Research Article

# Hazard Assessment Through Hybrid *In Vitro/In Silico* Approach: The Case of Zearalenone

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**Summary**

Within the framework of reduction, refinement and replacement of animal experiments, new approaches for identification and characterization of chemical hazards have been developed. Grouping and read-across has been promoted as a most promising alternative approach. It uses existing toxicological information on a group of chemicals to make predictions on the toxicity of uncharacterized ones. In the present work, the feasibility of applying *in vitro* and *in silico* techniques to group chemicals for read-across was studied using the food mycotoxin zearalenone (ZEN) and metabolites as a case study. ZEN and its reduced metabolites are known to act through activation of the estrogen receptor α (ERα). The ranking of their estrogenic potencies appeared highly conserved across test systems including binding, *in vitro* and *in vivo* assays. This data suggests that activation of ERα may play a role in the molecular initiating event (MIE) and be predictive of adverse effects, and it provides the rationale to model receptor-binding for hazard identification. The investigation of receptor-ligand interactions through docking simulation proved to accurately rank estrogenic potencies of ZEN and reduced metabolites, showing the suitability of the model to address estrogenic potency for this group of compounds. Therefore, the model was further applied to biologically uncharacterized, commercially unavailable, oxidized ZEN metabolites (6α-, 6β-, 8α-, 8β-, 13- and 15-OH-ZEN). Except for 15-OH-ZEN, the data indicate that in general, the oxidized metabolites would be considered of lower estrogenic concern than ZEN and reduced metabolites.

Keywords: biological activity, docking, read-across, endocrine disrupting compounds, zearalenone

## 1 Introduction

In the process of chemical risk assessment, the steps of hazard identification and hazard characterization refer to the qualitative description and dose-response analysis of toxic effects (Schilter et al., 2013). Traditionally, both have relied on *in vivo* animal studies. Because of ethical concerns and scientific rationales, there have been major efforts to replace the use of experimental animals for hazard identification and characterization (Thomas et al., 2013). Several avenues have been developed to achieve such a challenging goal, including the application of *in vitro* and computational toxicology tools (NRC, 2007; Bradbury et al., 2004). In this context, the technique of chemical grouping and read-across has been promoted as a most promising and pragmatic alternative approach. It consists of identifying analogs of the chemical under investigation (grouping) and extrapolating its toxic properties using the available toxicological data to the analogs (read-across) (Patlewicz et al., 2014; Schilter et al., 2013; Wu et al., 2010). Finding adequate analogs is not straightforward and has to be based on a number of structural and biological features (OECD, 2011; Patlewicz et al., 2014; Schilter et al., 2013; Wu et al., 2010).

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**List of Abbreviations**

List of abbreviations: AOP, adverse outcome pathway; CALUX, Chemical activated luciferase reporter gene assay; E2, 17 β-estradiol; EGFP, enhanced green fluorescent protein; ERα, estrogen receptor alpha; HCS, high content screening; LBD, ligand binding domain; μM, micro-molar; MIE, molecular initiating event; OH-ZEN, hydroxy-zearalenone; α- and β-ZAL, alpha- and beta- zearalanol; ZEN, zearalenone; α- and β-ZEL, alpha- and beta- zearalenol

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In this context, the concept of the Adverse Outcome Pathway (AOP) is thought to significantly strengthen analog identification and the chemical grouping process (Crofton et al., 2014; Patlewicz et al., 2014).

The AOP concept provides a framework linking knowledge on the molecular initiating event (MIE), in which a chemical interacts with a biological target, to a sequential series of cellular, anatomical and functional changes resulting in an adverse effect (OECD, 2011, 2013; Crofton et al., 2014). Obviously, AOP is likely to constitute a powerful tool to group chemicals for read-across, for example according to a common MIE. In addition, the AOP concept will likely provide significant guidance for the exploitation of *in vitro* and *in silico* data aiming to facilitate the characterization of MIEs and to anchor them to apical effects in the *in vivo* situation.

To explore the potential role of the AOP framework in order to improve chemical grouping and read-across, zearalenone (ZEN) and metabolites were selected as a case study. ZEN and metabolites are food relevant mycotoxins (EFSA, 2011; Kuiper et al., 1997; WHO, 2000). In most mammalian species ZEN undergoes reductive, oxidative and conjugative metabolism (EFSA, 2011; WHO, 2000). The main metabolites formed by enzymatic reduction are zearalenols (α- and β- ZEL) and zearalanols (α- and β-ZAL) (Fig. 1). The so far identified oxidized metabolites share hydroxylation at position 13, 15, 6 and 8 (Fig. 2). ZEN is known to produce an array of toxicological effects with the most critical resulting from estrogenic activity triggered by an activation of the estrogen receptor alpha (ERα) (EFSA, 2011; WHO, 2000; Shen et al., 2013). ERα is a transcriptional factor belonging to the nuclear receptor superfamily that mediates, together with the beta isoform (ERβ), many of the biological effects of estrogens (Kumar et al., 2011).

ZEN and its reduced metabolites are structurally very similar, but their estrogenic potencies differ significantly. There is currently no data on the potential estrogenicity of oxidized metabolites. It is interesting to note that based on currently available information, the estrogenic potency of ZEN and reduced metabolites ranks the same in *in vitro* binding assays (EFSA, 2011; Takemura et al., 2007), in cellular *in vitro* assays – which use endpoints such as transcriptional activation or cellular proliferation (Bovee et al., 2004; Frizzell et al., 2011; Molina-Molina et al., 2014; Shier et al., 2001) – and in animal oral *in vivo* estrogenicity tests (Everett et al., 1987). This strong correlation between binding affinity and bioactivity in *in vitro* and *in vivo* systems is compatible with the hypothesis that for this specific group of chemicals, the interaction of the ligand with ERα may be considered as the MIE. In other words, this provides a rationale that for uncharacterized chemicals structurally related to ZEN, the prediction of an early event such as ERα-binding properties will likely provide relevant mechanism-based information on their toxic effect and potency.

In the present work, the ranking of the estrogenic potency of ZEN and reduced metabolites was further confirmed using *in vitro* assays dealing with two different steps in the estrogenic response (receptor-ligand translocation and transcriptional activation). Then the feasibility to reproduce this ranking using an *in silico* docking method was evaluated. Since the docking data indicated a good power to predict estrogenic potency, the model

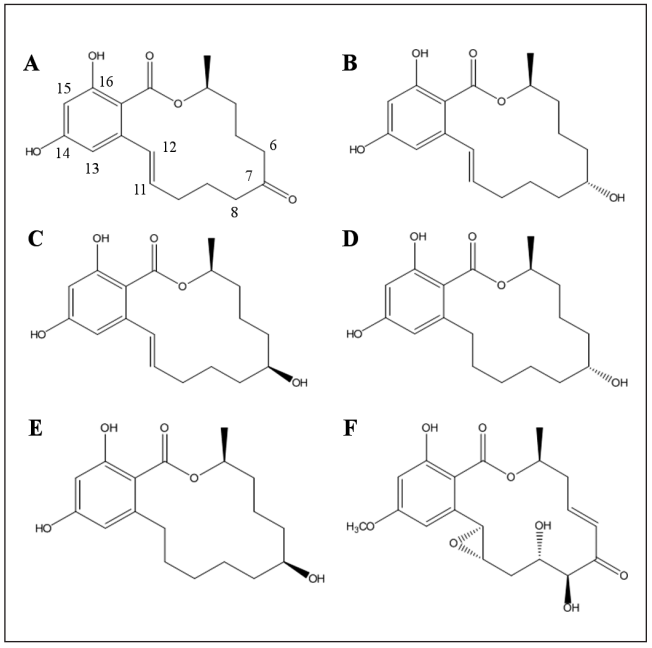


**Fig. 1: Zearalenone, reduced derivatives and related compound hypothemycin**
A, zearalenone (ZEN); B, α-zearalenol (α–ZEL);
C, β-zearalenol (β–ZEL); D, α-zearalanol (α–ZAL);
E, β-zearalanol (β–ZAL); F, hypothemycin

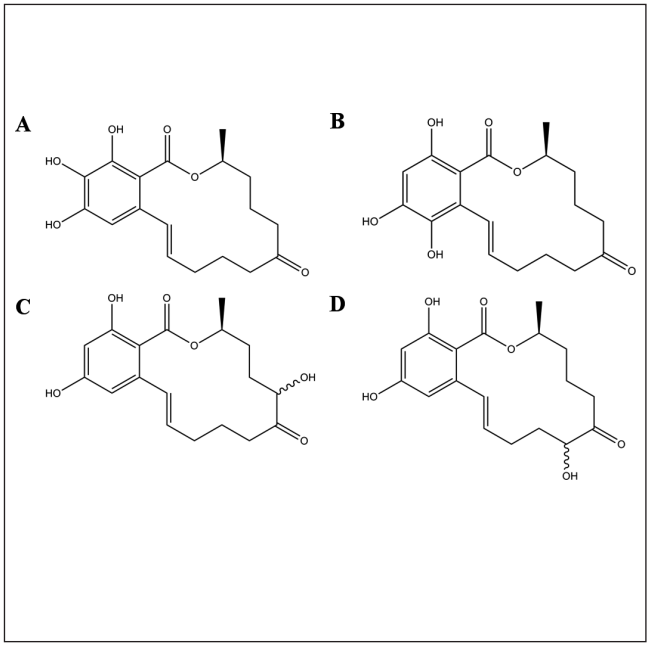


**Fig. 2: Oxidized metabolites of ZEN**
A, 15-hydroxy-zearalenone (15-OH-ZEN);
B, 13-hydroxy-zearalenone (13-OH-ZEN);
C, 6-alpha/beta-hydroxy-zearalenone (6α/β-OH-ZEN);
D, 8-alpha/beta-hydroxy-zearalenone (8αβ-OH-ZEN)

was then applied to investigate the activity of uncharacterized, commercially unavailable, oxidized ZEN metabolites, as well as hypothemycin, another natural fungal metabolite exhibiting a structure similar to ZEN (Fig. 1).

## 2 Material and methods

### 2.1 Chemicals

Zearalenone (ZEN), α and β zearalenol (ZEL), α and β zearalanol (ZAL), hypothemycin, 17 β-estradiol ($E_2$), dimethylsulfoxide (DMSO), penicillin/streptomycin (P/S), Dulbecco's phosphate buffered saline (PBS) and formalin solution neutral buffered 10% were purchased from Sigma Aldrich (Buchs, Switzerland); Dulbecco's Minimum Essential Medium (DMEM) high glucose with stable glutamine from PAA (Linz, Austria), charcoal stripped fetal bovine serum (FBS) and Hoechst 33258 were from Invitrogen (Eugene, Oregon, USA) and G418 from Roche (Mannheim, Germany).

### 2.2 Cell based tests – *in vitro* assays

*ERα redistribution assay*

Recombinant (U2OS) cells stably expressing human ERα fused to the C-terminus of enhanced green fluorescent protein (EGFP) from Thermo Fisher were used. U2OS cells are adherent epithelial cells derived from human osteosarcoma. The expression of EGFP-ERα is controlled by a standard promoter and continuous expression is maintained by addition of G418 (an aminoglycoside antibiotic used to select genetically engineered cells) to the culture medium.

Cellular activation and distribution of ERα upon treatment of the cells with the test compound was measured and compared to the activity of the reference compound $E_2$. Imaging and analysis was performed with the high content screening (HCS) platform ToxInsight (Thermo Scientific). Foci formation in the nuclear region (foci count, area and intensity) is the reported endpoint in this study. The agonistic version of the test was performed according to supplier instructions. Briefly, U2OS-ERα cells were seeded in 96-well plates, incubated for 24 h at 37°C and 5% $CO_2$ in Dulbecco's Modified Eagle Medium (DMEM) with high glucose and stable glutamine (PAA) supplemented with 10% charcoal stripped fetal bovine serum (FBS), 1% antibiotics (penicillin/streptomycin) and 0.5 mg/ml G418. Subsequently, cells were treated for 24 h with the test compounds (1 pM - 100 μM) and reference compound ($E_2$ 1.92 pM - 30 nM) in triplicates. At end of treatment, cells were fixed with 10% formalin solution, washed with phosphate buffered saline (PBS) and stained (DNA specific stain Hoechst 33258, 1 μM). Passage number of cells used was between 2 and 8. Scanning and data analysis: Plates were scanned with the ToxInsight imaging platform (Thermo Scientific) to measure viability through nuclei number (Hoechst stain) and receptor distribution as aggregation of GFP tagged nuclear foci. For threshold adjustment a fixed ratio was set between solvent (DMSO) and positive control (30 nM $E_2$). Raw data were extracted from the data visualization software and the value for each sample was normalized to positive control (100%, i.e., 30 nM $E_2$).

*ERα chemical activated luciferase reporter gene assay (CALUX®)*

For determination of full dose-response curves of the test compounds (agonistic activity), CALUX® cells (van der Burg et al., 2010) were seeded in 96 well plates in assay medium (DMEM/F12 medium with charcoal stripped serum and supplemented with non-essential amino-acids) at 37°C and 5% $CO_2$. After 24 h the cells were exposed in triplicate for another 24 h with dilution series of the pure compounds (ZEN, α- and β-ZAL, α- and β-ZEL: 0.05 pM - 0.5 μM; DMSO: 0.1% and $E_2$: highest concentration 0.1 nM as reference compound). Light emission was measured with a luminometer and quantified in relative light units, analysis results were interpolated in the calibration curve for determination of agonistic potential of the test compounds.

To determine antagonistic activity, cells were co-treated with test compound and the $EC_{50}$ of $E_2$ (6.2 pM); light emission was compared to cells treated only with $E_2$.

### 2.3 Computational analysis

With the aim to model estrogenic activity, the interaction between molecules and the active (agonistic) conformation of the ERα ligand binding domain (LBD) was evaluated by using the coupling of docking simulations and proper rescoring procedures. Specifically, the coupling of GOLD, as docking software, and HINT (Hydropathic INTeraction) (Kellogg and Abraham, 2000), as rescoring function, was chosen on the basis of previous studies demonstrating the higher reliability of HINT in respect to other scoring functions and the efficacy as re-scoring function to predict ligand interaction with several protein targets (Cozzini et al., 2002; Dellafiora et al., 2014; Fornabaio et al., 2003, 2004; Marabotti et al., 2008; Salsi et al., 2010), including estrogen receptors (Cozzini and Dellafiora, 2012; Dellafiora et al., 2013). The HINT scores provide empirical and quantitative evaluation of protein-ligand interaction as a sum of all single atomic contributions. Since they correlate with the free energy of binding, low or negative scores correlate with the thermodynamic disfavor of protein-ligand interaction. In particular, the interaction was considered appreciable when scored above 180 units, in accordance to Cozzini and Dellafiora (2012).

*Molecular modeling*

The model for human ERα-LBD was derived from the Protein Data Bank (http://www.rcsb.org) structures having PDB codes 2YJA (Phillips et al., 2011). Protein structure and ligands were processed according to Dellafiora et al. (2014).

*Docking simulations*

Docking simulations of all compounds were performed with the program GOLD version 5.0 (CCDC; Cambridge, UK; http://www.ccd.cam.ac.uk) on a double-quad cores machine equipped with 1.86 GHz processors. For each compound, 50 poses were generated and all co-crystalized ligands and crystallographic waters were removed. In this respect, it is worthy of mention that a specific water molecule, which lies aside and near to Glu353, has proved to contribute to the hydrogen-bonding network of ligands (REF) (Phillips et al., 2011). Nonetheless, its inclusion, which is actually paramount for the absolute calculation of the

free energy of binding, was not mandatory for the qualitative estimation of the protein-ligand interactions under investigation (*vide infra*). Software setting reported by Cozzini (Cozzini and Dellafiora, 2012) was used. GOLD uses a Lamarckian genetic algorithm; thus, poses and scores may change slightly from run to run. In order to avoid not-causative fluctuations in the case of proximal scores, we performed analysis in triplicate. From here we refer to scores as a mean value of three replicas.

*Rescoring procedure*

The software HINT was used as post-processing tool. All poses generated by GOLD were re-scored in order to better evaluate protein-ligand recognition and, among the 50 poses computed for each compound, we carried forth only the highest scored pose.

*Local minimization*

A mild local minimization of each best-scored binding architecture was performed using the software Sybyl. Each predicted pose was subjected to 200 iterations using the Powell algorithm within 5 Å around the ligand.

*Protomeric analysis*

The analysis of the protomeric state was performed using the software FLAP (Fingerprint for Ligand and Protein) (Baroni et al., 2007). Specifically, it was achieved by creating a database of all considered compounds taking into account the putative proportion of protomeric states at pH 7.4.

*Pharmacophore models*

The ligand binding site was defined by using the Flapsite tool of FLAP, while the GRID algorithm (Goodford, 1985) was used to investigate the corresponding pharmacophoric space. The DRY probe was used to describe the potential hydrophobic interactions, while the sp2 carbonyl oxygen (O) and the neutral flat amino (N1) probes were used to describe the hydrogen bond acceptor and donor capacity of the target, respectively. All images were obtained using the software PyMol version 1.4.1 (http://www.pymol.org).

## 3 Results

### 3.1 *In vitro* results

Figures 3A and 3B represent the dose response curves for ZEN, reduced metabolites, hypothemycin and $E_2$ on a logarithmic scale obtained in the ERα redistribution (Fig. 3A) and the CALUX assays (Fig. 3B). The CALUX assay was observed to be an order of magnitude more sensitive (up to 23x, depending on the compound tested). Respective $EC_{50}$ values were calcu-

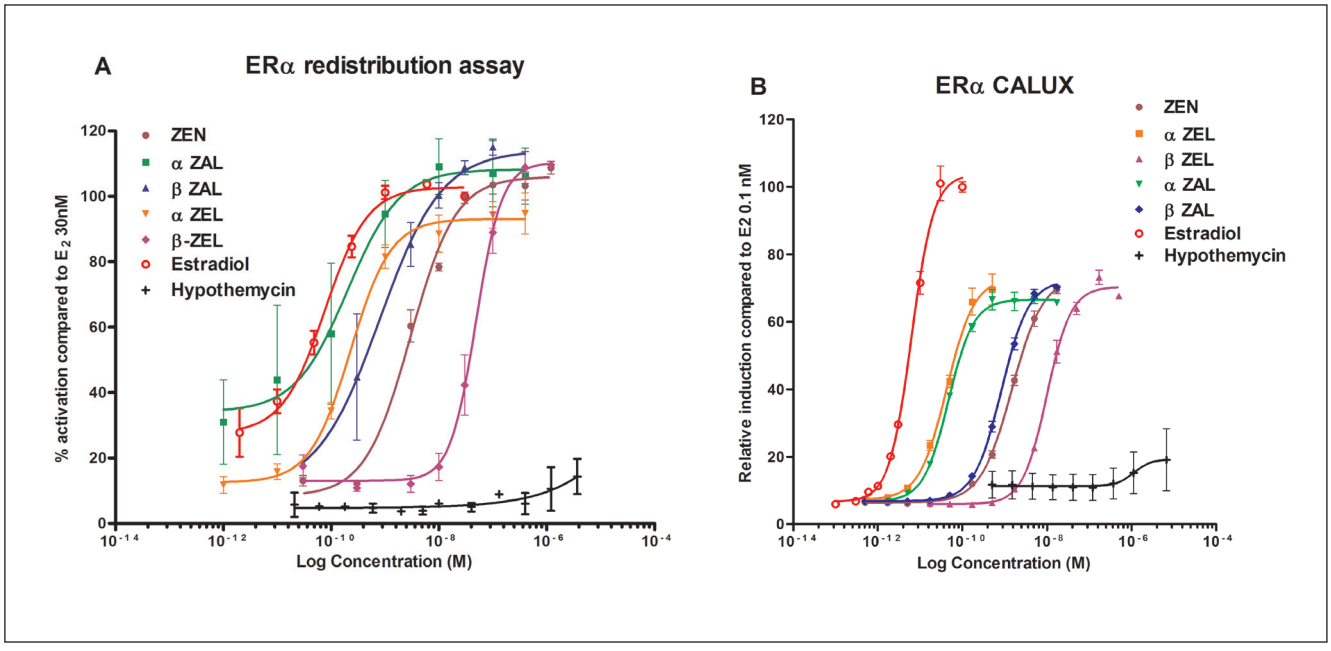


**Fig. 3: Results of ERα redistribution and CALUX assay**

Graphs show mean and standard deviation of each experimental point. Three experiments were performed per compound.
(A) Percentage of activation by ZEN and metabolites in comparison with 30 nM 17β-estradiol ($E_2$) measured using the redistribution assay. Dose range tested: 0.03 nM - 100 μM for ZEN, β-ZAL and β-ZEL; 1 pM - 100 μM for α-ZAL and α-ZEL; 0.021 mM - 100 μM for hypothemycin (cytotoxic concentrations were not used for the analysis); 1.92 pM - 30 nM for estradiol; DMSO: 0.1-1%. (B) Dose response curve for ZEN, reduced metabolites, $E_2$ and hypothemycin on a logarithmic scale measured with the CALUX assay. Dose range tested: 0.05 pM - 0.5 μM for ZEN and its metabolites; 0.5 nM - 10 μM for hypothemycin; highest concentration of $E_2$: 0.1 nM, DMSO: 0.1%. RLU: relative light units. Hypothemycin was tested at Nestlé Research Center (Lausanne, Switzerland) under the same conditions as BDS (Amsterdam, Netherlands).

lated (Tab. 1) and compared to the reference compound $E_2$. Relative potency is expressed as estradiol equivalents (EEQ, see Tab. 1). Ranking of ZEN and metabolites was the same in both test systems: α-ZEL and α-ZAL were the most potent substances; the lowest is β-ZEL ($E_2$ > α-ZAL ≈ α-ZEL > β-ZAL > ZEN > β-ZEL). Hypothemycin was inactive in both assays.

**Tab. 1: Half maximum effective concentration ($EC_{50}$) of ZEN and metabolites in the ERα redistribution and CALUX assay**

| Compound | ERα redistribution assay ($EC_{50}$) | CALUX assay ($EC_{50}$) |
|---|---|---|
| Estradiol | 0.14 nM | 0.0046 nM |
| α-ZEL | 0.22 nM | 0.0096 nM |
| α-ZAL | 0.20 nM | 0.019 nM |
| β-ZAL | 0.79 nM | 0.28 nM |
| ZEN | 4.27 nM | 0.49 nM |
| β-ZEL | 48.69 nM | 2.5 nM |

## 3.2 Molecular modeling results

*Anatomy of the binding pocket*

The pharmacophoric analysis of the binding site revealed that the pocket environment was prevalently hydrophobic with two constrained polar patches at opposite ends corresponding with Glu353 and Arg394 and His524, respectively (Fig. 4). Therefore, it is thought that polar groups of ligands should be placed in such patches to satisfy pharmacophoric requirements.

*Protomeric states*

The interaction with Glu353 and Arg394 through hydrogen bonds is a common feature found throughout the PDB structures. Consequently, the unavailability of hydroxyl groups due to protonation equilibria might affect the interaction with the receptor. For this reason, putative protomeric states were calculated for each molecule at neutral conditions and then assessed for their ability to interact with the receptor. Specifically, $E_2$ and hypothemycin occurred only as neutral. A similar occurrence of neutral and deprotonated forms were found for 15-OH-ZEN and 13-OH-ZEN (58% and 42%, respectively). All other molecules were mostly found as neutral (namely, 77% vs. 23%). As expected, all deprotonated forms were predicted to be unable to interact with protein. Accordingly, the protonation equilibria in solution might actually play a role in influencing the relative estrogenic potency of ZEN and derivatives.

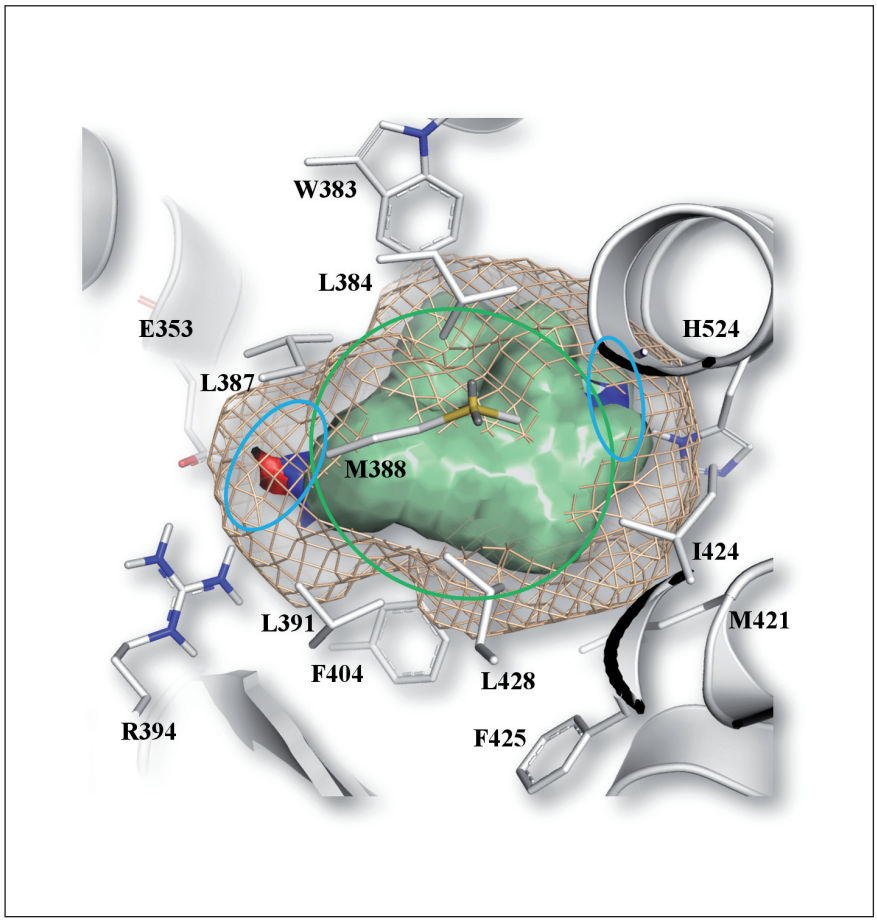


**Fig. 4: The anatomy of the binding site**
Green, red and blue contours identify regions sterically and energetically favorable for hydrophobic, H-bond acceptors and H-bond donors groups, respectively. The shape of the binding site is represented in mesh. The main residues involved in polar interactions and some hydrophobic residues are represented in sticks. Blue and green rings represent the region of the pocket suitable for receiving polar and hydrophobic groups, respectively.

*Feasibility assessment*

A fit-for-purpose training procedure was performed by comparing computed results with experimental data. The procedure was able to predict protein recognition by $E_2$, ZEN, α-ZAL, α-ZEL, β-ZAL and β-ZEL, while the inactive compound hypothemycin was predicted to be unable to interact (Tab. 2). It has to be noted that a mild local minimization of each binding architecture markedly improved the model by giving the same potency rank obtained with the ER-CALUX® bioassay. Notably, our findings showed a better quantitative correlation was achieved when the intraclass comparison considered solely ZEN and derivatives ($R^2$ of 0.96 with logarithmic regression) and not $E_2$ ($R^2$ of 0.52). The strong reliability of calculating on the ZEN scaffold thus became clear. Therefore, the pro-

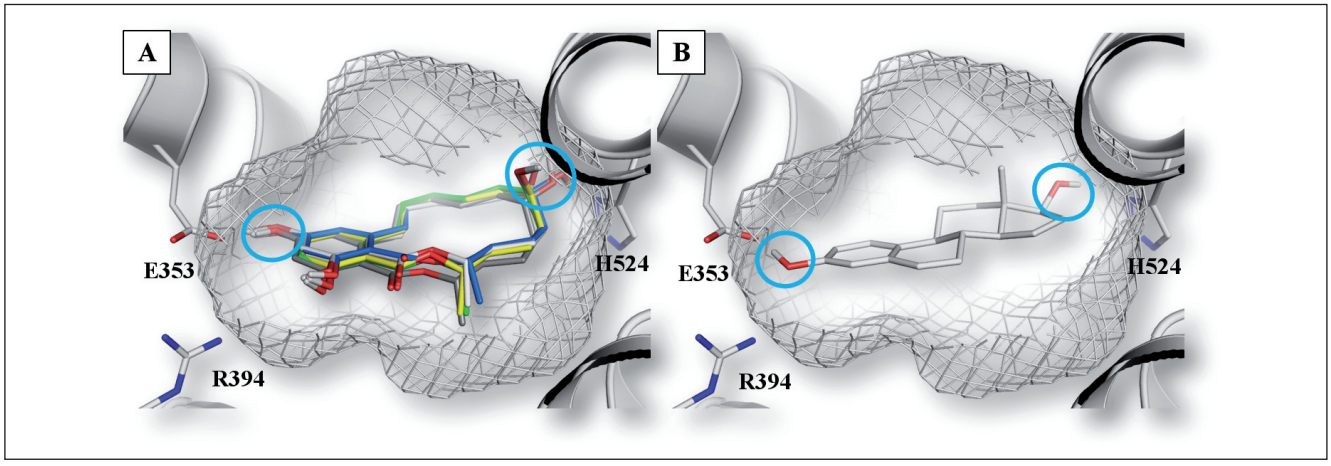


**Fig. 5: Binding mode of ZEN and reduced derivatives (A) in comparison with the crystallographic pose of $E_2$ (B)**
The shape of the binding site is represented in mesh while ligands and the residues involved in polar interactions are represented in sticks. The chemical groups of ligands involved in polar interactions with the pocket are ringed in blue.

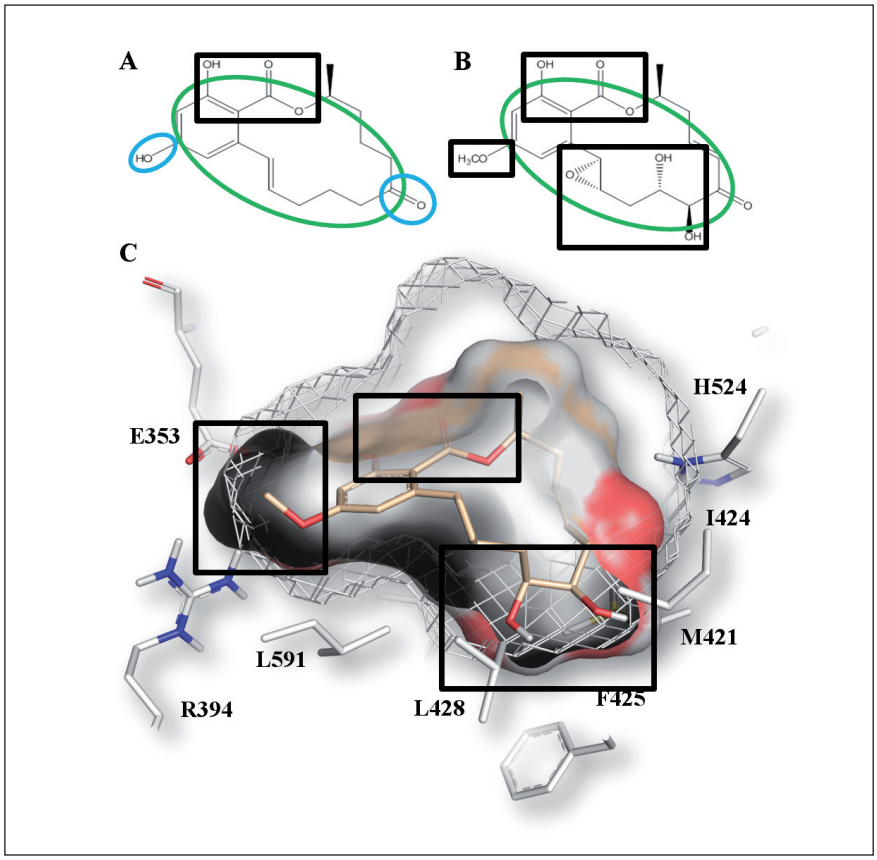


**Fig. 6: Structure-activity relationship of ZEN scaffold**
Blue and green rings indicate respectively the molecular moieties able to satisfy polar and hydrophobic requirements of the pocket. Conversely, interfering groups are highlighted in black boxes. (A) ZEN; (B) hypothemycin; (C) three-dimensional diagram of hypothemycin. The shape of the binding site is represented in mesh, and amino acids are shown in sticks. Hypothemycin is shown in sticks and cut surface to better underline the excess of volume in respect to pocket boundaries.

cedure was considered sufficiently reliable and sensitive to be applied to predict the potential estrogenicity of oxidized ZEN derivatives. From a structural point of view, a common frame of posing was found for ZEN and derivatives. The binding mode resembled that of $E_2$ with the aromatic ring facing Arg394 and Glu353 and keto/hydroxyl groups in position 7 facing the hydrophilic patch in correspondence to His524 (Fig. 5). It is worthy to note that this finding is in agreement with the crystallographic pose of α-ZAL recently reported by Delfosse and co-workers (Delfosse et al., 2014).

In particular, the main polar interaction is charged on the hydroxyl group in position 14 through a hydrogen bond with Glu353, while keto/hydroxyl groups in position 7 satisfied polar requirements at the opposite end of the pocket. The other polar groups (namely the hydroxyl group in position 16 and the lactone portion) caused hydrophobic/polar interferences being placed in correspondence to a non-suitable environment (Fig. 6A). With respect to hypothemycin, the simultaneous lack of a free hydroxyl group at position 14 and the hydrophilic gain on the core of the molecule caused the lack of interaction (Fig. 6B). Interestingly, the presence of a hydroxyl group instead of a ketone in correspondence to the His524 end seemed to be a preferable condition as previously observed for steroid ligands (Sonneveld et al., 2006). In this respect, alpha stereochemistry of ZEN derivative better satisfied pocket requirements, while for beta isomers (β-ZAL was 10-50 fold more potent than β-ZEL) it is thought that a greater flexibility – due to the carbon-carbon double bond reduction – is advisable for orienting the molecule properly.

*Query-set results*

Structurally, ZEN oxidized derivatives shared the same orientation and interaction pattern observed for ZEN and reduced derivatives. As a general outcome it was observed that oxidative metabolism might quench agonistic activity, preventing the whole ligand-receptor interaction in the case of 13-OH-ZEN and 8αOH-ZEN (Tab. 3). Such a pattern was mainly due to the increase of polar/hydrophobic interferences by gaining hydroxyl groups in positions 6, 8 or 13. Conversely, 15-OH-ZEN placed the 15-hydroxyl group in a suitable environment (i.e., in front of Arg394 and Glu353), thus engaging Glu353 and Arg394 similarly to $E_2$ (Fig. 7). This resulted in a slightly higher score, suggesting at first glance a potentially enhanced activity. Nevertheless, it should be kept in mind that a relevant abundance of the deprotonated form, for which the lack of interaction was calculated (see above), was predicted for the isolated molecule at pH 7.4. Therefore, the activity may get worse if the protonation equilibrium shifts toward the deprotonated form in physiological conditions.

**Tab. 2: Computed results of ZEN and reduced derivatives**
Experimental rank is obtained by using ERα-CALUX assay.

| Compound | Experimental rank | HINT score without minimization | HINT score with minimization |
|---|---|---|---|
| Estradiol | 1 | 1454.08 | 1380.22 |
| Hypothemycin | / | -1897.81 | -1314.35 |
| α-ZEL | 2 | 767.73 | 648.39 |
| α-ZAL | 3 | 671.44 | 632.53 |
| β-ZAL | 4 | 500.25 | 505.31 |
| ZEN | 5 | 492.82 | 499.77 |
| β-ZEL | 6 | 570.15 | 469.55 |

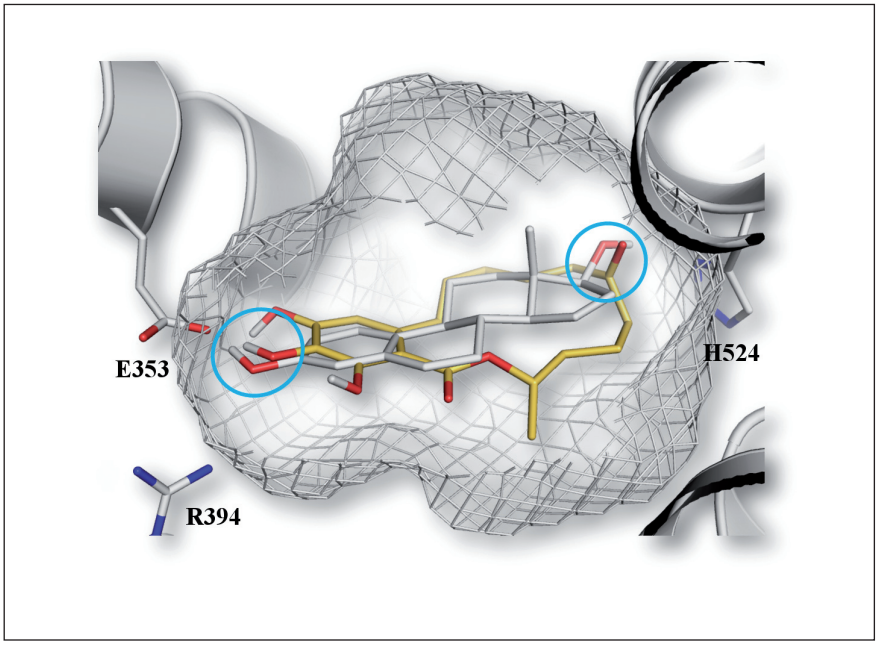


**Fig. 7: Binding mode of 15-OH-ZEN (green) in comparison with the crystallographic pose of $E_2$ (white)**
The shape of the binding site is represented in mesh while ligands and the residues involved in polar interactions are represented in sticks. The chemical groups of ligands involved in polar interactions with the pocket are ringed in blue.

**Tab. 3: Computed results of oxidized derivatives of ZEN**

| Compound | HINT score | Predicted activity |
|---|---|---|
| 13-OH-ZEN | -47.27 | Negative |
| 15-OH-ZEN | 545.20 | Positive |
| 8αOH-ZEN | -37.74 | Negative |
| 6αOH-ZEN | 197.87 | Positive |
| 8βOH-ZEN | 212.76 | Positive |
| 6βOH-ZEN | 280.99 | Positive |

In order to maintain agonistic activity of ZEN derivatives, hydrophilic modifications seem to be rigidly constrained. Orthogonal hydroxylation onto the longitudinal axis of the pseudo-symmetry of ZEN decreased its activity to the extent of preventing interaction. On the contrary, apical hydrophilic modifications (i.e., in positions 7 and 15) are better tolerated. According to what was reported for ER ligands by Katzenellenbogen (2011), the hydrophobic scaffold of resorcylic acid lactones satisfies spatial and pharmacophorical requirements of the pocket without exerting any substantial contact with the protein. Conversely, polar substituents may have different effects on the pocket-ligand interaction.

## 4 Discussion

There is an increasing demand to switch from high dose animal toxicology studies to alternative methods in order to identify and characterize chemical hazards (Thomas et al., 2013). In this context, one of the most promising and pragmatic approaches is read-across. This is a technique based on grouping and comparison of chemicals. It uses existing toxicological information on a group of chemicals to make a prediction of the toxicity of an uncharacterized structure (OECD, 2011; Patlewicz et al., 2014; Schilter et al., 2013; Wu et al., 2010). Obviously, for the prediction to be relevant, chemicals used for read-across must exhibit sufficient analogy with the chemical under investigation. Consequently, the reliability of read-across highly depends upon the selection of analogs. This is a very complex exercise. Indeed the analogs should present similarity on the grounds of structure, physicochemistry, metabolism as well as the biological mechanism of action (OECD, 2011; Patlewicz et al., 2014; Schilter et al., 2013; Wu et al., 2010). In this context, it is now widely recognized that the AOP concept could play an essential role to define the data required to facilitate the formation of relevant chemical categories for read-across: chemicals could be grouped according to key events, but more likely according to MIEs (Crofton et al., 2014; Patlewicz et al., 2014). Importantly, since MIEs reflect direct interactions between chemicals and biological/biochemical targets, they appear particularly suitable to be modelled by applying alternative methods based on *in vitro* and/or *in silico* techniques.

In the present work, we applied *in vitro* and *in silico* data, together with published information, to investigate the potential of these alternative methods to contribute to grouping and read-across. For this purpose, ZEN and metabolites were selected as a case study since they are structurally and biologically very similar (EFSA, 2011; WHO, 2000). Also, several works already pointed toward the feasibility of modeling the estrogenic activity of ligands through the evaluation of interaction with ER-LBD *in silico* (McRobb et al., 2014; Ng et al., 2014b). In the first step, available experimental data were reviewed to identify the most relevant endpoint to be used for the formation/characterization of a ZEN analog group. ZEN is well documented to exert an array of toxicological effects, mostly mediated by perturbations of the steroid hormonal system (EFSA, 2011; WHO, 2000). There are a number of endocrine actions reported for ZEN and metabolites which could play a role as MIE. It has been well established that ZEN and metabolites activate estrogen receptors α and β (Bovee et al., 2004; Bravin et al., 2009; EFSA, 2011; Frizzell et al., 2011; Shier et al., 2001; Takemura et al., 2007; WHO, 2000). It has also been suggested that these molecules may alter steroidogenesis (Frizzell et al., 2011; Gracia et al., 2007; Kolle et al., 2012; Ranzenigo et al., 2008; Yang et al., 2007) and may act as an antagonist of the androgenic receptor (Molina-Molina et al., 2014). Undoubtedly, ZEN and metabolites might perturbate several toxicological pathways, but based on the body of available data and reviews from regulatory agencies, the critical effects are thought to result from estrogenic activity mainly through an activation of ERα (EFSA, 2011; Kuiper et al., 1997; WHO, 2000). ERα activation/interactions has been evident at picomolar or nanomolar concentrations using tests covering different steps of the estrogenic response: binding affinity (Takemura et al., 2007), activation of transcriptional activation (Frizzell et al., 2011), proliferation of MCF7 cells (Molina-Molina et al., 2014; Shier et al., 2001) and yeast transfected with ERα (Bovee et al., 2004), as well as results on transcriptional activation (ERα CALUX®) and receptor-ligand translocation (ERα redistribution assay), as obtained in this study. Active concentrations (pico to nanomolar range: this study, and studies aforementioned) were found to be much lower than the levels required to alter steroid metabolism (micromolar range, for examples see: (Frizzell et al., 2011; Gracia et al., 2007; Kolle et al., 2012; Ranzenigo et al., 2008; Yang et al., 2007). Interestingly, estrogen may stimulate activities of enzymes of steroidogenesis such as aromatase via ER-mediated regulation (Lee et al., 2012). Therefore, the alteration of steroid hormone production by ZEN and metabolites might be a consequence of ERα activation.

We have ranked the parent compound ZEN and the four reduced metabolites α-, β-ZAL and α-, β-ZEL according to estrogenic potency with two independent *in vitro* estrogenicity tests (ERα redistribution and ERα-CALUX® assay). Activity was $E_2$ > α-ZAL ≈ α-ZEL > β-ZAL > ZEN > β-ZEL. Importantly, ranking appears highly similar across various

tests informing on different steps in the estrogenic response, including receptor binding (Takemura et al., 2007), receptor-ligand translocation (redistribution assay), transcriptional activation (CALUX-assay; other cell systems: e.g., Frizzell et al., 2011) and cell proliferation *in vitro* (MCF7 cells) (e.g., Molina-Molina et al., 2014; Shier et al., 2001). In addition, the ranking observed *in vitro* is fully in line with the ranking of estrogenic potencies measured in animals *in vivo* (Everett et al., 1987). It should be kept in mind, however, that the complexity of the molecular events triggering the estrogenic response is high (Kocanova et al., 2010; Lee et al., 2012) and involves a number of cofactors beyond the estrogen receptors, including, for instance, the interaction with serum binding proteins (Hong et al., 2012, 2015) – which may actually reduce the bioavailability of compounds. Nevertheless, the highly robust potency ranking observed with biological assays *in vitro* and *in vivo* as well as binding tests supports the role of the ligand-receptor interaction as the MIE in the estrogenic-mediated toxic effects induced by the members of this class of compounds. For this specific group of chemicals, the data available provides a strong rationale to model receptor-binding for hazard identification and characterization of analogs of unknown activity through read-across, based on both structural (ZEN analogs) and biological (ERα activation) properties. This can be achieved either through *in vitro* investigations or, when chemicals are not available through docking, as illustrated for the oxidized metabolites. This requires to demonstrate that the *in silico* model would predict the ranking with sufficient reliability.

By comparing computed and experimental results, the computational procedure proved to be sensitive to slight structural modifications on the ZEN scaffold: (i) the decoy (i.e., inactive compound sharing a degree of similarity with molecules under analysis) hypothemycin was properly predicted as inactive; (ii) reduced derivatives of ZEN were ranked in agreement with experimental data (i.e., $E_2$ > α-ZAL > α-ZEL > β-ZAL > ZEN > β-ZEL). Also, a good quantitative correlation with experimental activity was found. Taken together, this finding outlined the strong reliability of this model in calculating the xenoestrogenicity of ZEN and reduced derivatives. Therefore, the procedure was considered suitable to extend analysis also to the oxidized derivatives. The strong concordance of experimental and computational ranks may be due to the employment of a molecular model derived from active agonistic conformation of ERα-LBD. Taken together, these results suggest the possibility to employ docking simulations not only to compute the binding event, but also to analyze other kinds of biological activity if a relationship exists between protein architecture and a given function. This is the case for ERs. Indeed crystallographic data so far available suggest a unique and unambiguous LBD-dependent mechanism related to the agonistic response (e.g., Brzozowski et al., 1997; Spyrakis and Cozzini, 2009) where the LBD adopts a "closed" and active conformation able to promote the formation of pre-initiation complex assembly (Sabbah et al., 1998). Concerning ZEN oxidized derivatives, they presented variable estrogenic activity but, overall, a diminished potency is to be expected due to polar/hydrophobic mismatches. Because for ZEN analogs predicted estrogenic potencies were correlated to *in vivo* potencies, the present data indicate that in general oxidized metabolites would be considered of lower estrogenic concern than ZEN and reduced metabolites. This might not be true for 15-OH-ZEN, characterized by a high HINT score. Consequently, this specific oxidized metabolite should be considered of higher priority for further investigation, also in respect to the possible effects mediated by ERβ. Indeed, the ERβ binding site differs from ERα in only two residues (Ng et al., 2014a) – thus a similar calculated pattern of interaction is expected – but 15-OH-ZEN might exert tissue-specific actions through the activation of both ERs isoforms in living organisms.

Overall, the estrogenic potency ranking obtained by docking appears of biological significance and therefore may be applied to select the toxicological information to be used in order to characterize the hazard of oxidized ZEN metabolites through read-across. As shown previously, this process requires integrating all available experimental data to document that for the members of this group of chemicals, ligand-receptor binding is likely to play the role of MIE in the development of estrogen-mediated adverse effects *in vivo*. However, the relatively low concern for estrogenicity of ZEN oxidized metabolites, as suggested by the present work, is not sufficient to make any firm statement regarding safety. There is uncertainty regarding the possible involvement of other relevant and independent toxic mechanisms and MIEs. For example, it is documented that 13- and 15-OH-ZEN undergo equilibrium to quinones, which might redox-cycle and covalently modify biological macromolecules (Pfeiffer et al., 2009), causing oxidative stress, DNA damage or DNA adducts (EFSA, 2011; Metzler et al., 2010). The actual significance of these other mechanisms is difficult to anticipate but could impact apical toxicological endpoints relevant for hazard characterization. In addition, the possible presence of other mechanisms requires careful interpretation when evaluating structurally similar chemicals identified as non-active for the molecular target under investigation. For example, although structurally similar to ZEN, hypothemycin is not expected to induce any estrogenic effects, but is known to be an inhibitor of several kinases (Winssinger and Barluenga, 2007). The safety relevance of such a mechanism is currently unknown.

## 5 Conclusions

There is increasing awareness within the scientific community and government agencies that new approaches are needed to evaluate the safety of the relatively large number of chemicals in commerce and the environment. Toxicity testing and risk assessment are becoming more economical, less animal intensive and more relevant to human health by integrating new technologies such as *in silico* tools and mechanistic based *in vitro* assays (Thomas et al., 2013).

In the present work, *in vitro* assays identifying the MIE linked to an adverse outcome together with a careful review of the existing literature data were used to determine which chemical structures bind ERα and induce gene activation. As demonstrated by this case study with ZEN and metabolites, such an approach could be applied to assess the safety concern associated with mycotoxin exposure or to understand the potential role of metabolites in the toxicity of contaminants. It may also help to decide whether combination toxicology principles such as dose addition should be applied in case of exposure to a mixture of structurally related compounds.

In our case study, the effectiveness of the computational procedure to model estrogenic activity of ZEN and reduced metabolites was verified using experimental data. These results justify the application of our *in silico* model to generate highly relevant information on putative activity of non-characterized oxidized metabolites. Despite the prediction of an overall quenching effect, 15-OH-ZEN was identified as a potential concern which deserves more detailed investigation. The other oxidized ZEN metabolites were estimated to raise low estrogenic concern. The aim of this *in vitro* / *in silico* approach is to build tools which allow the user to fill data gaps by integrating experimental mechanistic with existing data for similar chemicals. Even if findings on oxidized derivatives belong to the field of activity prediction, the approach presented herein is particularly relevant when data are required quickly for compounds that are not commercially available, unstable and/or difficult to purify/synthetize.

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## Conflict of interest statement

The authors declare that they have no conflict of interest.

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