The dietary biogenic amines tyramine and histamine show synergistic toxicity towards intestinal cells in culture

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Running title: Synergistic cytotoxicity of tyramine and histamine

Abstract

Tyramine and histamine are the biogenic amines (BA) most commonly found at high concentrations in food; they may even appear together at toxic concentrations. The present work examines, via real-time cell analysis, whether histamine and tyramine show synergistic toxicity towards intestinal cell cultures. Employing a constant equipotency ratio, their interaction was examined via the combination index (CI) method of Chou & Talalay. Co-treatment with tyramine and histamine was associated with a stronger cytotoxic effect than was treatment with either BA on its own. Indeed, a synergistic interaction ($CI < 1$) was observed in the range of concentrations found in foods. The results also show that histamine at concentrations below the legal limit increases the cytotoxicity of tyramine at concentrations frequently reached in some foods. The synergistic cytotoxicity of tyramine and histamine should be taken into account when establishing legal limits designed to ensure consumer safety.

Keywords: Biogenic Amines; Histamine; Tyramine; Cytotoxicity; Synergy; Food Safety.

Chemical compounds studied in this article

Tyramine (PubChem CID: 66449); Histamine (PubChem CID: 5818)

1 Introduction

Biogenic amines (BA) are biologically active nitrogenous organic compounds that may accumulate in food via the enzymatic decarboxylation of their precursor amino acids by certain spoilage microorganisms (Alvarez & Moreno-Arribas, 2014; Linares, Martin, Ladero, Alvarez & Fernandez, 2011). Fermented foods and beverages, fish, and fish products, can accumulate high BA concentrations (Bover-Cid, Hugas, Izquierdo-Pulido & Vidal-Carou, 2001; Fernandez, Linares, del Rio, Ladero & Alvarez, 2007; Capozzi et al., 2011; Ladero et al., 2011; Ladero, Cañedo, Perez, Martin, Fernandez & Alvarez, 2012; Ladero, Fernandez, Cuesta & Alvarez, 2010b; Ladero, Linares, Fernandez & Alvarez, 2008; Silla Santos, 1996). The ingestion of BA-rich food can cause adverse toxicological reactions and intoxications harmful to health (Ladero, Calles-Enriquez, Fernandez & Alvarez, 2010a). Indeed, the Food and Agriculture Organization of the United Nations (FAO) (2014), has declared BAs in food to pose a biological hazard.

Tyramine and histamine are the most common BAs found in foods (Linares et al., 2011, 2012). The European Food Safety Authority (EFSA) deems them to be the most toxic of all BAs, and to have a negative impact on food safety (European Food Safety Authority, 2011). The consumption of food rich in tyramine can cause toxicological reactions (headaches, migraine, neurological disorders, nausea, vomiting, respiratory disorders and hypertension) together

referred to as the "cheese reaction" (Broadley, 2010; Finberg & Gillman, 2011; Shalaby, 1996). Similarly, the ingestion of histamine-rich food can cause scombroid syndrome, a condition characterized by adverse neurological, gastrointestinal, circulatory and respiratory symptoms, flushing, rashes and urticaria (Ladero et al., 2010a; Shalaby, 1996; Stratta & Badino, 2012; Visciano, Schirone, Tofalo & Suzzi, 2014).

Although the presence of BAs in foods poses a threat to human health, legislation establishing their maximum legal limit remains wanting. In the European Union, histamine is the only BA for which legal limits have been set (by the EFSA), and then only for scombrid fish (200 mg/kg) and fish products (400 mg/kg) (European Commission, 2005). The US Food and Drug Administration (FDA) suggests 500 mg/kg histamine in fish to be a health threat (Food and Drug Administration, 1996, 2001). For tyramine, only recommended limits have been proposed (European Food Safety Authority, 2011).

The EFSA Panel on Biological Hazards (BIOHAZ) indicates that further research is needed regarding BA toxicity (European Food Safety Authority, 2011). Our group recently examined the toxicity of tyramine and histamine in human intestinal cell cultures via real-time cell analysis (RTCA) (Linares et al., 2016). That work revealed i) both tyramine and histamine to be toxic towards HT29 intestinal cell cultures at concentrations found in BA-rich food, and that ii) tyramine cytotoxicity was due to the induction of necrosis, while histamine exerted its effects via the induction of apoptosis (Linares et al., 2016).

Importantly, tyramine and histamine commonly appear together (along with other BAs such as putrescine and cadaverine) at high concentrations in fermented foods such as cheese (Alvarez et al., 2014; Ladero et al., 2008, 2010b, 2011; Ladero, Martinez, Martin, Fernandez & Alvarez, 2010c; Linares et al., 2011). Knowledge of the toxicity of dietary BA combinations, however, is limited. The aim of the present work was therefore to examine, via RTCA, the effect of tyramine and histamine in combination on human intestinal cell cultures. The nature of their interaction (synergistic, additive or antagonistic) was determined via the combination index (CI) method of Chou & Talalay (1984). Finally, work was performed to determine whether histamine at concentrations below the legal limit increases the cytotoxicity of tyramine at the concentrations reached in some foods.

2 Material and Methods

2.1 Cell line and growth conditions

The intestinal cell line HT29 (ECACC 91072201), derived from a human colorectal adenocarcinoma, was purchased from the European Collection of Cell Cultures and used to create an *in vitro* model of the intestinal epithelium. These cells were routinely cultured in McCoy's 5a medium supplemented with 10% heat-inactivated foetal bovine serum plus a mixture of antibiotics (50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µg/ml gentamicin and 1.25 µg/ml amphotericin B). All media and reagents were purchased from Sigma-Aldrich

(Madrid, Spain). All manipulations required for culturing, passaging (144 to 149 passages were performed) and maintenance of the cell line were undertaken in a 5% CO₂ atmosphere at 37°C, within an SL Waterjacked CO₂ Incubator (Sheldon Mfg. Inc., Cornelius, OR, USA), following standard procedures (Ruas-Madiedo, Medrano, Salazar, de Los Reyes-Gavilan, Perez & Abraham, 2010).

2.2 Real-time cell analysis

The RTCA system is used to gather information on cell proliferation, migration and cytotoxicity via changes in cell morphology and adhesion (Atienzar et al., 2011). An xCelligence Real-Time Cell Analyzer (ACEA Bioscience Inc., Roche Applied Science, Germany) was used as previously described (Linares et al., 2016) to detect any changes in the proliferation, adhesion or morphology of the HT29 intestinal cells following their treatment with different doses of tyramine [4-(2-aminoethyl)phenol hydrochloride] (Acros Organics, Belgium), histamine [2-(4-Imidazolyl) ethylamine dihydrochloride] (Sigma-Aldrich) or their combination. Cells were seeded in 16-well E-Plates (Roche Applied Science) equipped with gold microelectrodes that generate an electric field (<20 mV). Real-time measurements of the electrical impedance (referred to as the cell index) across the interdigitated microelectrodes allows for the monitoring of the variables mentioned above.

Briefly, HT29 cells were seeded at a density of 2×10^4 cells/well in the above E-Plates containing 100 μ l of medium per well. They were then incubated and

monitored in a Heracell-240 Incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) at 37°C in a 5% CO₂ atmosphere (Hidalgo-Cantabrana et al., 2014). Stock solutions of tyramine and histamine were dissolved in water and adjusted to pH 6.8. Approximately 20 h after seeding, the proliferating cells were treated for 24 h with 14 different concentrations of tyramine, histamine or their combination (Table 1). Combination studies were performed employing a constant equipotency ratio (Chou & Talalay, 1984), thus ensuring that the contribution of each BA to the combined toxic effect was equal. To define the equipotency ratio and keep it constant, the assayed concentrations of histamine and tyramine were based on their previously assessed IC₅₀ values (the concentration of BA required to achieve half the most potent cytotoxic effect observed by RTCA): tyramine 3.2 mM, and histamine 26.0 mM (Linares et al., 2016). The tyramine:histamine equipotency ratio (IC_{50(T)}/IC_{50(H)}) was therefore 1:8.125. The concentrations assayed in the co-treatments were 0.03, 0.05, 0.07, 0.11, 0.17, 0.25, 0.33, 0.4, 0.5 and 0.67, 1, 1.5, 2, 2.5 times the IC₅₀ values (Table 1). Tyramine and histamine were also analyzed independently at these concentrations.

After addition of the BA, the cell index was monitored for 24 h; this was normalized to the time point just before the addition of the BA, and set to 1. All tests were performed in triplicate.

Dose-response curves for tyramine, histamine and their combination were constructed using RTCA software, plotting the normalized cell index at 24 h of treatment against the value for the corresponding BA concentration. The

IC₅₀ values for tyramine, histamine and their combination were calculated using RTCA software.

2.3 Live cell microscopy

Cells were seeded at a density of 2×10^4 cells/well and incubated in flat-bottomed 96-well microplates under identical conditions to those used in the RTCA studies. After 20 h of incubation the cells were treated with concentrations of tyramine and histamine at 0.33, 0.4, 0.5, 0.67 and 1 times their IC₅₀ values (Table 1). Tyramine and histamine were also tested independently at these concentrations. At 48 h post-treatment, live cells were visualised using an inverted LumaScope-600 Series optical microscope (Etaluma, Carlsbad, CA) with a 40 x objective.

2.4 Determination of combination index values

The nature of the tyramine-histamine interaction was determined via the CI method using CompuSyn software (ComboSyn, Inc, Paramus, NJ) as previously described (Chou, 2006). The normalized cell index values obtained in RTCA analysis were transformed to percentage cytotoxicity values, deeming the highest and the lowest indices to represent 0% and 100% cytotoxicity respectively. These percentages were treated using CompuSyn software to yield the CI values, which were plotted against the corresponding percentage cell toxicity to render the CI plot. The type of tyramine-histamine interaction was

scored as synergistic ($CI < 1$), additive ($CI = 1$) or antagonistic ($CI > 1$) as previously described (Chou et al., 1983, 1984).

2.5 Statistical analysis

Experimental results are presented as the means $\pm$ standard deviation of three independent replicates. Means were compared using the Student *t* test. Significance was set at $p < 0.05$.

3 Results

3.1 Dynamic cell responses of intestinal cells co-treated with tyramine and histamine

A dose-dependent cytotoxic effect of co-treatment with tyramine and histamine was seen via the progressive reduction in the normalized cell index. Co-treatment induced a significantly greater reduction in this index than did tyramine alone at all the concentrations tested (Fig. 1A to 1I; $*p < 0.05$). It also induced a significantly greater reduction compared to histamine alone, but only at concentrations ranging from $0.4 \times IC_{50}$ to $2 \times IC_{50}$ (Fig. 1C to 1H; $*p < 0.05$). No significant differences were observed at the lowest ($0.25 \times$ and $0.33 \times IC_{50}$; Fig. 1A and 1B) and the highest ($2.5 \times IC_{50}$; Fig. 1I) concentrations tested.

The same concentrations (Table 1) were used to construct dose-response curves for tyramine and histamine alone and in combination. The dose-response curve for the co-treatment always fell below those for both tyramine (Fig. 2A) and histamine (Fig. 2B) alone. In addition, the co-treatment IC_{50} value ($IC_{50(T+H)}$) was reduced by 2.4-fold compared to the IC_{50} for tyramine ($IC_{50(T+H)} = 1.64 \pm 0.02$ mM and $IC_{50(T)} = 3.99 \pm 1.28$ mM respectively), and showed a 2.02-fold reduction when compared to the IC_{50} for histamine ($IC_{50(T+H)} = 13.22 \pm 0.16$ mM and $IC_{50(H)} = 26.80 \pm 1.28$ mM respectively). These findings confirm the increased cytotoxicity of combined tyramine and histamine towards HT29 cell cultures over that of either BA alone.

3.2 Microscopy-based examination of cell cultures treated with combined tyramine and histamine

To observe the cytotoxic effects caused by the tyramine-histamine co-treatment, cell cultures were treated with different concentrations of tyramine, histamine and their combination (maintaining the equipotency ratio) (Fig. 3). The number and morphology of the cells remained apparently unaltered after the treatment with tyramine or histamine alone at concentrations below $0.67 \times IC_{50}$. Their cytotoxicity began to become visible at $1 \times IC_{50}$. The effect of co-treatment was visible at the lowest concentration assayed ($0.33 \times IC_{50}$); at concentrations above $0.4 \times IC_{50}$, drastic changes in cell morphology were seen along with reductions in cell numbers. In fact, almost all the cells showed altered morphology following co-treatment at $1 \times IC_{50}$. These observations

confirm the increased cytotoxicity towards HT29 cell cultures of the histamine and tyramine co-treatment.

3.3 Nature of the tyramine-histamine interaction

The CI plot (Fig. 4) revealed the CI values for tyramine and histamine concentrations below 1.60 mM and 13.00 mM respectively (Fig. 4; f, $0.5 \times IC_{50}$) - which induced <45% of the highest cytotoxicity observed - to fall below the additive line ($CI < 1$). This suggests the tyramine-histamine interaction to be synergistic. Thus, when together, lower concentrations of these BAs are needed to achieve the same degree of cell toxicity associated with either BA alone. The CI values for concentrations of tyramine and histamine above 4.80 mM and 39.00 mM respectively (Fig. 4; i, $1.5 \times IC_{50}$), which caused more than 81% of the highest cytotoxicity observed, were above the additive line ($CI > 1$), indicating antagonism between the BAs at these concentrations.

3.4 Cytotoxic effect of histamine concentrations below the legal limit when combined with concentrations of tyramine reached in certain foods

Tests were performed to determine whether the presence of histamine at concentrations below the lowest threshold established by the EFSA (200 mg/kg, set for scombrid fish) (European Food Safety Authority, 2011) increase the cytotoxicity of tyramine at concentrations reached in certain foods. For this, HT29 cells were treated with 1.71 mM (190 mg/kg) histamine plus 3.64 mM (500 mg/kg) tyramine, and the effect monitored by RTCA. Tyramine and

histamine were also assayed independently at these concentrations (Fig. 5). The curve for 1.71 mM histamine (Fig. 5) revealed continued cell proliferation, indicating this concentration not to be toxic for HT29 cells. A progressive reduction in the normalized cell index was seen, however, when the cell cultures were treated with 3.64 mM tyramine (Fig. 5). Moreover, co-treatment with 1.71 mM histamine and 3.64 mM tyramine caused a significant ($p < 0.05$) reduction in the normalized cell index compared to the tyramine treatment alone. These findings indicate that the cytotoxicity of tyramine is greater when histamine is present at concentrations below the legal limit for scombrid fish.

4 Discussion

Given that some fermented foods may contain several BAs (Linares et al., 2011, 2012), the interactions these may undergo, and how these may affect their toxicity, need to be investigated. To our knowledge, this is the first study to examine the combined toxicity of two dietary BAs - tyramine and histamine - and to investigate their interaction. The present results show that the combined toxic effects of tyramine and histamine towards intestinal cell cultures is greater than that of either BA alone. Indeed, the IC_{50} value for the combined BAs was reduced by 2.4-fold compared to that of tyramine alone, and by 2.02-fold compared to that of histamine alone.

We have recently described that histamine and tyramine have a different cytotoxic mode of action (Linares et al., 2016): while histamine leads cell death by apoptosis, tyramine has a more rapid effect that causes cell necrosis.

Consequently, tyramine might facilitate the access of histamine into the cells, increasing its toxicity. Although the different biochemical events behind their toxicity could explain the observed synergistic effect, further research will be needed to elucidate the precise molecular mechanisms underlying the synergistic toxicity between tyramine and histamine.

In any case, the present results clearly show a synergistic interaction between tyramine and histamine at concentrations of <1.60 mM and <13.00 mM respectively (corresponding to 219.49 mg/kg tyramine and 1445 mg/kg of histamine in food). This synergy is apparent over a range of concentrations easily reached in foods such as cheese (Fernandez et al., 2007; Mayer, Fiechter & Fischer, 2010; Redruello et al., 2013), fish products (Jiang, Xu, Li, Dong & Wang, 2014; Prester, 2011) and meat products (Ladero et al., 2010a).

At concentrations of tyramine and histamine above 4.80 mM and 39.00 mM respectively (corresponding to approximately 658 mg/kg and 4335 mg/kg in food), the interaction between tyramine and histamine was antagonistic. To our knowledge, the literature contains no report of foods containing such high concentrations of these BAs together, although the EFSA has reported very high concentrations of each on its own e.g., 2100 mg/kg for tyramine in blue cheese, and 8910 mg/kg for histamine in fish and fish products (European Food Safety Authority, 2011). Thus, it is unlikely that anyone would ingest food containing tyramine and histamine at antagonistic concentrations (i.e., concentrations that would cause the mutual reduction of the cytotoxic effect of

each BA). However, it should be remembered that, although antagonistic, such high concentrations of tyramine and histamine would still be dangerous to health.

The results also showed that a content of 190 mg/kg histamine - a concentration below the most restrictive (200 mg/kg) legal limit (European Food Safety Authority, 2011) - increased the toxicity associated with 500 mg/kg of tyramine (a concentration easily reached in some foods) (European Food Safety Authority, 2011). In fact, these BAs have been found together at such concentrations (and indeed higher) in cheese (Gosetti, Mazzucco, Gianotti, Polati & Gennaro, 2007; Redruello et al., 2013). In addition, foods that develop high tyramine concentrations, such as cheese (Linares et al., 2011; Spano et al., 2010) and dry fermented sausages (Suzzi & Gardini, 2003), might be consumed with foods that have high histamine concentrations, e.g., other cheeses, fish, fish products and meat products (European Food Safety Authority, 2011), in the same meal.

Further, the cytotoxicity of tyramine and histamine could be enhanced by the ingestion of fermented beverages; alcohol potentiates the toxic effect of BAs (Ladero et al., 2010a), and such beverages can be sources of these BA in their own right (European Food Safety Authority, 2011). In addition, the synergistic cytotoxic effect between tyramine and histamine could be potentiated in individuals that -either genetically or due to the intake of certain antidepressant treatments- have inhibited the amine oxidases responsible for BA detoxification

in the intestinal epithelium (Ladero et al., 2010a; McCabe-Sellers, Staggs & Bogle, 2006). An additional threat is the presence in the ingested food of other BA, such as putrescine, which competes with histamine for diamine-oxidase (Maintz & Novak, 2007) and cadaverine, which is an inhibitor of this enzyme (Lehane & Olley, 2000), increasing histamine toxicity. Besides putrescine and cadaverine, other BAs such as, phenylethylamine, tryptamine, spermine and spermidine can also be present in large amounts in certain foods and beverages (Alvarez et al., 2014). The interactions between these and histamine and tyramine also need to be investigated.

5 Conclusions

In summary, the present results reveal a synergistic cytotoxic effect between tyramine and histamine in an *in vitro* model of human intestinal epithelium. Non-toxic concentrations of histamine (below the safety threshold established by the EFSA) enhance the cytotoxicity of tyramine at concentrations commonly reached in food. This interaction should be taken into account when establishing the legal limits of both BAs in food.

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Table 1. Doses of tyramine and histamine used in co-treatment experiments. BAs were used either alone or combined at a fixed ratio of 1:8.125 (tyramine:histamine).

	<i>Fold reduction or increase in the IC₅₀^a</i>													
	0.03x	0.05x	0.07x	0.11x	0.17x	0.25x	0.33x	0.4x	0.5x	0.67x	1x	1.5x	2x	2.5x
Tyramine (mM)	0.11	0.16	0.24	0.36	0.53	0.80	1.10	1.30	1.60	2.10	3.20	4.80	6.40	8.00
Histamine (mM)	0.86	1.28	1.93	2.89	4.33	6.50	8.70	10.40	13.00	17.30	26.00	39.00	51.90	64.00

^a IC₅₀ values for tyramine and histamine were previously obtained via RTCA analysis [IC_{50(T)} = 3.2 mM and IC_{50(H)} = 26.0 mM respectively] (Linares et al., 2016)

Figure legends

Figure 1. Real-time cell analysis of tyramine, histamine and their combination on HT29 intestinal cells. Cells were exposed to different concentrations of tyramine (T, *circles*), histamine (H, *squares*) and their combination (T+H, *triangles*). The concentrations tested corresponded to the following proportions of the IC₅₀: (A) 0.25 x IC₅₀, (B) 0.33 x IC₅₀, (C) 0.4 x IC₅₀, (D) 0.67 x IC₅₀, (F) 1 x IC₅₀, (G) 1.5 x IC₅₀, (H) 2 x IC₅₀, and (I) 2.5 x IC₅₀ (see Table 1 for tyramine and histamine concentrations). The time of BA administration is marked by a vertical arrow. Data represent the means ± standard deviations of three experiments. **p*<0.05 T or H compared to T+H.

Figure 2. Dose-response curves for tyramine, histamine and the tyramine and histamine combination. HT29 intestinal cells were exposed to different concentrations of tyramine, histamine and their combination. The concentrations tested corresponded to the proportions of the IC₅₀ shown in Table 1. The dose-response curves for (A) tyramine (T, *circles*) and its combination with histamine (T+H, *triangles*), and (B) histamine (H, *squares*) and its combination with tyramine (T+H, *triangles*), were determined as the normalized cell index at 24 h of treatment at the corresponding concentration of the BA. Data represent the means ± standard deviations of three experiments.

Figure 3. Toxicity towards HT29 cells of tyramine and histamine co-treatment. The cells were exposed to different concentrations of tyramine, histamine and their combination. The concentrations tested corresponded to the following

proportions of the IC_{50} : $0.33 \times IC_{50}$, $0.4 \times IC_{50}$, $0.5 \times IC_{50}$, $0.67 \times IC_{50}$ and $1 \times IC_{50}$. Live cells were visualised using an inverted optical microscope (40 x magnification)

Figure 4. Combination index (CI) analysis of tyramine and histamine co-treatment. CI values were calculated for the tyramine and histamine combinations (T+H) used in the RTCA analysis, i.e., corresponding to the following proportions of the IC_{50} : a, $0.11 \times IC_{50}$; b, $1.17 \times IC_{50}$; c, $0.25 \times IC_{50}$; d, $0.33 \times IC_{50}$; e, $0.4 \times IC_{50}$; f, $0.5 \times IC_{50}$; g, $0.67 \times IC_{50}$; h, $1 \times IC_{50}$; i, $1.5 \times IC_{50}$; j, $2 \times IC_{50}$; and k, $2.5 \times IC_{50}$. CI values of <1 , $=1$ and >1 indicate synergy, additivity and antagonism respectively.

Figure 5. Real-time cell analysis of the cytotoxic effect of histamine at a concentration below the legal limit combined with a tyramine concentration commonly reached in food. Cells were co-treated with 1.71 mM histamine and 3.64 mM tyramine (T+H, *triangles*). Single tyramine (T, *circles*) and histamine (H, *squares*) were also tested. The BA administration point is shown by a vertical arrow. Data represent the means $\pm$ standard deviations of three experiments. $*p < 0.05$ for T+H compared to either T or H.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5