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Highlights

-We determined the in vitro genotoxicity of toxins, OA, DTX-1 and -2 on HepaRG cells

- OA, DTX-1 and -2 were classified as aneugens using the combination of pH3 and γH2AX

-BMD modelling demonstrated that DTX-1 is the most potent aneugen among toxins

ABSTRACT

The phycotoxins, okadaic acid (OA) and dinophysistoxins 1 and 2 (DTX-1 and -2), are protein phosphatase PP2A and PP1 inhibitors involved in diarrhetic shellfish poisoning (DSP) in humans. Data on the *in vivo* acute toxicity of the OA-group toxins show some differences and the European Food Safety Authority (EFSA) has determined toxicity equivalent factors (TEFs) of one for the reference toxin, OA, as well as for DTX-1 and 0.6 for DTX-2. However, recent *in vitro* studies indicated that DTX-1 seems to be more toxic than OA. As OA was described as apoptotic and aneugenic compound, we analyzed the DNA damage responses induced by the 3 toxins through γH2AX and pH3 biomarkers on proliferative HepaRG cells using High Content Analysis. We quantitatively examined the responses for γH2AX and pH3 by benchmark dose analyzing (BMD) using PROAST software. We found that the three toxins increased both γH2AX- and pH3-positive cells populations in a concentration-dependent manner. The 3 toxins induced mitotic arrest, characteristic of aneugenic compounds, as well as DNA strand-breaks concomitantly to cytotoxicity. BMD analysis showed that DTX-1 is the most potent inducer of DNA damage, followed by OA and DTX-2. The quantitative genotoxic data provided in this study are additional findings for reconsidering the estimated TEFs of this group of phycotoxins.

Keywords (3 to 6 keywords)

Genotoxicity, phycotoxins, HepaRG, γH2AX, pH3, BMD

1. Introduction

Diarrhetic Shellfish Poisoning (DSP) is a gastrointestinal intoxication characterized by gastro-intestinal symptoms such as diarrhea, nausea and vomiting. It is caused by ingestion of shellfish contaminated with okadaic acid (OA) and its analogues, the dinophysistoxins (DTXs), toxins synthesized by marine dinoflagellates such as *Dinophysis* and *Prorocentrum* [1,2]. OA is the major DSP toxin found in Europe, while the methylated derivative dinophysistoxin-1 (DTX-1) has been mainly found in Japan [2]. Some European countries (Ireland, Italy, Spain and Portugal) have also detected DTX-1 and the isomeric analogue DTX-2 in shellfish [3].

OA and its analogues inhibit serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A), which are essential in the regulation of intracellular processes in mammalian cells [4,5]. The structural difference between OA and its analogues with respect to the number and position of methyl groups has been shown to modulate the affinity of the toxin for the catalytic site of PP2A. For PP2A, the inhibition potency ranged DTX-1>OA>DTX-2, but differs for PP1 OA>DTX-1>DTX-2 [6–8].

The acute *in vivo* toxicity of these toxins has been primarily investigated by intraperitoneal injection in mice, and similar lethal dose 50 (LD50) values have been calculated for DTX-1 and OA, while DTX-2 was shown to be 0.6-times as potent. Therefore, the European Food Safety Authority (EFSA) has determined toxicity equivalent factors (TEFs) of 1 for the reference toxin, OA, as well as for the DTX-1 analogue and 0.6 for DTX-2 [9] . However, a recent paper showed that DTX-1 was more toxic than OA after intraperitonaeal injection (ip) in mice, with a LD50 of 150.4 and 185.6 µg/kg pc for DTX-1 and OA, respectively [10]. Similar results were obtained after oral administration of toxins in mice, with LD50 of 487, 760 and 2,262 µg/kg bw for DTX-1, OA and DTX-2 respectively [11]. Consequently, TEFs

values are rather expected as 1 for OA, 1.5 for DTX-1 and 0.3 for DTX-2 after oral administration, and 1 and 1.2 for OA and DTX-1, respectively after i.p administration.

In vitro OA was shown to induce DNA damage, including DNA adducts, DNA double-strand-breaks (DSBs), micronucleus induction and chromosome loss [12–15]. Less information are available concerning the genotoxic potential of DTX-1 and -2. Nevertheless, a multiparametric analysis recently showed that OA, DTX-1, and DTX-2 were highly cytotoxic on Caco-2 and HepaRG cells, inducing cell loss, activation of caspase-3 and γ-H2AX formation [16,17]. Moreover, if the three toxins induced similar *in vitro* effects, around five-fold lower DTX-1 concentrations compared to OA and DTX-2 were necessary to display the same effect level in human intestinal Caco-2 and HT-29 MTX cells [16].

The aim of this study was to investigate the genotoxic potencies of DSP toxins in proliferative human liver HepaRG cells using the combination of H2AX and pH3 assays. The combination of these two markers permits to detect genotoxic chemicals with DNA and no-DNA damaging properties as previously published [18–20]. The *in vitro* γH2AX and pH3 data obtained were used to determine the relative potencies of the three DSPs toxins through concentration-responses modelling using PROAST benchmark dose (BMD) software. The BMD concept was used as an alternative to the NOAEL to derive point of departure for toxicity data. The Benchmark procedure can be applied to various types of data and recently quantitative assessment with BMD modelling has been employed for in vitro genotoxicity data [21–23]. The data provide further inside in congener specific in vitro γH2AX and pH3 induction potencies of DSPs toxins, which can be used to refine current TEFs.

2. Methods

2.1.Chemicals and reagents

Dimethylsulfoxide (DMSO) and insulin were purchased from Sigma (St. Quentin-Fallavier, France). Okadaic acid, DTX-1 and DTX-2 were purchased from IMB/NRC (Halifax, NS Canada). Methylmethanesulfonate (MMS) was supplied by Acros Organics (Fairlawn, NJ). Colchicine (COL) was purchased by Serva. Williams' E medium, Fetal Bovine Serum Fetalclone II (FBS), penicillin and streptomycin were purchased from Invitrogen Corporation (Illkirch, France). Fetal bovine serum (FBS) was purchased by Dutscher (Brumath France). Hydrocortisone hemisuccinate was from Upjohn Pharmacia (Guyancourt, France). The primary and secondary antibodies were purchased from Abcam (Cambridge, UK): mouse monoclonal anti γH2AX ser139 (ab26350), rabbit monoclonal anti Histone H3 (phospho S10) (ab5176), goat anti-mouse IgG H&L AlexaFluor 647 (ab150115). goat anti-rabbit IgG H&L 488 (ab150077). Formaldehyde and Giemsa were provided by Fisher (Illkirch-Graffenstaden, France).

2.2.Cell Culture and treatment

HepaRG cells were cultured in Williams E medium (Eurobio, Les Ulis, France) supplemented with 10% FCS (Perbio, Brebières, France), 100 units/mL penicillin (Invitrogen Corporation, Illkirch, France), 100 µg/mL streptomycin (Invitrogen Corporation), 5 µg/mL insulin (Sigma-Aldrich, Lyon, France), 2 mM L-glutamine (Thermofisher, Waltham, MA, USA), and 25 µg/mL hydrocortisone succinate (Pharmacia & Upjohn, Guyancourt, France). Undifferentiated proliferative HepaRG cells (passages 13–19) were seeded at a density of 26,000 cells/cm2 in 96-well plates. Twenty-four hours after seeding, HepaRG cells were treated with different concentrations of toxins for 24 hours in culture medium free of FBS.

2.3.Cellular imaging and High Content Analysis (HCA)

After 24 h treatment with toxins, cells were fixed 10 min with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Plates were then incubated in blocking solution (PBS with 3% BSA and 0.05% Tween-20) for 30 min before addition of primary antibodies. All antibodies were prepared in blocking solution. Primary antibodies (1:1000) were incubated overnight at 4°C. After three washing steps with PBS, secondary antibodies (1:1000) were incubated for 45 min at room temperature. Cells were then washed three times with PBS + 0.05% Tween-20. Nuclei were stained with 1 µg/mL DAPI in PBS for 5 min for automated cell identification by HCA. Plates were scanned with an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, USA) and analyzed using the Target Activation module of the BioApplication software. For each well, 10 fields (10X objective) were scanned and analyzed for immunofluorescence quantification. Cell numbers were determined by quantification of cell nuclei from DAPI staining and were expressed as percentage of cells compared to control. **γ**H2AX and pH3 were quantified in cell nuclei and expressed as fold compared to control, and nuclear area was determined for each cell.

2.4.Data analysis and BMD modelling

All experiments were repeated at least 3 times. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc tests using Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). All error bars denote SEM. Statistical significance was depicted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The γH2AX, pH3 and nuclear area data were quantitatively evaluated by benchmark dose (BMD) analysis using PROAST Software (version 65.2, developed by the Dutch National Institute for Public Health and the Environment, RIVM) following the technical guidance [24]. Briefly, the Hill and the exponential models combined were used for modeling the continuous concentration-dependent data [23]. The best choice between the two model

families was made using the AIC (Akaike Index Criteria) as proposed by EFSA Guidance. The BMD20 values (20% increase over the vehicle control response) and the BMDL values (the upper and lower 95% CIs of the BMD) were calculated for each data set. Compared to classical BMR (benchmark response) values (5% or 10% in risk assessment), that choice of a 20% BMR could seem strange. However, considering that the dose-effects curves are sigmoïds with a long horizontal beginning, choosing a BMR of 20% highly decreases the uncertainty around the BMD and balances this uncertainty around the BMD value. In that case the ratio BMD/BMDL is as efficient as BMDU/BMDL or any other in evaluating that uncertainty. In order to determine the RPF of a given compound compared to the value of OA, the ratio of the BMD and BMDL of OA and the BMD and the BMDL of selected compound were determined. RPFs larger than 1 point to a higher potency than OA and below 1 to a lower potency.

3. Results

3.1.Cytotoxicity of phycotoxins

After 24 h of treatment, cytotoxicity of phycotoxins, MMS and COL was determined by HepaRG cell count compared to negative control (**Fig.1**). DTX-1 was the most cytotoxic compound with an IC50 of 6.75 nM, followed by OA and DTX-2, with IC50 of 13.97 and 18.43 nM, respectively (**Table 1**). The IC50 of the the positive aneugenic (COL) and clastogenic (MMS) compounds were calculated to 65.48 nM and 163.8 µM respectively (**Table 1**).

3.2.γH2AX and pH3 induction on HepaRG cells

Proliferative HepaRG cells were exposed to increasing concentrations of phycotoxins OA, DTX-1 and DTX-2. COL and MMS were used as positive aneugenic and clastogenic compounds, respectively. A concentration-dependent increase of H2AX and pH3 was observed for the three phycotoxins after 24 h of exposure (Figure 2). The positive aneugen

COL increased pH3 from 78 nM, while γH2AX also increased but concomitantly with cytotoxicity. Statistically significant increases of pH3 were observed at 20, 5 and 10 nM for OA, DTX-1 and DTX-2 respectively. COL induced pH3 at 150 nM. γH2AX was also statistically increased with toxins at 20, 5 and 20 nM for OA, DTX-1 and DTX-2, respectively. The positive clastogen MMS increased γH2AX from 200 µM. COL increased also γH2AX, concomitantly with pH3 induction. These data showed that the three toxins seem to have a similar genotoxic mechanism of action and could be classified as aneugenic rather than clastogenic. We also observed that the DSP toxins decreased nuclear area at the same concentrations.

3.3.BMD modelling of γH2AX and pH3 induction and nuclear area data and comparison of DSP toxins potency

In order to compare the potency of DSP toxins in the pH3 and γH2AX assays, the PROAST software was applied to analyze concentration-response data and to determine BMD and BMDL20. Figure 3 illustrates the two curves on the pH3 data fitted by PROAST for OA and DTX-1. The BMD, BMDL20 and RPF values that were determined on the 2 endpoints with the 3 toxins are summarized in Table 2. For pH3 induction, we observed a similar BMDL value around 0.36 nM for the three toxins, whereas COL was 50 fold less potent. However, the BMD value was lower for DTX-1 and similar for AO and DTX-2 and the RPF values for pH3 induction based on BMD values were 2.11 and 1.04 for DTX-1 and -2, respectively. For γH2AX, RPFs for DTX-1 and -2 were calculated as 2 and 5.25 based on BMDL and 2.33 and 1.42 based on BMD. For nuclear area, only OA and DTX-1 induced an effect on this parameter and RPFs were calculated as 119 and 8.4 based on BMDL and BMD, respectively.

4. Discussion

The aims of the present study were to determine the concentration-dependent genotoxic effect of DSP toxins using the combination of γH2AX and pH3 assays, to characterize their aneugenic potential and to compare their genotoxic potencies with BMD analysis. We found that the combination of γH2AX and pH3 provided a relevant surrogate readout to identify clastogenic and aneugenic compounds as previously published [19,20,25,26]. In fact, COL and MMS were clearly classified as aneugenic and clastogenic, respectively, on proliferative HepaRG cells with these two markers. However, in our experimental conditions, we observed that COL also increased γH2AX, as previously published with other aneugenic compounds in HepG2 cells [20], whereas such increase of γH2AX with aneugens failed to be detected in TK6 cells [25]. This discrepancy could be explain by the fact that, during flow analysis of TK6 cells, highly fluorescent γH2AX-positive cells that could be apoptotic cells were excluded [27,28], whereas we did not exclude them. In the same way, we observed an increase of pH3 with the clastogen MMS at high concentrations, that could also be due to cytotoxicity [27].

We found that OA and its analogues DTX-1 and -2 highly increased pH3-positive HepaRG cells, illustrating their aneugenic potential. OA has yet been demonstrated as aneugenic in previous studies [13,29], but it is the first time that DTX-1 and -2 are clearly classified as aneugenic also. Considering their similar molecular initiating event, the inhibition of PP1 and PP2A, it is not surprising that they induced the same cellular effects such as mitotic arrest followed by chromosome loss. We also observed that the three toxins, as well as colchicine, induced γH2AX certainly due to apoptosis as previously observed [14,17,28]. In fact, OA, DTX-1 and -2 were found to induce γH2AX after 24-hrs of treatment on intestinal Caco-2 and HT-29 MTX cells [17].

The BMD has been used recently in genetic toxicology for concentration-dose-response modeling to estimate the point of departure [21,22,30,31]. In fact, this approach is less dependent on dose selection and spacing and takes into account the shape of the dose-response curve [32]. In the present study, we performed the BMD analysis on γH2AX, pH3 and nuclear area markers for each toxin and for the positive compounds to rank the genotoxicity potential of DSP toxins. The BMR specified is one standard deviation from the control and we also calculated a BMDL of 20% over the control. Then the BMD and the BMDL calculated here are in line with NOAEL and LOAEL, respectively. Based on pH3 data, we found that DTX-1 is the most potent aneugenic compound with a BMD calculated at 0.89 nM, followed by OA and DTX-2 with a similar potential. The resulting RPFs based on BMD values, using OA as reference toxin, were 2.1 and 1 for DTX-1 and -2, respectively. Therefore, DTX-1 is 40-fold and OA and DTX-2 20-fold more genotoxic than the positive aneugenic compound colchicine. Similar results were obtained with γH2AX: DTX-1 was two-fold more potent than OA, whereas a RPF of 1.42 was determined for DTX-2 based on BMD values. The nuclear area gives indication of the effect on the size of the nuclei. Only OA and DTX-1 exhibited a decrease of this parameter. RPF was calculated as 8.4 for DTX-1 based on BMD modeling. Taken together, these results showed that DTX-1 is a more potent genotoxin than OA and DTX-2, with a RPF around 2, whereas DTX-2 exhibited an equal genotoxic potential to OA.

These results confirmed some in vitro and in vivo data demonstrating that DTX-1 was more toxic than OA. DTX-1 was found to induce cytotoxic effects at five-fold lower concentrations than OA and DTX-2 on intestinal Caco-2 and HT-29 MTX cells [17]. However, instead of the BMD approach, this comparison was influenced by the concentration-spacing selection. Although EFSA fixed a TEFs equal to 1 for DTX-1, *in vivo* data showed also that DTX-1 was more toxic than OA in particular after oral administration in mice [10,11]. The higher toxicity

of DTX-1 could be partially explained by a slightly higher inhibition potency on PP2A of DTX-1 compared to OA, with a RPF determined as 1.3 [11]. Nevertheless, OA seems to be a more potent PP1 inhibitor than DTX-1 [8]. Another non-exclusive explanation for the higher oral toxicity of DTX-1 compared to OA is the highest efficiency of intestinal absorption of DTX-1 as observed in vitro with Caco-2 cells monolayer [33].

In conclusion, our results suggest that DSP toxins, OA, DTX-1 and -2, seem to have an identical genotoxic mode of action and could be considered as aneugenic compounds based on the combination of pH3 and γH2AX endpoints. However, considering also the increases of γH2AX marker concomitantly with pH3, further investigations are required to determine if these toxins could induced DSBs independently to mitotic arrests and apoptosis. Moreover, BMD modelling demonstrated that DTX-1 has a more in vitro genotoxic potential than OA and DTX-2 and that this additional finding should be taken into account when revising TEFs values for DSP toxins.

AUTHOR CONTRIBUTIONS

LLH conceived the study and designed the experiments; LLH performed the experiments; LLH and ACR analyzed the data; LLH, ACR and VF wrote the manuscript. All authors reviewed the manuscript and finally approved this version to be published.

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Table 1: IC50 values of OA, DTX-1, DTX-2, COL and MMS on HepaRG cells after 24h of treatment

Compounds	IC50 (nM)	95% CI
OA	13.97	4.33-45.05
DTX1	6.75	3.82-11.95
DTX2	18.43	8.97-37.88
COL	65.48	49.03-87.46
MMS	163.8*	22.34-1201

*µM

Table 2: BMD20, BMDL20 and RPF values determined on different endpoints with the tested compounds

	pH3				γH2AX				Nuclear area			
	BMDL (nM)	**RPF**	**BMD20 (nM)**	**RPF**	**BMDL (nM)**	**RPF**	**BMD20 (nM)**	**RPF**	**BMDL (nM)**	**RPF**	**BMD20 (nM)**	**RPF**
OA	0.36	1	1.86	1	0.84	1	2.56	1	2.97	1	14	1
DTX-1	0.38	0.95	0.89	2.11	0.42	2	1.1	2.33	0.025	118.80	1.72	8.14
DTX-2	0.38	0.95	1.8	1.04	0.16	5.25	1.8	1.42	/	/	/**	/**
COL	19.4	0.02	37	0.05	15.7	0.05	33	0.08	/	/	/*	/*
MMS	513 000	0.000001	95 000	0.00002	202 000	0.000004	50 000	0.000051	/	/	/*	/*

BMD20 = benchmark dose for a 20% BMR

BMDL 20 =Benchmark dose lowest limit of confidence interval for a 20% BMR,

*no effect, **effect inferior to 20%

Figure legends

Fig. 1. Dose response curves represent the cell count of HepaRG compared to vehicle control conditions after 24-h of treatment.

Fig. 2. Concentration-response curves for pH3, γH2AX and nuclear area induction effects in proliferative HepaRG cells exposed with increasing concentration of OA, DTX-1 and -2 toxins for 24 h (mean ± SD).

Fig.3. Results of BMD modelling of concentration-response data for pH3 induction by OA (A, B) and DTX-1 (C, D). The figure shows the files generated by PROAST, with the best fitted expermimental model (A,C) and the best fitted Hill model (B, D).

Fig. 1.

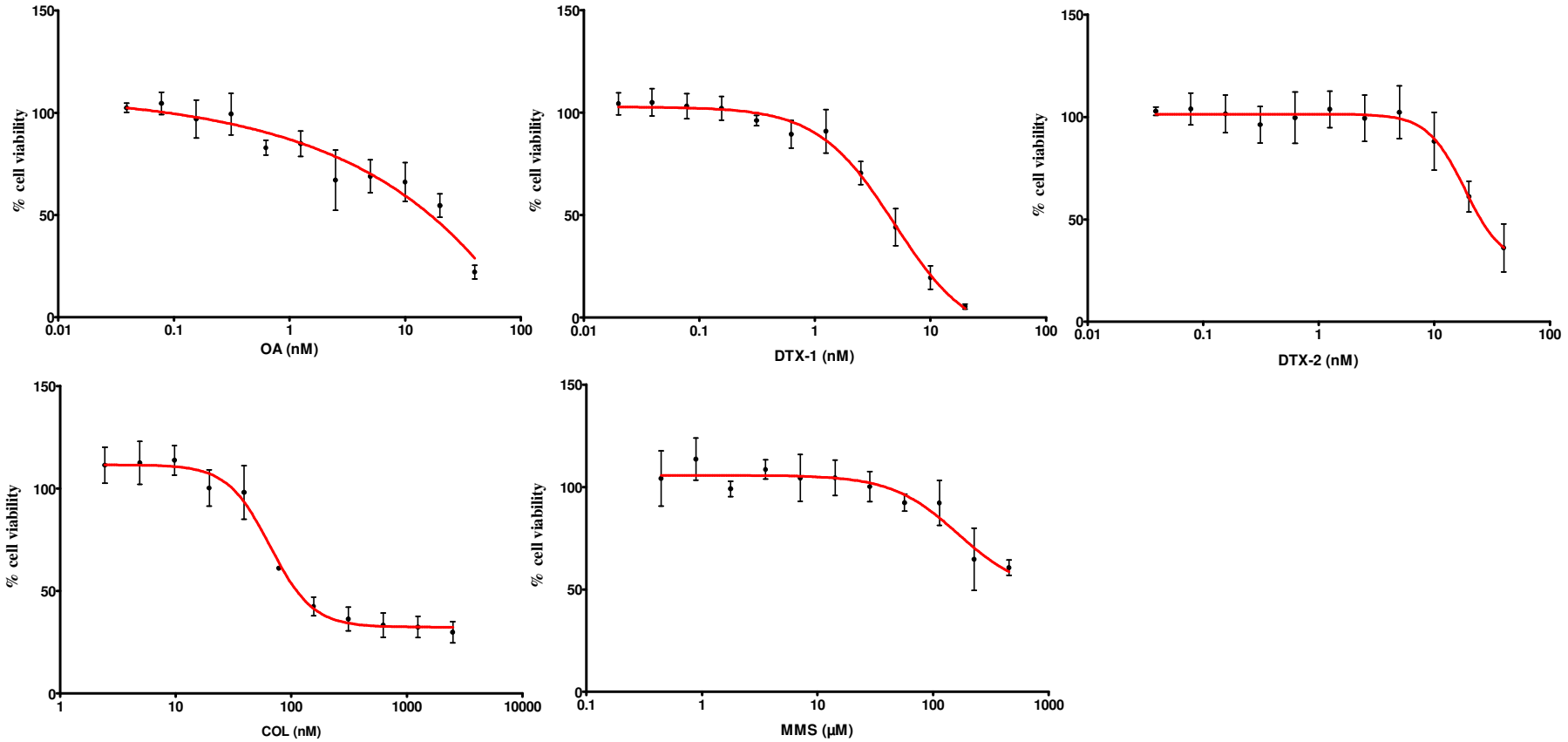

% cell viability
150
100
50
0
0.01
0.1
1
10
100
OA (nM)
% cell viability
150
100
50
0
0.01
0.1
1
10
100
DTX-1 (nM)
% cell viability
150
100
50
0
0.01
0.1
1
10
100
DTX-2 (nM)
% cell viability
150
100
50
0
1
10
100
1000
10000
COL (nM)
% cell viability
150
100
50
0
0.1
1
10
100
1000
MMS (µM)

Fig. 2.

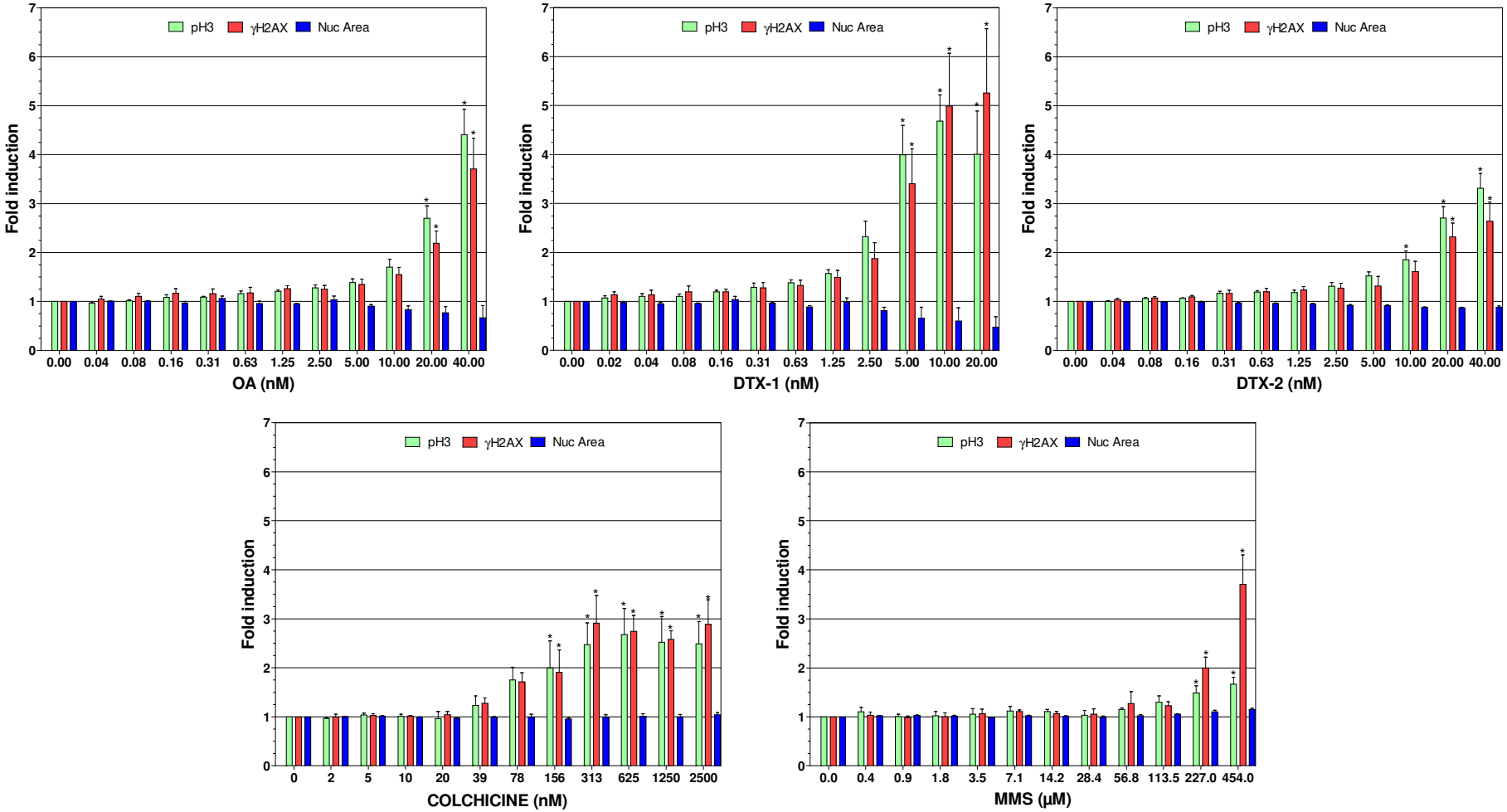
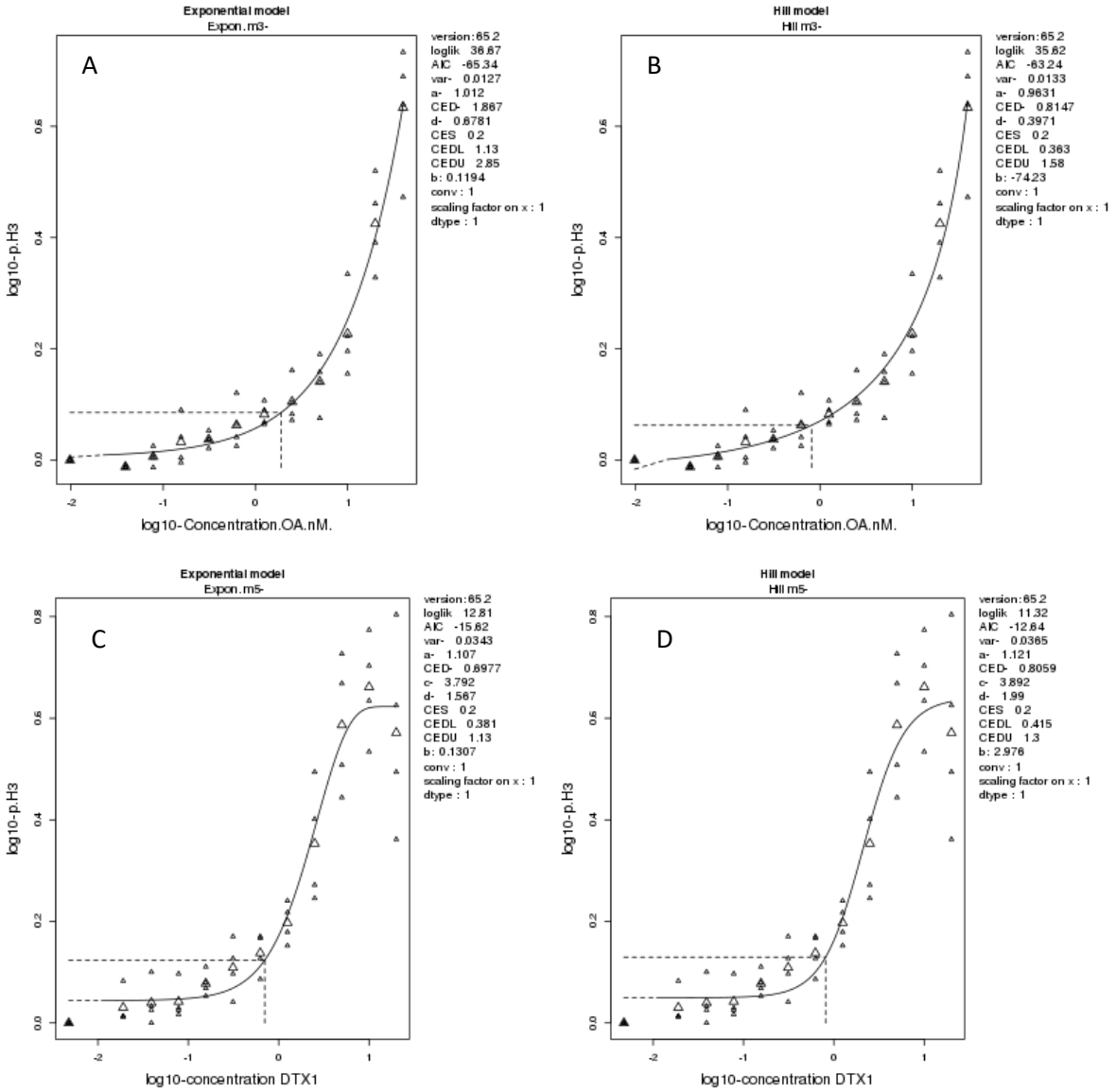

pH3
γH2AX
Nuc Area
Fold induction
OA (nM)
0.00 0.04 0.08 0.16 0.31 0.63 1.25 2.50 5.00 10.00 20.00 40.00
DTX-1 (nM)
0.00 0.02 0.04 0.08 0.16 0.31 0.63 1.25 2.50 5.00 10.00 20.00
DTX-2 (nM)
0.00 0.04 0.08 0.16 0.31 0.63 1.25 2.50 5.00 10.00 20.00 40.00
COLCHICINE (nM)
0 2 5 10 20 39 78 156 313 625 1250 2500
MMS (µM)
0.0 0.4 0.9 1.8 3.5 7.1 14.2 28.4 56.8 113.5 227.0 454.0

Fig. 3.


Exponential model
Expon.m3-
A
version:65.2
loglik 36.87
AIC -65.34
var- 0.0127
a- 1.012
CED- 1.867
d- 0.8781
CES 0.2
CEDL 1.13
CEDU 2.85
b: 0.1194
conv : 1
scaling factor on x : 1
dtype : 1
log10-p.H3
log10-Concentration.OA.nM.
Hill model
Hill m3-
B
version:65.2
loglik 35.82
AIC -63.24
var- 0.0133
a- 0.9631
CED- 0.8147
d- 0.3971
CES 0.2
CEDL 0.363
CEDU 1.58
b: -74.23
conv : 1
scaling factor on x : 1
dtype : 1
log10-p.H3
log10-Concentration.OA.nM.
Exponential model
Expon.m5-
C
version:65.2
loglik 12.81
AIC -15.62
var- 0.0343
a- 1.107
CED- 0.8977
c- 3.792
d- 1.567
CES 0.2
CEDL 0.381
CEDU 1.13
b: 0.1307
conv : 1
scaling factor on x : 1
dtype : 1
log10-p.H3
log10-concentration DTX1
Hill model
Hill m5-
D
version:65.2
loglik 11.32
AIC -12.64
var- 0.0365
a- 1.121
CED- 0.8059
c- 3.892
d- 1.99
CES 0.2
CEDL 0.415
CEDU 1.3
b: 2.976
conv : 1
scaling factor on x : 1
dtype : 1
log10-p.H3
log10-concentration DTX1