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1 Comparative analysis of the *in vitro* cytotoxicity of the dietary biogenic
2 amines tyramine and histamine
3
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22 Running title: *In vitro* cytotoxicity of tyramine and histamine
23
24
25

26 **Abstract**

27

28 Tyramine and histamine, the most toxic biogenic amines (BA), are often
29 found in high concentrations in certain foods. Prompted by the limited
30 knowledge of BA toxicity, and increasing awareness of the risks associated
31 with high intakes of dietary BA, the *in vitro* cytotoxicity of tyramine and
32 histamine was investigated. Tyramine and histamine were toxic for HT29
33 intestinal cell cultures at concentrations commonly found in BA-rich food, as
34 determined by real-time cell analysis. Surprisingly, tyramine had a stronger
35 and more rapid cytotoxic effect than histamine. Their mode of action was
36 also different, while tyramine caused cell necrosis, histamine induced
37 apoptosis. To avoid health risks, the BA content of foods should be reduced
38 and legal limits established for tyramine.

39

40 **Keywords**: Biogenic amines; Cytotoxicity; Food security; Histamine;
41 Tyramine.

42

43 **Chemical compounds studied in this article**

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

45

46 **1. Introduction**

47

48 Biogenic amines (BA) are described as biologically active, low molecular
49 weight organic bases that may accumulate in some foods owing to the
50 undesirable microbial decarboxylation of certain amino acids (Linares,
51 Martin, Ladero, Alvarez, & Fernandez, 2011; Naila, Flint, Fletcher, Bremer, &
52 Meerdink, 2010). BA-producing microorganisms may be contaminants, but
53 may also belong to groups that participate in food fermentation. In fact,
54 certain bacterial species widely used in the dairy industry, and which enjoy
55 'generally regarded as safe' (GRAS) status, have been typified as BA
56 producers (Fernandez, Linares, & Alvarez, 2004; Ladero, Rattray, Mayo,
57 Martin, Fernandez, & Alvarez, 2011; Linares, del Rio, Ladero, Redruello,
58 Martin, Fernandez, et al., 2013).

59

60 Foods likely to contain high BA concentrations are fish and fish products,
61 along with fermented foods and beverages such as cheese and wine
62 (European Food Safety Authority, 2011; Linares, del Rio, Ladero, Martinez,
63 Fernandez, Martin, et al., 2012). The presence of BA in food is deemed a
64 biological hazard by the Food and Agriculture Organization of the United
65 Nations (FAO) (Food and Agriculture Organization of the United Nations,
66 2014). Although cheese has been implicated in outbreaks of histamine
67 poisoning, such incidents have long been associated with the ingestion of
68 spoiled fish of the *Scombroidae* family (which gave rise to the term
69 'scombroid syndrome') (Food and Drug Administration, 2001; Visciano,
70 Schirone, Tofalo, & Suzzi, 2014). Typically, such poisoning is manifested as

71 allergic reactions, acute gastrointestinal distress, and perhaps neurological
72 and cutaneous symptoms (Eastaugh & Shepherd, 1989). The term 'cheese
73 reaction' was coined to refer to the symptoms - hypertension, migraine,
74 headaches and other neurological problems - induced following the ingestion
75 of cheese with high tyramine concentrations (Price & Smith, 1971; Shalaby,
76 1996; Stratton, Hutkins, & Taylor, 1991). These problems can be particularly
77 severe in people who are susceptible owing to a reduced capacity to detoxify
78 BA in the gut (Ladero, Calles-Enriquez, Fernandez, & Alvarez, 2010; Spano,
79 Russo, Lonvaud-Funel, Lucas, Alexandre, Grandvalet, et al., 2010). This
80 capacity may vary due to genetic reasons, but risk factors such as
81 gastrointestinal disease, the taking of certain medications and the intake of
82 alcohol can reduce the activity of the detoxifying enzymes (Ladero, Calles-
83 Enriquez, Fernandez, & Alvarez, 2010).
84
85 Although it is generally accepted that BAs should not be allowed to
86 accumulate in food products, legislation in this respect is insufficient. It is
87 very difficult to establish a uniform maximum limit for ingested BAs since their
88 toxic effect depends on the type of BA in question, the presence of
89 modulating compounds, and on the efficiency of each person's detoxification
90 system. The only BA for which maximum limits have been legally set by the
91 European Food Safety Authority (EFSA) is histamine, and then only in
92 scombroid-like fish (200 mg/kg) and fish products (400 mg/kg) (European
93 Comission, 2005). The US Food and Drug Administration (FDA) suggested
94 histamine concentrations in fish of over 500 mg/kg to be a danger to health

95 (Food and Drug Administration, 1996, 2001). No further legislation exists
96 anywhere to regulate histamine or the remaining BAs in any other food.
97
98 Recently, the EFSA Panel on Biological Hazards (BIOHAZ) conducted a
99 qualitative risk assessment for BAs in fermented foods, and concluded that
100 our present knowledge of their toxicity was limited, and that further research
101 was needed (European Food Safety Authority, 2011). Using real-time cell
102 analysis (RTCA), the present work examines the *in vitro* toxicity of tyramine
103 and histamine (the predominant BA in food) on human intestinal cells. The
104 gastrointestinal mucosa, which is composed mainly of a monolayer of
105 intestinal epithelial cells (IEC), represents the first point of contact with orally
106 ingested BA. Cells belonging to the HT29 intestinal cell line, used as an *in*
107 *vitro* model of IEC, were therefore exposed to different doses of tyramine and
108 histamine and their cytotoxicity, including their capacity to induce necrosis
109 and apoptosis, determined. The IC_{50}, the 'non-observed adverse effect level'
110 (NOAEL) and the 'lowest observed adverse effect level' (LOAEL) values
111 were calculated for each BA.
112
113 **2. Material and Methods**
114
115 *2.1. Cell line and growth conditions*
116
117 The intestinal cell line HT29 (ECACC 91072201), derived from a human
118 colorectal adenocarcinoma, was purchased from the European Collection of
119 Cell Cultures and used to create an *in vitro* model of the intestinal epithelium.

120 The HT29 cells were routinely cultured in McCoy's 5a medium supplemented
121 with 10% heat-inactivated foetal bovine serum plus a mixture of antibiotics
122 (50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 1.25
123 μg/ml amphotericin B). All media and reagents were purchased from Sigma-
124 Aldrich (Madrid, Spain). All manipulations required for culturing, passaging
125 (144 to 149 passages were performed) and maintenance of the cell line were
126 undertaken in a 5% CO_2 atmosphere at 37°C, in a SL Waterjacked CO_2
127 Incubator (Sheldon Mfg. Inc., Conrnelius, OR, USA), following standard
128 procedures (Ruas-Madiedo, Medrano, Salazar, De Los Reyes-Gavilan,
129 Perez, & Abraham, 2010).
130
131 *2.2. Real-time cell analysis*
132
133 The RTCA system is used to gather information on cell proliferation,
134 migration and cytotoxicity via changes in cell morphology and adhesion
135 (Atienzar, Tilmant, Gerets, Toussaint, Speeckaert, Hanon, et al., 2011). An
136 xCelligence Real-Time Cell Analyzer (ACEA Bioscience Inc., Roche Applied
137 Science, Germany) was used as previously described (Limame, Wouters,
138 Pauwels, Fransen, Peeters, Lardon, et al., 2012) to detect any changes in
139 the proliferation, migration adhesion or morphology of the HT29 intestinal
140 cells following their treatment with different doses of tyramine [4-(2-
141 aminoethyl)phenol hydrochloride] (Acros Organics, Belgium) and histamine
142 [2-(4-Imidazolyl)ethylamine dihydrochloride] (Sigma-Aldrich). Cells were
143 seeded in 16-well E-Plates (Roche Applied Science) equipped with gold
144 microelectrode sensors at the bottom. These sensors generate an electric

145 field when a low voltage (<20 mV) is applied between the electrodes. Real-
146 time measurements of the electrical impedance (referred to as the cell index)
147 across the interdigitated microelectrodes allows for dynamic monitoring.
148
149 Briefly, HT29 cells were seeded at a density of 2 x 10^4 cells/well (for studies
150 on proliferating cells) or 2 x 10^5 cells/well (for studies on confluent cells) in
151 16-well E-Plates containing 100 µl of medium per well, and then incubated
152 and monitored in a Heracell-240 Incubator (Thermo Electron LDD GmbH,
153 Langenselbold, Germany) at 37°C and with a 5% CO_2 atmosphere (Hidalgo-
154 Cantabrana, Kekkonen, de los Reyes-Gavilan, Salminen, Korpela,
155 Gueimonde, et al., 2014). Stock solutions of tyramine and histamine were
156 dissolved in water and adjusted to pH 6.8. Approximately 20 h after seeding,
157 the cells were treated with one of 13 concentrations of tyramine (0 to 28.9
158 mM) or histamine (0 to 129.7 mM). The cell index was monitored for 24 h for
159 proliferation studies, and for 30 h for confluence studies, under the same
160 conditions. The cell index was normalized to the time point just before the
161 addition of the BA and set to 1. For each condition, measurements were
162 performed in triplicate.
163
164 Dose-response curves for tyramine and histamine were constructed using
165 RTCA software, plotting the normalized cell index at 24 h of treatment
166 against the $\log_{10}$ value of the corresponding BA concentration. Non-linear
167 regression trend lines were fitted to sigmoid dose-response (variable slope)
168 curves using SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA,
169 USA). This software also provided the coefficient of determination (R^2) as a

170 measurement of the goodness of fit of the data to the curves, as well as the
171 Hill slope value, which indicates the steepness of the curve.
172
173 The IC_{50} was defined as the concentration of BA required to achieve half of
174 the strongest cytotoxic effect observed by RTCA. IC_{50} values for tyramine
175 and histamine were calculated in proliferating cultures using RTCA software
176 to construct a sigmoid dose response curve for arbitrary time points (8 h, 12
177 h, 18 h and 24 h) of BA exposure.
178
179 *2.3. Fluorescence microscopy: nuclear DNA staining*
180
181 Cells were seeded at a density of 2 x 10^5 cells/well, and incubated in flat-
182 bottom microplates under identical conditions to those used in the RTCA
183 studies. After 20 h of incubation the cells were treated with one of six
184 different concentrations of tyramine (0 to 17.3 mM) or histamine (0 to 21.7
185 mM). At 24 h post-treatment, the cells were washed with 0.1 M PBS pH 7.2,
186 fixed with 3.7% paraformaldehyde for 15 min, permeabilised by immersion in
187 absolute ethanol for 5 min, and then labelled with 4'6-diamino-2-phenylindole
188 dihydrochloride (DAPI) (Thermo Fisher Scientific Inc., Madrid, Spain) by
189 incubation for 5 min with the stain (DAPI 300 nM in 0.1 M PBS pH 7.2). Once
190 the labelling solution was aspirated, the cells were rinsed three times in PBS,
191 and each well of the microplate was viewed using a 3-colour LumaScope-
192 600 epifluorescence microscope (Etaluma Inc., CA, USA).
193
194 *2.4. Cell apoptosis*

195

196 One of the most established hallmarks of apoptosis is DNA fragmentation
197 (Saraste & Pulkki, 2000). The commercially available Cellular DNA
198 Fragmentation ELISA Kit (Roche Applied Science) was used to measure
199 apoptosis-associated DNA fragments in the cytoplasm. This assay is based
200 on the quantitative "sandwich enzyme immunoassay" (ELISA) principle, and
201 involves two mouse monoclonal antibodies directed against DNA and 5'-
202 bromo-2'-deoxyuridine (BrdU). Briefly, HT29 cells were seeded at 2×10^4
203 cells/well in 96-well plates and incubated with BrdU for 20 h at 37°C in a 5%
204 CO_2 atmosphere. After this time, proliferating cells were exposed to the
205 corresponding BA dose for 24 h. In parallel with the BA-treated samples, a
206 negative control (untreated cells) was established to determine the
207 spontaneous release of DNA fragments, and a positive control to estimate
208 the incorporation of BrdU into the genomic DNA (cells treated with 1% Triton-
209 X100). Cells were collected after centrifugation at 250 *g* for 10 min and lysed
210 with the incubation solution for 30 min at 25ºC. After further centrifugation at
211 250 *g* for 10 min, the resulting supernatant was transferred to a microplate
212 precoated with anti-DNA antibodies, followed by a standard ELISA assay to
213 detect the BrdU-labelled DNA fragments. Finally, apoptosis-associated DNA
214 was determined at 450 nm in a Benchmark Plus Microplate
215 Spectrophotometer (Bio-Rad, Madrid, España). DNA fragmentation was
216 expressed as the fold increase of the control values.

217

218 *2.5. Cell lysis assay*

219

220 RTCA cell culture supernatants collected after 24 h of incubation with the
221 corresponding dose of tyramine or histamine were tested for the presence of
222 lactate dehydrogenase activity (LDH) using the LDH Assay Kit (Roche Applied
223 Science), following the manufacturer´s instructions. The release of LDH into the
224 medium is a marker of the cell membrane damage that occurs in necrosis.
225 Negative (no lysing reagent) and positive controls (with lysis reagent) were run
226 in parallel.

227

228 *2.6. Data and statistical analysis*

229

230 The results obtained in all experiments are expressed as the means ±
231 standard deviation of three independent replicates. Statistical treatment
232 involved ANOVA followed by Fisher's multiple comparison test; this was
233 performed using SigmaPlot software. Significance was set at $p<0.05$
234 (indicated in figures with an asterisk).

235

236 The NOAEL value was identified as the highest concentration of BA that
237 caused no detectable adverse effect on the target cells. The LOAEL value
238 was defined as the lowest concentration of BA that produced a detectable
239 adverse effect.

240

241 **3. Results**

242

243 *3.1. Dynamic cell responses of tyramine and histamine-treated intestinal cells*

244

245 To determine whether tyramine and histamine treatments perturbed cell
246 homeostasis and induced cytotoxic effects in the HT29 cells, dose-response
247 curves were constructed in real time by the RTCA system. Approximately 20
248 h after seeding, different concentrations of each BA were added and the cells
249 monitored for 24 h (Fig. 1). It was found that the concentration of histamine
250 and tyramine remained the same after the 24 hours (data not shown),
251 suggesting insignificant amino oxidase activities in HT29 cell cultures.
252
253 The curves showed that cells proliferated over the 20 h of culture before BA
254 treatment. After this time, the curves for BA-untreated cultures showed
255 continued proliferation. However, a drastic reduction in the normalized cell
256 index was seen when the cells were exposed to increasing concentrations of
257 tyramine (Fig. 1A) or histamine (Fig. 1B). Both BAs were associated with a
258 dose-dependent cytotoxic effect. The effect of tyramine was very acute and
259 rapid: the normalized cell index of proliferating cells treated with
260 concentrations above 2.9 mM dropped within a few minutes. The cytotoxicity
261 of the histamine was less severe than that of tyramine: cells treated with 5
262 mM histamine showed no change in their proliferation curves. However,
263 doses of 10.8 mM or higher were cytotoxic, although more moderately and
264 less acutely so than observed for cytotoxic doses of tyramine. The cytotoxic
265 effects of both BAs on confluent HT29 cells (data not shown) were similar to
266 those seen on proliferating cells.
267
268 Dose-response curves for tyramine and histamine were constructed 24 h
269 after BA exposure (Fig. 2). Both that of tyramine (Fig. 2A) and histamine (Fig.

270 2B) fitted a sigmoid curve with a R^2 of 0.9823 and 0.9942 respectively. The
271 Hill slope of the curve for tyramine (-3.14) was steeper than that for histamine
272 (-1.44). Thus, small increases in tyramine concentration had a greater toxic
273 effect than small increases in histamine.
274
275 The greater cytotoxicity of tyramine was confirmed when the IC_{50} values for
276 each BA were compared at 8 h, 12 h, 18 h and 24 h (Table 1). For each time
277 point, the RTCA data fitted a sigmoid dose-response curve with an R^2 of
278 >0.995 in all cases. The IC_{50} values showed both BAs to have a dose-
279 dependent cytotoxic effect on the HT29 cells (Table 1). Tyramine was
280 approximately 10 times more toxic than histamine (IC_{50} of tyramine after 24 h
281 of treatment 3.2 ± 0.04 mM, compared to 26.0 ± 1.2 mM for histamine).
282 Thus, a 10-times lower concentration of tyramine than histamine is needed to
283 achieve half of the latter's strongest cytotoxic effect. The results also
284 indicated that the cytotoxic effects of tyramine did not increase with
285 incubation times beyond 18 h. In contrast, sensitivity to histamine increased
286 over time.

287 The NOAEL and LOAEL values for tyramine (Fig. 2A) were 1.8 mM and 2.2 mM
288 respectively, and 3 mM and 4 mM respectively for histamine. Thus, the cells
289 showed a higher toxicological threshold for histamine.
290
291 *3.2. Fluorescence microscopy examination of tyramine- and histamine-*
292 *treated cell cultures*
293

294 To visualize cytotoxic effects, cell cultures treated for 24 h with a range of
295 tyramine or histamine concentrations were stained with DAPI and observed
296 by fluorescence microscopy (Fig. 3). The number of cells decreased
297 inversely with the concentration of both BAs, although the tyramine had a
298 more severe effect. Tyramine doses above 2.9 mM had a more serious effect
299 on the cells, indeed, at the two highest concentrations tested (11.5 mM and
300 17.3 mM) very few cells were observed. The cytotoxicity of histamine began
301 to become evident at higher concentrations (above 5 mM), but even beyond
302 the maximum concentration of tyrosine used, the cytoxicity of histamine was
303 less severe.

304

305 The chromatin of the histamine-treated cells appeared more condensed - a
306 characteristic of apoptosis - than the chromatin of the tyramine-treated cells
307 (Fig. 3). This suggests that the mode of action of these BAs might be
308 different. Further experiments to examine this were therefore performed.

309

310 *3.3. Apoptosis assays*

311

312 Intracellular DNA fragments typical of apoptotic cells were sought using the
313 cellular DNA fragmentation assay. To ensure the meeting of the necessary
314 cell death test conditions, endpoint times of 24 h were employed (the time
315 needed by each BA to induced maximum cell damage). Intermediate doses -
316 5.8 mM for tyramine and 16.3 mM for histamine – were used to ensure that
317 cells would be damaged though not all would be killed. After BA-treatment of
318 the BrdU-labelled cells, DNA fragments were extracted from the cytoplasm

319 and quantified. DNA-fragmentation detected in these cells was slightly
320 increased (12.3%) in comparison with controls. In contrast, DNA
321 fragmentation in the cells exposed to histamine increased by 43.9% (Fig. 4).
322 This strongly suggests that histamine, much more so than tyramine, exerts
323 an apoptotic effect.
324
325 *3.4. Assessment of cytolytic (necrotic) effect of tyramine and histamine*
326
327 The cytotoxic mode of action of tyramine and histamine was further assessed
328 using the LDH assay. LDH is a cytosolic enzyme released into the medium
329 after necrotic cell death. Based on the RTCA results, an LDH assay end-
330 point of 24 h was chosen. Assays were performed using the concentration
331 range employed to construct the dose-response curves for tyramine and
332 histamine. Figure 5 shows the percentage cytolysis induced by tyramine and
333 histamine. Negligible LDH activity was detected in cell cultures exposed to
334 histamine, except at the highest concentration tested (127.9 mM) in which
335 cytolysis reached 45%. In contrast, a dose-dependent increase in LDH
336 leakage was observed with tyramine, indicating that, unlike histamine,
337 tyramine caused the necrosis of the HT29 cells.
338
339 **4. Discussion**
340
341 Dose-dependent toxic effects were observed both in proliferating and
342 confluent cell cultures exposed to either tyramine or histamine. However,
343 tyramine was more cytotoxic than histamine at all time points, as indicated by

344 its lower IC_{50}, NOAEL and LOAEL values compared to histamine. To our
345 knowledge, this is the first report of IC_{50}, NOAEL and LOAEL values for
346 tyramine and histamine recorded for *in vitro* cultures of intestinal cell lines.
347 Tyramine had an acute, rapid effect; the cell index dropped abruptly after
348 treatment with 14.4 mM tyramine or higher doses, whereas a similar dose of
349 histamine induced more moderate effects. An explanation for this can be
350 found in the different mode of action of tyramine and histamine. LDH release
351 from tyramine-exposed cells increased significantly, particularly at higher
352 concentrations, indicating that this BA has a necrotic effect. In contrast,
353 negligible cytolytic activity was detected in cultures exposed to histamine,
354 suggesting that this BA causes cell death without affecting membrane
355 stability. In addition, cells exposed to histamine showed significantly
356 increased DNA fragmentation, strongly suggesting that the biochemical event
357 behind the cell death induced by it is apoptosis rather than necrosis.
358 Whereas necrosis is an unexpected, unordered and accidental form of cell
359 death, apoptosis is a form of programmed cell death (Kanduc, Mittelman,
360 Serpico, Sinigaglia, Sinha, Natale, et al., 2002). It has been reported that
361 histamine is deeply involved in apoptosis triggering and progression
362 (Toninello, Salvi, Pietrangeli, & Mondovi, 2004). Histamine is a key mediator
363 in the development of allergic reactions, which evoke activation and
364 degranulation of eosinophils and airway epithelial tissue injury; apoptosis is
365 believed to be a major mechanism for the clearance of eosinophilic
366 inflammation (Hasala, Giembycz, Janka-Junttila, Moilanen, & Kankaanranta,
367 2008; Ling, Ngo, Nguyen, Thurmond, Edwards, Karlsson, et al., 2004).
368

369 The *in vitro* approach used in this work was shown to be a useful way of
370 assessing the risk of toxicity after the consumption of food containing
371 tyramine and histamine. These compounds are the most toxic of all BAs, and
372 of particular importance with respect to food safety (European Food Safety
373 Authority, 2011). However, in Europe legal limits have only ever been set for
374 histamine, and then only in fish and fish products (European Comission,
375 2005; established by the EFSA), and in the USA, the FDA has only
376 established a guidance histamine concentration of 50 mg/kg for the
377 consumption of scombroid or scombroid-like fish (Food and Drug
378 Administration, 2001). There is no legal limit at all for histamine in other
379 foods, yet it can reach concentrations of 1850 mg/kg in cheese, 400 mg/kg in
380 fermented sausages, and 758 mg/kg in sauces (European Food Safety
381 Authority, 2011). These levels are much higher than the cytotoxicity threshold
382 detected in the present work for histamine (4.0 mM, equivalent to 440.6
383 mg/kg). This result supports previous assumptions that concentrations of
384 histamine above 400 mg/kg are probably dangerous to health (Taylor, 1985).
385 More recently, other authors have suggested histamine concentration of 500
386 mg/kg in food to be hazardous to human health (Askar & Treptow, 1986;
387 Rauscher-Gabernig, Grossgut, Bauer, & Paulsen, 2009; Shukla, Kim, & Kim,
388 2011). It is generally assumed that histamine is the most toxic BA; however,
389 to our knowledge there are no scientific data to substantiate it. The EFSA
390 considers tyramine and histamine as the most toxic BA, but indicates that
391 there is no available information to conduct quantitative risk assessment of
392 BA (European Food Safety Authority, 2011). No legal limit for tyramine in
393 food has ever been set anywhere, yet the present results indicate that this

BA is even more toxic than histamine. The recorded cytotoxicity threshold of 2.2 mM is equivalent to 301.8 mg/kg, a concentration easily exceeded in cheese (in which concentrations of 2519 mg/kg have been detected (Redruello, Ladero, Cuesta, Alvarez-Buylla, Martin, Fernandez, et al., 2013), fermented sausages (up to 1740 mg/kg), fish and fish products (up to 634 mg/kg) and fish sauces (up to 741 mg/kg) (European Food Safety Authority, 2011). Concentrations below this threshold might also cause adverse reactions, although not so severe. However, people with less powerful BA-detoxification systems caused by genetic deficiencies, gastrointestinal disease, mono- or diamine oxidase inhibitor medication, or who have ingested alcohol or other potentiating factors (Maintz & Novak, 2007), might be at greater risk.

In summary, the present results reveal that unexpectedly, tyramine was more cytotoxic than histamine on an *in vitro* model of the human intestinal epithelium. Nevertheless, in both cases the concentrations found to be toxic are commonly reached in BA-rich foods. Thus, it might be advisable to establish measures aimed at reducing their concentrations to below the cytotoxic levels here determined.

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422

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542 **6. Figure legends**

543

544 **Figure 1.** Real-time cell analysis of the effects of tyramine and histamine on
545 HT29 cells. Cells were seeded in a 16-well E-plate (20,000 cells/well) and
546 after 20 h of growth were exposed to the indicated concentrations of tyramine
547 (A) or histamine (B) or control medium (0 mM). The point of administration of
548 tyramine or histamine is shown by vertical arrows on the graph. Changes in
549 the normalized cell index were dynamically monitored every 15 min for 24 h.
550 Data are the means of three replicates; vertical bars represent standard
551 deviations.

552

553 **Figure 2.** Dose-response curves for tyramine and histamine in HT29
554 intestinal cells. Cell cultures were treated with a range of tyramine (A) or
555 histamine (B) concentrations for 24 h. Data represent the means ± standard
556 deviations of three experiments. The non-linear regression trend lines fitted a
557 sigmoid dose-response curve (variable slope). An asterisk indicates the first
558 significant difference with respect to the minimal dose of BA assayed (0.7
559 and 0.2 mM for tyramine and histamine respectively) and represents the
560 LOAEL concentration (*p<0.05). Numeric values for NOAEL, LOAEL, Hill
561 slope and R^2 are also indicated.

562

563 **Figure 3.** Toxicity of tyramine and histamine on intestinal cells. HT29 cells
564 were grown in flat-bottomed microplates for 20 h, and then treated with
565 increasing concentrations of (A) tyramine or (B) histamine for 24 h. They
566 were then fixed, stained with DAPI, and viewed with an epifluorescence

567 microscope. Apoptotic cells are marked with white arrows.

568

569 **Figure 4.** Apoptotic DNA-fragmentation induced by tyramine and histamine
570 in HT29 intestinal cells. BrdU-labelled DNA fragments released from the
571 nucleus to the cytoplasm due to apoptosis were quantified in HT29 cells
572 exposed to 5.8 mM of tyramine or 16.3 mM histamine for 24 h. The
573 percentage of DNA fragmentation was calculated as follows: 100 X [(DNA
574 fragmentation of treated samples – DNA fragmentation of negative control) /
575 (DNA fragmentation of positive control – DNA fragmentation of negative
576 control)]. The data represent the means of three replicates; vertical bars
577 represent standard deviations.

578

579 **Figure 5.** Determination of the cytolytic effect of tyramine (A) and histamine
580 (B) on intestinal cells. HT29 cells were seeded in 96-well plates and after 20
581 h of growth in different concentrations of the corresponding BA were added
582 to each well incubated for 24 h. Necrosis was then measured via lactate
583 dehydrogenase activity (LDH). The data represent the percentage of cells
584 lysed by treatment with different concentrations of tyramine or histamine. The
585 percentage of cells lysed was calculated as follows: 100 X [(492 nm
586 absorbance of treated samples – absorbance of negative control) /
587 (absorbance of positive control – absorbance of negative control)]. Data
588 represent the means of three replicates; vertical bars represent standard
589 deviations.

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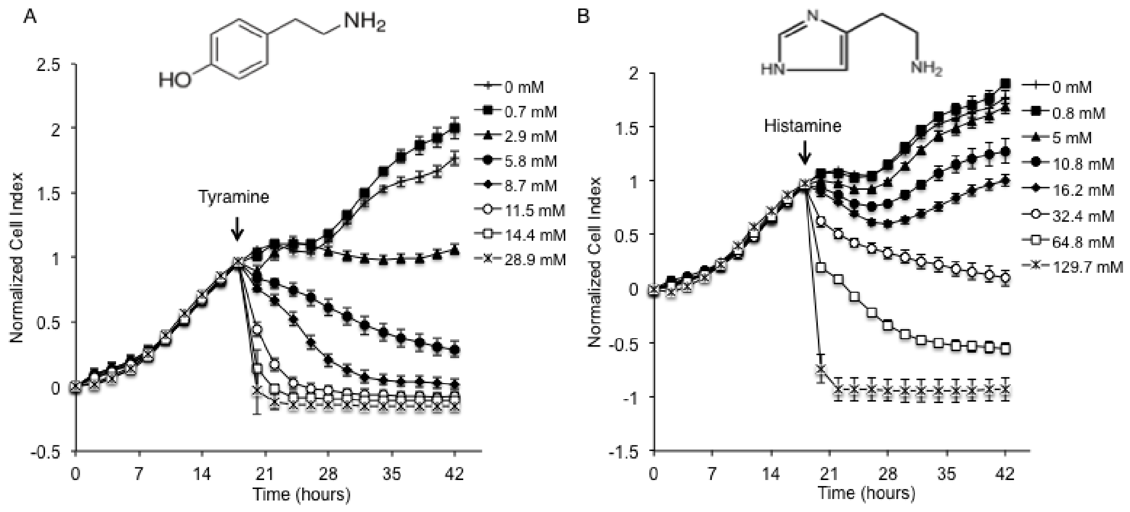

Figure 1

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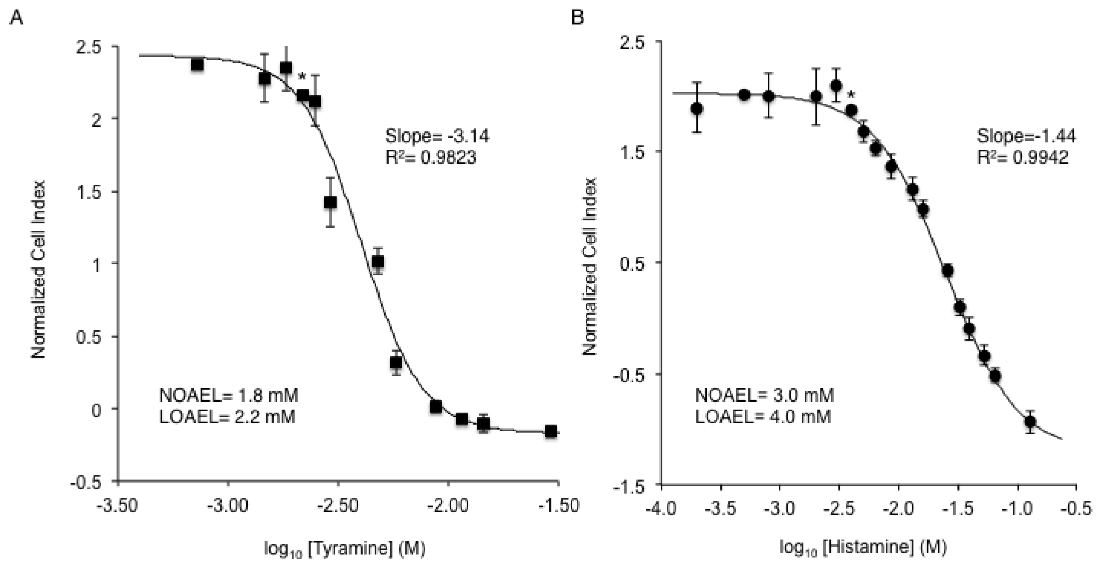

Figure 2

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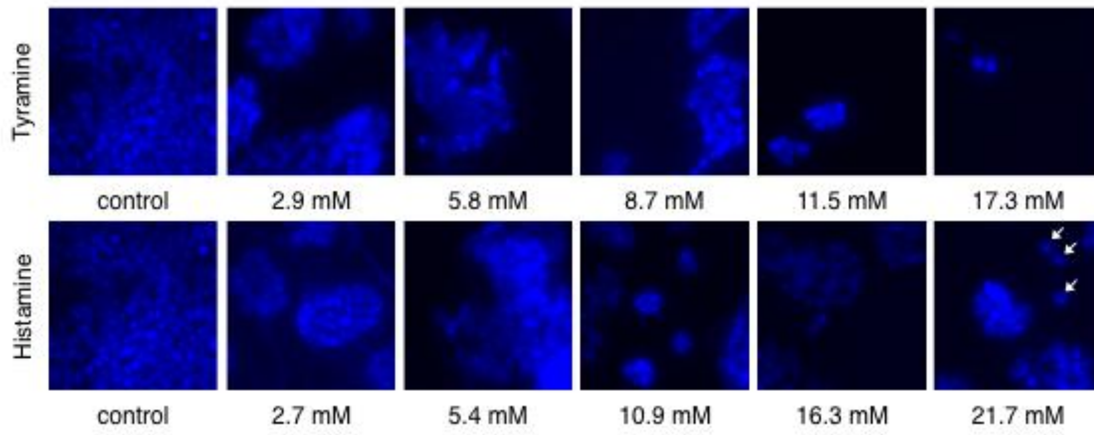

Figure 3

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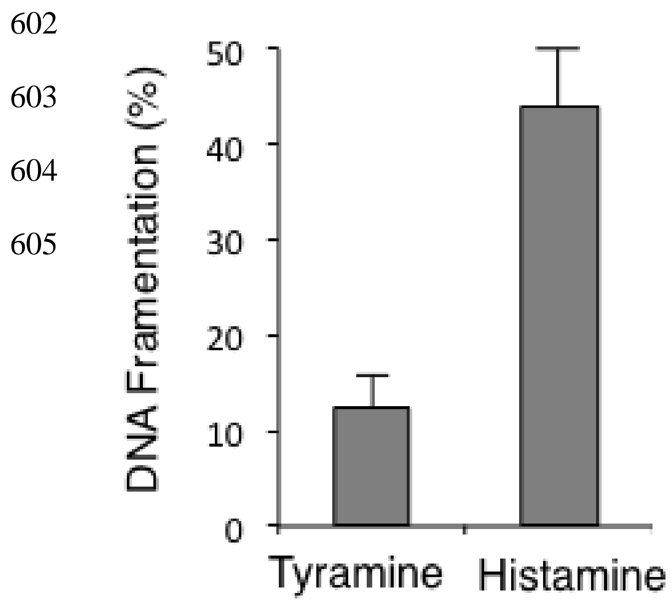

Figure 4

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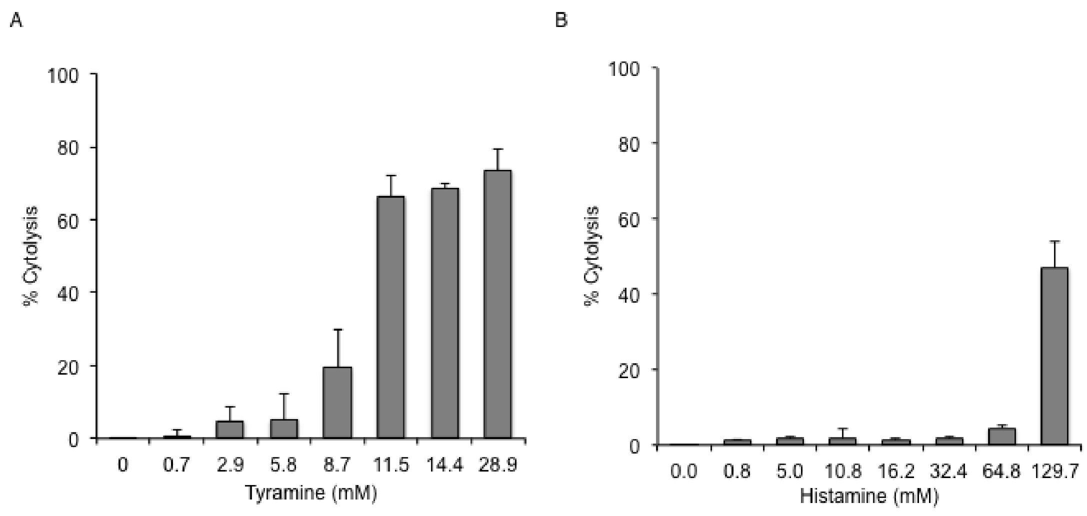

Figure 5

610

611 **Table 1**. IC_{50} values (mean ± standard deviation) for tyramine and histamine

612 after exposure of HT29 intestinal cells for different times.

613

Time (h)	Tyramine (IC_{50})	Histamine (IC_{50})
8	6.9 ± 0.30	261.9 ± 147.50
12	5.0 ± 0.10	55.4 ± 6.30
18	3.4 ± 0.04	31.0 ± 1.90
24	3.2 ± 0.04	26.0 ± 1.20

Values are the mean ± standard deviation in mM