Evaluation of the genotoxic potential of apoptosis inducers with the γH2AX assay in human cells

Laure Khoury, Daniel Zalko, Marc Audebert

Version of Record: https://www.sciencedirect.com/science/article/pii/S1383571820300358
Manuscript_a298d9a9ec5f5d62471bf4d2eef6b399

Evaluation of the genotoxic potential of apoptosis inducers with the γH2AX assay in human cells

Laure Khoury[1, #], Daniel Zalko[1], and Marc Audebert[1,*]

[1] Toxalim, Université de Toulouse, INRAE, INP-ENVT, INP-EI-Purpan, Université de Toulouse 3 Paul Sabatier, Toulouse, France

[#] Present address: PrediTox, Toulouse, France

Short title: Apoptotic inducers genotoxic potential

*** Corresponding author:**

Marc AUDEBERT, PhD

ORCID 0000-0001-7898-6912

INRAE-UMR1331, Toxalim, 180 chemin de Tournefeuille BP 93173

31027 Toulouse Cedex 3, France, Phone +33 582 066 311

E-mail address: marc.audebert@inrae.fr

Abstract

Human risk assessment of genotoxic chemicals is an important area of research. However, the specificity of *in vitro* mammalian genotoxicity assays is sometime low, as they yield to misleading positive results that are not observe in *in vivo* studies. Apoptosis can be a confounding factor in the interpretation of the results. Recently, a new strategy for genotoxicity screening, based on the combined analysis of phosphorylated histones H2AX (γH2AX) and H3 (pH3), was proposed to discriminate efficiently aneugenic from clastogenic compounds. However, γH2AX biomarker could also be induce by apoptosis. The aim of the present study was to investigate the specificity of this genotoxic biomarker. For this purpose, we analyzed 26 compounds inducing apoptosis by different mechanism of action, with the γH2AX assay in three human cell lines after 24 h treatment. Most of the tested chemicals were negative in the assay, whatever the cell line tested. The few compounds that generated positive data have also been report positive in other genotoxicity assays. The data presented here demonstrate that the γH2AX assay is not vulnerable to the generation of misleading positive results by apoptosis inducers. Currently, no formal guidelines have been approve for the γH2AX assay for regular genotoxicity studies, but we suggest that this biomarker could be used as a new standard genotoxicity assay.

Keywords: Genotoxicity, apoptosis, H2AX, micronucleus, cell lines.

1. Introduction

In vitro genotoxicity assays demonstrate sometime low specificity and it is difficult to differentiate a true genotoxic effect from cytotoxicity. In the micronucleus (MN) assay, apoptosis can be a confounding factor in the interpretation of the MN induction [1, 2]. Moreover, most genotoxicity assays are low throughput and time-consuming, and do not permit to distinguish the genotoxic mode of action of the tested compounds (as aneugens or clastogens). Recently, a new strategy for genotoxicity screening has been propose, based on the combined analysis of γH2AX and pH3 biomarkers, which permit to discriminate efficiently clastogens, aneugens and misleading cytotoxic chemicals [3-6].

Apoptosis process has an important role during the development process and in normal tissue homeostasis for the elimination of altered cells or cells that are no longer needed [7]. This process is also the last choice for cells if repair of DNA damage is slow or incomplete. The cell death triggered by apoptosis is the result of two important apoptotic pathways [8]. The extrinsic signaling pathway mediated by death receptors that are the target of specific ligands like TRAIL [9, 10]. The intrinsic pathways governed by mitochondria with different pro-apoptotic proteins like Bcl-2 or Bax [11, 12] or by endoplasmic reticulum stress signaling [13-15]. Some compounds can induce apoptosis by a direct interaction with the different components of the apoptotic signaling pathways. Numerous apoptotic mechanism of action induced by chemicals have been demonstrate, targeting mitochondria and associated proteins, endoplasmic reticulum or proteasome [16]. Apoptosis is also the consequence of genotoxin insults, as revealed in cells treated with various genotoxic agents, including anticancer drugs [17].

Phosphorylation of histone H2AX, named γH2AX, is a marker of DNA damage [18]. This phosphorylation event is amplified after DNA double-strand breaks [19-21], and promote cell cycle checkpoint arrest, DNA repair and apoptosis [22-24]. Indeed, the function of H2AX is believed to be associated primarily with repair of DNA damage, but this histone play also a role

in the apoptotic process. The H2AX phosphorylation is required for DNA ladder formation, but not for the activation of caspase-3; and the JNK/H2AX pathway cooperates with the caspase-3/CAD pathway resulting in cellular apoptosis [25]. The γH2AX genotoxicity biomarker is now currently used in numerous genotoxicity assays and demonstrated a high predictivity [6]. Although different genotoxicity studies have demonstrated the high specificity of the γH2AX biomarker [5, 6, 26], no study has been specifically perform to assess the effect of apoptosis inducers on the γH2AX biomarker.

The aim of the present study was to study the effect on the γH2AX biomarker of a panel of apoptosis inducer compounds with different mechanism of action (Table 1). The screening has been realize on three human cell lines with p53 wild type status and distinct biotransformation properties: the hepatic cell line HepG2, the colon cells LS-174T and the renal cell line ACHN. Both HepG2 and LS-174T cell lines have important bioactivation capabilities, whereas ACHN cells have poor metabolizing capacities [27, 28]. For all the chemicals, the phosphorylation of H2AX and the cytotoxic potential was simultaneously examine with the ICW technique after 24 h treatment.

2. Materials and methods

2.1. Chemicals and reagents

All stock solutions of the compounds were prepared in 100 % dimethyl sulfoxide (DMSO). From the stocks, 3-fold dilution series were prepared. All the compounds were of analytical grade, and were purchase from Sigma-Aldrich (Saint Quentin Fallavier, France). Penicillin, streptomycin, trypsin, PBS, RNAse A, and Triton X-100 were purchase from Sigma-Aldrich. The phosphatase inhibitor cocktail tablets ("PHOSSTOP") were purchased from Roche, and the blocking solution (MAXblock Blocking Medium) was purchased from Active Motif (Belgium). CF770 antibody (goat antibody anti-rabbit and anti-mouse) and RedDot2 were purchase from Biotium (Hayward, California, USA).

2.2. Selection of compounds

The identities of the 26 chemicals are listed in Table 1, along with current knowledge of their toxic mechanism of action and previous MN induction report. All the compounds were select from the literature and represent a broad range of apoptosis inducing activities. The highest concentration tested for most of the compounds was 100 µM, in accordance with the concentration currently used in *in vitro* genotoxicity tests [29].

2.3. Cell culture

HepG2 human hepatoblastoma cells (ATCC N° HB-8065), ACHN human renal adenocarcinoma cells (ATCC N° CRL 1611) and LS-174T human epithelial colorectal adenocarcinoma cells (ATCC N° CL-188) were grown in αMEM medium supplemented with 10 % FBS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Cultures were maintain in a humidified atmosphere with 5 % CO2 at 37 °C and the medium was refresh every two to three days during sub-culturing.

2.4. In-cell western γH2AX assay

The γH2AX assay using the in-cell western technique was performed as previously described [5, 26, 30, 31]. Different primary antibodies were used: rabbit monoclonal anti-γH2AX (clone 20E3) from Cell Signaling technology, rabbit monoclonal anti-H2AX (clone 12D1), and anti-H1 (39708) from Active Motif (Belgium). For cytotoxicity determination, the DNA content (as a surrogated to the number of cells) recorded in the different experiments was compare with the DNA content in the control vehicle treatment. All experiments were perform at least in triplicate, independently.

2.5. Data analysis

Genotoxicity was consider positive when a compound induced a statistically significant 1.5-fold histone phosphorylation γH2AX at a level of cytotoxicity below 50% compared to the control. These parameters were based on our previous studies [5] and are similar to those used by other groups who use γH2AX quantification [3, 32]. Statistical analyses were perform using Student's t-test with Excel 2010 Software. Error bars represent SEM (the standard error of the mean). After treatment, statistically significant decreases in the phosphorylation of H2AX or non-phosphorylated H2AX and H1, were compared with controls using Student's test; *, $p < 0.05$; **, $p < 0.01$.

3. Results

First, we tested a compound activating apoptosis through an exogenous signaling pathway. A TRAIL death receptor ligand was test in the three cell lines (HepG2, LS-174T and ACHN) and we observed cytotoxicity in the ACHN cells (Figure 1). No induction of γH2AX was detected with this compound whatever the cell lines used (Table 2). Then, five compounds targeting apoptotic proteins like Bax, Bak or PKC (Table 1), were evaluate for their effects on histone H2AX phosphorylation status and cytotoxicity, in the three human cell lines. Results are listed in Table 2. In the three cell lines used, embelin was not genotoxic but cytotoxic at 100 µM (Figure 1). Triton X100 decreases the H2AX phosphorylation in HepG2 and LS-174T cell lines but not in ACHN cells. At the same time, triton X100 was cytotoxic in HepG2 and ACHN cell lines at 100 µM and at 30 µM in LS-174T cells (Figure 1). ICRF-193 induced γH2AX in HepG2 and ACHN cell lines and was not cytotoxic in the three cell lines tested (Figure 1). In HepG2 and LS-174T cell lines, staurosporine and zapotin increase γH2AX (Figure 1). No cytotoxic effect was observe for zapotin in HepG2 or ACHN cells. Staurosporine did not induced apoptosis in HepG2 cells. However, this compound was cytotoxic in ACHN and LS-174T cell lines.

We tested two compounds that interact, directly or not, with the Bcl-2 protein family (Table 1). In the three cell lines, YC137 increased the H2AX phosphorylation at the same concentration (0.3 µM) and was cytotoxic at 100 µM (Figure 2). The BH3 domain inhibitor (BH3i-1) was cytotoxic in the three cell lines used. We tested two protein synthesis inhibitors. Emetine increases γH2AX at 1µM in HepG2 and ACHN cells and was cytotoxic at 0.1 µM in LS-174T cell lines. Reveromycin A was cytotoxic in LS-174T cells at 3 µM but not genotoxic in any cell lines (Figure 2).

A set of four compounds that interact with the mitochondria were tested (Table 2). Carboxyatractyloside potassium salt (CAT) and sodium iodoacetate were neither genotoxic nor

cytotoxic in the three cell lines used at the highest concentration tested (100 µM). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and costunolide did not induced γH2AX and were cytotoxic in the three cell lines used (Figure 3).

Three endoplasmic reticulum (ER) stress inducers (17-AAG, thapsigargin and tunicamycin) were analyse for their genotoxic and cytotoxic effects (Table 2). None of these chemicals was genotoxic in any cell line. However, we noted that the cytotoxicity of these chemicals was higher in LS-174T cells compared to HepG2 and ACHN cell lines (Figure 4).

A set of five proteasome inhibitors was then test in the three cell lines (Table 2). For the 20S proteasome inhibitors, gliotoxin and tributyltin were not genotoxic and highly cytotoxic to the three cell lines at 3 and 1 µM, respectively. The three 26S proteasome inhibitors (bortezomib, MG-115 and MG-132) were not genotoxic in any cell line. However, we noted that the cytotoxicity of these chemical was higher in LS-174T cells compared to HepG2 and ACHN cell lines (Figure 5).

Finally, we tested a set of four compounds that target the p53 pathway (Table 2). Pifithrin α and nutlin-3 did not induced γH2AX and were cytotoxic equivalently in the three cell lines used (Figure 6). On the opposite, RITA and P5091 induced significantly γH2AX. RITA was observed genotoxic in LS-174T and HepG2 cells at 10 nM but had no genotoxic effect in the ACHN cell line. P5091 treatment increased γH2AX in the three cell lines at 10 µM. Moreover, these two chemicals induced cell death (Figure 6).

4. Discussion

Recently, a new *in vitro* strategy for genotoxicity screening was propose based on the combined analysis of histones γH2AX and pH3, permitting to discriminate efficiently clastogens, aneugens and misleading cytotoxic chemicals [3-6]. However, i*n vitro* genotoxicity assays demonstrate sometime low specificity and difficultly to efficiently differentiate true genotoxic chemical from cytotoxic compound [1, 2]. It is therefore of value to expose new genotoxicity assay to a variety of cytotoxic stimuli in order to assess any potential limitations.

The aim of the present study was to test a panel of apoptosis inducer compounds with different mechanism of action, to evaluate the specificity of the γH2AX assay to differentiate apoptotic from genotoxic agents. The screening has been realize on three human cell lines after 24 h treatment. Cytotoxicity could be a confounding factor in genotoxicity analysis and limit the cytotoxicity to 50 % in data analysis has been proposed to avoid false-positive genotoxic results [33]. From the 26 chemicals tested, only seven (ICRF-193, Staurosporine, zapotin, YC137, Emetine, P5091 and RITA) were detected as genotoxic, demonstrating that apoptosis induction was not a systematic confounding factor in this assay. Moreover, it was recently demonstrate that mild apoptosis induction could leads to DNA damage that, in turn, could promotes genomic instability, cellular transformation, and tumorigenesis.[12].

We decided to investigate in more detail the toxicological effects of the seven positive chemicals in the γH2AX assay. ICRF-193 has been shown to be a topoisomerase II inhibitor [34] and to induce γH2AX [35], so our result is coherent with the mode of genotoxic action of this compound. Staurosporine is frequently use as positive control for apoptosis induction through protein kinase C epsilon inhibition. There are controversial data about the genotoxicity of staurosporine with notably negative [36] and positive MN results [37, 38]. Nevertheless, different studies have observed γH2AX with staurosporine treatment [39-41] and importantly,

this chemical was described to have a tumor-promoting activity [42]. Zapotin, like staurosporine, is a protein kinase C epsilon inhibitor [43]. The observed induction of γH2AX in your study may be linked to this property. We also noted that, as with staurosporine, zapotin induced genotoxicity only in HepG2 and LS-174T cells and over a large concentration range. YC137 is an inhibitor of anti-apoptotic Bcl-2 family members. In agreement with the present study, this chemical has demonstrate a genotoxic property in a MN assay [38]. Emetine is an anti-parasitic compound with few genotoxicity data. However, this compound has demonstrate to be an *in vivo* genotoxin in Drosophila [44]. Concerning the two compounds targeting the p53 pathway observed as genotoxic in your study, P5091 has been demonstrate to induce reactive oxygen species [45]. We previously observed that oxidative stress induced γH2AX [46]. Concerning RITA, this compound have been characterized as an inducer of MN [38] and γH2AX [47]. DNA-protein and DNA-DNA cross-links have been observe in treated cells with RITA [48, 49]. Based on our previous studies, due to the fact that RITA genotoxicity was only observe in cell lines with bioactivation properties (HepG2 and LS-174T cells), we hypothesized that RITA metabolization should be necessary for DNA damage induction [5, 46]. However, we could not excluded that this discrepancy between cell lines could also be linked to disparity in antioxidant capacities, apoptosis signaling, DNA repair proficiency or expression levels of drug transporters. Overall, we concluded that the seven positive compounds in your assay should not be interpret as false-positive results.

We observed a marked decrease in γH2AX with some studied compounds, without a marked decrease in cytotoxicity. We confirmed a more general histone dissociation phenomenon with a decrease of also histones H2AX and H1 after MG-132, MG-115 and 17-AAG treatments in HepG2 cells (Fig. S1). This effect may result from general toxicity and linked to the loss of higher order chromatin compaction and corresponding histone dissociation [50].

One of the interesting result of your study was the negative genotoxic potential of the nutlin-3 compound. This chemical is an inhibitor of the mdm2/p53 protein complex, resulting in the induction of p53. Conflicting genotoxicity results have been published notably with positive gadd45 and MN induction [38]. No γH2AX induction was observe in two other studies [3, 49]. Recently, this chemical was also mentioned as a false-positive genotoxic compound using a transcriptomic approach [51].These results highlight the high specificity of the γH2AX assay.

The observations presented here allow the conclusion that the γH2AX assay is not prone to misleading positive results caused by apoptosis inducer compounds. The majority of the apoptosis-inducing chemicals tested were negative in the γH2AX assay and those that were positive demonstrated genotoxic mechanisms of action. They were also positive in other routinely used *in vitro* genotoxicity tests. The present study confirmed the high specificity (95%) of the γH2AX genotoxicity biomarker reveal by analyzing data with 135 non-genotoxic chemicals [6] and it is capacity to distinguishing genuine genotoxins from general apoptotic stimuli. Currently, no formal guidelines have been approve for the γH2AX assay for regular genotoxicity studies, but we suggest that this biomarker could be used as a new standard genotoxicity assay.

Conflict of interest statement

The authors declare no competing interests.

Author contributions

L.K. performed all of the experiments, data analysis and interpretation through discussions with M.A.. M.A. planned and supervised the project. The first draft of the manuscript was written by L.K.. All the authors discussed the results and contributed to editing of the manuscript.

References

[1] S.M. Galloway, Cytotoxicity and chromosome aberrations in vitro: experience in industry and the case for an upper limit on toxicity in the aberration assay, Environ Mol Mutagen, 35 (2000) 191-201.

[2] C.A. Hilliard, M.J. Armstrong, C.I. Bradt, R.B. Hill, S.K. Greenwood, S.M. Galloway, Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons, Environ Mol Mutagen, 31 (1998) 316-326.

[3] S.M. Bryce, J.C. Bemis, J.A. Mereness, R.A. Spellman, J. Moss, D. Dickinson, M.J. Schuler, S.D. Dertinger, Interpreting in vitro micronucleus positive results: simple biomarker matrix discriminates clastogens, aneugens, and misleading positive agents, Environmental and Molecular Mutagenesis, 55 (2014) 542-555.

[4] S.M. Bryce, D.T. Bernacki, J.C. Bemis, S.D. Dertinger, Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach, Environ Mol Mutagen, 57 (2016) 171-189.

[5] L. Khoury, D. Zalko, M. Audebert, Complementarity of phosphorylated histones H2AX and H3 quantification in different cell lines for genotoxicity screening, Arch Toxicol, 90 (2016) 1983-1995.

[6] B. Kopp, L. Khoury, M. Audebert, Validation of the gammaH2AX biomarker for genotoxicity assessment: a review, Arch Toxicol, 93 (2019) 2103-2114.

[7] T.G. Cotter, Apoptosis and cancer: the genesis of a research field, Nat Rev Cancer, 9 (2009) 501-507.

[8] M.O. Hengartner, The biochemistry of apoptosis, Nature, 407 (2000) 770-776.

[9] A. Ashkenazi, V.M. Dixit, Death receptors: signaling and modulation, Science, 281 (1998) 1305-1308.

[10] J.D. Twomey, S.R. Kim, L. Zhao, W.P. Bozza, B. Zhang, Spatial dynamics of TRAIL death receptors in cancer cells, Drug Resist Updat, (2015).

[11] L. Milane, M. Trivedi, A. Singh, M. Talekar, M. Amiji, Mitochondrial biology, targets, and drug delivery, J Control Release, (2015).

[12] G. Ichim, J. Lopez, S.U. Ahmed, N. Muthalagu, E. Giampazolias, M.E. Delgado, M. Haller, J.S. Riley, S.M. Mason, D. Athineos, M.J. Parsons, B. van de Kooij, L. Bouchier-Hayes, A.J. Chalmers, R.W. Rooswinkel, A. Oberst, K. Blyth, M. Rehm, D.J. Murphy, S.W. Tait, Limited mitochondrial permeabilization causes DNA damage and genomic instability in the absence of cell death, Molecular cell, 57 (2015) 860-872.

[13] D.G. Breckenridge, M. Germain, J.P. Mathai, M. Nguyen, G.C. Shore, Regulation of apoptosis by endoplasmic reticulum pathways, Oncogene, 22 (2003) 8608-8618.

[14] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat Rev Mol Cell Biol, 8 (2007) 519-529.

[15] I. Tabas, D. Ron, Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress, Nat Cell Biol, 13 (2011) 184-190.

[16] G. Ichim, S.W. Tait, A fate worse than death: apoptosis as an oncogenic process, Nat Rev Cancer, 16 (2016) 539-548.

[17] B. Kaina, DNA damage-triggered apoptosis: critical role of DNA repair, double-strand breaks, cell proliferation and signaling, Biochem Pharmacol, 66 (2003) 1547-1554.

[18] S. Singh, V. Kumar, S. Thakur, B.D. Banerjee, S. Chandna, R.S. Rautela, S.S. Grover, D.S. Rawat, S.T. Pasha, S.K. Jain, R.L. Ichhpujani, A. Rai, DNA damage and cholinesterase activity in occupational workers exposed to pesticides, Environ. Toxicol. Pharmacol., 31 (2011) 278-285.

[19] A. Kinner, W. Wu, C. Staudt, G. Iliakis, γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin, Nucl. Acids Res., 36 (2008) 5678-5694.

[20] S. Takahashi, H. Sato, Y. Kubota, H. Utsumi, J.S. Bedford, R. Okayasu, Inhibition of DNA-double strand break repair by antimony compounds, Toxicology, 180 (2002) 249-256.

[21] G.P. Watters, D.J. Smart, J.S. Harvey, C.A. Austin, H2AX phosphorylation as a genotoxicity endpoint, Mutat. Res., 679 (2009) 50-58.

[22] P.A. Jeggo, M. Lobrich, DNA double-strand breaks: their cellular and clinical impact?, Oncogene, 26 (2007) 7717-7719.

[23] K.C. Summers, F. Shen, E.A. Sierra Potchanant, E.A. Phipps, R.J. Hickey, L.H. Malkas, Phosphorylation: the molecular switch of double-strand break repair, Int J Proteomics, 2011 (2011) 373816.

[24] E.P. Rogakou, D.R. Pilch, A.H. Orr, V.S. Ivanova, W.M. Bonner, DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139, J Biol Chem, 273 (1998) 5858-5868.

[25] C. Lu, F. Zhu, Y.Y. Cho, F. Tang, T. Zykova, W.Y. Ma, A.M. Bode, Z. Dong, Cell apoptosis: requirement of H2AX in DNA ladder formation, but not for the activation of caspase-3, Molecular cell, 23 (2006) 121-132.

[26] L. Khoury, D. Zalko, M. Audebert, Validation of high-throughput genotoxicity assay screening using γH2AX in-cell western assay on HepG2 cells, Environmental and Molecular Mutagenesis, 54 (2013) 737-746.

[27] M. Audebert, L. Dolo, E. Perdu, J.P. Cravedi, D. Zalko, Use of the γH2AX assay for assessing the genotoxicity of bisphenol A and bisphenol F in human cell lines, Archives of Toxicology, 85 (2011) 1463-1473.

[28] M. Audebert, A. Riu, C. Jacques, A. Hillenweck, E.L. Jamin, D. Zalko, J.P. Cravedi, Use of the γH2AX assay for assessing the genotoxicity of polycyclic aromatic hydrocarbons in human cell lines, Toxicology Letters, 199 (2010) 182-192.

[29] M.M. Moore, M. Honma, J. Clements, T. Awogi, G.R. Douglas, F. van Goethem, B. Gollapudi, A. Kimura, W. Muster, M. O'Donovan, R. Schoeny, S. Wakuri, Suitable top concentration for tests with mammalian cells: mouse lymphoma assay workgroup, Mutat. Res., 723 (2011) 84-86.

[30] V. Graillot, N. Takakura, L.L. Hegarat, V. Fessard, M. Audebert, J.-P. Cravedi, Genotoxicity of pesticide mixtures present in the diet of the French population, Environmental and Molecular Mutagenesis, 53 (2012) 173-184.

[31] V. Graillot, F. Tomasetig, J.-P. Cravedi, M. Audebert, Evidence of the in vitro genotoxicity of methyl-pyrazole pesticides in human cells, Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 748 (2012) 8-16.

[32] D.J. Smart, Genotoxicity of topoisomerase II inhibitors: An anti-infective perspective, Toxicology, 254 (2008) 192-198.

[33] M.D. Fellows, M.R. O'Donovan, E. Lorge, D. Kirkland, Comparison of different methods for an accurate assessment of cytotoxicity in the in vitro micronucleus test. II: Practical aspects with toxic agents, Mutat Res, 655 (2008) 4-21.

[34] K.C. Huang, H. Gao, E.F. Yamasaki, D.R. Grabowski, S. Liu, L.L. Shen, K.K. Chan, R. Ganapathi, R.M. Snapka, Topoisomerase II poisoning by ICRF-193, J Biol Chem, 276 (2001) 44488-44494.

[35] I. Park, H.K. Avraham, Cell cycle-dependent DNA damage signaling induced by ICRF-193 involves ATM, ATR, CHK2, and BRCA1, Exp Cell Res, 312 (2006) 1996-2008.

[36] S.M. Bryce, J.C. Bemis, S.L. Avlasevich, S.D. Dertinger, In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity, Mutat Res, 630 (2007) 78-91.

[37] S. Meintieres, A. Biola, M. Pallardy, D. Marzin, Using CTLL-2 and CTLL-2 bcl2 cells to avoid interference by apoptosis in the in vitro micronucleus test, Environ Mol Mutagen, 41 (2003) 14-27.

[38] C.H. Topham, N. Billinton, R.M. Walmsley, Nongenotoxic apoptosis inducers do not produce misleading positive results in the TK6 cell-based GADD45a-GFP genotoxicity assay, Toxicol Sci, 128 (2012) 79-91.

[39] D. Rybaczek, M. Kowalewicz-Kulbat, Premature chromosome condensation induced by caffeine, 2-aminopurine, staurosporine and sodium metavanadate in S-phase arrested HeLa cells is associated with a decrease in Chk1 phosphorylation, formation of phospho-H2AX and minor cytoskeletal rearrangements, Histochemistry and cell biology, 135 (2011) 263-280.

[40] G. Harris, T. Palosaari, Z. Magdolenova, M. Mennecozzi, J.M. Gineste, L. Saavedra, A. Milcamps, A. Huk, A.R. Collins, M. Dusinska, M. Whelan, Iron oxide nanoparticle toxicity testing using high-throughput analysis and high-content imaging, Nanotoxicology, 9 Suppl 1 (2015) 87-94.

[41] T.J. Gaymes, R.A. Padua, M. Pla, S. Orr, N. Omidvar, C. Chomienne, G.J. Mufti, F.V. Rassool, Histone deacetylase inhibitors (HDI) cause DNA damage in leukemia cells: a mechanism for leukemia-specific HDI-dependent apoptosis?, Molecular cancer research : MCR, 4 (2006) 563-573.

[42] S. Yoshizawa, H. Fujiki, H. Suguri, M. Suganuma, M. Nakayasu, R. Matsushima, T. Sugimura, Tumor-promoting activity of staurosporine, a protein kinase inhibitor on mouse skin, Cancer Res, 50 (1990) 4974-4978.

[43] E. Toton, N. Lisiak, B. Rubis, J. Budzianowski, P. Gruber, J. Hofmann, M. Rybczynska, The tetramethoxyflavone zapotin selectively activates protein kinase C epsilon, leading to its down-modulation accompanied by Bcl-2, c-Jun and c-Fos decrease, European journal of pharmacology, 682 (2012) 21-28.

[44] M.L. Carbajal Rivera, M.G. Ordaz Tellez, A. Castaneda Sortibran, R. Rodriguez-Arnaiz, Emetine and/or its metabolites are genotoxic in somatic cells of Drosophila melanogaster, Journal of toxicology and environmental health. Part A, 70 (2007) 1713-1716.

[45] G. Lee, T.I. Oh, K.B. Um, H. Yoon, J. Son, B.M. Kim, H.I. Kim, H. Kim, Y.J. Kim, C.S. Lee, J.H. Lim, Small-molecule inhibitors of USP7 induce apoptosis through oxidative and endoplasmic reticulum stress in cancer cells, Biochem Biophys Res Commun, 470 (2016) 181-186.

[46] L. Khoury, D. Zalko, M. Audebert, Evaluation of four human cell lines with distinct biotransformation properties for genotoxic screening, Mutagenesis, 31 (2016) 83-96.

[47] A. Wiegering, N. Matthes, B. Muhling, M. Koospal, A. Quenzer, S. Peter, C.T. Germer, M. Linnebacher, C. Otto, Reactivating p53 and Inducing Tumor Apoptosis (RITA) Enhances the Response of RITA-Sensitive Colorectal Cancer Cells to Chemotherapeutic Agents 5-Fluorouracil and Oxaliplatin, Neoplasia, 19 (2017) 301-309.

[48] W. Nieves-Neira, M.I. Rivera, G. Kohlhagen, M.L. Hursey, P. Pourquier, E.A. Sausville, Y. Pommier, DNA protein cross-links produced by NSC 652287, a novel thiophene derivative active against human renal cancer cells, Molecular pharmacology, 56 (1999) 478-484.

[49] M. Wanzel, J.B. Vischedyk, M.P. Gittler, N. Gremke, J.R. Seiz, M. Hefter, M. Noack, R. Savai, M. Mernberger, J.P. Charles, J. Schneikert, A.C. Bretz, A. Nist, T. Stiewe, CRISPR-Cas9-based target validation for p53-reactivating model compounds, Nature chemical biology, 12 (2016) 22-28.

[50] K. Wojcik, M. Zarebski, A. Cossarizza, J.W. Dobrucki, Daunomycin, an antitumor DNA intercalator, influences histone-DNA interactions, Cancer biology & therapy, 14 (2013) 823-832.

[51] J.C. Corton, K.L. Witt, C.L. Yauk, Identification of p53 Activators in a Human Microarray Compendium, Chem Res Toxicol, 32 (2019) 1748-1759.

[52] A. Kimura, A. Miyata, M. Honma, A combination of in vitro comet assay and micronucleus test using human lymphoblastoid TK6 cells, Mutagenesis, 28 (2013) 583-590.

[53] S.M. Bryce, S.L. Avlasevich, J.C. Bemis, M. Lukamowicz, A. Elhajouji, F. Van Goethem, M. De Boeck, D. Beerens, H. Aerts, J. Van Gompel, J.E. Collins, P.C. Ellis, A.T. White, A.M. Lynch, S.D. Dertinger, Interlaboratory evaluation of a flow cytometric, high content in vitro micronucleus assay, Mutat Res, 650 (2008) 181-195.

[54] D.T. Bernacki, S.M. Bryce, J.C. Bemis, D. Kirkland, S.D. Dertinger, gammaH2AX and p53 responses in TK6 cells discriminate promutagens and nongenotoxicants in the presence of rat liver S9, Environ Mol Mutagen, 57 (2016) 546-558.

[55] S.L. Avlasevich, S.M. Bryce, S.E. Cairns, S.D. Dertinger, In vitro micronucleus scoring by flow cytometry: differential staining of micronuclei versus apoptotic and necrotic chromatin enhances assay reliability, Environ Mol Mutagen, 47 (2006) 56-66.

[56] M. Ljungman, The DNA damage response--repair or despair?, Environ Mol Mutagen, 51 (2010) 879-889.

[57] W.P. Roos, B. Kaina, DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis, Cancer Lett, 332 (2013) 237-248.

Table 1. Chemicals studied with their proposed mechanism of apoptosis induction and micronucleus report.

Name of compound	CAS No.	Mode of action	*In vitro* MN genotoxicity data
TRAIL ligand	Not available	Death receptor ligand	No data available
ICRF-193	21416-68-2	Apoptotic proteins induction	No data available
Embelin	550-24-3		No data available
Triton X100	9002-93-1		Negative data [33, 52]
Staurosporine	62996-74-1	Protein kinase C inhibitors	Discordant data [36-38, 53]
Zapotin	14813-19-5		No data available
BH3i-1	300817-68-9	Bcl-2 family proteins targets	No data available
YC137	810659-53-1		Positive data [38]
Emetine	316-42-7	Protein synthesis inhibitor	No data available
Reveromycin A	134615-37-5		No data available
Carboxyatractyloside potassium salt (CAT)	77228-71-8	Mitochondria uncouplers	No data available
Sodium Iodoacetate	305-53-3		No data available
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	555-60-2		Negative data [3, 4, 54]
Costunolide	553-21-9		No data available
Tanespimycin (17-AAG)	75747-14-7	Endoplasmic reticulum stress inducers	No data available
Thapsigargin	67526-95-8		Negative data [4, 54]
Tunicamycin	11089-65-9		Negative data [4, 54]
Gliotoxin	67-99-2	20S proteasome inhibitors	Discordant data [37]
Tributyltin	1067-52-3		Negative data [3, 55]
Bortezomib	179324-69-7	26S proteasome inhibitors	No data available
MG-115	133407-86-0		No data available
MG-132	133407-82-6		No data available
Nutlin-3	548472-68-0	p53 targets	Discordant data [54-57]
P5091	882257-11-6		No data available
Pifithrin α	63208-82-2		No data available
RITA	213261-59-7		Positive data [38]

Table 2. **Summary results of *in vitro* genotoxicity and cytotoxicity.** Phosphorylation of histone H2AX (γH2AX) and % of relative cell count (RCC) in HepG2, LS-174T and ACHN cell lines treated with chemicals with different mode of apoptotic inducing action. Orange and upwards arrows represent an increase in γH2AX compared to the control. Green and downwards arrows represent a significant decrease in γH2AX or a decrease of RCC superior to 50 % compared to the control. Concentrations (µM), excepted trail ligand in µg/mL, correspond to the lowest effective concentration (LEC) observed. No color boxes indicate no biomarker variation and the highest concentration tested is indicated in brackets.

Cell lines		HepG2		LS-174T		ACHN	
Mode of action	**Name of compound**	**γH2AX**	**RCC**	**γH2AX**	**RCC**	**γH2AX**	**RCC**
Death receptor ligand	TRAIL ligand	(10)	(10)	(10)	(10)	↘ 3	↘ 10
Apoptotic proteins induction	ICRF-193	↗ 0.3	(100)	(100)	(100)	↗ 1	(100)
	Embelin	(10)	↘ 100	(10)	↘ 100	(10)	↘ 100
	Triton x100	↘ 3	↘ 100	↘ 3	↘ 30	(30)	↘ 100
Protein kinase C inhibitors	Staurosporine	↗ 0.3	(10)	↗ 0.01	↘ 3	(1)	↘ 3
	Zapotin	↗ 0.3	(100)	↗ 0.3	↘ 100	(100)	(100)
Bcl-2 family proteins target	BH3i-1	(30)	↘ 100	(10)	↘ 30	(30)	↘ 100
	YC137	↗ 0.1	↘ 3	↗ 0.1	↘ 1	↗ 0.1	↘ 3
Protein synthesis inhibitor	Emetine	↗ 1	(100)	↘ 0.1	↘ 0.3	↗ 1	(100)
	Reveromycin A	↘ 10	(10)	(1)	↘ 3	↘ 10	(10)
Mitochondria uncouplers	CAT	(100)	(100)	(100)	(100)	(100)	(100)
	Sodium Iodoacetate	(100)	(100)	(100)	(100)	(100)	(100)
	CCCP	↘ 30	↘ 300	↘ 30	↘ 100	(100)	↘ 100
	Costunolide	(10)	↘ 30	(10)	↘ 30	(10)	↘ 30
Endoplasmic reticulum stress inducers	17-AAG	↘ 10	(10)	↘ 0.3	↘ 10	(10)	(10)
	Thapsigargin	↘ 0.03	(1)	(0.03)	↘ 0.1	↘ 1	(1)
	Tunicamycin	↘ 1	↘ 10	↘ 0.03	↘ 0.1	↘ 0.1	↘ 100
20S proteasome inhibitors	Gliotoxin	(0.3)	↘ 1	(1)	↘ 3	(1)	↘ 3
	Tributyltin	↘ 0.3	↘ 1	(0.3)	↘ 1	(0.3)	↘ 1
26S proteasome inhibitors	Bortezomib	↘ 0.01	(0.1)	(0.01)	↘ 0.03	↘ 0.01	(0.1)
	MG-115	↘ 3	(10)	(1)	↘ 3	↘ 0.3	(10)
	MG-132	↘ 0.3	(1)	(0.3)	↘ 1	↘ 0.1	(1)
p53 target	Nutlin-3	↘ 1	↘ 100	↘ 3	↘ 100	↘ 1	↘ 100
	P5091	↗ 10	↘ 100	↗ 10	↘ 30	↗ 10	↘ 30
	Pifithrin α	↘ 300	↘ 1000	↘ 300	↘ 1000	↘ 300	↘ 1000
	RITA	↗ 0.01	↘ 3	↗ 0.003	↘ 0.3	(100)	(100)

Figure legends

Fig. 1. *In vitro* cytotoxicity and genotoxicity of TRAIL ligand, triton X100, ICRF-193, zapotin and staurosporine tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).

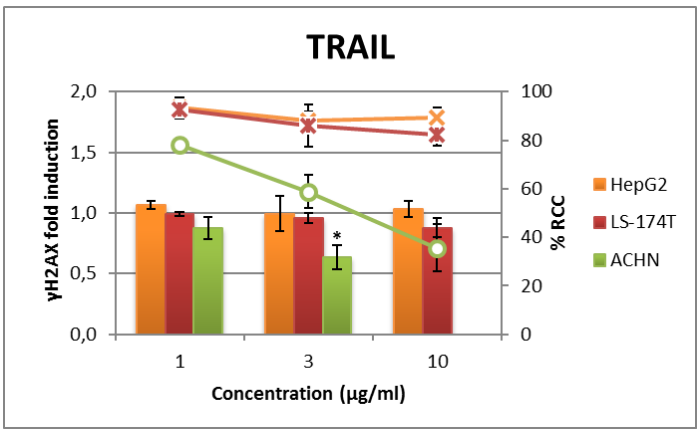
Fig. 2. *In vitro* cytotoxicity and genotoxicity of BH3i-1, YC137, emetine and reveromycin A tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).

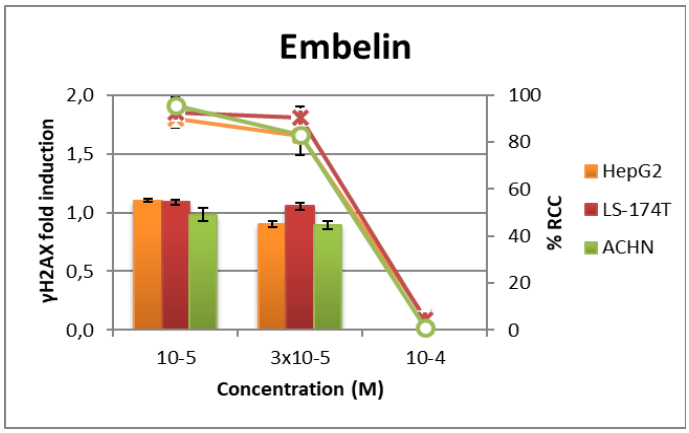
Fig. 3. *In vitro* cytotoxicity and genotoxicity of embelin, carboxyatractyloside, CCCP and costunolide tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).

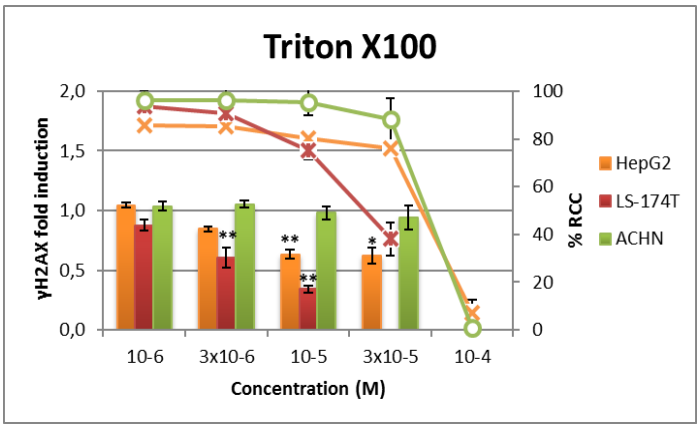
Fig. 4. *In vitro* cytotoxicity and genotoxicity of 17-AAG, tunicamycin and thapsigargin tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).

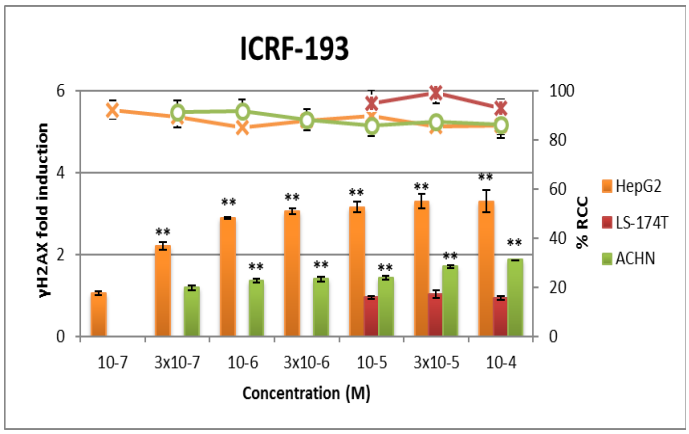
Fig. 5. *In vitro* cytotoxicity and genotoxicity of gliotoxin, tributylin, Bortezomib, MG-132 and MG-115 tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).

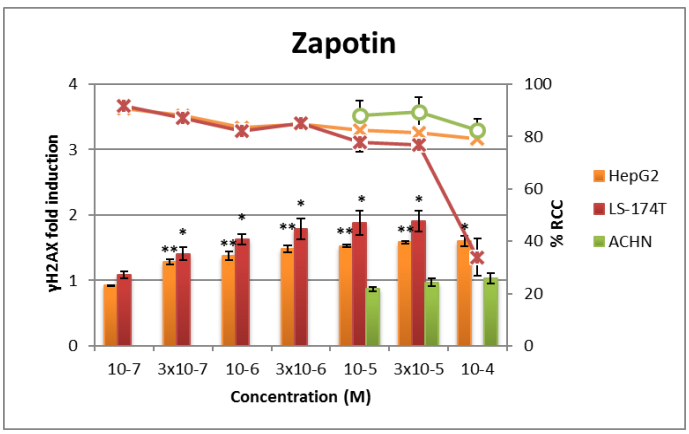
Fig. 6. *In vitro* cytotoxicity and genotoxicity of pifithrin α, P5091, RITA and nutlin-3 tested for γH2AX in the HepG2, LS-174T and ACHN cell lines. Cytotoxicity is represented by the % RCC. Each value represents the mean ± SEM (n≥3) after 24 h of treatment. Significant differences were observed between controls and matched groups (*, p≤0.05; **, p≤ 0.01).











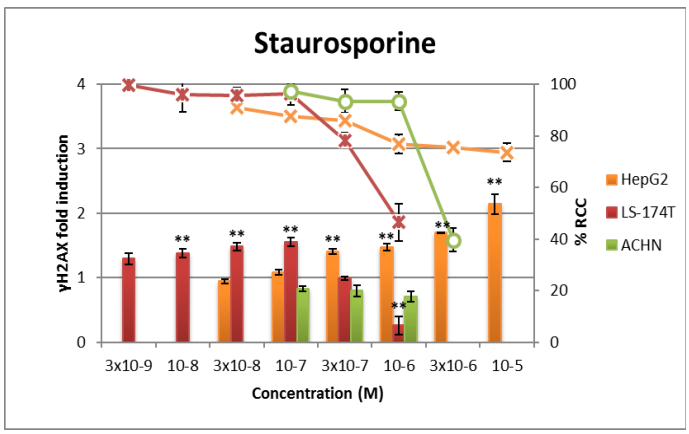
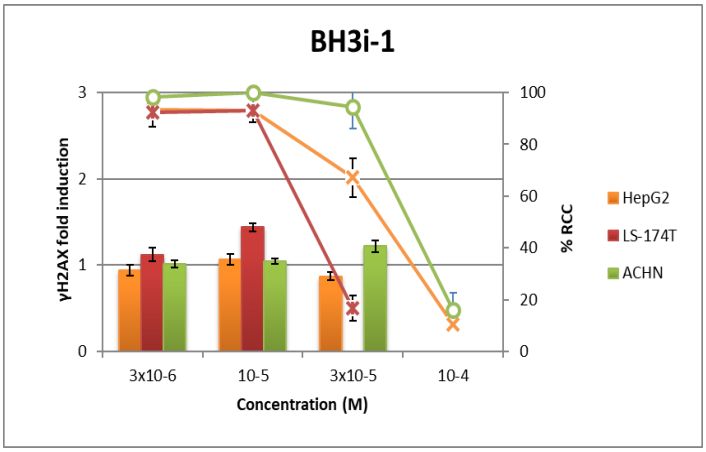
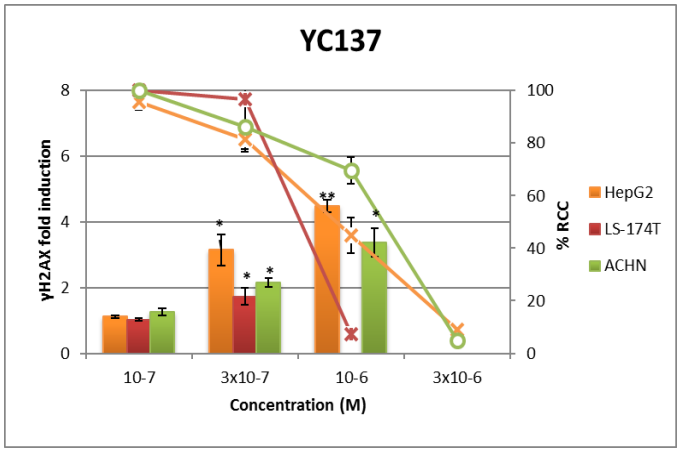
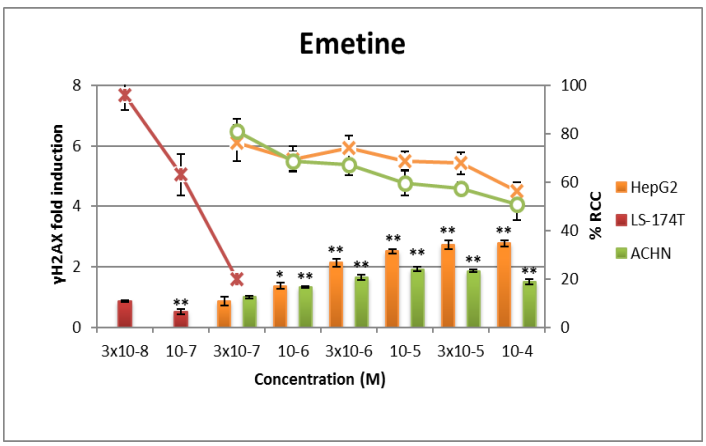


Figure 1 Khoury et Al.







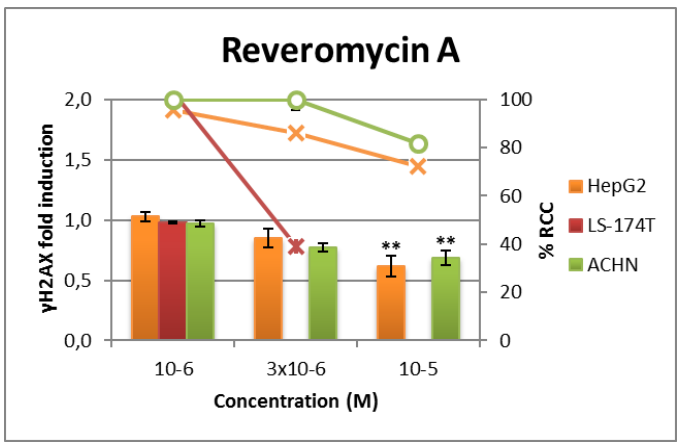
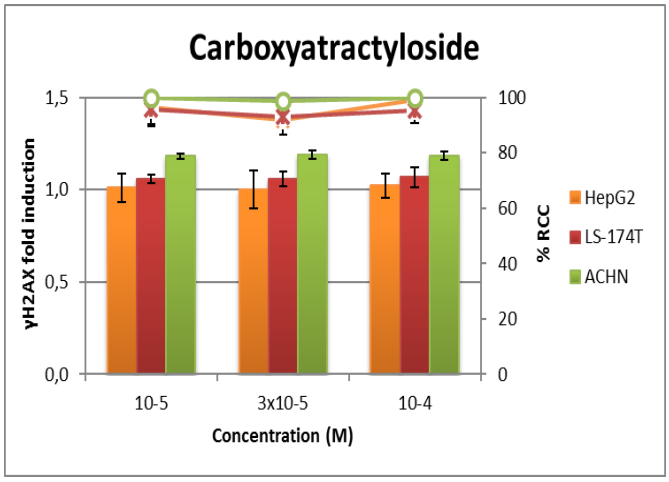
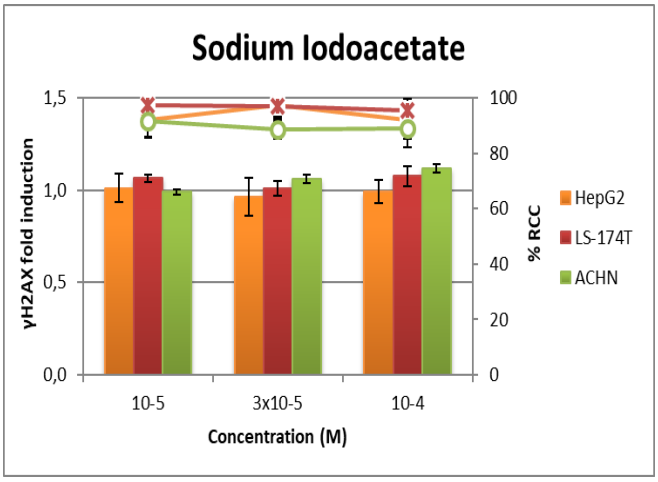
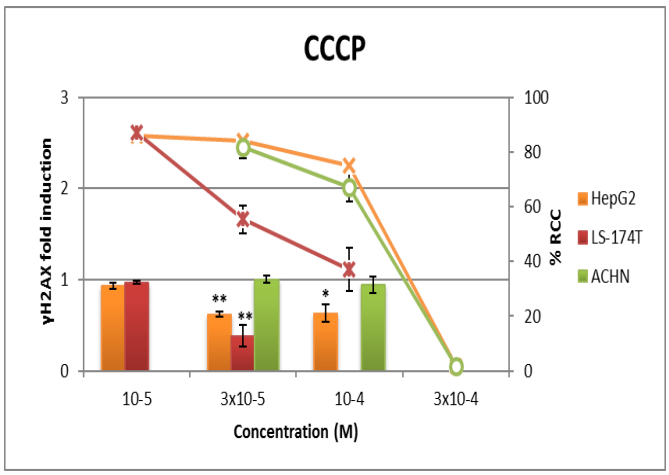


Figure 2 Khoury et Al.







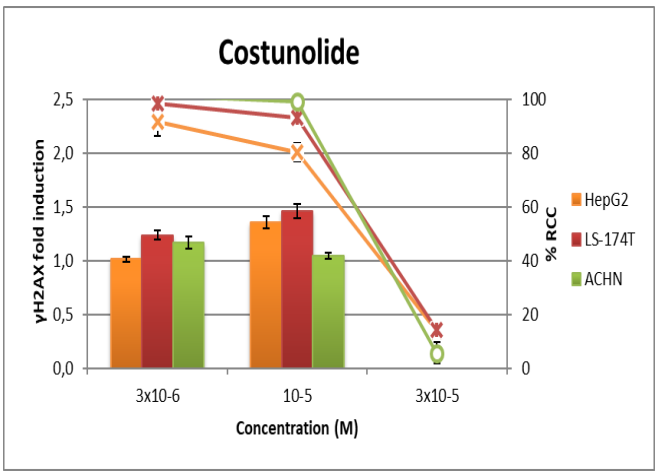
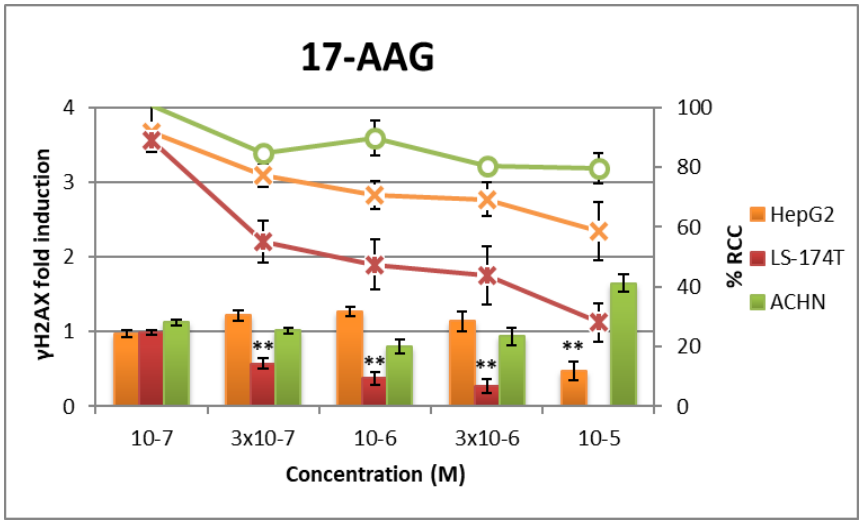
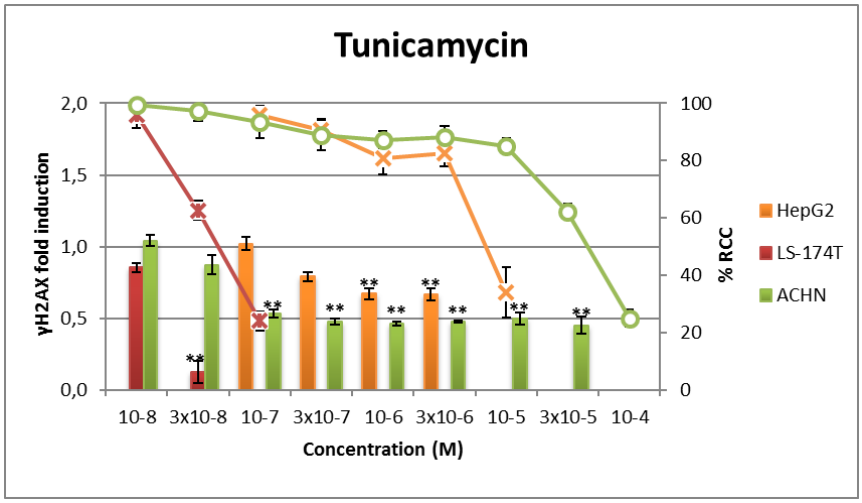


Figure 3 Khoury et Al.





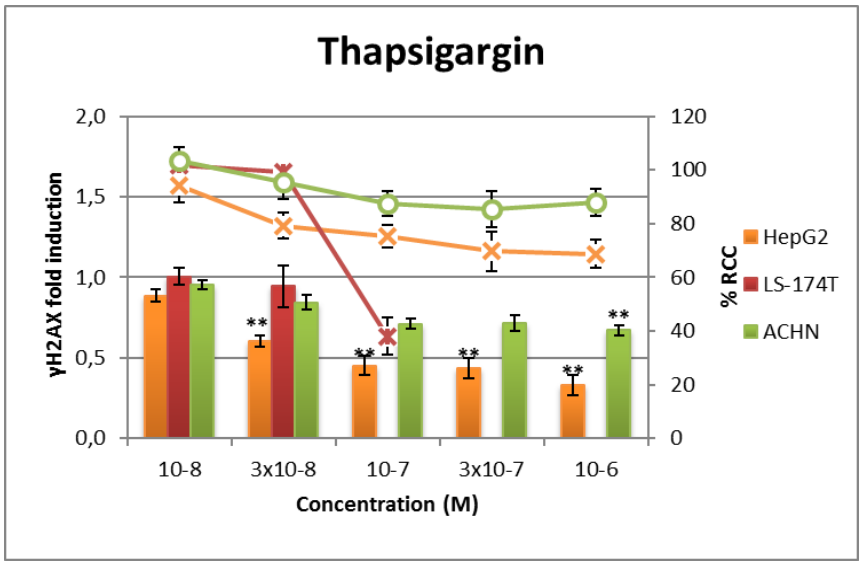
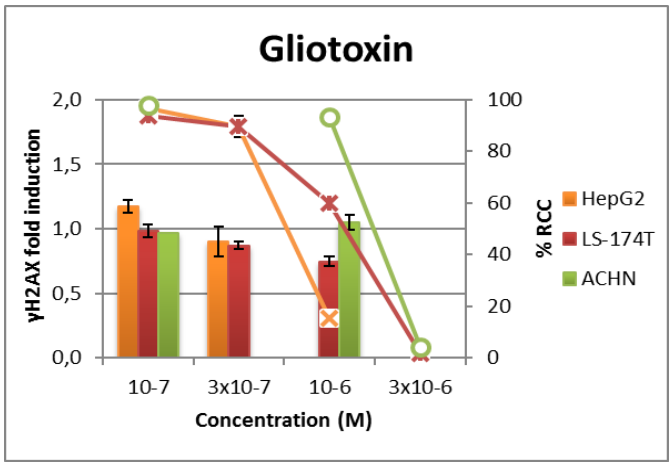
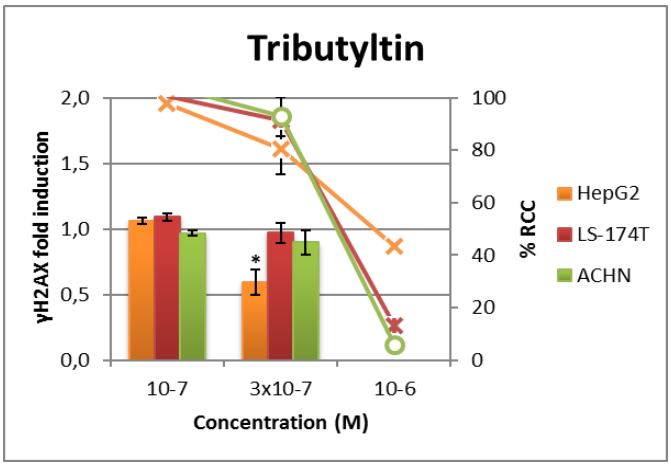
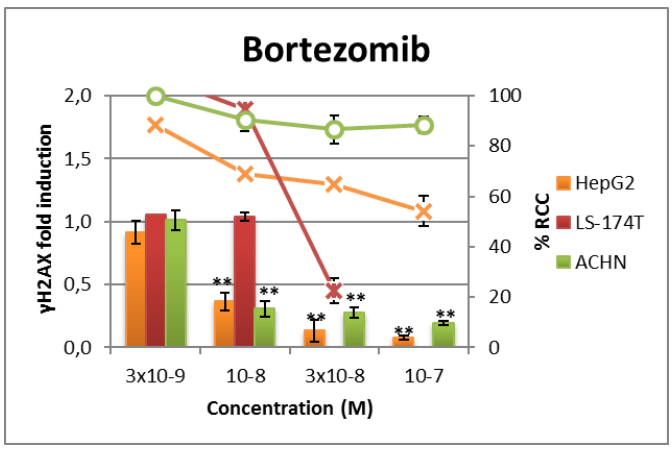
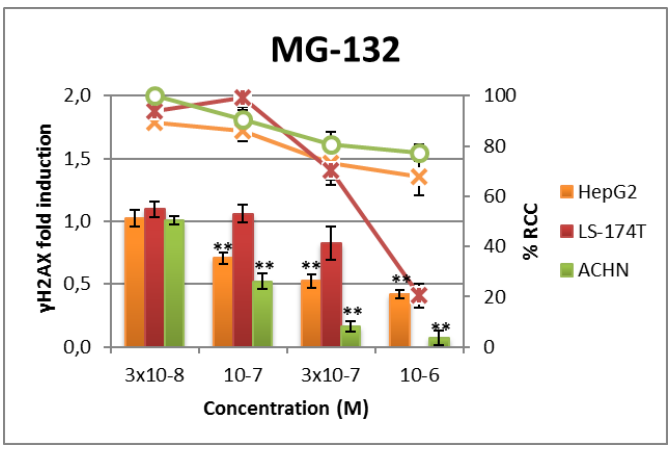


Figure 4 Khoury et Al.









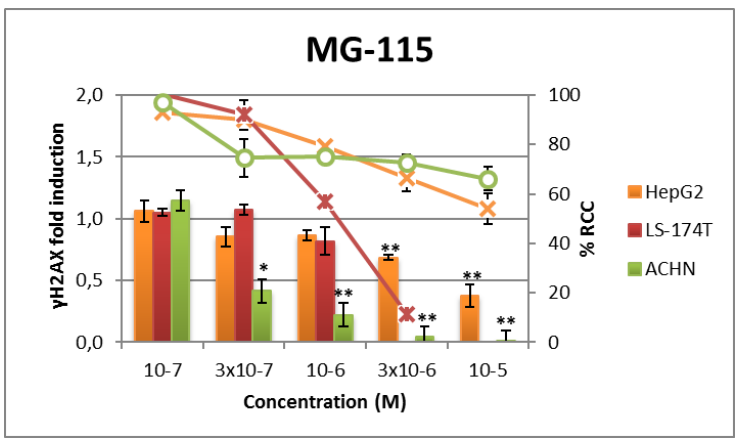


Figure 5 Khoury et Al.

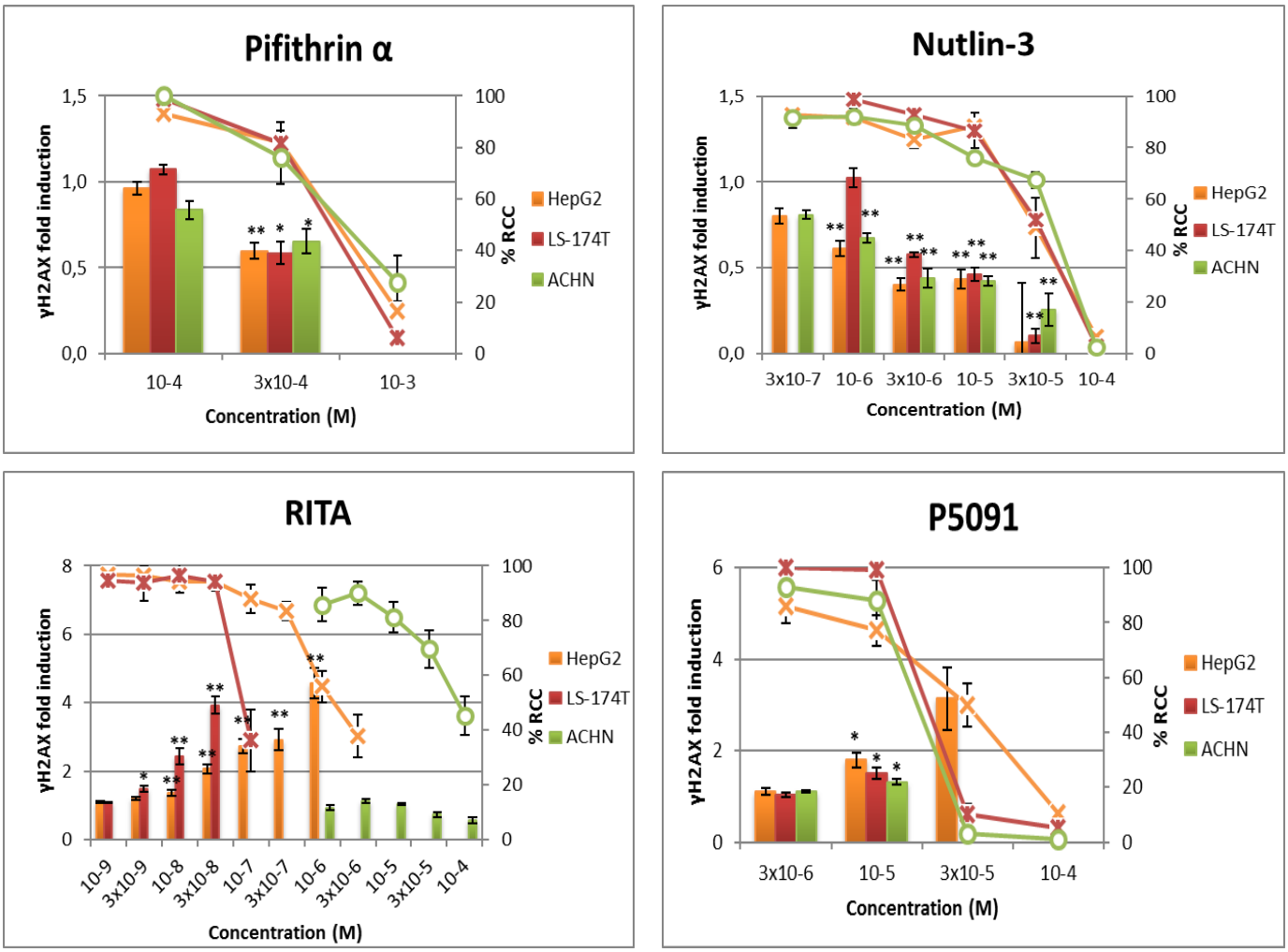


Figure 6 Khoury et Al.