skin	2D gels/MALDI	[151]
uranium	In vitro (human)	Kidney cell line	2D gels/MALDI	[152]
uranium	In vitro (human)	lung cell line	2D gels/MALDI	[153]
zinc	In vitro (human)	Colon cancer cell line	2D gels/MALDI	[154]
Zinc	In vitro (human)	HeLa	2D gels	[138]

The proteomic responses observed with metals, metalloids and their organic derivatives are similar to the ones observed with small organic molecules (Table 3). Heat shock proteins are found modulated in more than half of the papers ([133, 136, 137, 140, 142, 144, 145, 146, 148, 151, 152]). Enolase is also found modulated frequently ([132, 133, 135, 139, 142, 144, 147, 151]), as well as ER stress ([133, 135, 140, 142, 147, 148]), proteasome ([132, 135, 136, 145, 147, 148]), or peroxiredoxins ([135, 137, 139, 140, 148]). It must be emphasized that peroxiredoxins are found in all the papers mentioned in Table 4 and dealing with strongly thiophilic metals such as mercury or cadmium, which is consistent with their

thiol-containing active site.

2.5. Toxicoproteomics of responses to nanoobjects

Nanoobjects, mostly nanoparticles and nanofibers, are more and more a potential concern for human health, and are therefore under intense scrutiny for possible adverse health effects. As it is difficult to forecast the behavior of nanoobjects in biological environments and their interaction with living cells, toxicoproteomics is almost ideal in this field to figure out which cellular responses are at play when cells encounter nanoobjects. It is therefore not surprising that toxicoproteomics studies have developed in this field, as shown in Table 5

Table 5 : Toxicoproteomic studies on nanoparticles and nanofibers

Nanoobject	In vivo/ in vitro	Organ/cell type	Proteomic setup	Reference
gold	In vitro (human)	Leukemia cell line	2D gels/LCMS	[155]
nanodiamonds	In vitro (human)	Stem cells	ITRAQ	[156]
carbon black	In vivo (mouse)	Lung	Shotgun (label free)	[157]
carbon nanotubes	In vitro (human)	Monocyte cell line	2D gels/MALDI	[158]
carbon nanotubes	In vivo (mouse)	Lung	Shotgun (label free)	[157]
carbon nanotubes	In vivo (rat)	Lung	2D gels/MALDI	[159]
carbon nanotubes	In vitro (human)	Hepatoma line	ITRAQ	[160]
graphene	In vitro (human)	Hepatoma line	ITRAQ	[160]
copper oxide	In vitro (mouse)	macrophages	2D gels/LCMS	[161]
iron oxide	In vivo (rat)	Lung	2D gels/MALDI	[159]
iron oxide	In vitro (human)	Stem cells	ITRAQ	[156]
silica	In vivo (rat)	Lung	2D gels/MALDI	[159]
silica	In vitro (human)	Lung carcinoma line	Shotgun (label free)	[162]
titanium dioxide	In vitro (human)	Bronchial cell line	2D gels/LCMS	[163]
titanium dioxide	In vitro (human)	Bronchial cell line	DIGE/MALDI	[164]
titanium dioxide	In vivo (mouse)	Lung	2D gels/LCMS	[165]
titanium dioxide	In vivo (mouse)	kidney	2D gels/LCMS	[166]
zinc oxide	In vitro (mouse)	macrophages	2D gels/LCMS	[167]

undefined (wear particles)	In vitro (human)	macrophages	2D gels/MALDI	[168]
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In this area, the results of toxicoproteomic studies are much less constant and consistent than in the previous sections. Sometimes usual players are found, such as the ER stress markers [155, 160], proteasome subunits ([158, 163, 167, 168]) or peroxiredoxins ([155, 160, 161, 164]). In other cases none of these classical proteins are found [162, 166], which can be related to the weak toxicity of the precise nanoobject in the tested biological system. Nonetheless, all the toxicoproteomic studies carried out on nanoobjects have provided new mechanisms well beyond the oxidative stress that is almost always searched for in this area. Such mechanisms include interference with the central metabolism for ZnO nanoparticles, [167], with glutathione metabolism and mitochondria for Cu and CuO nanoparticles [161], or the more classical unfolded protein response [155, 162] for silica and nanogold, respectively.

3. Proteomics for finding molecular targets of toxicants

Because of its wide scope and depth of analysis, proteomics can be happily used to identify molecular targets of toxicants, which is one of the major stakes in toxicology. Compared to the study on cellular responses, the name of the game is no longer to compare series of biological samples treated with different doses of a toxicant, but to analyze a few samples in which the molecular targets of the toxicant will have been made selectively detectable. Depending on the type of interaction between the targets to be found and the toxicant, i.e. covalent vs. non-covalent interactions, very different experimental schemes are used.

3.1. Finding targets by non-covalent interactions: chemical proteomics

In this type of studies, a very classical affinity chromatography setup is usually implemented (Figure 3). The molecule of interest is immobilized on a solid phase, on which a cell or tissue lysate prepared under non-denaturing conditions is loaded. After interaction and washes to remove the non-specifically bound proteins, elution is performed either by protein denaturation (thorough but less specific) or competition with a soluble form of the bait (lower yields but more specific) and the released proteins are analyzed by proteomic techniques.

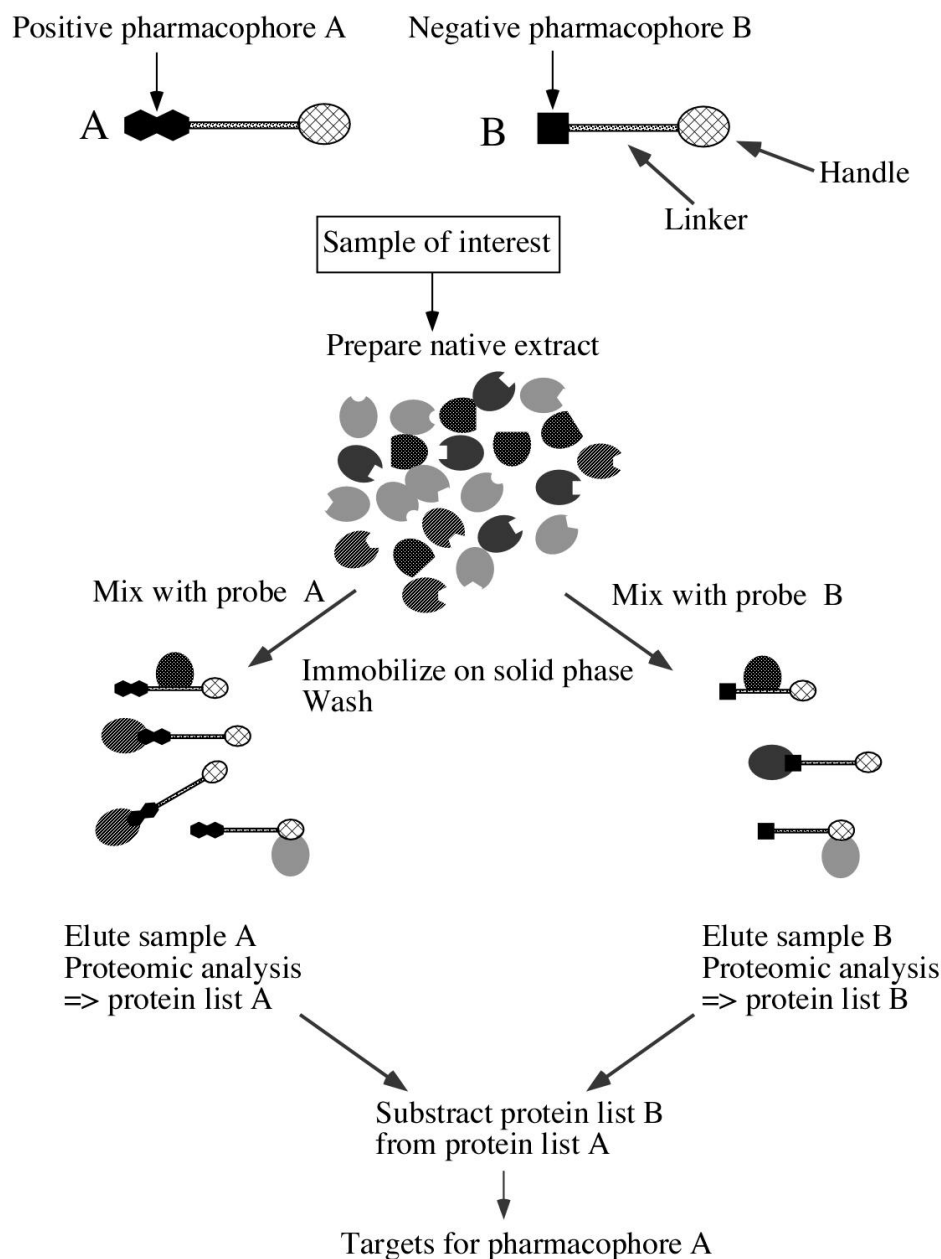


Figure 3: Scheme of principle of chemical proteomics (non-covalent interactions)

The purpose of a chemical proteomic is to identify the proteins that bind to a pharmacophore of interest. For technical reasons this pharmacophore is bound to a handle (clickable chemical group or directly a solid bead) via a linker (probe A). The handle allows retrieving selectively the proteins bound to the pharmacophore from the complex biological extract, and the linker is necessary to decrease the interferences between the pharmacophore and the handle. However, some proteins can bind to the linker or to the handle, and thus can be unspecifically retrieved. To alleviate this problem common to all affinity-based proteomic approaches, a negative control (probe B) must be used in parallel to subtract the background proteins.

Examples of this approach are compiled in Table 6. As can be seen, the hot topic in this area is to find drug targets (known targets and off targets), and this new field has been already reviewed [169-171].

Table 6: Detection of protein targets of chemicals through non-covalent interaction

Chemical	Biological system	Affinity type	Proteomic setup	Reference
abl inhibitors	HeLa cells	immobilized	ITRAQ	[172]
abl inhibitors	K562 cells	immobilized	shotgun	[173]
andrographolide	Various human cell lines	In solution	ITRAQ	[174]
bosutinib	Various human cell lines	immobilized	shotgun	[175]
CK2 inhibitors	HeLa cells	Immobilized (indirect)	2D gels/MALDI	[176]
dasatinib	Various human cell lines	Immobilized vs. In solution	shotgun	[177]
E-3180	Ovary cancer cell line	immobilized	SILAC	[178]
gelfitinib	Human myeloma cell line	immobilized	SILAC	[179]
HDAC inhibitors	Various human cell lines	immobilized	ITRAQ & TMT	[180]
INNO-406	Human leukemia and normal blood cells	immobilized	shotgun	[181]
PBT-1	Human lung cancer cell lines	immobilized	shotgun	[182]
copper and zinc	Various human hepatoma cell lines	immobilized	2D gels/LCMS	[183]
copper	human hepatoma cell line	immobilized	2D gels/MALDI	[184]
zinc	Mouse macrophage cell line	immobilized	2D gels/LCMS	[167]

It is clear from Table 6 that shotgun proteomics is used for finding drug targets while 2D gels are applied to find targets of metals. There is however an exception to this rule [176], in which the binding screen is not a positive one (small molecule of interest bound to the resin) but a negative one (pharmacological inhibitor in solution and competition with the natural ligand for the target bound to the resin). This general trend is due to the widely different affinities of the two classes of molecules for their targets. Affinity of drugs for their targets, even their off-targets, is usually very high, in the low submicromolar range. This means in turn that in an affinity chromatography screen, strong washes can be implemented to remove all the contaminants before the final elution of the targeted proteins. In this frame, shotgun proteomics excels by

its exquisite sensitivity. In the case of metals, the situation is very different. The affinity of proteins for metals is usually quite poor. This affinity must not be confused with the affinity of metalloproteins for their metals, which can be very high, e.g. in the zinc finger proteins. However, metalloproteins with such strongly bound metals are not demetallated when they are prepared before to binding to the immobilized metal, so that the affinity chromatography will really reveal the binding to proteins of excess metal, which is the case looked at in toxicoproteomics of metals. Thus, with such low affinities, excessive washes on the column would result into excessive losses of metal-binding proteins. This means in turn that the eluates of immobilized metal columns usually contain a non-negligible proportion of background proteins. In this frame, 2D gels bring their unique ability of an intra sample quantitative analysis, something that shotgun proteomics does not easily perform. 2D gels also bring a very easy comparison between the eluate and the various controls that are needed to find the metal-binding proteins in this experimental setup.

It must also be kept in mind that affinity chromatography is not the ideal situation for finding targets. First, the binding of the affinity probe to the bead may introduce steric constraints that prevent some protein targets from binding to the column. A classical example in the non-drug field is represented by the quest for ATP-binding proteins by affinity chromatography. Immobilization of the ATP by the base is the easiest but also the most-interfering one, as the protein must differentiate ATP from the other NTPs in the cell and therefore "read" the base moiety of ATP [185]. Thus, immobilization of ATP by the terminal phosphate with spacer arms must be used to improve the representation of the ATP-binding proteome [186, 187]. Transposed to the chemical proteomic field, this means that a good knowledge of the binding of at least some target proteins must be earned prior to designing the affinity adsorbent.

Second, and in addition to this problem, affinity chromatography is usually performed on whole lysates. This means for example that some of the proteins that will see the bait in the affinity chromatography setup would never be in contact with the drug in vivo because of the cellular compartmentalization. In addition, the binding assay will be performed under a single condition in terms of ionic strength and buffer composition (e.g. presence or absence of divalent cations) and this precise condition may not be appropriate for all targets.

This means that schemes in which the interaction of the bait with the proteins are made under in vivo conditions, following the principles developed in B. Cravatt's group [188], prior to final immobilization of the bait-target complexes, e.g. by click chemistry [189] or avidin chromatography [190], are usually more performing than classical affinity chromatography setups for finding drug targets [177].

3.2. Targets of chemicals determined by covalent binding

Covalent binding of chemicals to biomolecules is also a very important toxic mechanism. Besides the obvious binding of electrophilic chemicals to the various nucleophilic sites present in biomolecules (e.g. bases in nucleic acids, lysine and cysteine side chains in proteins), it is also known for decades that chemicals that are not electrophilic per se, such as aromatic hydrocarbons, can undergo in vivo an oxidative metabolism that can produce electrophilic species such as epoxides [191], which are the real causative agents of the toxic phenomena observed, such as necrosis [192] or tumorigenesis [193]. This bioactivation process is not restricted to aromatic hydrocarbons and can happen for many molecules (e.g. in [194]). It is of course worth to be investigated in order to determine how toxicity arises when such

reactive metabolites are produced, and to identify their molecular targets. In the protein world, the fact that the core technologies used in proteomics have the potential to detect these targets is known since even before the word proteomics was even coined [195, 196].

In this type of toxicoproteomic analysis, the name of the game is to detect and identify the proteins that have bound the metabolites of the chemicals of interest. In an ideal world, the analysis should go down to the modified peptide, so that the modified site can be identified. This is however not often possible, as the chemical adduction often alters strongly the physico-chemical properties of the modified peptides. Murphy's law being what it is, this means that the modified peptides are often very difficult to obtain and identify. First, the exact adducting entity must be known to be able to select the modified peptides in the mass spectrometer, and this is often uneasy. Second, adduction on cysteines is not a real problem for the production and extraction of the modified peptides, but adduction on lysines is a problem, as it causes an automatic miscleavage with trypsin, sometimes with a charge loss, so that extracting and finding the modified peptide becomes almost impossible. Shotgun proteomics approaches have been described to identify modified peptides following exposure to reactive chemicals. However, the biological relevance of the data generated from such large screens needs to be cautiously evaluated [197, 198]

Thus, is often much safer to stop at the modified protein level, the bulk of the unmodified residues in the protein acting as a buffer to keep everything soluble and amenable to analysis (Figure 4).

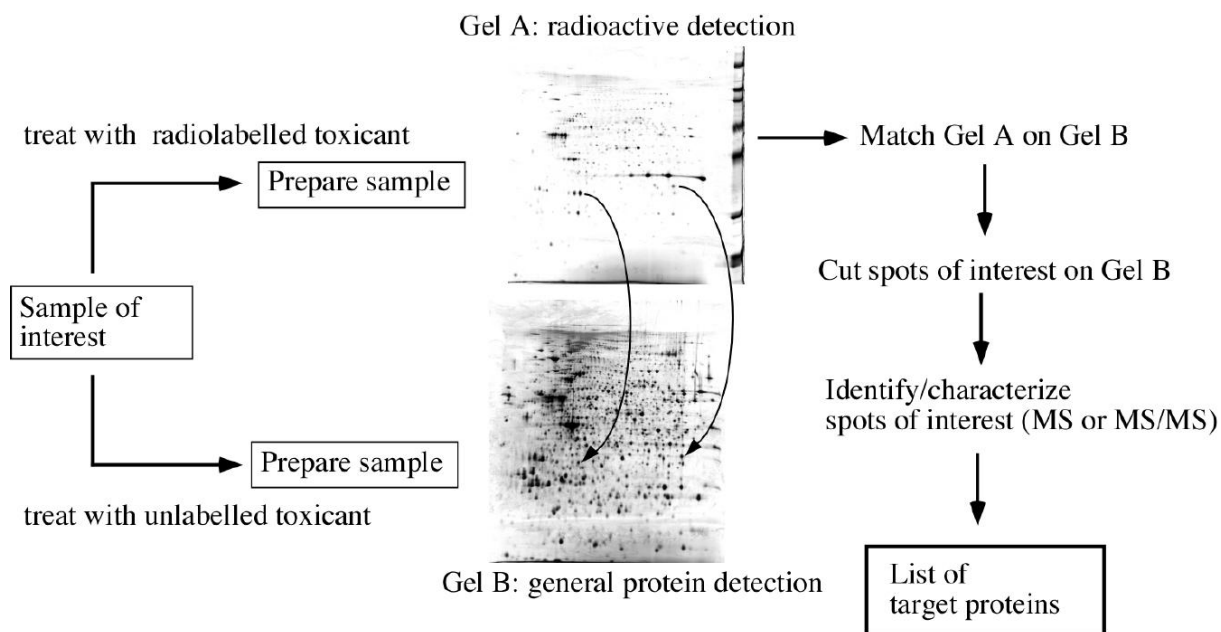


Figure 4: Scheme of principle of covalent binding proteomics

The purpose of the experiment is to identify the proteins that bind a chemical of interest or its reactive metabolites produced by bioactivation. The most classical, 2D gel-based process is illustrated on the figure. In this process the two critical steps are:

- (i) the correct matching of the labelled spots on the general 2D map. This can be made much easier by a low sensitivity, permanent staining prior to radioactivity detection (e.g. Coomassie blue staining)
- (ii) the handling of the multiple protein identifications from a single spot by mass spectrometry.

Some measures (e.g. high resolution pH gradients in IEF, third dimension electrophoresis)

can be taken to alleviate this problem when the modified peptide is not detected.

In this frame, the modified proteins can sometimes be detected just by the pI shift on 2D gels [195, 196]. When the metabolite is large enough to be used as an hapten, detection of the modified proteins by antibodies is also feasible [199]. However, in most of the cases, the chemical is given as a radioactive precursor and the modified proteins are detected through detection of radioactivity. Positional alignment on gels where a similar but nonradioactive extract has been loaded allows picking up the proteins for identification by standard techniques. This rather cumbersome setup is required by the fact that it is not possible to chemically modify the molecule to be investigated, as this would result into an altered metabolism. There are therefore very few options to detect the molecule of interest when bound to the target proteins.

Examples of this strategy are given in Table 7 below

Table 7: Detection of protein targets of chemicals through covalent labelling

Chemical	Target cell	Proteomic setup	Detection method	reference
acrolein	Mouse heart (in vivo)	2D gels/MALDI	blotting	[200]
acrylamide	Rat neurons(in vitro)	Shotgun	Mass adduct	[197]
bromobenzene	Rat liver (in vivo)	2D gels/MALDI	radioactivity	[201]
bromophenol	Rat liver (in vivo)	2D gels/MALDI	radioactivity	[202]
dopamine	Human neuroblastoma (in vitro)	2D gels/MALDI	radioactivity	[203]
furan	Rat liver (in vivo)	2D gels/LCMS	radioactivity	[204]
monocrotaline	Human endothelial cells(in vitro)	2D gels/MALDI	radioactivity	[205]
mycophenolate	Rat kidney (in vivo)	2D gels/LCMS	blotting	[199]
naphthalene	Mouse liver microsomes (in vitro)	2D gels/MALDI	radioactivity	[206]
quinones	Human bronchial cells(in vitro)	2D gels/MALDI	radioactivity	[207]
thioacetamide	Rat hepatocytes (in vitro)	2D gels/LCMS	radioactivity	[208]
thiobenzamide	Rat liver (in vivo)	2D gels/MALDI	radioactivity	[209]

This strategy builds in an important caveat, however, which is the comigration problem in 2D gels [210]. This comigration problem is not always a major issue in classical proteomics [211], but in this precise toxicoproteomic frame, the real target protein can be a minor component and not the major one. As the modified peptide is often not detected, there is always a doubt in the assignment of the target proteins. Thus, the implicit gamble that is made in the studies cited in Table 7 is that the reactivity of all proteins present within the same 2D spot is similar, so that the most abundant one is the real target. In the absence of functional validation (e.g. showing that the activity of the identified protein is lower when the cells have been treated with the target chemical) this gamble appears rather risky. There are however means to address the comigration problem, either by using a third separation [212], or by using narrow pH gradients in which the comigration probability becomes negligible [213], [214]. However, all these strategies require a second round of experiments, which is not very practical in setups that are already rather slow because of the time needed to detect the radioactive signal.

Thus, the optimal strategy in this case is probably to use directly multiple narrow-range pH gradients in the first round of experiments. Although requiring more experiments and more sample at once, the time saved by carrying out only one round of long experiments instead of two is probably worth the effort.

Once a given target protein has been identified, specific proteomic investigations can sometimes be carried out to characterize the modified residues. Reliable MS-based protocols have been recently described for this purpose [215, 216]

Compared to the situation of chemical proteomics in the previous section, the specificity issues in the case of covalent binding do not arise from the general experimental setup, as everything is made in vivo with the required molecule only, but arise from the readout system that is used.

3.3 Indirect targets

For some toxicants, generic toxic mechanisms such as oxidative stress are implied. In such cases, one possible aim of toxicoproteomic studies is to find the targets of the reactive oxidant species produced during the oxidative stress. These targets are identified through the chemical modifications induced by the oxidative stress, and the modifications most commonly used are tyrosine nitration and protein carbonylation, which are identified by immunoblotting either directly (tyrosine nitration) or after derivatization with a suitable hydrazine used as a hapten (protein carbonylation). Only a few examples are present in the toxicoproteomic literature, one on tyrosine nitration [217], and two on protein carbonylation [218, 219].

The rather low popularity of these studies can be explained by the fact that they require blotting and gel alignment steps that make them more cumbersome to carry out than classical response-type studies. In addition, and oppositely to what is encountered in other types of target research, they often yield indirect targets, which is not felt as interesting as direct targets.

4. A critical analysis of the achievements of toxicoproteomics

With almost 20 years of toxicoproteomics and several tens of papers published in the field, it is now possible to make a critical appraisal of the literature, which gives quite interesting insights regarding the technical choices and the value of the results

4.1. Technical issues in toxicoproteomics

On a technical point of view, there is a strong overrepresentation of 2D gel-based proteomics in toxicoproteomics compared to other areas of proteomics in cell biology. This question arises in the toxicoproteomics of cell responses and not really in the targets finding area, where there is a clear-cut distinction between non covalent interactions (dominated by shotgun proteomics) and covalent binding (dominated by 2D gels).

This overrepresentation of 2D gels may be linked in part to the historical perspective inherent to a review paper. However, as most of the papers cited here are less than five years old, this preeminence of 2D gels is likely to be linked to their higher reproducibility when large series of samples are analyzed. The high reproducibility of 2D gels has been demonstrated over the years, with median coefficient of variations in the 20-30% range [220-222], even in large sample series. Such coefficient of variations have only recently been accessible to shotgun proteomics. For label-free methods, there are still limited to either short series of experiments [223], or to a moderate depth of analysis [224]. In methods using stable isotope labelling, the intraexperiment coefficients of variations are low but limited to the multiplex ability of the method [224, 225], thereby limiting the number of samples that can be analyzed. Moreover, the missing value problem is still a major issue in shotgun proteomics [226], so that sophisticated and still questionable imputation strategies must be implemented [157]. In addition, deriving a variation in a protein amount from several variations at the peptide level is still a difficult issue [227]. Conversely, 2D gels are able to handle similar sample series with high reproducibility [167] provided that a modern image analysis software is used [228].

This high sensitivity to reproducibility issues may be specifically increased in the context of toxicoproteomics. First, many toxicoproteomic studies are carried out on animal tissues after an *in vivo* exposure. As animal tissues are much more variable than pure *in vitro* models, adding a lot of technical variability on top of this biological variability is likely to decrease strongly the power of the study. Second, even for *in vitro* models and as shown in the very basic dose-survival curves presented in Figure 5, the reproducibility is excellent when the cells are all alive or all dead. In the middle, and especially in the high slope zone around the EC50, the experimental variability increases dramatically. As most of the toxicoproteomic studies are carried out between EC10 and EC50 in order to visualize an effect, there are also carried out in this zone of high biological variability. In this context, adding a high technical variability to this already high biological variability would just blur, statistically speaking, the effects to be observed.

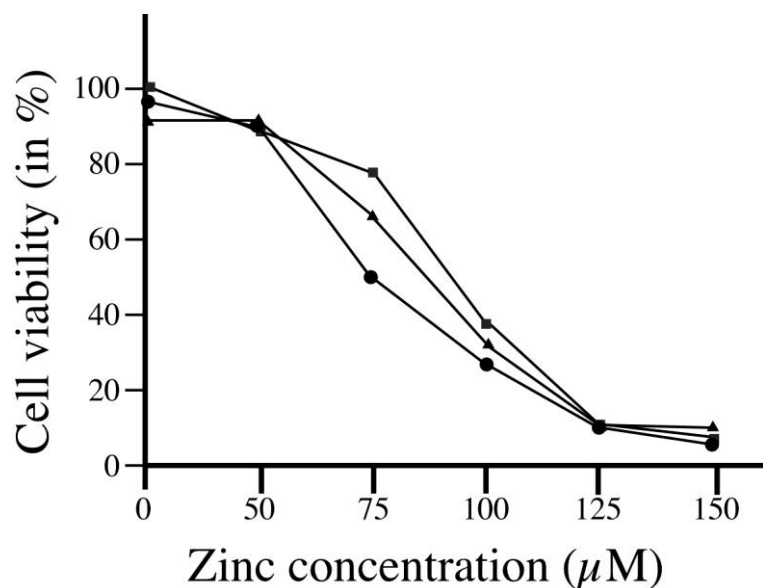


Figure 5: variability of survival curves.

J774 mouse macrophage cells are grown in 12-cell plates and exposed to zinc ion for 24 hours at the given concentration. Their viability after exposure is measured by the neutral red intake test. The same experiment is carried out in triplicate, and the results are displayed together on the same graph. The dispersion of the viability data in the EC20-EC75 range is easily seen, showing that cells exposed to a given concentration in replicate experiments can be in a fairly different physiological state.

However, the reproducibility afforded by 2D gels come at a price, which is the limited depth of analysis of the proteomes. It is therefore not surprising that most of the toxicoproteomic studies published to date converge on shared mechanisms dealing with rather abundant proteins.

4.2. The results hierarchization issue

Apart from this technical point, toxicoproteomic studies, as all proteomic studies, suffer from a major concern that roots in the lack of hierarchization intrinsic to the approach. Taking the example of the interaction of small molecules with their protein targets, classical biochemical studies provide a list of K_d together with the list of proteins, helping to sort which proteins will bind the molecule first. When transposed to a proteomic setup, in this case a chemical proteomic one, all proteins with a low enough K_d to keep the binding throughout the experiment will show up exactly the same way and with no hierarchization at all. This problem is not limited to chemical proteomics. In the case of cellular biology, any cellular response is a mixture of essential responses (essential meaning here required to survive the toxic challenge) and of "fitness" responses (responses increasing the performance of the cells but not mandatory to survive). Proteomic experiments do not bring any information in sorting the essential responses from the fitness ones, while this information is much needed to derive biologically relevant conclusions.

In this respect, it is tempting to sort the proteomic results by the significance index provide by the statistical tests (maximal fold change and or minimal risk value). However, it should be kept in mind that the extent of a change in terms of induction or repression fold is not necessarily correlated with its importance in cell survival processes. For example, making 20% more of an already abundant protein may represent a major effort for a cell and be important for its survival.

There is also a tendency to consider as important what is specific and as unimportant what is frequently encountered in the response to different toxicants. Here again there is no evidence backing this rule, and oppositely a commonly encountered response may well be common exactly because it is useful to increase survival under several circumstances.

4.3. The validation issue

Thus, the only way to differentiate essential responses from non-essential ones is to perform followup, validation experiments that will aim not at confirming the biochemical changes already seen in proteomics, but at determining how the changes observed in the proteomic screen correlate or not, experimentally speaking, with the cell survival or cell damage upon the toxic challenge.

However, in toxicoproteomics as well as in proteomics in general, studies embedding their own functional validation experiments are uncommon, to say the least. Nevertheless, it is quite informative to dissect what concepts and experiments have been used to carry out functional validation.

When an enzyme appears as modulated in a proteomic screen, there are two possible levels of validation. The first one is to measure the intracellular levels of the products of the enzymes, in order to check whether the observed change in the enzyme level correlates with a change in activity. This approach has been carried out in the case of A549 cells treated with benzene and toluene [99]. Several proteins involved in the central metabolism were modulated in response to these aromatic hydrocarbons, and the authors measured the levels of several metabolites to get a better picture of the real metabolic alterations going on. With the development of metabolomic techniques, such approaches should become even easier and greatly informative. In the same trend, enzyme activities can be measured to confirm that changes observed in the proteomic screen do translate in the same changes in the enzyme activity, and are not just attempts of compensation of a toxicant-induced decrease in activity. Such approaches have also been described for metabolic enzymes [91, 167].

When possible, an even more interesting validation, on a toxicological point of view, is to feed the cells challenged with the toxicant with the product of this enzyme. There are a few examples of this approach in toxicoproteomic studies. When HL60 leukemia cells are treated with arsenite, a down regulation of fatty acid synthase is observed [132]. Feeding the arsenite-treated cells with palmitic acid increased cell survival, hereby showing that at least part of the arsenite toxicity was due to the inhibition of fatty acid synthesis.

In the same trend, it has been shown that several central metabolism enzymes are modulated in macrophage cells treated with zinc [167] or in ovary cancer cells treated with a gold complex [36]. Feeding the cells with extra pyruvate, the end product of the glycolysis and pentose phosphate pathways, increases cell survival upon zinc or organogold challenge, respectively.

Maybe one of the most exquisite examples based on activity validation is also one of the oldest toxicoproteomic piece of work. In a series of papers, S. Steiner's group showed first that one of the major effects of cyclosporine A on kidney was to decrease the level of calbindin [10]. They subsequently showed that this decrease was translated into impaired calcium repumping in the kidney, leading to calcium mineralization in the renal tubules [11, 229].

Another interesting path for validation studies is to use pharmacological inhibitors (or, more rarely, stimulators) of the pathways that have been evidenced in the proteomic screen. In some instances, the rationale is to add a pharmacological inhibition on top of the inhibition observed with the toxicant tested, and to check whether this results in a higher mortality. An example of this approach can be found with proteasome inhibition in response to zinc [167].

In other cases, the pharmacological inhibitor is used to counter the cellular response and to check the effects on cell survival. When macrophage cells are treated with copper oxide nanoparticles, both the glutathione biosynthesis and the mitochondrial respiratory chain are up-regulated [161]. Opposing these effects with non toxic concentrations of buthionine sulfoximine (an inhibitor of glutathione biosynthesis) or respiratory chain inhibitors, respectively, enhances the toxicity of the copper oxide nanoparticles. The same holds true with pharmacological inhibition of HSP90 with LiCl in celastrol-treated cells [83], or with 17AAG in arsenic oxide-treated cells [131].

ER stress has been described and put forward in many of the studies cited in this review. However, only two studies used the ER stress inhibitor salubrinal to check whether ER stress was instrumental in cell death/survival upon the toxic challenge or was just a side effect [83, 93]. In both cases, treatment with salubrinal decreased cell death, showing the pivotal role of the ER stress in the toxic process.

Besides the use of pharmacological inhibitors, another way of manipulating the activity of a protein is to change its level through genetic engineering techniques, either by transfection (increase of the level) or more and more frequently by siRNA techniques (decrease of the level). Compared to chemical inhibitors, these techniques are less easy to implement but should work for each and every protein. These techniques have seldom been used in toxicoproteomics, but their success rate is high. In most cases, these approaches target proteins that are upregulated in response to toxicants and that may represent survival factors. For example, the stress protein hsp60 is induced in response to digoxin [86], and an increase of the level of this protein by transfection decreases the toxicity of digoxin. Conversely, Grp78 is induced in response to troglitazone [59], and its forced decrease by siRNA increases the toxicity of troglitazone. The same dynamics holds true for valosin-containing protein in response to chromium [141]. In a more subtle way, silencing of Hsp90 during treatment of macrophages with calcium oxalate interferes with the phagocytic activity of macrophages [137]. There is also one example of a study targeting a protein that is decreased in response to a toxicant, namely the 14-3-3 zeta protein in response to arsenic trioxide [131]. Quite interestingly, further decrease of the protein level by siRNA increases the sensitivity of the cells to the toxicant, while increasing the protein level by transfection decreased the action of the toxicant. This decrease of the 14-3-3 zeta protein when cells are exposed to arsenic trioxide is therefore very likely to be a direct effect of the toxicant, inducing a strong pro-death effect in the cells.

4.4. The specificity issue

In addition to this validation issue, there is another question that percolates the field of toxicoproteomics, which is the question of specificity. This question can be

approached at two levels. The first level is to delineate the specific vs the generic responses in a given system. As shown in this review, the vast majority of the toxicoproteomic papers deal with a simple control vs toxicant-treated situation, so that the issue of specificity just cannot be addressed. Too few studies include "intermediary controls", i.e. toxicants of the same or of another chemical family, which is the only way to address the specificity issue. In the same trend, authors investigating serially a few toxicants in a series of papers usually do not use their previous results to address this issue.

The second level is more a problem of relevance. In other words, can a generic response or a response seeming unrelated to the process under investigation still be relevant? The almost instinctive answer to this question is that when this happens, this means that proteomics unravels only the more visible, generic responses, and that the specific responses are not highlighted by the proteomic screen and remain undescribed. However, this intuitive view is sometimes wrong, as shown by the work on cell chemoresistance to anticancer drugs. For example, old toxicoproteomic work showed that cells resistant to drugs overexpress proteins such as cofilin [79], while there is no obvious relation between resistance to drugs that induce DNA breaks and the overexpression of a cytoskeletal protein. Nevertheless, it was recently shown that simple cofilin overexpression confers resistance to cisplatin [230], of course by a yet completely unknown mechanism, but this validates the relevance of the proteomic work carried out 15 years ago. Besides cofilin, the same holds true for fatty acid binding proteins [79, 231].

To conclude this section, it is a pity that so few proteomic papers in general and toxicoproteomic papers in particular include their own validation studies. Such an absence can be understood from ancient papers, e.g. from a decade ago, when the validation means were not what they are now. For more recent papers, with the analytical tools currently at hand and with the simplicity of the output to measure in the case of toxicoproteomic studies (i.e. cell death/survival), too many proteomic papers stop in the middle of the river, leaving the reader short of solid hypotheses or conclusions and just with speculations.

4.5. What can we think of shared/transversal mechanisms

Examination of the toxicoproteomic outcomes shows that some molecular mechanisms, such as metabolic activation (e.g. with enolase and other enzymes of the glycolytic pathway), ER stress, chaperones induction or mitochondrial stress are found in a high proportion of the toxicoproteomic papers, almost independently of the type of toxicant and thus probably independently of the initial mechanism of action. This phenomenon is reminiscent of the well-known *déjàvu* in proteomics, i.e. proteins which amounts is found to be modulated in many different experimental contexts [97, 232]. It is therefore important to interpret it and figure out whether this is a core phenomenon or just a background effect.

In order to perform this interpretation, we need to figure out what is going on in a cell exposed to a toxic. The toxicant will first distribute within the cell, but not uniformly, depending on its physico-chemical characteristics. For example, cationic species, whether inorganic cations or hydrophobic organic cations, are pumped in the mitochondria because of their transmembrane potential. Then the toxicant will bind to its molecular targets and inhibit them with several possible mechanisms. Because of the various feedback mechanisms present in cells, the cell will react on two initial levels:

(i) it will try to correct the molecular defect. If the target is DNA the cell will try to repair it, if the target is a protein the cell will synthesize some extra protein to restore the function

(ii) it will try to remove the toxicant. This can be made by several mechanisms, such as pumping the molecule out of the cell, conjugating it to a biological acceptor such as glutathione to prevent its binding to the target (so that induction of glutathione transferases is commonly observed), or oxidizing it (e.g. with cytochromes P450 which are almost ubiquitously expressed [233]) here again to prevent it from binding. The point is that nothing comes for free in a cell, so that all of these processes call for extra energy, both redox energy (NADPH for the functioning of cytochromes P450 and of glutathione reductase) and chemical energy (NTPs to produce RNA and proteins). It is therefore no surprise to observe an induction of the energy-producing metabolism. This increased energy production comes at a price, however, which is an increased oxidative stress due to the incomplete reduction of oxygen. This oxidative stress is of course more important if energy production is one of the targets of the toxicant, i.e. a situation where the cell tries to activate a malfunctioning machinery. It is therefore no surprise either to see an induction of antioxidant proteins such as peroxiredoxins.

Secondary to this oxidative stress but also to the structural destabilization that is sometimes induced by the toxicant, more proteins than usual get unfolded, so that a response to this accumulation of unfolded proteins is often induced, detected by an increase in the proteasome pathway and in the chaperones of various types.

Last but not least, why are the ER and mitochondrial stress response so commonly activated? In other words, why are the ER and mitochondria more stressed than other parts of the cells?

For the mitochondria, this is due both to their ability to pump various cationic species that become concentrated within the mitochondrial matrix, and to their sensitivity to oxidative stress if anything goes wrong with the energy metabolism, which is activated.

For the ER, the prevalence of the ER stress may be related to the fact that ER is also the site of localization of cytochromes P450. These molecules do degrade organic toxicants but they may release even more toxic products. In addition, for performing their oxidative chemistry cytochromes P450 activate the molecular oxygen into very reactive transient species. Normally these species are not released but any leak, even small, will create an intense oxidative stress and thus an unfolded protein response in the ER.

Thus, as well as for the classical déjà vu in proteomics [97], the common mechanisms frequently encountered in toxicoproteomic correspond to core stress response mechanisms to exogenous toxicants, and they probably play a central role in the mechanisms that cell activate to survive the toxic stress. It is therefore not surprising that the few studies that have attempted to validate these aspects of cellular responses have succeeded and demonstrated their relevance to cell resistance to toxicants.

5. What future for toxicoproteomics ?

First of all, it should be mentioned that toxicoproteomics follows the rapid development of proteomics in general, and almost two thirds of the papers cited in this review have been published in the last five years. This reflects the fact that proteomics is improving rapidly and has never been as good, fast and easy as now. More importantly, examination of the deliverables of the toxicoproteomic literature makes reasonable to state that proteomics is a sound approach in the field of toxicology, as it does deliver molecular targets and/or molecular mechanisms occurring in cells or tissues treated with toxicants, as shown by the papers presenting an experimental validation of the proteomic findings. This includes (but is not limited to) off target kinases [177], fatty acid synthase [132], glutathione biosynthesis [161] or chaperones [59, 86, 131, 141].

Despite these recent progresses, it can be reasonably stated that proteomics is strongly underused in mechanistic toxicology, although it has the strong advantage over transcriptomics to integrate all the phenomena that occur post transcriptionally and are an integral part of the biological response, e.g. chemical modifications, induced proteolytic degradation, impaired translation etc...

There are multiple reasons to this rather limited use. First, even if becoming easier, proteomics is still perceived as cumbersome and very far from being a high throughput technique in the sense of toxicologists or pharmacologists. It should rather be seen as a high content technique that can deliver a lot of information, but on a limited set of conditions.

Second, even in this specific frame, the question of the real impact of proteomics in toxicology should be raised. In the absence of functional validation, the impact is always limited. When the final output of a toxicoproteomic paper is just a list of proteins, and especially a list of modulated proteins instead of a list of direct targets, the reader is left in a difficult situation. Even when the list is short, it is usually full of proteins that do not show an obvious link with the anticipated action of the toxicant. And even if validation of these findings should be relatively easy, this distance to expectations is not tempting to carry out a validation, which burden should rest on the scientists having initiated the study. When the list of modulated proteins is very large, as afforded by transcriptomic or shotgun proteomic studies, additional pitfalls are encountered. First the lack of hierarchization in the results make it very difficult to decide where the validation effort should go in priority, given the fact that it will be impossible to validate so many protein changes. Second the multiple testing issue increases, and it has been shown that classical correction strategies often remove interesting changes and leave noise [234]. Third, because of the missing value problem and of the difficulties in deriving a protein abundance change from several changes in peptide abundances (e.g. in [227]) the robustness of the data decreases concomitantly with the increase in the number of proteins analyzed. Thus, the probability of having data that look statistically correct but are based on weak proteomic experimental evidences increases, and such data are bound to fail at the validation stage.

All these reasons explain why proteomics has often been perceived as disappointing regarding its impact/investment ratio.

In addition, specificity will remain a problem because of the difficulty of analyzing very large series of samples with proteomic techniques, but the impact depends on the purpose of the study. When the goal is to find biomarkers [235, 236] specificity is an issue, but it can be addressed by careful and ambitious study design, including not

only a control and a single toxicant, but also several chemicals chosen to address the specificity issue (e.g. in [26, 63, 237]). However, specificity is not necessarily a condition for usefulness, and oppositely defining shared targets/reactions can be extremely useful in several areas.

One of these areas is the field of anticancer chemotherapy, where toxicoproteomics can be very useful. At the level of target finding, determining if different drugs have or not the same targets is a very valuable information for designing combined chemotherapies. At the level of cellular reactions, knowing how a cancer cell responds to a drug can give insights to counter this reaction and thus sensitize the cancer cell to the drug or decrease the side effects of the drug. Examples of this strategy are appearing in the literature [66, 67]. As another example, the fact that organogold compounds do induce a proteasome response [36] brings to the front the idea to combine organogold with proteasome inhibitors therapies. In this general frame, the goal of toxicoproteomic studies is to provide enough information to build combined chemotherapies based on molecular informations not only at the level of mode of action, but also at the level of cellular responses. The promise of such informed combined chemotherapies is to decrease dramatically the level of relapses and at the same time to decrease the toxic effects on healthy cells.

This conceptual frame will also be very useful in other areas of toxicology where toxicity is an unwanted event, whether occupational toxicology or drug toxicology. In fact, toxicoproteomics will be very useful to address one of the major challenges in modern toxicology, namely the toxicology of mixtures. Synergistic effects, either additive or through crosstalks, are a major issue, and addressing them requires a good molecular knowledge of the molecular effects of the toxicants, which proteomic can provide. For example, just by using knowledge already at hand, it would be interesting to verify if the completely different toxicants inducing ER stress show an additive toxicity, even if their primary mode of action is different.

This concept can also be used at a finer level. In particular, knowing what a cell induces in response to a toxicant also tells where it becomes more vulnerable, and thus sensitive to other toxicants acting not additively, but through synergic mechanisms (e.g. celastrol and lithium as in [83]).

Thus, toxicoproteomics finds itself at the heart of predictive and mechanistic toxicology at two levels:

(i) by extracting as much information as possible from in vitro systems, it maximizes their usefulness and is part of the general move aiming at limiting the use of animals for toxicological screening (e.g. in [26, 237])

(ii) by performing a screen of targets/responses without preconception, it helps at defining new molecular mechanisms and thus at making predictions about toxicity of combinations of chemicals, which is another sense of the word "predictive toxicology"

Thus, it can be concluded that toxicoproteomics will be an important part of the roots from which the much-needed tree of toxicology, and especially toxicology of mixtures will grow, so that the future of the field should be bright. It will be even brighter if the toxicoproteomists take the good habit of pushing their studies to the end at all levels, as derived from the critical analysis outlined in section 4. On a proteomic technical point of view, it will mean to be very strict with the proteomic setups used. If 2D gels

are preferred, they shall be pushed to their maximum in order to increase the depth of analysis and thus the probability to go beyond the usual players so often described in toxicoproteomics. If shotgun proteomics is preferred, special care should be taken to the statistical quality of the data. The analysis should be restricted to the proteins for which robust quantitative data can be derived, without resorting to imputation strategies to mask the missing values. Such poor data should be discarded without regrets, as there will stay more than enough proteins to be analyzed.

On a more general point of view, no toxicoproteomic paper should appear without an experimental validation of the main findings, and the classical scheme of a binary comparison control vs treated with one toxicant should decrease in favor of more complex comparison including a non-treated sample and samples treated with several different products to address the specificity issue. Fulfilling these conditions will be the only way for proteomics to have a real impact in toxicology.

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