In vitro assessment of emerging mycotoxins co-occurring in cheese: A potential health hazard

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Abstract:

Some *Penicillium* strains used in cheese ripening produce emerging mycotoxins, notably roquefortine C (ROQC) and cyclopiazonic acid (CPA), as well as enniatins (ENNs) and beauvericin (BEA). Co-occurrence of these mycotoxins in natural samples has been reported worldwide, however, most studies focus on the toxicity of a single mycotoxin. In the present study, the effects of ROQC and CPA alone and in combination with BEA and ENNs A, A1, B, and B1 were analysed in human neuroblastoma cells. ROQC and CPA reduced cell viability, with IC_{50} values of 49.5 and 7.3 µM, respectively, and induced caspase-8-mediated apoptosis. When ROQC and CPA were binary combined with ENNs, an enhancement of their individual effects was observed. Furthermore, a clear synergism was produced when ROQC and CPA were mixed with the four ENNs. An additive effect was also described for the combination of CPA + ENNs (A, A1, B, B1) + BEA. Finally, the effects of commercial cheese extracts containing the mentioned mycotoxins were evaluated, finding a strong reduction in cell viability. These results suggest that the co-occurrence of emerging mycotoxins in natural matrices could pose a potential health risk.

Keywords: Roquefortine C, cyclopiazonic acid, toxicity, enniatins, apoptosis, synergism

Statements and Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the conception and design of the study. Nadia Pérez-Fuentes carried out the research and was involved in writing the original draft and in reviewing and editing the manuscript. Rebeca Alvariño and Amparo Alfonso contributed to the conception, methodology and drafting, including review and editing. Jesús González-Jartín carried out the research and was involved in writing the manuscript. Mercedes R. Vieytes contributed to the methodology. Luis M. Botana was responsible for fundraising and supervision.

Data Availability

All data used in this study are included in the manuscript Figs.

Acknowledgments

The research leading to these results has received funding from the following grants. From Conselleria de Cultura, Educacion e Ordenación Universitaria, Xunta de Galicia, GRC (ED431C 2021/01). From Ministerio de Ciencia e Innovación, PID 2020-11262RB-C21, Grant CPP2021-008447 funded by MCIN/AEI/10.13039/501100011033 and by The European Union NextGenerationEU/PRT. From European Union, Interreg EAPA-0032/2022 – BEAP-MAR, HORIZON-MSCA-2022-DN-01-MSCA Doctoral Networks 2022 101119901-BIOTOXDoc, and HORIZON-CL6-2023-CIRCBIO-01 COMBO - 101135438.

Disclosure Statement

The authors do not have conflicts of interest to declare.

1. Introduction

Fungal-derived compounds represent a great percentage of active natural products. Some of these metabolites, produced by species from the genera *Aspergillus*, *Fusarium* and *Penicillium*, are toxic to animals and humans. These molecules, named mycotoxins, are naturally present in contaminated matrices, so they can be found in cereals such as maize or rye, but also in other foods like milk, cheese or even fish (El-Sayed et al., 2022; Marin et al., 2013). *Penicillium* genus represents the most common contaminating mould in dairy products, followed by *Aspergillus*, and it is particularly well adapted to grow in cheese matrix (Kure and Skaar, 2019). Some *Penicillium* species that are used in cheese elaboration, such as *P. roqueforti* or *P. camemberti,* produce emerging mycotoxins, including roquefortines, cyclopiazonic acid (CPA) or even regulated mycotoxins like patulin (Izzo et al., 2022; Perrone and Susca, 2017). Emerging mycotoxins are molecules not routinely assessed, but their presence in food and feed is increasing. In the case of cheese, a recent study detected several non-regulated mycotoxins in commercial preparations, standing out the presence of CPA and roquefortine C (ROQC) in combination with other emerging mycotoxins like beauvericin (BEA) or enniatins (ENNs) (Rodríguez-Cañás et al., 2023).

ROQC is a 2,5-diketopiperazine (Figure 1a), usually detected in cheese at low concentrations and mainly produced by *P. roqueforti*. A neurotoxic effect after ROQC administration has been described in mice and its presence has been associated with intoxications in dogs and cattle (Tiwary et al., 2009; N. Hymery et al., 2018). However, there are not enough data on ROQC toxicity, and its mechanism of action remains unclear. CPA, an indole tetrameric acid mycotoxin (Figure 1b), is one of the most common and stable mycotoxins in cheese (Casquete et al., 2021; Nolwenn Hymery et al., 2014). This compound inhibits the Ca^{2+}-ATPase of the sarcoplasmic reticulum and produces hepatotoxic, neurotoxic, and nephrotoxic effects in different animal models (Fliszár-Nyúl et al., 2022). CPA is produced by several *Aspergillus* species and has presented cytotoxicity at high concentrations in different cell lines, so it is not considered a health issue (Chang et al., 2009) . However, its combination with aflatoxins produces additive effects, so its toxicity could be currently underestimated since its mixed effects with other mycotoxins have not been extensively studied (Chang et al., 2009; Pier et al., 1989).

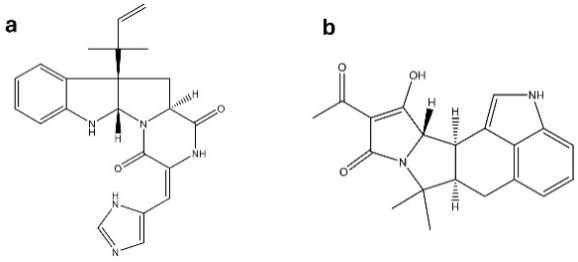

Figure 1. Chemical structures of roquefortine C (a) and cyclopiazonic acid (b).

In this sense, we have recently described that the mechanism of action of ENNs is mediated by an effect in calcium fluxes, so the co-occurrence of CPA with these emerging mycotoxins could represent a health risk that is not being considered.

ENNs, along with BEA, are cyclic hexadepsipeptides produced by *Fusarium* spp. that have been considered as ionophores for a long time, but the exact mechanism of action of each toxin has not been widely explored (Shin et al., 2009). Both ENNs and BEA have been described to be cytotoxic and neurotoxic in several cell lines (Agahi et al., 2021; Krug et al., 2018; Manyes et al., 2018). In recent works, we have observed that the four most common ENNs (A, B, A1 and B1) present a different mode of action despite their structural similarities. All the toxins alter calcium homeostasis, but by affecting different intracellular pools (N. Pérez-Fuentes et al., 2022; Nadia Pérez-Fuentes et al., 2023). Moreover, ENNs interfere with mitochondrial function and induce apoptosis through mechanisms involving oxidative stress and cell cycle disruption (Qinghua Wu et al., 2018; Hamza Olleik et al., 2019b). Regarding BEA, it forms ion-selective channels with several metallic cations leading to an increase in cell membrane permeability (Caloni et al., 2020; Kouri et al., 2003). BEA has a wide range of biological properties, including the induction of cytotoxicity, oxidative stress, and disruption of mitochondrial function in several cell lines (Mallebrera et al., 2018; Hasuda et al., 2023; Søderstrøm et al., 2022; Caloni et al., 2020).

Although ROQC, CPA, ENNs and BEA presence is increasing and they naturally co-contaminate different types of cheese, there are not enough data on their combined toxicity. In this context, the objective of this study was to evaluate the combined effect of these naturally occurring emerging toxins in SH-SY5Y human neuroblastoma cells. To this end, the single effects of ROQC and CPA on cell viability were initially assessed, followed by the evaluation of dose-response combinations of the emerging mycotoxins. Finally, contaminated cheese extracts were tested in order to analyse the effect of these toxins in a natural sample.

2. Methods and material

2.1. Chemicals and solutions

CyQUANT™ lactate dehydrogenase (LDH) Cytotoxicity Assay Kit, Dulbecco's Modified Eagle Medium: F-12 nutrient Mix (DMEM/F-12), trypsin/EDTA (0.05%), glutamax, penicillin-streptomycin (10,000 U/mL), SuperSignal West Pico, SuperSignal West Femto, Pierce TM Protease Inhibitor Mini Tablets and Pierce TM Phosphatase Inhibitor Mini Tablets were bought in Thermo Fisher Scientific (Madrid, Spain). Durapore membrane centrifugal filters (0.22 µm pore size), ENN A, ENN A1, ENN B, ENN B1, CPA and ROQC (purity >95 %), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), saponin (SAP), staurosporine (STS), Annexin V-FITC Apoptosis Detection Kit, anti-beta actin monoclonal antibody and another chemical reagent grade were purchased from Merck (Madrid, Spain). Cheese samples were obtained in local supermarkets and storaged at 4ºC until analysis. Cyclosporin A (CsA) and anti-caspase 9 antibody were obtained from Abcam (Cambridge, UK). Anti-caspase-8 antibody was purchased in Cell Signalling Technology (Massachusetts, USA).

The composition of Locke´s buffer was: 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$ 1.3 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES. Phosphate buffered saline (PBS) was composed of (in mM): 137.0 NaCl, 8.2 Na_2HPO_4, 1.5 KH_2PO_4 and 3.2 KCl (pH 7.4). Hypotonic lyses buffer was composed of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1 mM EDTA. Stock solutions of mycotoxins were prepared in DMSO, and serial dilutions were carried out in culture medium. DMSO concentration was kept under 0.8% in all experiments.

2.2. Cheese extracts and analysis

Cheese samples were analysed utilizing a method previously validated for the simultaneous detection of 32 mycotoxins in this matrix (Rodríguez-Cañás et al., 2023; Jesús M. González-Jartín et al., 2020). Briefly, samples were homogenized, and 1 g of each cheese was subjected to extraction using a QuEChERS (quick, easy, cheap, effective, rugged, and safe) protocol. Initially, 4 mL of 2% acetic acid solution were added, followed by 4 mL of acetonitrile, vigorous mixing was applied after each solvent addition using a vortex mixer. Phase separation was achieved with 1.6 g of MgSO4 and 0.4 g of NaCl. Subsequently, the organic phase was diluted 1:2 with 2% acetic acid and filtered through a 0.22 µm centrifugal filters. The resulting extracts were then analysed using an ultra-high liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) protocol. Cheese extracts (500 µL) were further subjected to drying in a centrifugal evaporator, followed by reconstitution in 500 µL of DMSO.

2.3. Cell Culture

SH-SY5Y human neuroblastoma cell line was purchased from American Type Culture Collection (ATCC), number CRL2266. Cells were cultured in DMEM/F-12 supplemented with 1% glutamax, 10% fetal bovine serum (FBS) and 10,000 U/mL penicillin-streptomycin. Cells were maintained at 37 ℃ in a humidified atmosphere of 95% air and 5% CO_2 and dissociated once a week using 0.05% trypsin/EDTA.

2.4. Cell viability assay

SH-SY5Y cells were cultured in 384-well plates at concentration of 2.5×10^4 cells/well in complete cell growth medium. After 24 h of incubation, cell medium was changed to DMEM/F-12 supplemented with 1% glutamax, 2% fetal bovine serum (FBS) and 10,000 U/mL penicillin-streptomycin.
Concentrated cheese extracts were prepared as described above and SH-SY5Y cells were treated at 1:200, 1:300, 1:400, 1:500 and 1:1000 dilutions. The less contaminated cheese sample (corresponding to camembert cheese) was used as a control, as no mycotoxin-free matrix was found. In individual mycotoxin assays, cells were treated at concentrations ranging from 0.01 to 100 µM. In the case of mycotoxin combinations, the ratio among different mycotoxins was selected based on the most frequent proportions found in contaminated cheese (Rodríguez-Cañás et al., 2023). In binary mixtures, CPA and ROQC were combined at 10:1 ratio with ENN A, ENN A1, ENN B or ENN B1. Then, the effects of a 1:1:1:1 mixture of ENNs A, A1, B and B1, and a combination of the four ENNs with BEA (1:1:1:1:1) were assessed. Finally, ROQC and CPA were combined with ENNs A, A1, B, and B1 at 10:1:1:1:1 proportion, and with the four ENNs and BEA at 10:1:1:1:1:1 ratio. Combined concentration ranges were selected based on the results obtained with individual mycotoxins to include their half maximal inhibitory concentration (IC_{50}) values in the concentration range analysed.

The effect of individual and mixed mycotoxins on SH-SY5Y cell viability was determined using the MTT assay, as previously described (Alvariño et al., 2017; Mosmann, 1983). Cells were treated for 24 h and SAP from *Quillaja bark* at 1mg/mL was used as cell death control. After treatment, SH-SY5Y cells were washed three times with Locke´s solution and 200 µL of MTT (500 µg/mL) were added per well. Then, cells were incubated at 37ºC for 1 h and 300 rpm in an orbital shaker. After incubation, MTT was removed, and 5%

sodium dodecyl sulphate was added to lysate the cells. Absorbance was read in a spectrophotometer plate reader at 595 nm. All the experiments were performed in triplicate at least three independent times.

2.5. Cytotoxicity assay

Cell cytotoxicity induced by mycotoxins, alone and in combination, was determined by CyQUANT™ LDH Cytotoxicity Assay Kit test, following manufacturer´s instructions (N. Pérez-Fuentes et al., 2021; Weyermann et al., 2005). Cells were treated with cheese extracts, single toxins and mixtures as described above for 24 h. Then, 50 µL of cell medium were transferred to a 96-well flat-bottomed well and the kit reaction mixture was added. After 30 minutes, the stop solution was added, and LDH release was assessed. SAP from *Quillaja bark* at 1mg/mL was used as cell death control. Absorbance was read at 490 and 680 nm in a plate reader. Finally, the background signal (680 nm absorbance) was subtracted from the 490 nm absorbance to determine LDH release into the medium. Experiments were performed three independent times in triplicate.

2.6. Cell death type assessment

Annexin V-FITC Apoptosis Detection Kit was used to determine the cell death type induced by ROQC and CPA, following manufacturer´s instructions (Alvariño et al., 2020; Crowley et al., 2016). For this assay, cells were seeded in 12-well plates at 5×10^5 cells per well. After 24 h, cells were treated with ROQC and CPA and incubated for 24 h. STS at 0.1 µM was used as a positive control. The cells were then washed with PBS, resuspended in annexin binding buffer containing annexin V-FITC and propidium iodide (PI) and incubated for 15 minutes at room temperature. Then, cells were resuspended in commercial PBS (pH 7.2) (Thermo Fisher Scientific), filtered, and kept on ice. Fluorescence was then analysed by flow cytometry using the ImageStreamMKII instrument (Amnis Corporation, Luminex Corp, Austin, TX, USA). IDEAS Application 6.0 software was used to determine the fluorescence of 10,000 events (Amnis Corporation, Luminex Corp).

2.7. Western blot analysis

Cells were seeded in a 12-well plate at 5×10^5 cells per well and allowed to attach for 24 h. After this time, CPA and ROQC were added for 24 h. SH-SY5Y cells were then rinsed twice with cold PBS, cells were scrapped and 100 µL of ice-cold hypotonic lysis buffer containing protease and phosphatase inhibitor cocktails were added. Samples were sonicated for 1 min and centrifugated for 20 min at 4 °C and 12000 rpm. Then, the supernatant was collected as the cytosolic fraction. Electrophoresis was performed as previously described on 4-20% sodium dodecyl sulphate polyacrylamide gels containing 15 µg of cytosolic protein and transferred onto PVDF membranes (Begum et al., 2022; Alvariño et al., 2020). Precision Plus Protein Standards Kaleidoscope molecular weight marker was used to determine the protein size. The membranes were then blocked with 0.5% BSA and antibody incubation was performed using the SNAP i.d. protein detection system. Caspase-8 was detected with anti-caspase-8 antibody (1:1000) and anti-caspase 9 (1:2000) antibody was used to detect caspase-9. Signal was normalized by β-actin with anti-β-actin antibody (1:5000). Immunoreactive bands were detected using Supersignal West Pico or Supersignal West Femto and Diversity GeneSnap software (Syngene). Experiments were performed three independent times by duplicate.

2.8. Statistical analysis

Data are expressed as mean ± SEM of three independent experiments. Differences were assessed by one-way ANOVA followed by Dunnett's or Tukey's multiple comparison tests. Statistical significance was set at *p< 0.05, **p< 0.01 and ***p< 0.001. IC_{50} and half maximal effective concentration (EC_{50}) values were calculated using GraphPad Prism 8 software by fitting the data with a log(inhibitor) *vs* response or log(agonist) *vs* response model, respectively.

The combination index was calculated with Chou & Talalay equation (Ting-Chao Chou and Talalay, 1983):

$$Combination\ index = \frac{D1}{D(X)1} + \frac{D2}{D(X)2}$$

where (DX)1 and (DX)2 are the IC_{50} values of each mycotoxin alone, and D1 and D2 are the IC_{50} values of each mycotoxin in binary combination. According to Chou & Talalay method, synergism, additivity, and antagonism were considered when the combination index was <1, =1 and >1, respectively.

3. Results

3.1. Single effects of ROQC and CPA in human neuroblastoma cells

Based on a previous analysis of cheese samples, in which CPA and ROQC were frequently detected, these toxins were selected to test their toxicity in SH-SY5Y cells (Rodríguez-Cañás et al., 2023). At first, the effects of these two metabolites on cell viability and cytotoxicity were determined. Cells were treated with ROQC and CPA at concentrations ranging from 0.1 to 100 µM for 24 h. MTT was used to analyse the effects of these toxins on cell viability while cytotoxicity was determined by measuring the LDH release into the culture medium.

In MTT assay, ROQC reduced cell viability between 16.3 – 99.4 % at concentrations over 40 µM (Figure 2a), while CPA seemed to be more toxic, decreasing cell viability between 64.6 – 96.0 % at doses higher than 5 µM (Figure 2b). With the data obtained in MTT assays, IC_{50} values for each toxin were calculated by fitting the data with a log(inhibitor) *vs* response model. ROQC presented an IC_{50} of 49.5 µM (CI: 45.6 – 53.5 µM) and CPA was more toxic, with a value of 7.3 µM (CI: 5.9 – 8.8 µM).

Regarding LDH assay, the results were consistent with those obtained when measuring cell viability, as LDH release was observed at the highest concentrations used (Figure 2 c, d). ROQC increased LDH at concentrations above 40 µM, reaching a reduction of 100 % at the 100 µM dose. Although CPA seemed to affect more the cell metabolism, its effects on LDH release were lower (84.5 ± 5.9 % at 100 µM). With the data obtained with this assay, EC_{50} values for each toxin were calculated by fitting the data with a log(agonist) *vs* response model. ROQC showed an EC_{50} value of 49.5 µM (CI: 45.4– 53.0 µM). In the case of CPA, its EC_{50} was higher (84.1 µM, CI: 77.3 – 91.5 µM).

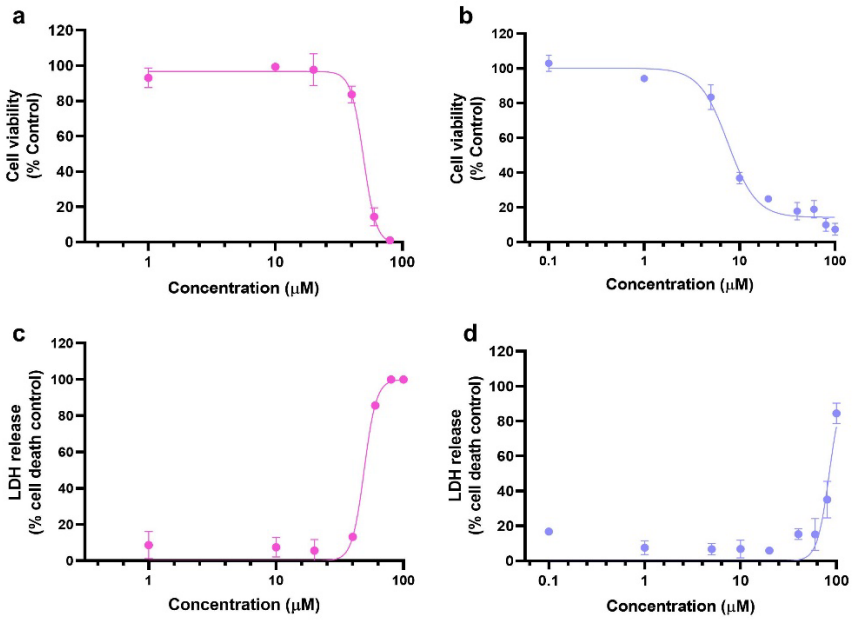

Figure 2. Single effects of ROQC and CPA on cell viability and cytotoxicity of SH-SY5Y cells. Cells were treated with mycotoxins for 24 h. (a) Effect of ROQC and (b) CPA on cell viability, assessed by MTT test. (c) Cytotoxicity of ROQC and (d) CPA, determined by LDH assay. Saponin at 1 mg/mL was used as cell death control. Data are mean ± SEM of three independent replicates expressed as percentage of untreated control cells. Data were fitted with a log (inhibitor) *vs* response and a log (agonist) *vs* response model for cell viability and LDH assays, respectively.

Next, the type of death induced by ROQC and CPA was assessed. Cells were treated for 24 h with mycotoxins at IC_{50} concentrations determined by MTT assay. Therefore, ROQC at 49.5 µM and CPA at 7.3 µM were added to cells for 24 h. The apoptotic inducer STS was used at 0.1 µM as a positive control. Samples were then co-stained with Annexin V-FITC and PI and the fluorescence was analysed by flow cytometry (Figure 3a). The percentages of necrotic cells (Annexin V-FITC negative and PI positive) and apoptotic cells (Annexin V-FITC positive and PI positive and negative) were calculated. ROQC and CPA induced a significant increase in apoptotic cells, with percentages of 52.7 ± 1.9 % ($p<0.01$) and 43.8 ± 8.0 % ($p<0.05$), respectively, without changing the proportion of necrotic cells compared to the control. These effects were similar to the results of the apoptotic inducer STS, that increased the number of cells undergoing apoptosis until 59.5 ± 3.6% ($p<0.001$).

Then, to decipher the type of apoptosis produced by ROQC and CPA, the expression of caspase-8 and caspase-9 were determined. The first one is involved in the extrinsic apoptotic pathway, whilst caspase-9 participates in the intrinsic or mitochondrial pathway. As Figure 3b shows, the expression of cleaved caspase-8 (41-43 KDa band) was increased by CPA (31.0 ± 16.8 %, $p<0.05$), while active caspase-8 expression (18 KDa band) was augmented by ROQC a 61.1 ± 36.9 %, compared to control cells. No significant differences were found in the expression of caspase-9 after treatment with these toxins.

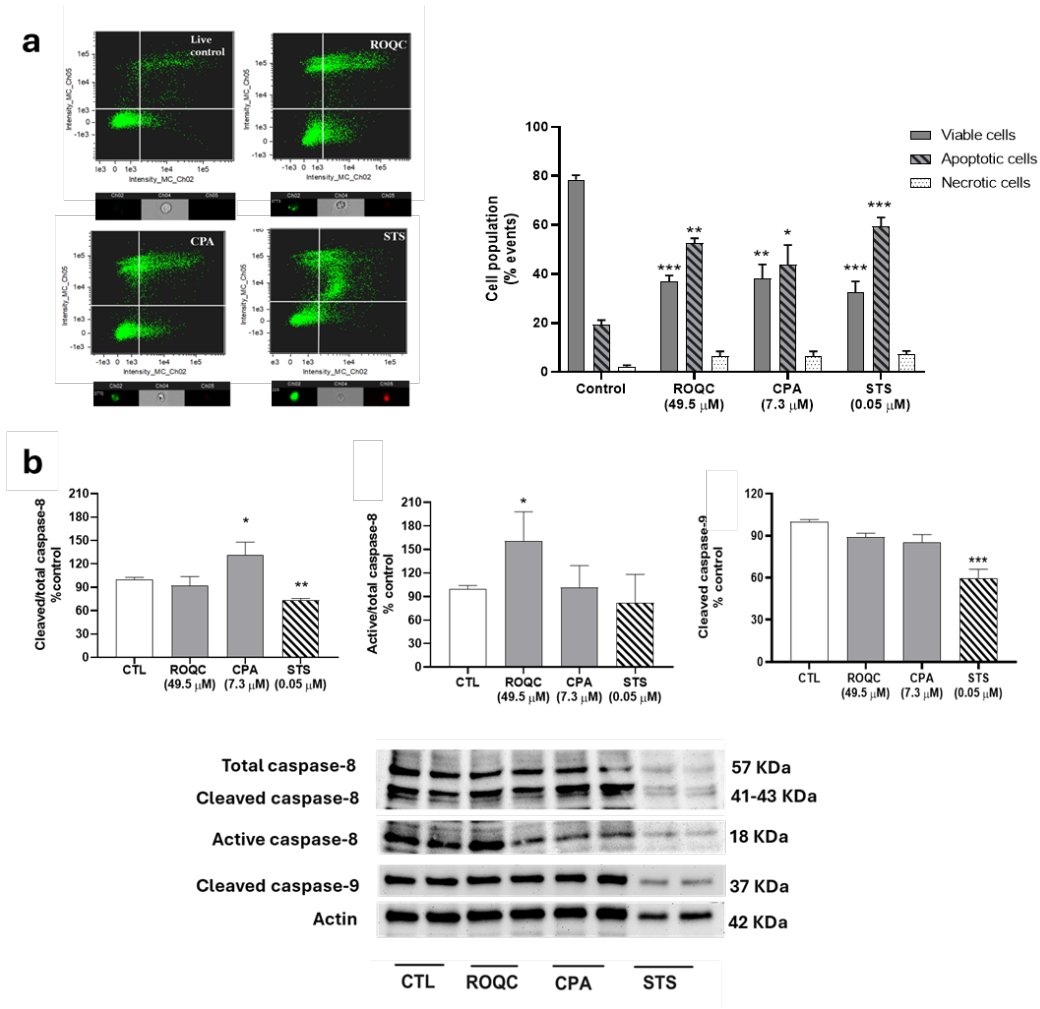

Figure 3. Cell death type induced by ROQC and CPA. (a) Results obtained by flow cytometry of SH-SY5Y cells treated with ROQC and CPA for 24 h at IC_{50} values, co-stained with Annexin V-FITC and PI. Fluorescence of 10000 cells was monitored. Percentages of viable cells (Annexin V-FITC -/PI), apoptotic cells (Annexin V-FITC + /PI – and +), and necrotic cells (Annexin V-FITC – /PI+) were calculated. STS was used as a positive control. (b) Expression of cleaved caspase -8, active caspase-8 and cleaved caspase-9 measured by western blot. Cleaved and active caspase-8 are expressed as ratio between its total levels. Protein band expression is normalized by actin levels. Values are mean ± SEM of three independent replicates, carried out by duplicate, and compared to control cells by one-way ANOVA followed by Dunnett test (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$).

3.2. Effects of combined emerging mycotoxins on viability and cytotoxicity of SH-SY5Y cells

As cheese samples are commonly contaminated with ROQC and CPA in combination with other emerging mycotoxins, these two compounds where binary combined with ENNs A, A1, B and B1 at 10:1 ratio. The proportion was selected based on the most common proportions found in cheeses (Rodríguez-Cañás et al., 2023).

Before starting with the combined effects, the cytotoxicity of ENNs was tested. We had previously performed dose-response assays with these toxins in SH-SY5Y cells cultured in medium with 10% FBS (N. Pérez-Fuentes et al., 2021; 2022). Recently, we observed that the reduction of FBS concentration to

2%improves the results and reduces the variability between replicates (Figure 1S)(Mengual Gómez et al., 2010; Khasawneh et al., 2019; Lopes et al., 2010). In this sense, both MTT and LDH assays were repeated with ENNs A, A1, B and B1 in cell medium with 2% FBS (Figure 2S). No significant variations were observed in the data obtained with MTT assay, except for ENN B. This toxin did not produce a complete inhibition of cell viability in our previous results, but with a lower percentage of FBS we observed a total reduction at the highest dose tested, obtaining an IC_{50} of 1.5 µM (CI: 0.93 – 3.5 µM), in the same range than the previous one (0.43 µM). Regarding LDH release, no differences were detected, as EC_{50} values are similar to the previously reported for ENNs A, A1 and B1, whilst ENN B did not produce cell leakage(N. Pérez-Fuentes et al., 2022; 2021).

After confirming that FBS concentration did not affect ENNs results, the effects on cell viability and cytotoxicity of ROQC binary combined with ENNs (10:1) were determined. Regarding cell viability, the results obtained with the combination ROQC + ENN A were higher than those of ROQC alone, but similar to those of ENN A alone, which caused cell damage at concentrations higher than 2 µM (Figure 4 a). In ROQC + ENN A1 mixture, the effect of both toxins alone was enhanced, with a reduction in cell viability of 42.7 ± 13.5% at a concentration of 20 µM + 2 µM (Figure 4 b). In the case of ROQC + ENN B and ROQC + ENN B1 combinations, a decrease in cell viability similar to the effect produced by both ENNs alone was obtained. However, this reduction in cell survival was higher than the induced by ROQC alone (Figure 4 c, d).

Data obtained with LDH assay for ROQC combinations were consistent with those of cell viability. ROQC + ENN A and ROQC + ENN A1 mixtures showed greater effects than the emerging mycotoxins alone, inducing cytotoxicity at concentrations higher than 20 µM + 2 µM (Figure 4 e, f). ROQC + ENN B combination produced complete death at the highest concentrations used (80 µM + 8 µM and 100 µM + 10 µM), potentiating the effects of ENN B alone (Figure 4 g). For ROQC + ENN B1 mixture, the effects observed were slightly higher than those of both mycotoxins alone, showing a LDH release of 46.2 ±15.5 % at 40 µM + 4 µM (Figure 4 h).

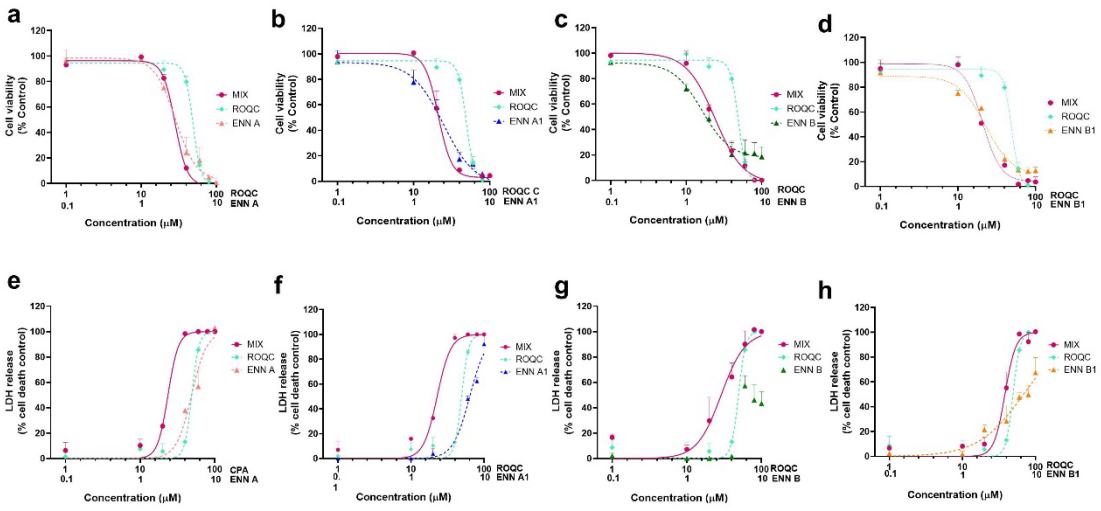

Figure 4. Effects on cell viability and cytotoxicity of binary combinations of ROQC with ENNs A, A1, B and B1. Cells were treated with binary mixtures of mycotoxins for 24 h. (a) Effect of ROQC + ENN

A (10:1), (b) ROQC+ ENN A1 (10:1), (c) ROQC + ENN B (10:1) and (d) ROQC + ENN B1 (10:1) on cell viability, assessed by MTT test. (e) Cytotoxicity of ROQC + ENN A (10:1), (f) ROQC+ ENN A1 (10:1), (g) ROQC + ENN B (10:1) and (h) ROQC + ENN B1 (10:1), determined by LDH assay. Purple line represents the mixture (MIX) of toxins, light green pointed line corresponds to ROQC, red pointed line represents ENN A, blue line represents ENN A1, dark green pointed line corresponds to ENN B and ENN B1 is represented by an orange pointed line. Saponin at 1 mg/mL was used as cell death control. Pointed lines show the effects of each toxin alone while purple line represents the mixture. Data are mean ± SEM of three independent replicates. Data were fitted with a log (inhibitor) *vs* response and a log (agonist) *vs* response model for cell viability and LDH assays, respectively.

Then, the effects of CPA and ENNs (10:1) binary mixtures were determined. In MTT assay, all combinations showed a dose-response curve similar to the produced by CPA alone. The effects of ENNs were slightly increased since a reduction on cell viability was induced at concentrations above 1 µM + 0.1 µM (Figure 5 a-d). By contrast, when cytotoxicity was determined, CPA effects were intensified in all combinations, reaching a 100% of LDH release in CPA + ENN A and CPA + ENN A1 mixtures (Figure 5 e-h).

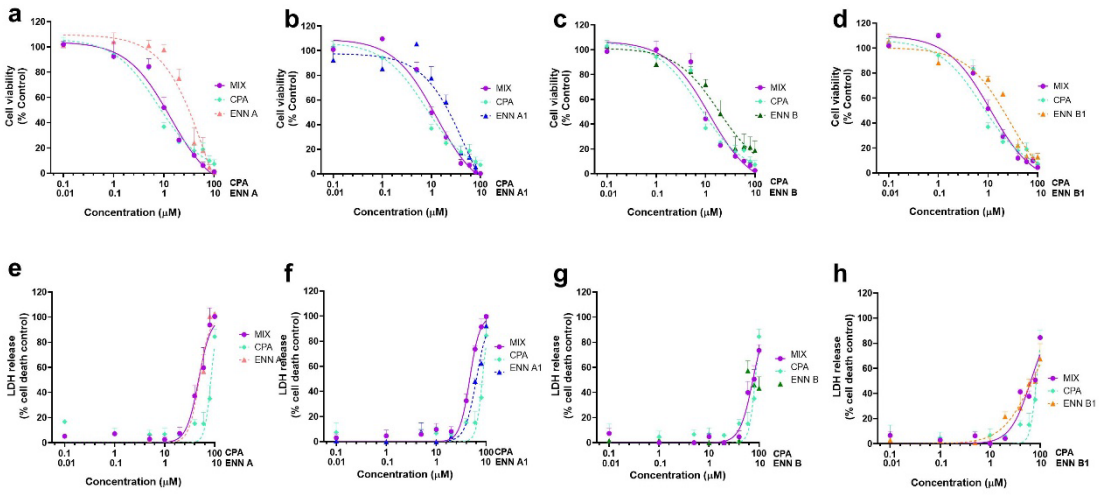

Figure 5. Cell viability and cytotoxicity of CPA binary combinations with ENNs A, A1, B and B1. SH-SY5Y cells were treated with binary mixtures of mycotoxins for 24 h. (a) Effect of CPA + ENN A (10:1), (b) CPA+ ENN A1 (10:1), (c) CPA + ENN B (10:1) and (d) CPA + ENN B1 (1:10) on cell viability, assessed by MTT test. (e) Cytotoxicity of CPA + ENN A (10:1), (f) CPA+ ENN A1 (10:1), (g) CPA + ENN B (10:1) and (h) CPA + ENN B1 (10:1), determined by LDH assay. Purple line represents the mixture (MIX) of toxins, pointed lines corresponds to each individual compound. Saponin at 1 mg/mL was used as cell death control. Pointed lines show the effects of each toxin alone while purple line represents the mixture. Data are mean ± SEM of three independent replicates. Data were fitted with a log (inhibitor) *vs* response and a log (agonist) *vs* response model for cell viability and LDH assays, respectively.

Then, IC_{50} and EC_{50} values were calculated for each mixture by fitting the data with a log(inhibitor) *vs* response model and with a log(agonist) *vs* response model, respectively (Table 1S). With these results,

combination index was obtained following Chou & Talalay method, and the value obtained was higher than 1 in all cases, indicating antagonism (Ting-Chao Chou and Talalay, 1983).

Since ROQC and CPA are usually detected in cheese combined with ENNs A, B, A1 and B1, as well as with BEA, all the toxins were tested together at ratios according to their natural proportions. As MTT test is more sensitive, it was performed to determine the effects of mixtures on cell viability. Then, IC_{50} values and combination index for each mixture were calculated as described above.

Before proceeding with the combination of all toxins, the four ENNs (A, A1, B and B1) were mixed in a 1:1:1:1 ratio at concentrations ranging from 0.01 to 10 μM. We had previously tested binary combinations of these toxins at 1:1 proportion and most of them produced antagonism, except ENN B1 and ENN A mixture, that resulted in an additive effect (Nadia Pérez-Fuentes et al., 2023; N. Pérez-Fuentes et al., 2022). As Figure 6 a shows, treatment with the four ENNs together enhanced the effects of the single toxins, presenting IC_{50} values of 1.1 μM (CI: 0.6 – 2.0 μM), slightly lower than the IC_{50} of each toxin alone (among 1.5 and 2.7 μM). Nevertheless, the combination index for this mix was 2.0, indicating antagonism. Then, ROQC was mixed with the four ENNs at 10:1:1:1:1 ratio (ROQC+ENN A+ENN A1+ENN B+ENN B1). With this combination, an increase greater than 15% on their individual effects was observed (Figure 6 b). The IC_{50} obtained was 3.0 μM (CI: 1.2 - 5.5 μM) for ROQC and 0.3 μM (CI: 0.12 - 0.55 μM) for each ENN. Then, a combination index of 0.6 was obtained, revealing a synergistic effect among these toxins. CPA was also combined with ENNs A, A1, B and B1 at a 10:1:1:1:1 proportion. In this case, CPA effects were potentiated about a 9%. The IC_{50} for CPA was 0.82 μM (CI: 0.1 – 1.7 μM) and a value of 0.08 μM (CI: 0.01 - 0.17 μM) was obtained for each ENN (Figure 6 c). In this case, the combination index was 0.26, indicating synergism.

Next, BEA was added to the previously described mixtures at the same proportion as ENNs. Combinations were performed as follows: ENN A+ ENN A1+ ENN B+ ENN B1 + BEA (1:1:1:1:1), ROQC+ ENN A+ ENN A1+ ENN B+ ENN B1 + BEA (10:1:1:1:1:1) and CPA + ENN A+ ENN A1+ ENN B+ ENN B1 + BEA (10: 1:1:1:1:1). The effects observed for the first mixture were similar to the obtained with the four ENNs, showing an IC_{50} of 1.1 μM (CI: 0.6 – 1.8 μM) for each toxin (Figure 6 d). The same occurred when ROQC was combined with ENNs and BEA, whose individual effects were increased (Figure 6 e). The IC_{50} of this combination was 4.8 μM (CI: 2.3 – 8.4 μM) for ROQC and 0.5 μM (CI: 0.23 – 0.84 μM) for ENNs and BEA. The mixture of CPA with ENNs and BEA also intensified the effects of individual mycotoxins, but it seems to present a lower effect than CPA and ENNs combination (Figure 6 f). In this case, the IC_{50} was 2.4 μM (CI: 0.74 – 5.0 μM) for CPA and 0.24 μM (CI: 0.07 – 0.5μM) for each ENN and BEA, respectively. Although potentiation of the individual effects of the mycotoxins was observed in all BEA mixtures, the combination index was higher than 1 in all cases, except for the mixture of CPA with ENNs and BEA, which presented a value of 0.9, indicating an additive effect.

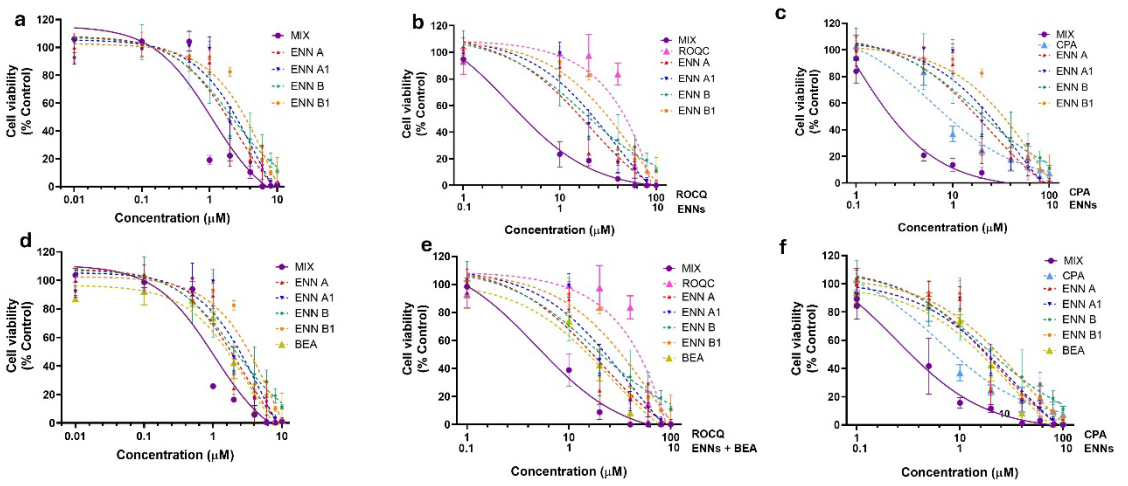

Figure 6. Effects of emerging mycotoxins multiple combinations on viability of SH-SY5Y cells. Cells were treated with combined mycotoxins for 24 h and MTT assay was carried out. Pointed lines showed the effects of each toxin alone while purple line represents the mixture of all. (a) ENN A+ ENN A1+ ENN B + ENN B1 (1:1:1:1), (b) ROQC + ENN A+ ENN A1+ ENN B + ENN B1 (10:1:1:1:1), (c) CPA + ENN A+ ENN A1+ ENN B + ENN B1 (10:1:1:1:1), (d) ENN A+ ENN A1+ ENN B + ENN B1 + BEA (1:1:1:1:1), (e) ROQC + ENN A+ ENN A1+ ENN B + ENN B1 + BEA (10:1:1:1:1:1) and (f) CPA + ENN A+ ENN A1+ ENN B + ENN B1 + BEA (10:1:1:1:1:1). Purple line represents the mixture (MIX) of toxins, pointed lines corresponds to each individual compound. Mean ± SEM of three independent replicates expressed as percentage of untreated control cells. Data were fitted with a log (inhibitor) *vs* response model

3.3. Analysis of mycotoxin content and cytotoxicity of three cheese samples

In view of the results obtained with mycotoxin combinations, the cytotoxicity of cheese extracts was studied in the same cell model to test the effect of a real mixture. At first, cheese samples were analysed using an in-house validated method designed for the quantification of 32 mycotoxins, including regulated, emerging, and modified compounds. Notably, the method exhibits high sensitivity, enabling the detection of compounds at concentrations below the maximum residue levels specified in European regulations. Moreover, the method demonstrates satisfactory accuracy and precision, meeting the performance criteria defined in legislation (Jesús M. González-Jartín et al., 2020; EC, 2006).

Several mycotoxins were detected in the extracts (Figure 3S), and their quantification was done by using linear 1/X calibration curves. Results were adjusted for matrix effects and recovery, and expressed in nM, as shown in Table 2S. No regulated or modified mycotoxins were detected, however, several emerging compounds were identified, including trace amounts (< 20 µg/kg) of *Fusarium* toxins namely BEA and ENNs, which were consistently found across all cheese types. Additionally, low levels of alternariol methyl-ether (AME) and sterigmatocystin (STG) were detected. Furthermore, ROQC was detected in the Emmental sample at levels close to 2500 µg/kg and CPA was found in blue cheese at 800 µg/kg.

Then, the three cheese extracts were tested in human neuroblastoma cells. Camembert sample was used as a matrix control, since it was the less contaminated cheese, and it did not present ROQ and CPA. The effects

of the extracts on cell viability and cytotoxicity were determined with MTT and LDH, respectively. Cells were treated with the extracts at dilutions between 1:200 and 1:1000 for 24 h. In MTT assay, Emmental extract reduced cell viability between 20.0 and 99.9% at concentrations above 1:500 compared to the Camembert control. On the other hand, blue cheese extract caused a smaller reduction in viability (40.1 - 66.0% at concentrations between 1:400 and 1:200) (Figure 7 a). LDH release was increased by 84.8 ± 6.4 % and 13.9 ± 4.8 % at the highest concentration used for Emmental and blue cheese extracts, respectively, in agreement with the results obtained in the MTT test (Figure 7 b).

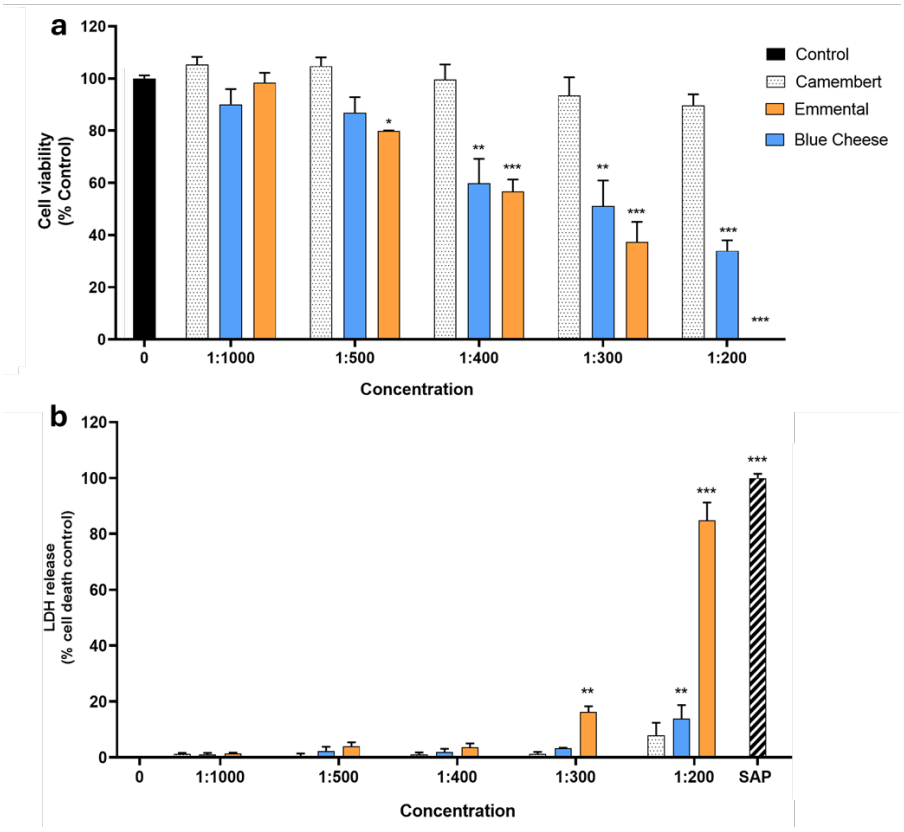

Figure 7. Effect of cheese extracts on cell viability and cytotoxicity of SH-SY5Y cells. Cells were treated with dilutions between 1:200 - 1:1000 of each cheese extract for 24 h. Effects of emmental extract (orange), blue cheese extract (blue) and camembert extract as blank (white) where determined on (a) cell viability and (b)cytotoxicity by MTT test and LDH assay, respectively. Saponin (SAP) at 1 mg/mL was used as cell death control. Data are mean ± SEM of three independent replicates expressed as percentage of untreated control cells. IC_{50} values were determined by fitting the data with a log (inhibitor) vs response model. EC_{50} values were determined by fitting the data with a log (agonist) vs response model.

4. Discussion

Mould growth on cheese is a major concern as it raises both food quality and safety issues. While several genera of fungi can affect cheese in terms of decomposition, some species are used in the ripening process. *Penicillium* is the most commonly used genus in this process. These cheese-contaminating moulds have the potential to produce mycotoxins such as ochratoxin A, STG and CPA, that remain stable under processing

conditions, retaining their cytotoxic activity (Izzo et al., 2022; Rodríguez-Cañás et al., 2023; Kure and Skaar, 2019). To date, the EU legislation does not regulate levels of mycotoxins in cheese, and only aflatoxin M1 is regulated in milk and milk-derived products (EC, 2023). However, recent studies have highlighted the presence of emerging mycotoxins in both milk and cheese at a wide range of concentrations, including ENNs, BEA, ROQC and CPA (Izzo et al., 2022; Rodríguez-Cañás et al., 2023; J. M. González-Jartín et al., 2021). In this sense, we have previously analysed the effects of two milk extracts, contaminated with ENNs and BEA at concentrations in the picomolar range, observing that their co-occurrence had a greater effect on cell viability than expected (N. Pérez-Fuentes et al., 2021).

In the present study, the neuronal cell line SH-SY5Y was used because it has been described that ENNs, BEA, ROQC and CPA present neurotoxic effects(Aninat et al., 2001; Fliszár-Nyúl et al., 2022; Agahi et al., 2021; Krug et al., 2018). The first step was to study the single effects of ROQC and CPA, as these toxins have been detected in different cheese samples (Rodríguez-Cañás et al., 2023). Neither ROQC nor CPA are considered a potential health concern because they do not cause significant toxicological effects at the concentrations commonly found in food. However, data on their cytotoxicity are insufficient (N. Hymery et al., 2018; Maragos et al., 2023). In our assays, CPA was more toxic than ROQC, presenting an IC_{50} of 7.3 µM. This finding contrasts with a recent study on the same cell line which reported an IC_{50} of 40.8 µM for CPA after 72 h treatment (de Sá et al., 2024). It is important to note that the discrepancy in IC_{50} values could be due to differences in experimental conditions between the two studies. Key variables such as the solvent used, the number of cells, the percentage of FBS in the culture medium and the duration of the assay were not consistent between the experiments. These factors can significantly influence cell viability results (Ghasemi et al., 2021; Mengual Gómez et al., 2010; Khasawneh et al., 2019). As the toxin stock in the current work was prepared in DMSO, which has the ability to interact with phospholipids in cell membranes and enables the diffusion of molecules across the cell membrane, the use of this vehicle could facilitate the uptake of the toxin into the cell, allowing it to exert its effects more rapidly (Nguyen et al., 2020; Nolwenn Hymery et al., 2014). Indeed, in a study using DMSO as a solvent for CPA, a nanomolar reduction in cell viability was observed in several cell lines (Nolwenn Hymery et al., 2014).Then, to support the cell viability data, a cytotoxicity assay was performed. In this sense, CPA had an EC_{50} of 84.1 µM. The cytotoxicity method used measures the release of LDH into the culture media after cell membrane disruption, whereas MTT assay is more sensitive because it measures the cellular metabolic activity (Fotakis and Timbrell, 2006). Therefore, our data may indicate that CPA interrupts cellular metabolism without compromising plasma membrane integrity until high concentrations are reached. In the case of ROQC, it shows the same value in both assays (49.5 µM), indicating that this compound produces metabolic and cell membrane disruption simultaneously, which suggest a different mechanism of action than CPA. The exact mode of action of ROQC toxicity is not yet understood, but it inhibits rat and human cytochrome P450, involved in cellular metabolism and homeostasis, which could be related to the effects observed (Metin, 2023; Zhao et al., 2021).Both ROQC and CPA induced apoptotic cell death, involving the extrinsic pathway of apoptosis, as they increased the expression of active and cleaved caspase -8, respectively (Y. Wu et al., 2016). While there are no data on the type of death induced by ROQC, CPA has been described to induce p53-dependent apoptosis and to enhance IL-1β apoptosis in several cell lines, supporting our findings (Suzuki et al., 2024; Bonyadi et al., 2021; Miani et al., 2013).

On the other hand, ENNs and BEA, common mycotoxins found in cheese samples, have also been described to induce apoptosis of human neuroblastoma cells (N. Pérez-Fuentes et al., 2021; 2022; Rodríguez-Cañás et al., 2023; Izzo et al., 2022). ENNs and BEA have been the subject of recent studies due to their increasing prevalence in various foods. These toxins induce cytotoxicity in the micromolar range, affect mitochondrial function and alter calcium homeostasis in various cell lines (N. Pérez-Fuentes et al., 2022; Nadia Pérez-Fuentes et al., 2023; Alonso-Garrido et al., 2020; Aufy et al., 2023; Caloni et al., 2020). Although some studies reported additive or synergistic effects of these toxins in combination with other mycotoxins, these data are insufficient (EFSA, 2014). In a recent study, we observed an additive effect of the combination of ENN A + ENN B1 in SH-SY5Y cells (N. Pérez-Fuentes et al., 2022). However, the toxicity of all ENNs (A, A1, B and B1) together has not yet been evaluated in this cell line. In this sense, the present study describes for first time a greater combined effect of ENNs A, A1, B and B1 mixture than the observed when each toxin was added alone. Given their cytotoxicity and high prevalence in many natural samples, the co-occurrence of ENNs in food could pose a significant health risk. It should be noted that these four ENNs often contaminate natural matrices together, increasing the need to further evaluate their effects in combination in other cell lines and even in animal models (Izzo et al., 2022; Chalyy et al., 2021; H. Olleik et al., 2019a; Maranghi et al., 2018; Fraeyman et al., 2017).

Regarding CPA, it has shown additive effects with aflatoxins, but data on its joint toxicity with other mycotoxins is even scarcer than for ENNs (Pier et al., 1989). In the case of ROQC, to our knowledge, there are no studies about its combinations. Considering that both compounds are used in cheese ripening and that they are cytotoxic by themselves, we first tested their effects in binary combination with ENNs.

In all the mixtures, a shift in the combination curve is observed, suggesting an enhancement of their individual effects. Nevertheless, when the combination index was calculated, the value was greater than 1 in all cases, indicating antagonism. This discrepancy could be due to the fact that synergism is mutual for all compounds, whereas potentiation, enhancement or augmentation is unilateral (T. C. Chou, 2010). In some of the binary mixtures performed, we observed potentiation for one of the compounds but not for the other, as occurs in ROQC and ENN A mixture or in the combination of ROQC, ENNs and BEA, in which the combined IC_{50} is much lower than the individual value of each toxin. Synergism was only achieved in two of the mixtures tested (ROQC+ ENN A+ENN A1+ ENN B+ ENN B1 and CPA+ ENN A+ENN A1+ ENN B+ ENN B1), in which the combined IC_{50} was at least 9 times lower than the individual IC_{50} of each toxin. In the case of CPA+ ENN A+ENN A1+ ENN B+ ENN B1+ BEA mixture, an additive effect was observed. While ROQCs and CPAs are not considered a potential health hazard, existing toxicity data are limited to studies of these compounds alone without contemplating potential interactions with other mycotoxins (N. Hymery et al., 2018; Suzuki et al., 2024; Nolwenn Hymery et al., 2014). Here we observed that the co-occurrence of ENNs with CPA and ROQC, often observed in cheeses, significantly increases the effects of these two toxins, up to concentrations that could be found in natural samples (Rodríguez-Cañás et al., 2023; Maragos et al., 2023; Izzo et al., 2022). Hence, as they could be a health concern, it would be interesting to deepdive into their combined effects.

Considering that mycotoxins are liposoluble substances and cheese is mainly fat, the effects of the combination of these toxins could be further enhanced by the matrix (Taevernier et al., 2016; Krug et al., 2018; Alonso-Garrido et al., 2021). Interestingly, the analysis of extracts from cheese commercial samples

showed a strong reduction in cell viability, especially with the Emmental extract. As an academic exercise with no real-world implications, the 1:200 concentration of this extract rich in ROQC would be equivalent to approximately 45 g of cheese consumed. This estimation is based on a 60 kg woman with a body composition of 60% water. Therefore, even a small amount of cheese could potentially cause cellular damage. Although this calculation does not reflect a food risk evaluation, which was not the purpose of the article, it is worth mentioning that an extreme value from a commercial product could pose cytotoxic levels. Therefore, the role of emerging mycotoxins in food, cheese in this article, is clearly in need of risk evaluation, as their combined presence increases several folds the toxicity of certain mycotoxins. Also, this article provides experimental evidence that cheese should be included in the list of mycotoxins-regulated products. However, it would be necessary to evaluate the effects of other contaminated cheese matrix in different cell lines and animal models to confirm the hypothesis presented here.

In conclusion, our results indicate that ROQC and CPA are cytotoxic to human neuroblastoma cells and induce apoptosis via the extrinsic pathway. Their binary combination with ENNs A, A1, B and B1 results in an enhancement of the individual effects of each toxin. In addition, the mixture of emerging mycotoxins produced a higher effect than the cytotoxicity induced by ROQC, CPA, ENNs A, A1, B and B1 alone, and even greater damage was observed when they co-occurred on a natural matrix. In view of these results, it would be of particular interest to compare their effects on different natural matrices, which would help to better understand their impact on human health and consequently decide whether legal limits should be set in food.

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