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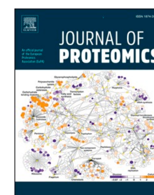

Research article

Systematic analysis of chemical-protein interactions from zebraffsh embryo by proteome-wide thermal shift assay, bridging the gap between molecular interactions and toxicity pathways

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A R T I C L E I N F O

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A B S T R A C T

The molecular interaction between chemicals and proteins often promotes alteration of cellular function. One of the challenges of the toxicology is to predict the impact of exposure to chemicals. Assessing the impact of exposure implies to understand their mechanism of actions starting from identification of specific protein targets of the interaction. Current methods can mainly predict effects of characterized chemicals with knowledge of its targets, and mechanism of actions. Here, we show that proteome-wide thermal shift methods can identify chemical-protein interactions and the protein targets from bioactive chemicals. We analyzed the identified targets from a soluble proteome extracted from zebrafish embryo, that is a model system for toxicology. To evaluate the utility to predict mechanism of actions, we discussed the applicability in four cases: single chemicals, chemical mixtures, novel chemicals, and novel drugs. Our results showed that this methodology could identify the protein targets, discriminate between protein increasing and decreasing in solubility, and offering additional data to complement the map of intertwined mechanism of actions. We anticipate that the proteome integral solubility alteration (PISA) assay, as it is defined here for the unbiased identification of protein targets of chemicals could bridge the gap between molecular interactions and toxicity pathways.

Significance: One of the challenges of the environmental toxicology is to predict the impact of exposure to chemicals on environment and human health. Our phenotype should be explained by our genotype and the environmental exposure. Genomic methodologies can offer a deep analysis of human genome that alone cannot explain our risks of disease. We are starting to understand the key role of exposure to chemicals on our health and risks of disease. Here, we present a proteomic-based method for the identification of soluble proteins interacting with chemicals in zebrafish embryo and discuss the opportunities to complement the map of toxicity pathway perturbations. We anticipate that this PISA assay could bridge the gap between molecular interactions and toxicity pathways.

1. Introduction

We are exposed to individual chemical or mixtures *via* air, water, food, and consumables and it is difficult to evaluate how the exposure would affect to human health. Toxicology aims to predict the impact of exposure to chemicals by understanding the possible mechanism of actions of the bioactive compounds on cells. Scientists, regulatory agencies, and public health authorities have agreed about our partial understanding of possible effects of exposure to chemicals and in particular, complex mixtures. Therefore exploring methodologies with larger capability to analyze chemicals and identify possible targets could provide a comprehensive understanding of the impact of exposure to chemicals, and predicting adverse outcome pathways and health outcomes [1].

Current methods in toxicology have achieved comprehensive knowledge from the main toxicity pathways and detailed structure information its receptor for a few chemical and classes [2]. When the

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Abbreviation

Abbreviation	Definition
PISA	Proteome integral solubility alteration
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
BPA	bisphenol A
TPP	thermal proteome profiling
bTPP	bioactive thermal proteome profiling
REACH	registration, evaluation, authorization and restriction regulation
dpf	days post fertilization
POP	persistent organic pollutant
EDC	endocrine disruptor compound
hpa	13^2-hydroxy-pheophytin a
DMSO	dimethyl sulfoxide
nLC-MS/MS	nano liquid chromatography-tandem mass spectrometry analysis
FASP	Filter aided sample preparation
ACN	acetonitrile
FA	formic acid
FDR	false discovery rate
ER	endoplasmic reticulum
Trap1	TNF receptor-associated protein 1
Calr3b	Calreticulin 3b precursor
Calr	Calreticulin
Mdka	midkine a
S100w	s100 calcium-binding protein w
RhoA-D	Rho-related GTP-binding protein RhoA-D
L1cama	neural adhesion molecule L1.2
Iglon5	zgc:110372
Tnni2a.4	fast muscle troponin I
Hrc	histidine-rich calcium-binding protein
Aldh2.2	aldehyde dehydrogenase 2
Pkmb	pyruvate kinase
Gaphs	glyceraldehyde-3-phosphote dehydrogenase
Npc2	npc intracellular cholesterol transporter 2
Ckmt2a	creatine kinase, mit 2a
Dbi	diazepam-binding inhibitor
Glo1	glyoxalase1
Mat2aa	methionine adenosyltransferase II
Fkbp9	peptidylprolyl isomerase
Anxa2b	annexin
Nap1l1	neuronal system, Nap1l1 protein
Crygm2d14	lens proteins crystallin gamma M2d14
Crygm2d2	crystalline gamma M2d2
Tpma	tropomyosin alpha-1 chain
Rplp1	ribosomal protein large P1
Negr1	kilon
Luc7l	LUC7-like
Cfhl4	complement factor H-like 4
Ubqln4	ubiquitilin 4
Grk7a	rhodopsin kinase grk7a
Mtap	ribosomal protein S27a; S-methyl-5′-thioadenosine phosphorylase
ER	endoplasmic reticulum
Prkcsh	Protein kinase C substrate 80 K-H
Lamb2	laminin beta 2

current knowledge or structural similarity could not lead to any educated guess, individual assays are applied, such measuring the alteration on the membrane stability, mitochondrial activity or increases in free radical species [3]. Targeted assays could only be applied to confirmed mechanism of action, such as measuring the induction of chemical-metabolizing enzymes that can have significant consequences on toxicity [4]. In that direction, collaborative projects like Tox21 have been working since 2008 for the developing and validation of individual cell-based assays to rapid screen for individual chemicals. The Tox21 project has complied over 100 bioassays that are used to screen several thousands of chemicals until now [3]. Unfortunately, there is not a simple solution to evaluate over 100,000 chemicals that are included in consumables, agriculture, manufactures, and industrial activities [3]. At the European Union, the legislation is based on the registration, evaluation, authorization, and restriction of chemicals in the frame of REACH regulation (EC2006) that is directly submitted by the producers. There is a recent agreement that the process of evaluation has serious limitation to cope with the amount of chemicals and diversity of mixtures [5].

More than 2 decades ago the major challenge of toxicology was the safety evaluation of chemical mixtures and determination of the lowest concentration of the individual chemicals of no health concern [6]. The mechanism of action of chemicals mixtures are not easily explained but a review from recent studies found that are relatively rare synergisms [7]. The studies of cocktail of chemicals have been leading by a few chemical classes and well-characterized combinations whereas the information from uncharacterized or more diverse combination of compounds are very scarce [7]. The aim to identify mechanisms underlying the adverse health effects of exposure to chemical mixtures is still pending [8]. Thus, the implementation of the current toxicological approaches with new methods that could rapidly evaluate the cellular response and efficiently predict the risk of exposure to chemicals is very timely [9].

Any strategy to assess the effects of exposure starts by elucidating the mechanism of action of the bioactive compound in a cellular milieu. It involves understanding the molecular interaction between the chemical and the molecular drivers of cellular functions, the proteins. The proteins are shaped to interact with small molecules in the cells to modulate cellular response [10]. Thus, proteins are prompted to establish molecular interaction with chemical compounds that unexpectable enter in the cell. The molecular interaction between chemicals with proteins initiates most of the alteration of cellular functions. Several proteomics-based methods have recently developed with the aiming to scrutinizing any proteome for specific protein-drug interactions that would define the targets of interest, reducing to a small numbers of candidate ligands to evaluate in more details through additional assays [11–14]. Those methods have received the interest of pharma [15,16] as well as medicine to study basic biological processes such as capturing intracellular signaling [17–19].

The first high-throughput assay for drug target deconvolution, named cellular thermal shift assay, was based on the application of the thermal shift principles at a proteome scale coupled with antibody-based identification [20]. However, key implementation that increased the opportunities of the proteome-wide thermal shift assay was the introduction of mass spectrometry for protein identification. The thermal proteome profiling (TPP) method incorporated mass spectrometry-based proteomic analysis by isobaric tandem mass tags increasing the high-throughput and the specificity of drug targets identified. The most common goal with this method is to find protein with alteration in the thermal profile [11]. The application of TPP to novel bioactive compounds, partially hydrophobic, pointed out the importance of a soluble proteome free from microsomes at the thermal shift assay. The hydrophobic compounds, as many chemicals, could easily integrate into the lipidic microsomal vesicles changing the concentration of compounds and proteome under the thermal shift assay. The modification introduced in the bioactive thermal proteome profiling (bTPP) method facilitates its application to chemicals [21]. Chemical compounds, such as endocrine disrupting chemicals, that are known to bind to proteins including receptors are chemical compounds have been considered for

evaluation by thermal proteome profiling approaches in cell lines [22]. Finally, the proteome integral solubility alteration (PISA) method moved one step forward eliminating the sigmoidal fitting of the protein melting curves. In PISA method, the main concept is to measure curve integrals under perturbation and control instead of the curve shape parameters [12].

Aiming to find a high-throughput methodology to tackle this challenge, we evaluated the applicability of the PISA assay to identify the proteins interacting with chemicals from the soluble proteome of zebrafish embryos. Toxicology also requires to circumvent the problem of use laboratory animals, and zebrafish (*Danio rerio*) embryo, a research model (zfin.org), has been considered sensitive enough to provide data to risk assessment [23] and it is considered an alternative to animal testing by the Eu Commission [24]. Here, we presented the application of PISA assay to identify protein targets, that are the molecular links to predict mechanism of actions, and we discussed four cases: i) a single chemical; ii) a simple chemical mixture; iii) a novel chemical from marine biodiscovery, and iv) a novel drug. This study with zebrafish embryo proteome evaluates the opportunities of PISA assay for human and environmental toxicology and remarks some differences from its application for drug target deconvolution.

2. Materials and methods

2.1. Sample preparation

Reagents and medium were purchased from Sigma-Aldrich unless otherwise noted. PBS was purchased from Trevigen. Zebrafish (*Danio rerio*) embryos from strain AB were obtained from zebrafish core facility, Karolinska Institute, Stockholm, Sweden. Embryos were collected 5 days post fertilization (dpf) and stored at −80 °C until protein extraction. The work with zebrafish embryos under 5 dpf can be considered an alternative to animal testing according to the Eu Commission Directive from 20/09/2010 where experiments with earliest life-stages of zebrafish up to 5 dpf are not regulated for animal experimentation [24].

2.2. Selection of different test compounds and concentrations

The bioactive compounds analyzed in this study and the corresponding concentrations were: i) 25 nM 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) a persistent organic pollutant (POP) and endocrine disruptor compound (EDC); ii) a chemical mixture composed by three POPs and EDCs with different mechanisms of action: 25 nM TCDD, 10 μM alpha-endosulfan and 50 μM bisphenol A (BPA); iii) 9.2 μM 13^2-hydroxy-pheophytin a (hpa), isolated from the marine cyanobacterial strain LEGE 07175 and the growing conditions, extraction, isolation, and characterization has been previously reported [25]; iv) 10 μM EG-011 (PCT/EP2018/057678) a new small molecule, with *in vitro* and *in vivo* anti-cancer activity in lymphoma models.

The rationale for the selection of the test compounds was to include chemicals previously studied individually or as mixtures. Therefore, we included compounds and concentrations from previously studied mixture for *in vitro* exposures to human hepatic cell line HepRG. This mixture originally contained TCDD, alpha-endosulfan, and we added BPA to gain in complexity. Moreover, this mixture was selected due to their relevance on human and environmental health, as all of them are POPs and EDCs. Further, TCDD and alpha- endosulfan can be found in the environment as a mixture because dioxins (TCDD) may contaminate chlorinated industrial products, as pesticides (alpha-endosulfan). Concentration selection was based on the translation of external intakes into internal doses in hepatic cells [26]. The hpa is a novel compound from marine biodiscovery recently characterized [25]. The concentration has selected was the EC_{50} based on phenotypic screening and from our previous experiments of thermal proteome profiling with hepatocytes cell lines [21,25]. Finally, EG-011 has been recently developed and it is an early phase of characterization. The concentration utilized was the EC_{50} provided by the developers of the compound.

2.3. PISA assay

Zebrafish embryos were resuspended in a buffer containing 20 mM Tris-HCl and 20 mM NaCl, at pH 8.5, and mechanically homogenized using a TissueLyser (Qiagen) for 3 min at 25 Hz. The cells were lysed by sonication in cycles of 10s/5s for 3 min at 6–10 μm amplitude at 50% intensity from an exponential ultrasonic horn of 3 mm in a Soniprep 150 MSE (MSE Ltd., Lower Sydenham, London, UK). The insoluble parts were sedimented by centrifugation at 100,000*g* for 60 min at 4 °C [21]. Protein concentration was determined by BCA assay [27]. The soluble proteome was used to perform the PISA assay, as described in Gaetani et al. [12] with some modifications. Briefly, the soluble proteome and the studied chemical were incubated for 10 min at 25 °C. The incubation was performed with the following compounds at the corresponding concentrations: i) 25 nM TCDD, ii) 25 nM TCDD, 10 μM alpha-endosulfan, 50 μM BPA, iii) 9.2 μM hpa, and iv) 10 μM EG-011. The control samples were incubated in the presence of the vehicle, dimethyl sulfoxide (DMSO), utilized for the solubilization of the compounds. Fifteen specific temperatures were selected for the thermal assay: 37, 40, 43, 46, 48, 50, 51, 52, 53, 54, 56, 58, 61, 64 and 67 °C. These temperatures were selected to ensure that at least 90% of the studied proteins have their melting point within this range [11]. Aliquots containing 25 μg of protein (one for each of the temperatures in the entire range covered in the thermal shift assay) were independently heated at the corresponding temperature for 3 min, followed by 3 min at room temperature. The samples were centrifuged at 100,000*g* for 20 min at 4 °C, to remove the proteins that had altered in solubility after the thermal shift assay [11]. The collection of soluble fractions in the supernatants were processed using a general bottom-up proteomics workflow and the purified peptides were analyzed by label-free nano liquid chromatography-tandem mass spectrometry analysis (nLC-MS/MS). Three biological replicates were performed for each experiment [28].

2.4. Filter aided sample preparation (FASP)

The samples were digested following the FASP method. First, the protein samples corresponding to the supernatants after centrifugation were prepared with SDT buffer (2% SDS, 100 mM Tris-HCl, pH 7.6 and 100 mM DTT), according to Wiśniewski et al. (2012) [29]. To perform FASP, the samples were diluted with 200 μl of 8 M urea in 0.1 M Tris/HCl, pH 8.5 (UA) in 30 kDa microcon centrifugal filter units. The filter units were centrifuged at 14,000 *g* for 15 min at 20 °C. The concentrated samples were diluted with 200 μl of UA and centrifuged at 14,000 *g* for 15 min at 20 °C. After discharging the flow-through 100 μl of 0.05 M iodoacetamide was added to the filter units, mixed for 1 min at 600 rpm on a thermo-mixer, and incubated static for 20 min in dark. The solution was drained by spinning the filter units at 14,000 *g* for 10 min. The filter units were washed three times with 100 μl buffer UA and centrifuged at 14,000 *g* for 15 min. The filter units were washed three times with 100 μl of 50 mM ammonium bicarbonate. Endopeptidase trypsin solution in the ratio 1:100 was prepared with 50 mM ammonium bicarbonate, dispensed, and mixed at 600 rpm in the thermomixer for 1 min. These units were then incubated in a wet chamber at 37 °C for about 16 h to achieve effective trypsinization. After 16 h of incubation, the filter units were transferred into new collection tubes. To recover the digested peptides, the tubes were centrifuged at 14,000 *g* for 10 min. Peptide recovery was completed by rinsing the filters with 50 μl of 0.5 M NaCl and collected by centrifugation. The samples were acidified with 10% formic acid (FA) to achieve pH between 3 and 2. The desalting process was performed by reverse phase chromatography in C18 top tips using acetonitrile (ACN; 60% v/v) with FA (0.1% v/v) for elution, and vacuum dried to be stored at −80 °C till further analysis.

2.5. Nano LC-MS/MS analysis

The desalted peptides were reconstituted with 0.1% FA in ultra-pure milli-Q water and the concentration was measured using a Nanodrop (Thermo Scientific). Peptides were analyzed in a QExactive quadrupole-orbitrap mass spectrometer (Thermo Scientific). Samples were separated using an EASY nLC 1200 system (Thermo Scientific) and tryptic peptides were injected into a pre-column (Acclaim PepMap 100 Å, 75 um × 2 cm) and peptide separation was performed using an EASY-Spray C18 reversed-phase nano LC column (PepMap RSLC C18, 2 um, 100 Å, 75 um × 25 cm). A linear gradient of 6 to 40% buffer B (0.1% FA in ACN) against buffer A (0.1% FA in water) during 78 min and 100% buffer B against buffer A till 100 min, was carried out with a constant flow rate of 300 nl/min. Full scan MS spectra were recorded in the positive mode electrospray ionization with an ion spray voltage power frequency (pf) of 1.9 kV (kV), a radio frequency lens voltage of 60 and a capillary temperature of 275 °C, at a resolution of 30,000 and top 15 intense ions were selected for MS/MS under an isolation width of 1.2 m/z units. The MS/MS scans with higher energy collision dissociation fragmentation at normalized collision energy of 27% to fragment the ions in the collision induced dissociation mode.

2.6. Peptide and protein identification and quantification

Proteome Discoverer (v2.1, Thermo Fischer Scientific) was used for protein identification and quantification. The MS/MS spectra (. raw files) were searched by Sequest HT against the Zebrafish database from Uniprot (UP000000437; 46,847 entries). A maximum of 2 tryptic cleavages were allowed, the precursor and fragment mass tolerance were 10 ppm and 0.6 Da, respectively. Peptides with a false discovery rate (FDR) of less than 0.01 and validation based on q-value were used as identified. The minimum peptide length considered was 6 and the FDR was set to 0.1. Proteins were quantified using the average of top three peptide MS1-areas, yielding raw protein abundances. Common contaminants like human keratin and bovine trypsin were also included in the database during the searches for minimizing false identifications. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE [30] partner repository with the dataset identifier PXD021670.

2.7. Analysis of PISA assay

According to the original publication of this method [12], PISA assay measures the protein abundance (Sm) of each pooled sample instead extracting the relative melting temperatures Tm by curve fitting, as in TPP. Therefore, the protein abundance represents the integral of the area under its melting curve within the used temperature interval. If Sm is the value for the control sample and Sm' is the corresponding value for the exposed sample, then the PISA analogue of ΔTm is ΔSm = Sm'-Sm [12]. To obtain ΔSm, we normalized the quantitative values for each protein on the average value for the control sample. Two-tailed Student's *t*-test (with equal or unequal variance depending on F-test) was applied to calculate *p*-values. Proteins with p-values < 0.05 were considered as protein targets. The data was represented in a volcano plot combining the calculated ΔSm and *p*-values, that facilitated to visualize the protein targets [12].

2.8. Pathway analysis

Pathway analysis was performed by protein interaction enrichment using STRING 11.0 [31]. Protein sequences were BLAST searched against the RefSeq human databases, and BLAST results were analyzed in a map of functional pathways.

3. Results

3.1. Implementation of PISA assay to analyze the soluble proteome from zebrafish embryo

The zebrafish embryo at the stage 5 dpf is broadly utilized in toxicology to assess the effects of drugs and substances and to understand mechanisms of toxicity poorly understood [24,32]. Here, the soluble proteome from zebrafish embryos was analyzed by PISA assay to identify proteins interacting with chemicals. The solubilization conditions were selected to maintain during the thermal shift assay two parameters invariable: proteome, and the concentration of chemicals. This is an important consideration for this methodology that aim to identify the protein-chemical interactions based on the alteration on protein solubility.

Firstly, several buffers were tested and a buffer contained 20 mM Tris-HCl and 20 mM NaCl, at pH 8.5 was selected. The proteome in this solution rendered the highest efficiency and reproducibility in the protein extraction, facilitated proteins in their native structure in solution, the stability of the proteome through the thermal assay [21]. This buffer yield between 2 and 3 μg protein/embryo after disrupting the tissues by mechanical press utilizing a homogenizer or a grinder with abrasive glass beads. The cell lysed with ultrasonic horn on top of the initial shearing process increased the extraction from 3 to 5.3 μg/embryo and this was the selected method for the study (Table 1). Secondary, the hydrophobic compounds, such as many chemical compounds, will tend to incorporate into the lipids from microsomes reducing the active concentration of compound in solution [33]. Therefore, the soluble proteome was obtained after sedimentation of the microsomal vesicles with the classical fractionation step at 100,000 *g* for 60 min at 4 °C [34,35] (Fig. 1).

3.2. Systematic analysis of chemical-protein interactions in the soluble proteome from zebrafish embryos

We evaluated the PISA assay to identify the proteins interacting with individual chemicals using TCDD as test compound and the zebrafish embryo soluble proteome. This compound is classified as persistent environmental pollutant, and as EDC. This chemical offers a well-known mechanism of toxicity based on the initial interaction to aryl hydrocarbon receptor. This protein could not be detected in the proteome that was analyzed in this study. We remind that the focus of this study is the unbiased identification of any interaction between the chemicals and all the proteins in the studied proteome. We used three biological replicates per condition, including the chemical perturbation with TCDD at 25 nM, and the corresponding control with a vehicle.

The soluble proteome analyzed yielded 2422 proteins identified and quantified across the replicates, and 840 proteins quantified in all three replicates were included in the PISA analysis. The analysis of the TCDD-protein interactions identified 9 protein targets. It has been evaluated the targets increasing and decreasing in solubility named as stabilized or destabilized proteins (Table S1). The protein targets can be depicted from the volcano plot (Fig. 2a). Six out them were destabilized, being Zgc:162944, TNF receptor-associated protein 1 (Trap1), Calreticulin 3b precursor (Calr3b), and Calreticulin (Calr), the ones with the highest significance negative difference in ΔSm compared to control. The remaining, midkine a (Mdka), s100 calcium-binding protein w (S100w) were destabilized and Rho-related GTP-binding protein RhoA-D (RhoA-D), neural adhesion molecule L1.2 (L1cama) and zgc:110372 (Iglon5) were stabilized by TCDD in the PISA assay. The analysis of the functional protein association of the targets by STRING identified 3 interactions with confidence score higher than 0.5 and 3 interactions with low confidence (score < 0.3). Trap 1, Calr, and Calr3b (Calrl) interactions corresponded to the ones with the highest confidence (Fig. 2b). After analyzing the network pathways connected with the identified targets, and the pathways connectivity, we can predict that TCDD-proteins

Table 1
Cell disruption techniques and factors evaluated to extract protein under native conditions from 5dpf zebrafish embryos.

Solid shear	Liquid shear	Buffer	Volume (μL)	Final volume (μL)	Protein concentration (μg/μL)	Total protein (μg)	Yield (μg /embryo)
Mechanical press homogenizer	No	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	60	41.5 ± 3.5	2.2 ± 0.5	90.2 ± 11.0	1.8 ± 0.2
Grinding with abrasives (10 beads - 0.1 mm)	No	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	100	79.0 ± 0	0.8 ± 0	63.8 ± 3.9	1.3 ± 0
Grinding with abrasives (10 beads - 0.1 mm)	No	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	125	94.0 ± 0	1.0 ± 0.1	98.6 ± 4.9	2.0 ± 0.1
Grinder (16 beads - 0.1 mm)	No	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	150	141.5 ± 0.7	1.2 ± 0	172.6 ± 5.9	3.5 ± 0.1
Grinding with abrasives (16 beads - 0.1 mm)	No	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	200	166.5 ± 3.5	0.9 ± 0	150.7 ± 9.2	3.0 ± 0.2
Grinding with abrasives (16 beads - 0.1 mm)	No	10 mM sodium phosphate, 145 mM NaCl, pH = 7.4 (PBS)	150	141.0 ± 1.4	0.5 ± 0.2	69.8 ± 23.4	1.4 ± 0.5
Grinding with abrasives (16 beads - 0.1 mm)	Sonication	20 mM Tris-HCl, 20 mM NaCl, pH = 8.5	150	139.5 ± 0.7	1.9 ± 0.1	265.0 ± 13.8	5.3 ± 0.3

All the trials were performed with 50 embryos. Instruments utilized: grinder type TissueLyser, 25 Hz, during 3 min; homogenizer type MiniPotter, 1 min strokes on bath ice; and sonicator type, ultrasonic horn of 3 mm, amplitude 6–10 μm.

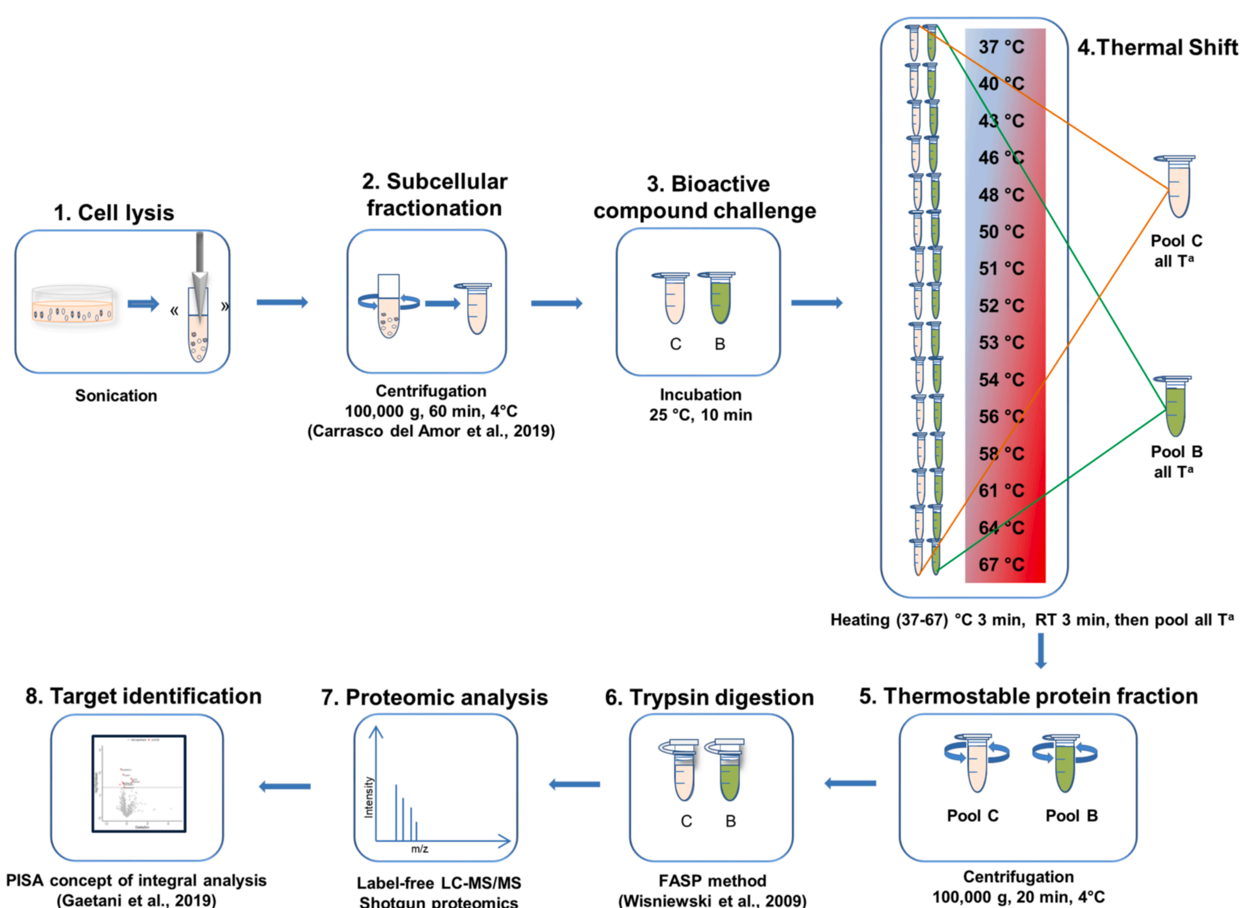

Fig. 1. Workflow of PISA method including modifications in the definition of the soluble fraction. The soluble proteome analyzed by the thermal shift assay in this study was obtained after sedimentation of microsomal vesicles by centrifugation at 100,000 *g* for 60 min as previous described [21].

binding events can interfere developmental processes on zebrafish embryos, including protein folding, cell adhesion, axonal growth, and neural tube formation (Fig. 2c, Table S1).

3.3. Systematic analysis of chemical mixture-protein interactions in the soluble proteome from zebraffsh embryos

After identifying the proteins interacting with a single chemical, we evaluated the applicability of the PISA method to identify the proteins interacting with a chemical mixture composed by TCDD, alpha-endosulfan, and BPA.

The soluble proteome analyzed yielded 2364 proteins identified and quantified across the biological replicates, and 768 proteins quantified in all three replicates were analyzed in the PISA assay. In total, 44 protein targets were identified corresponding to 42 proteins that decreased in solubility, and 2 proteins that increased in solubility (Fig. 3a). The proteins with the highest significance negative difference in ΔSm compared to control were fast muscle troponin I (Tnni2a.4) and

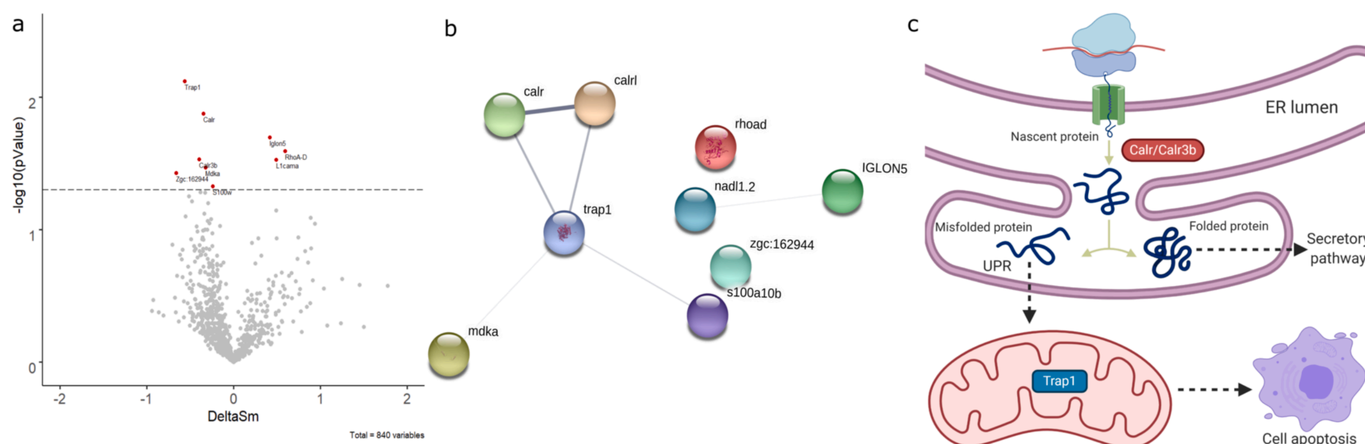

Fig. 2. Protein target identified by PISA method for TCDD and soluble proteome from zebrafish embryos. The concentration in the assay was 25 nM TCDD. Sections of the figure: (a) volcano plot with identified targets shown in red and labeled; (b) string analyses of the protein targets, 3 interactions with medium confidence (0.4 < score < 0.7) and 3 interactions with low confidence (0.15 < score < 0.4); and (c) main protein targets and predicted mechanisms of action (Pathway representation were created by Biorender.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

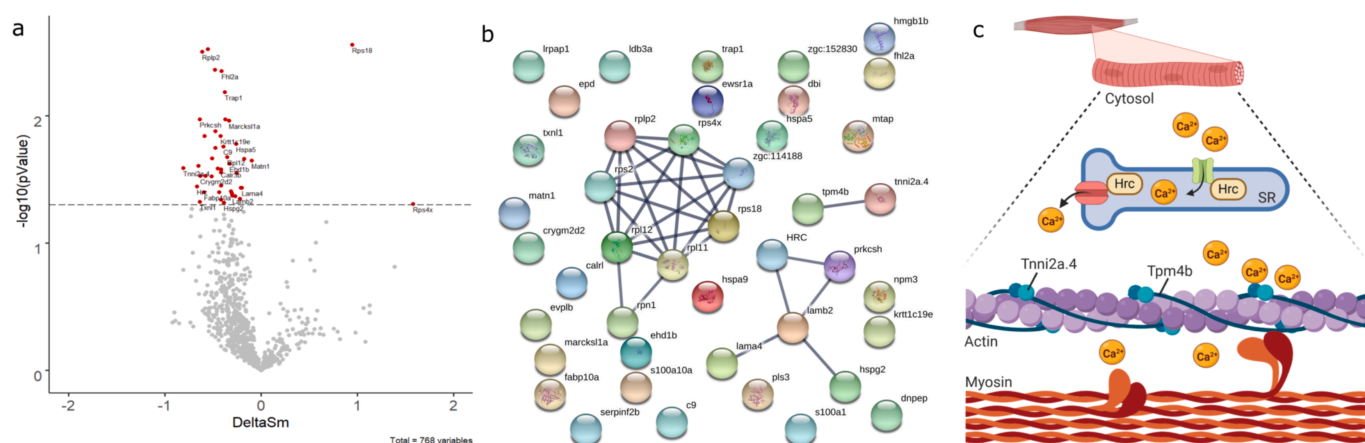

Fig. 3. Protein targets identified by PISA method for a chemical mixture composed by TCDD, alpha-endosulfan, and BPA and the soluble proteome from zebrafish embryos. The concentrations in the assay were 25 nM TCDD, 10 μM alpha-endosulfan and 50 μM BPA. Sections of the figure: (a) volcano plot with identified targets shown in red and labeled; (b) string analyses of the protein targets, 3 groups of interactions with highest confident (score > 0.9); and (c) main protein targets and predicted mechanisms of action (Pathway representation were created by Biorender.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

histidine-rich calcium-binding protein (Hrc). Three groups of protein interactions with highest confidence (score > 0.9) were identified after STRING analysis of protein targets and 2 groups corresponded to interactions with Tnni2a.4 and Hrc. Other targets protein kinase C substrate 80 K-H (Prkcsh) and laminin beta 2 (Lamb2) could also predict altering pathways (Fig. 3b, Table S2). There was another group of targets also identified in the only TCDD case such as Trap1 and Calr3b. In addition, some ribosomal proteins are also expected to find among the targets as those proteins expressed not identically but in all the tissues [36], and are directly involved in tissue biogenesis in the case of an organism in developmental stages [37]. Altogether, the protein targets identified can be used to predict the interference of the chemical mixture on several biological pathways specially muscle contraction, protein metabolism and turnover, and cell adaptation to stress (Fig. 3c).

3.4. Systematic analysis of novel compound-protein interactions in the soluble proteome from zebraffsh embryos

Chemical compounds extracted from marine biomass are thoroughly examined aiming to explore its beneficial or harmful effects. We tested hpa, a partially characterized compound extracted from a marine cyanobacterial strain, that was selected for its potential interests as nutraceutical after phenotypical screenings, and *in vitro* targeted assays [25]. We applied the PISA assay as described for previous chemicals. We identified 2318 proteins and the 790 proteins presented in all the biological replicates were included in the analysis. From the 40 protein targets identified, 37 of them corresponded to proteins decreasing in solubility after the thermal assay, and just 3 showed an increase in solubility (Fig. 4a).

Several identified proteins were involved in metabolic pathways such as aldehyde dehydrogenase 2 (Aldh2.2), pyruvate kinase (Pkmb), glyceraldehyde-3-phosphote dehydrogenase (Gaphs), intracellular cholesterol transporter 2 (Npc2), creatine kinase, mit 2a (Ckmt2a), diazepam-binding inhibitor (Dbi), glyoxalase1 (Glo1), methionine adenosyltransferase II (Mat2aa), peptidylprolyl isomerase (Fkbp9). Among the protein targets that showed high values of ΔSm, structural proteins such as fast muscle troponins I (Tnni2a.4), and annexin (Anxa2b) were included. A group of targets were involved in organ-related functions including neuronal system, Nap1l1 protein (Nap1l1), lens proteins crystallin gamma M2d14 (Crygm2d14), and crystalline gamma M2d2

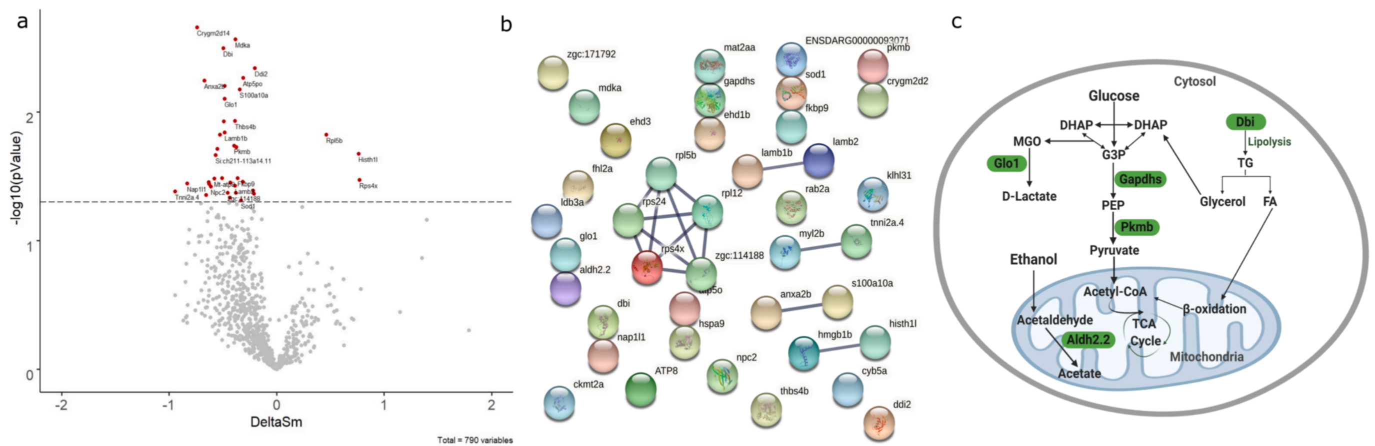

Fig. 4. Protein targets identified by PISA method for hpa and the soluble proteome from zebrafish embryos. The concentration in the assay was 9.2 μM hpa. Sections of the figure: (a) volcano plot with identified targets shown in red and labeled; (b) string analyses of the protein targets, 15 interactions with the highest confidence (score > 0.9), and 23 interaction with high confidence (0.7 < score < 0.9): and (c) main protein targets and predicted mechanisms of action (Pathway representation were created by Biorender.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Crygm2d2). In addition, several of the ubiquitous and high abundant proteins such as those related to translation are also among the identified targets (ribosomal family, Rps4x, Rpl5b, Rps24, zgc:114188, and Rpl12; Fig. 4b, Table S3). In summary, the protein targets involved in metabolic pathways could be connected to the preliminary observation from phenotyping screening of the compounds [25] (Fig. 4c).

3.5. Systematic analysis of novel drug-protein interactions in the soluble proteome from zebraffsh embryos

In the case of novel drug under development, the target identification is usually performed in a specific cell type and by target engagement assays or more recently by thermal and solubility proteome profiling methods. However, next step is discharging any unspecific binding to the non-target tissues that could be performed on a battery of cell lines or *in vivo* animal models. We suggested here to apply the proteome from zebrafish embryo and the PISA assay to scrutinize if there are additional protein interactions at non-target tissues. We evaluated this question with a cancer drug under development named EG-011 that aims to offer anti-cancer activity in lymphoma models.

The soluble proteome identified and quantified 2360 proteins across the replicates, and 861 proteins were quantified in all three replicates were included in the data analysis. In total, 10 protein targets were identified: 5 stabilized, and 5 destabilized proteins (Fig. 5a). The

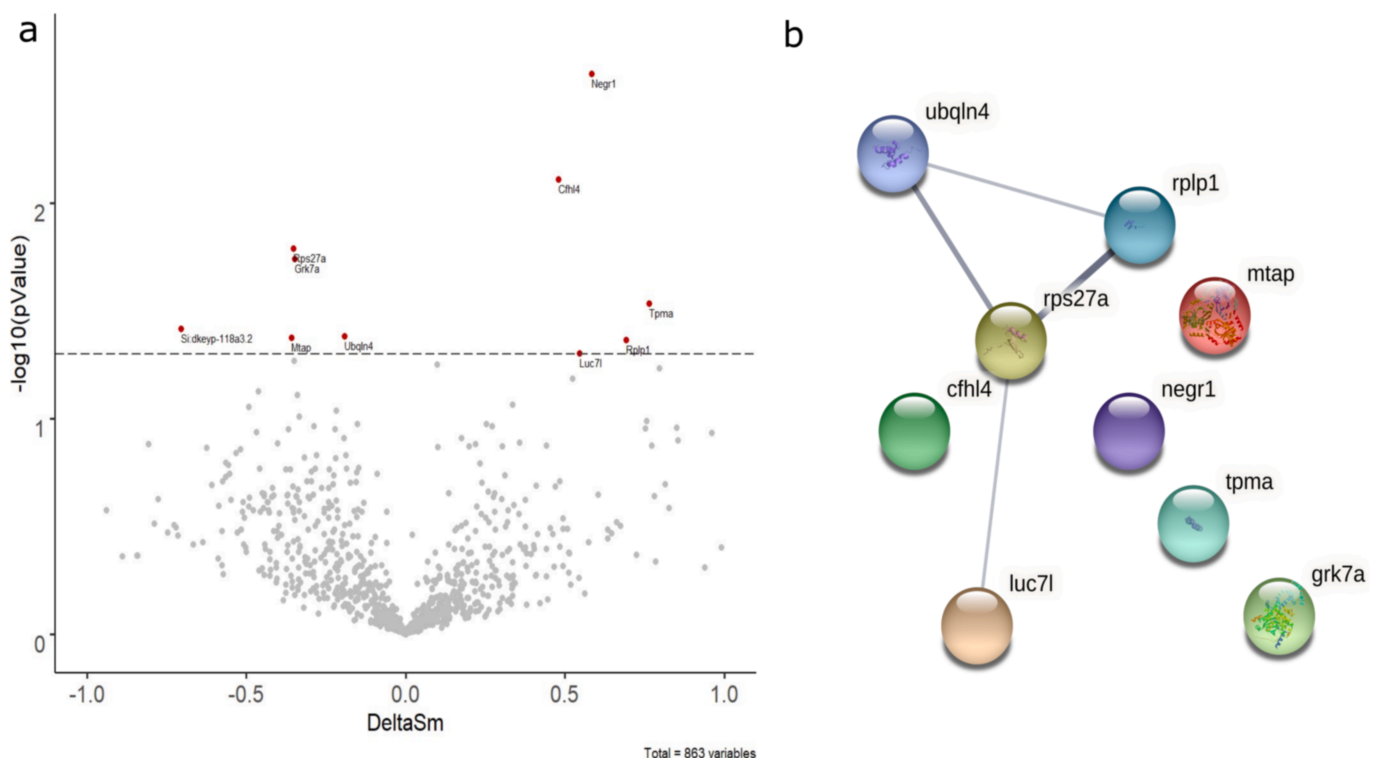

Fig. 5. Protein targets identified by PISA method for EG-011 and the soluble proteome from zebrafish embryos. The concentration in the assay was 10 μM EG-011. Sections of the figure: (a) volcano plot with identified targets shown in red and labeled; and (b) string analyses of the protein targets, 5 interactions with low confidence (0.15 < score < 0.4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stabilized proteins were tropomyosin alpha-1 chain (Tpma), ribosomal protein large P1 (Rplp1), kilon (Negr1), and LUC7-like (Luc7l) and complement factor H-like 4 (Cfhl4) and the destabilized ones: ubiquitilin 4 (Ubqln4), rhodopsin kinase grk7a (Grk7a), ribosomal protein S27a, S-methyl-5′-thioadenosine phosphorylase (Mtap) and a multispanning integral membrane protein without assigned function (Table S4). The targets were used to build a map of functional pathways only depicted a node around Rplp1, that is connected to Ubqln4, Rplp1, Luc7l covering functions around transcription, translation, and targeting protein to degradation (Fig. 5b).

4. Discussion

We discuss a few considerations for the applicability of PISA assay for the systematic identification of chemical-protein interactions from a zebrafish embryo proteome. The solutions most frequently used to extract proteins from those larvae contain detergents and chaotropic agents that could extract proteins at high yield but are not compatible with the principles of the thermal shift assay [38,39]. First, it is expected that the detergent micelles will change in size and shape in the thermal shift assay as the temperature increases over the detergent cloud temperature [40,41]. The temperature-dependent micellar transition is dominated by the properties of the head groups of the detergents, and specially, in the case of non-ionic detergents, the most frequently used in this context. This transition could lead to the removal of water molecules associated with the polar head groups at the interface, breaking intermolecular hydrogen bonds, and reducing the micelle curvature to planar, that cause a growth of the micelles, forming long cylindrical micelles [41]. Second, chaotropic agents such as urea can cause protein carbamylation above 37 °C, which makes its use incompatible with a thermal shift assay that should increase to 67 °C [42]. Therefore, we defined the conditions to solubilize a zebrafish embryo proteome compatible with a thermal shift assay.

The purpose of this study was to define a thermal shift assay for toxicology that should capture the thermal alteration of protein stability and the perturbation exclusively introduced by the chemical-protein interaction. Thus, the introduction of any temperature-sensitive variable altering the concentration of chemical, or the intrinsic properties of the proteome would reduce the value of the method for the unbiased identification of chemical-protein targets. We observed that the soluble proteome described in TPP, or PISA methods contains microsomal vesicle in solution [11,12]. It is well-known that hydrophobic compounds, such as many chemical compounds, are prompted to incorporate into the microsomal lipid vesicles. If the study of protein-chemical interaction is performed in a soluble proteome containing microsomes, the available chemical concentration in solution could decrease as the compound is embedded in the microsomal vesicles [33]. Moreover, thermal-shift assay is a scenario that mimics the temperature-induced membrane phase transition and fission of vesicles [43]. The thermal modifications in lipid fluidity and fission of the vesicles can change the sedimentation coefficient of the microsomes. Thus, the presence of lipidic vesicles in the studied proteome introduces a variable that contributes to alteration of protein solubility, and it is not only based on chemical-protein interaction [21]. Therefore, we have eliminated the vesicles from our studied proteome by introducing a centrifugation step that sediment the microsomal membranes before the thermal shift assay. This protocol gained in specificity and sensitivity to study hydrophobic compounds [34].

The proteins that increased in solubility are the objective in experiments for target engagement in drug discovery [20]. In our study with chemicals, we identified both stabilized and destabilized proteins. It has been studied that the increase of protein thermostability is also connected an enhanced rigidity, reduction of the flexible parts of the structure, facilitating proteins to stay in different conformational states, and reducing unfolded and precipitation [44]. As conformational flexibility is an essential factor for the dynamic of the molecular events, we hypothesized that the interactions that stabilized proteins could reduce the protein function [45]. Therefore, the stabilized and destabilized proteins interacting with chemicals could predict interference connected to partial disfunction of functional pathways that is the oppositive to identification of drug targets with an increase in solubility that is usually associated to activation of target function [12].

In our first study, we analyze the identified proteins interacting with one chemical in the zebrafish embryo soluble proteome. Three of those proteins, Trap1, Calr3b and Calr are chaperones involved in protein quality control pathways and are ubiquity distributed among tissues and organs. The function of Trap1, heat shock protein with ATPase activity, has been initially associated only to the mitochondrial transition pore and its integrity regulation. However, the Trap1 functional network has been enlarged by the discovery of the multiple subcellular localization. At the endoplasmic reticulum (ER) associated, the Trap1 offers a quality control mechanism related to proteasome subunit (TBP7) for proteins with mitochondrial localization [46]. This antiapoptotic activity should reduce the proteotoxic stress generated by accumulation of misfolded proteins [47]. On the other hand, Calr, and Calr3b are calcium-binding chaperones involved in protein folding through the glycoprotein-specific quality control machinery and in growth control [48–51]. In absence of calreticulin, misfolded proteins could accumulate driving ER stress. When this stress is severe and irreparable the unfolded protein response turns to a pro-death response through mitochondrial involvement, leading apoptosis, where Trap1 could be a potential regulator [47,50,52]. We hypothesize that those chemical-protein interactions could contribute to alteration of mitochondrial integrity and apoptosis [46,53], and compromise ER functionality decreasing protein folding efficiency and Ca2+ homeostasis [54,55]. The method also identified Mdka and L1cama that are tissue specific proteins with higher expression in spinal cord, brain, adrenal glands. The Mdka protein in zebrafish is a heparin-binding growth factor that initiates signaling pathways that promote repair, proliferation, and regeneration. Mdka plays a key role in the initial stages of zebrafish neurogenesis [56] and is implicated in organs development such as heart and retina [57]. L1cama is essential in neural migration, axonal growth and maturation of brain and neural structures [58]. Those tissue specific mechanisms that could be partially impaired by the interaction are related to the protein quality control pathways. This is in concordance with TCDD toxic effects described for zebrafish embryos, like embryonic brain development capacity reduction and craniofacial malformations [59], blood flow reduction and apoptosis incrementation in the midbrain [60,61], and heart malformation [62–64]. The elucidation of these mechanisms has begun by observations in developmental processes where pathways for midbrain circulation failure, circulatory failure and jaw malformation were suggested [65]. Those phenotypic outcomes previously described are compatible with the contribution from alteration of the functional pathways connected with the identified targets.

In this second study with a chemical mixture, Tnni2a and Tpm4b are proteins targets identified that regulate muscle contraction *via* calcium binding, and the attachment of actin to myosin filaments facilitating muscle contraction of cardiac and skeletal muscle. The mutations on Tnni2a.4 in zebrafish embryos showed myofibril degradation, inability to swim, and failure to inflate the swim bladder [66]. Studies utilizing zebrafish embryos exposed to a different type of POP mixtures also detected by transcriptomic analysis altered level of troponin [67]. Another group of targets, Hrc, Prkcsh, and Lamb2 can also be linked with muscle contraction at another level layer of the tissular mechanism. Hrc may play a role in the regulation of Ca + 2 storage in the sarcoplasmic reticulum of skeletal and cardiac muscle, in humans. As a result, a deficiency in Hrc provokes heart failure [68]. This PISA analysis also identified common targets, such as Trap1 and Calr3b, of TCDD as single chemical and as targets of the mixture also containing TCDD. However, it is also expected that the global impact of the same targets in mixtures could differ from the single chemical case. Considering that chemicals in a mixture could interact both with the other chemicals and proteins and

may present synergistic or antagonistic interactions [1,69].

In the third study, we have selected a new compound from marine biodiscovery with only partial chemical and biological characterization. The priority for those compounds is to reduce the length and complexity of applying numerous individual assays of get sufficient data to predict if any beneficial or harmful effects should be expected. For hpa, there was only initial phenotypic screenings that indicated possible reduction in lipid content in zebrafish embryo [25]. Several of the identified proteins were related to metabolic pathways, Gapdh and Pkmb from the glycolytic pathway could interfere in the main cellular energy conversion pathway and compromise cellular growth and survival. The target Aldh2.2 is key enzyme in ethanol degradation pathway with functional similarities to other ALDH enzymes previously identified as hpa target with bTPP using hepatic cell line HepG2 [21]. In human, ALDH2 is a mitochondrial enzyme that not only metabolizes aldehyde into acetic acid but also is involved on detoxification of toxic aldehydes. The increase of toxic aldehydes has been correlated with obesity and insulin resistance [70]. ALDH2 deficiency aggravate liver injury and has a crucial role in the pathogenesis of liver fibrosis and has been postulated for pharmacological strategies against alcohol liver disorders [71]. On the other hand, alterations in Glo1, another identified target, could reduce the detoxification of methylglyoxal and reactive aldehydes from the metabolism. The methylglyoxal mainly derives from oxidation of glycolytic intermediates dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate. It is a concern the defective methylglyoxal detoxification under high nutrition intake is an indicator of susceptibility to develop type 2 diabetes. In zebrafish, the knockout of Glo1 induces impaired glucose tolerance but does not cause vascular organ alterations [72]. The reduction in stability of Dbi, zebrafish target with similarity to human acyl-CoA binding protein (ACBP) also suggested interaction that would promote functional impairment. A role of appetite regulator factor from ACBP has been recently found to be conserved through evolution. In human and rodent, it is a key player in the hyperphagia that could be associated to obesity. The attenuation of ACBP is correlated in studies *in vivo* with increase of lipolysis and reduce food intake and therefore this target is a suitable candidate for intervention in lipid related metabolic alterations [73]. In summary, protein targets involved in metabolic pathways could be connect with the phenotype from the biodiscovery screening associated with the regulation of lipid metabolism and could be related with obesity and obesity-related comorbidities.

The last study aims to offer a rapid evaluation of possible the side-effects of a novel chemical in non-target tissues that is another challenge of toxicology. We evaluate a new drug in developmental phase with promising anti-cancer activity named as EG-011. The 5 identified proteins were ubiquitous in the organism and related to protein turnover, synthesis and degradation that are abundant proteins of the developmental stage of the zebrafish embryo. The specific drug targets that are under study both, *in vitro* and *in vivo* in lymphoma models were not any of the identified proteins in zebrafish proteome. Safety pharmacology and quality control of new drugs are another pillar of regulatory toxicology. To assess the quality of any novel drugs it is required the assessment of any unexpected or harmful effects in the non-target organs and this application could simplify those studies at the early stage of drug development.

We have discussed the results of this methodology for our cases in the context of their specific aims. However, the overarching goal for toxicology is providing a comprehensive evaluation of a proteome for possible targets of chemicals, providing knowledge that could bridge the gap between molecular interactions and toxicity pathways. It is important to remark that most of the proteins scrutinized in this context with PISA would have never been studied as target with a single protein assay, enhancing this methodology the chances to unravel novel information. In spite of that, the validation of solid knowledge is not the primary goals of this methodology for toxicology. *A priori*, applying high-throughput methods is apparently not the most efficient strategy for validation of well-known targets and mechanism of actions. We have previously mentioned case of aryl hydrocarbon receptor for TCDD as a well-known target that is not into the studied proteome and therefore would not be an identified target with this methodology [74]. Finally, when newly scrutinized targets are obtained, it is recommendable to validate by an alternative, and ideally orthogonal, methodology. This should be the directions to explore for the results obtained by this method. Unfortunately, the validation strategies are not free from drawbacks. It not easy validate proteins that are not frequently required for functional assays or interaction assays. Orthogonal validation has been presented for protein targets from drugs analyzed by thermal proteome profiling methods. In that study the novel targets identified by isothermal shift assay, only protein kinases, could directly validate by a kinase affinity enrichment assay [75]. Any orthogonal validation should also evaluate the interaction in the proteome wide context, where complex milieu is ruling affinities for binding. In drug discovery work, purified proteins have been validated by differential scanning fluorimetry that could also assay chemical unfolding but it has recently described as prone to false positives and negatives [76].

5. Conclusions

We showed that the systematic analysis of chemical-protein interactions by PISA could identify the molecular interactions, that could cover gaps to define the interference on cellular functions, and aid in predicting the biological consequence in zebrafish embryo. This analysis should incorporate both the protein increasing and decreasing in solubility as all contribute to offer an overview of the interference. This application of a high-throughput thermal shift assay could contribute to address several challenges of toxicology including to predict the impact of exposure to individual chemical, mixtures of chemical, new chemicals from biodiscovery, or side-effects of newly developed chemicals. We anticipate that this PISA assay could bridge the gap between molecular interactions and toxicity pathways.

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Declaration of Competing Interest

No.

Data availability

Public reposition: T "Data are available via ProteomeXchange with identifier PXD021670."

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