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1 **1. Title page**

2 **HISTAMINE INHIBITS ADRENOCORTICAL CELLS PROLIFERATION BUT DOES NOT AFFECT**
3 **STEROIDOGENESIS**

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20 **Short title:** Histamine inhibits adrenal cell proliferation

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23 **2. Abstract**

24 Histamine (HA) is a neurotransmitter synthesized in most mammalian tissues exclusively by histidine
25 decarboxylase enzyme. Among the plethora of actions mediated by HA, the modulatory effects on steroidogenesis
26 and proliferation in Leydig cells (LC) have been recently described. In order to determine if the effects reported in
27 LC could be extrapolated to all steroidogenic systems, we studied the effect of this amine on proliferation and
28 steroidogenesis of the adrenal cortex, using two adrenocortical cell lines as experimental models, the murine Y1
29 and the human NCI-H295R cells.

30 Even when steroidogenesis was not modified by HA in adrenocortical cells, the biogenic amine inhibited the
31 proliferation of H295R cells. This action was mediated by the activation of the HRH1 receptor subtype and an
32 increase in the production of inositol phosphates as second messengers, causing a cell cycle arrest in the G2/M
33 phase. These results indicate a new role of HA on human adrenocortical cells proliferation that could contribute to
34 a better understanding of tumor pathology as well as to the development of new therapeutic agents.

35

36 **3. Introduction**

37 Histamine (HA) is a monoamine neurotransmitter synthesized exclusively by histidine decarboxylase (HDC) in
38 most mammalian tissues. It carries on its function through the activation of four different subtype receptors,
39 namely HRH1, HRH2, HRH3 and HRH4, all of them members of the G-protein coupled receptor (GPCR) family
40 and associated with different signal transduction pathways (Jones and Kearns 2010).

41 As regards steroidogenesis, HA was shown to stimulate this process in testicular parenchyma of the golden
42 hamster (Mayerhofer, et al. 1989). Our group extended those observations reporting a dual concentration-
43 dependent effect of the amine on steroidogenesis in MA-10 murine Leydig cells and in purified rat Leydig cells
44 (Mondillo, et al. 2005). These results revealed a novel biological activity of HA, namely, the negative modulation
45 of testicular steroid synthesis via HRH1. In addition, our results have also shown that NOS activation is the main
46 intracellular mechanism by which HA exerts its anti-steroidogenic effects (Mondillo, et al. 2009).

47 Over the last years, proliferative actions of HA have become more relevant as is evidenced by the increasing
48 number of scientific publications (Falus, et al. 2011). In this respect, it has been shown that HA can act as both
49 anti-mitogenic (Cricco, et al. 2006; Meng, et al. 2011; Petit-Bertron, et al. 2009) and mitogenic agent (Francis, et
50 al. 2009; Medina, et al. 2011; Molina-Hernandez and Velasco 2008; Stoyanov, et al. 2012) depending on the cell
51 type and the HA receptor pattern expressed. Particularly, our recent studies showed, for the first time, the
52 proliferative effect of HA in MA-10 Leydig cells, mediated *via* HRH2 activation, increased cAMP production and
53 ERK phosphorylation (Pagotto, et al. 2012).

54 Among steroidogenic tissues, the adrenal cortex is responsible for the production of steroid hormones essential for
55 life. It has been demonstrated that HA is able to regulate adrenal steroidogenesis in rat and dog by acting on CNS
56 via the HRH1 by an ACTH-independent mechanism (Bugajski 1984; Tsujimoto, et al. 1993). Furthermore, it is
57 known that the chromaffin cells of the adrenal medulla are capable of responding to HA via the HRH1,
58 stimulating the secretion of catecholamines and neuropeptides which, in turn, act in a paracrine way on cortical
59 cells regulating adrenal cortisol secretion (Bunn and Boyd 1992). Concerning the presence of HA in the adrenal
60 gland, it has been identified in guinea pig and rat adrenal glands, most being present in the cortex (Endo and
61 Ogura 1974). Possible sources of cortical HA would be those from subpopulations of adult chromaffin cells

62 present in the medulla (Tuominen, et al. 1993), the endings of the splanchnic nerve and the contribution of mast
63 cells that are arranged surrounding adrenal arterioles, near the capsule (Borges 1994; Hinson, et al. 1989). This
64 background supports an indirect effect of HA on the regulation of adrenal steroidogenesis. However, the literature
65 concerning a possible direct effect of the amine on adrenocortical cells is controversial, in part because they come
66 from studies on different species and utilize experimental approaches in which adrenocortical cells are partially or
67 even not isolated. For example, studies with perfused dog adrenal glands or guinea-pig primary cultures refer to a
68 direct effect of HA on cortisol secretion (Aikawa, et al. 1986; Matsumoto, et al. 1981) while others have
69 postulated the direct action of this amine only on chromaffin cells, using a bovine model (Orso, et al. 1997;
70 Yoshida, et al. 1997). To date, no studies have been reported that include the study of a possible direct action of
71 HA on pure adrenocortical cell lines, which would define the situation unequivocally.
72 About human adrenocortical proliferation and HA, Szabó et al (Szabo, et al. 2009) have recently published that
73 HDC expression and HA content were highest in normal tissues, lower in benign tumors, and significantly lower
74 in Adrenocortical Carcinoma (ACC).
75 Considering the information above and our previous findings about the ability of HA to regulate testicular
76 steroidogenesis, the aim of this work was to study the direct effect of this amine on adrenal steroidogenesis and
77 proliferation. To reach this goal, we used two well-characterized adrenocortical cell lines, human NCI-H295R and
78 murine Y1, which serve as established models for studies of adrenal cortical neoplasia and human adrenal
79 steroidogenesis (Gazdar, et al. 1990; Rainey, et al. 2004; Rodriguez, et al. 1997).
80

81 **4. Materials and Methods**

82 **4.1. Materials**

83 Histamine dihydrochloride, HRH1 agonist 2-((3-Trifluoromethyl)phenyl)histamine dimaleate (FMPH), HRH1
84 antagonist Pyrilamine, HRH2 agonist Amthamine (AMTH), HRH3 agonist Imetit (IMET), HRH4 agonist VUF
85 8430 (VUF), TME-cAMP, BSA, MTT, transferrin, selenium, glutamine and $NaHCO_3$, phospholipase C inhibitor
86 (U-73122), PLC inactive analog inhibitor (U-73343), doxorubicin, epigallocatechin gallate (EGCG), mouse
87 monoclonal anti-β tubulin and caspase-3 antibodies were purchased from Sigma Chemical Co. (St. Louis, MO).
88 [3H]-Pyrilamine, Na[125I]-I, [3H]-Thymidine and Myo-[3H]-inositol were purchased from NEN (Boston, MA).
89 Cell culture supplies were from Gibco-BRL (Gaithersburg, MD). Dowex AG-I-X8 resin was from Bio-Rad
90 (Hercules, CA). TME-cAMP was radiolabeled with $Na^{125}I$ in our laboratory by the method of chloramine-T
91 (specific action 600 Ci/mmol). Antibody for cAMP was provided by Dr. A.F. Parlow (NHPP). Specific antibodies
92 for progesterone and StAR, were gifts from Dr Bussmann (IBYME-CONICET-Argentina) and Dr Miller
93 (University of California, San Francisco), respectively. Anti HA antibody was from Alpha Diagnostic (San
94 Antonio, USA). Rabbit anti-HDC antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
95 Secondary conjugated anti-rabbit antibody coupled with peroxidase was from Vector Labs (Burlingame, USA).
96 DMEM/F12 medium (GIBCO) and Hyclone supplemented calf serum (Thermo Scientific) were from Invitrogen.
97 Insulin was a gift from Laboratorios Beta (Buenos Aires, Argentina). Other reagents used were of the best grade
98 available and were obtained from commonly used suppliers.

99

100 **4.2. Cell line cultures**

101 Cell lines used in this study were obtained from the ATCC (Rockville, MD, USA) and were used with no more
102 than 20 passages.

103 1- Human adrenocortical cancer cell line NCI-H295R (ATCC, CRL-2128) was cultured as monolayer in
104 DMEM/HAM'S F12 medium supplemented with 6.25 μg/ml transferrin, 6.25 μg/ml insulin, 6.25 ng/ml selenium,
105 5.35 μg/ml linoleic acid, 5% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). Cultures
106 were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C and harvested weekly. The mostly

107 secreted steroids are cortisol and dehydroepiandrosterone sulfate (DHEAS) (Rainey et al, 2004) by stimulation
108 with db-cAMP or forskolin or with ACTH at a lesser extent. For this reason, stimulation of steroidogenesis was
109 done in the presence of db-cAMP. In addition, aldosterone was determined by stimulation with 10^{-7} M
110 Angiotensin II, in the absence or presence of two different concentrations of HA (10^{-5} or 10^{-9} M). Cortisol and
111 DHEAS were measured by RIA with commercial kits (Coat-a-Count, Siemmens Healthcare Diagnostic, LA,
112 USA). Aldosterona was quantified as described (Mele, et al. 2012).
113 2- Y1 cells (ATCC, CCL-79) are an ACTH- and cAMP-responsive subclone of the mouse adrenocortical tumor
114 cell line (Yasumura 1968) and produce mainly progesterone. Cells were grown as monolayer in Ham's F10
115 medium containing heat-inactivated fetal bovine and horse serum (2.5% and 12.5% respectively), 200 U/ml
116 penicillin G, and 270 µg/ml streptomycin sulphate, in a humidified atmosphere of 5% CO_2 in air at 37°C.
117 Progesterone was measured by RIA as previously described (Piotrkowski, et al. 2009). The intra and inter-assay
118 variations were 8.0% and 14.2% respectively.
119
120 **4.3. Determination of intracellular cAMP production**
121 Cells were seeded in 24 well microplates (5 x 10^5 cell/well) in complete medium. After 24 h, media was replaced
122 with the assay medium (DMEM/HAM'S F12 and 0.1 % BSA). After 20 min incubation with the corresponding
123 stimulus (10^{-5} M HA, 10^{-5} M FMPH or 5 x 10^{-3} M forskolin) cells extracted with 0.5 ml of cold ethanol. After
124 centrifugation for 15 min at 9000 x g, supernatants were evaporated and pellets were resuspended using 50 mM
125 sodium acetate buffer (pH 6.0). Unknown samples and standards were acetylated and assayed by RIA as
126 described (Del Punta, et al. 1996). The inter and intra-assay variations of coefficients were lower than 10%.
127
128 **4.4. Determination of [3H]-Inositol Phosphates production**
129 Cells were incubated in a 6 well microplate (1 x 10^6 cell/well) with 2 µCi of myo-[3H]-inositol for 48 h before the
130 experiment. At the end of the labeling period, cells were washed with assay medium (DMEM/HAM'S F12 and
131 0.1 % BSA) and preincubated for 15 min with 20 mM LiCl. At the end of this period, 10^{-5} M HA, 10^{-5} M FMPH

132 or 10^{-3} M NaF (as positive control) was added. After 30 min incubation, total inositol phosphates (InsPn) were
133 measured as previously described (Ascoli, et al. 1989) by using Dowex columns.
134 Results were expressed as the ratio obtained when [3H]-InsPn activity was normalized to total [3H]-inositol
135 recovered from the initial wash of the Dowex columns corresponding to the intracellular [3H]-inositol pool
136 (Mondillo, et al. 2005).
137
138 **4.5. Ligand binding assays for HRH1 subtype histamine receptor**
139 Cells were seeded in 24 well microplates (5 x 10^5 cell/well) and cultured for 48 h in complete medium. The cells
140 were rinsed twice with PBS and incubated for 40 min at 4ºC in 200 ml of 50 mM Tris/HCl pH 7.5 containing
141 increasing concentrations of [3H]-Pyrilamine (1 to 1000 nM). Nonspecific binding was defined with 100 mM
142 cold pyrilamine. After incubation, cells were washed with ice-cold Tris/HCl 50 mM at 4ºC and scraped to remove
143 them from the wells; radioactivity was determined by liquid-scintillation counting.
144
145 **4.6. [^{3}H]-Thymidine incorporation assay**
146 DNA synthesis was evaluated according to the amount of [3H]-Thymidine incorporated into the H295R cells.
147 Cells were seeded in 96-well microplates (3 x 10^4 cells/well) in complete medium. After 18 h, media was replaced
148 with DMEM/F12 with reduced serum (1%) and incubated with different concentrations of HA and the indicated
149 compounds for 24 h, with a pulse of 0.25 µCi/ml [3H]-Thymidine for the last 12 h. At the end of the pulse period,
150 cells were frozen at -20°C and harvested in glass fiber discs by filtration. Samples were washed with 95% ethanol,
151 dried, and counted by liquid scintillation counting.
152
153 **4.7. MTT assay**
154 This assay is based on the transformation and colorimetric quantification of 3-(4,5-dimethylthiazol-2-yl)-2,5-
155 diphenyltetrazolium bromide (MTT). Herein a linear relationship between cell number and signal produced was
156 established, thus allowing the quantification of cell proliferation. In brief, cells were plated in 96-well microplates
157 (3 x 10^4 cells/ well), and 24 h later they were treated with the indicated compounds. After 24 h, MTT was added

158 (final concentration 0.5 mg/ml), and cells were incubated at 37°C for 2 h. To stop the coloring reaction and
159 dissolve the formed formazan crystals, a solubilization solution (isopropanol with HCl) was added, and the
160 mixture was incubated overnight at room temperature. The color intensity was measured at 570 nm using a
161 multiplate ELISA reader.

162

163 **4.8. Cell cycle analysis**

164 H295R cells were seeded in 6-well microplates (1.5 x 10^6 cell/well) in complete culture medium. After 18 h,
165 culture medium was replaced with DMEM/ F12 with reduced serum (1%) and incubated with HA or FMPH, both
166 at a concentration of 10^{-5} M for 24 h. After the incubation period, cells were harvested by trypsinization,
167 centrifuged, washed twice in PBS and fixed in PBS ice-cold ethanol (1:3). After centrifugation at 4°C, cells were
168 finally resuspended in 3.8 mM sodium citrate buffer, containing 40 µg/ml PI and 100 ug/ml DNase-free RNase A.
169 After 30 min incubation, samples were measured with a FACsARIA Flow Cytometer. The percentage of cells in
170 the G1, S, and G2/M phases of the cell cycle were determined with WinMDI 2.8 and Cylchred analytical
171 software.

172

173 **4.9. Tunel assay**

174 H295R cells were cultured in 6-well microplates (1.5 x 10^6 cell/well) in complete media. After 18 h, culture
175 medium was replaced with DMEM/F12 with reduced serum (1%) and cells were incubated with HA or FMPH,
176 both at a concentration of 10^{-5} M for 24 h or 72 h. After incubation, nuclear DNA fragmentation was detected by
177 Tunel method using the cell death detection kit (Roche Applied Science, Germany) according to the
178 manufacturer's instructions. Apoptosis was analyzed by flow cytometry and data were processed with WinMDI
179 2.8 software.

180

181 **4.10. Western Blot analysis**

182 Total cellular protein was obtained by placing cells in lysis buffer (10 mM Tris-HCl, 1% Tritón X-100, 0.5 mM
183 EGTA , pH 7.4) containing a protease inhibitor cocktail (5 ug/ml leupeptin, 25 mM NaF, 25 mM sodium

184 orthovanadate, 400 μM PMSF, 5 μg/ml pepstatin and 5 μg/ml aprotinin), followed by 30 passages trough a 1ml
185 syringe. Protein concentrations were measured using the Bradford assay for total protein. Equal amounts of
186 protein per sample (50 μg) were loaded onto a 10% (w/v) SDS-polyacrylamide gel (Mini Protean III System; Bio-
187 Rad, Hercules, CA). Electrophoresis, transfer of proteins to PVDF membranes, and immunodetection of StAR,
188 HDC, caspase-3 and β-tubulin were performed under optimized conditions.
189

190 **4.11. Immunocytochemistry of HA content**

191 Y1 and H295R cells were seeded onto a 12-mm diameter round glass coverslip, precoated with polylysine ($3x10^4$
192 cell/coverslip) and after 3 days were washed and fixed with 4% formaldehyde for 15 min at room temperature.
193 Cells were permeabilized for 10 min with 0.25 % TritonX-100 and 0.3 M glycine in PBS (PBST) and unspecific
194 binding was blocked with 1% BSA in PBST for 30 min. Coverslips were incubated for 24 h with primary
195 antibody against HA (1:100) or normal rabbit serum in PBS (negative control) overnight at 4ºC, followed by
196 incubation with a secondary conjugated anti-rabbit antibody coupled with peroxidase (1:4000) for 1,5 h at room
197 temperature. Immunoreactivity was detected with 2.7 mM 3,3-diaminobenzidine tetrahydrochloride in PBS with
198 0.03% hydrogen peroxide (w/v). For quantification of HA immunocytochemical staining, representative cells
199 were chosen and visualized by a 40 x 10 magnification through a Zeiss-Axiophot (Zeiss Oberkochen, Germany)
200 with Olympus DP70 digital camera. Five hundred cells for each cell type of three independent experiments, were
201 subjected to histogram analysis by using Photoshop CS 8.0.1. Mean gray values from negative controls were
202 substracted from mean gray values determined from cells stained for HA to exclude background staining.
203

204 **4.12. Statistical Analysis**

205 All experiments reported herein were repeated at least three times. If heterogeneity of variance was detected by
206 Bartlett's test, this was reduced by logarithmic transformation of the data before analysis. These data were then
207 subjected to Student test or one-way ANOVA followed by Bonferroni test for multiple range comparisons. *P*
208 values < 0.05 were accepted as significant.
209

210 **5. Results**

211 **5.1. Effect of HA on steroid production and StAR expression in Y1 and H295R adrenocortical cells**

212 Y1 and H295R cells were incubated with increasing concentrations of HA (10^{-11} to 10^{-5} M) for 5 or 24 h, in the
213 absence or in the presence of 1mUI/ml ACTH or 0.5 mM db-cAMP, for each cell line, respectively. Figure 1, A
214 and B shows that HA treatment did not modify the steroid synthesis in any cell type, unstimulated or stimulated,
215 at any HA concentration or incubation time. Figure only shows 24 h-treatment and cortisol quantification in
216 H295R cells. At previously mentioned, DHEAS and aldosterone concentrations were also measured, but no
217 differences were observed. Aldosterone production was increased after stimulation with Angiotensin II but
218 different HA concentrations did not modify the steroidogenesis.

219 To discard a possible simultaneous activation of different receptors with antagonistic effects, the steroid
220 production was assessed for 24 h in the presence of different specific agonists for each receptor subtype in the
221 absence or presence of stimulus (ACTH 1mUI/ml for Y1 cells or 0.5 mM dibutyryl cAMP for H295R cells).
222 Compounds used were: FMPH as agonist HRH1, AMTH as HRH2 agonist, IMET as HRH3 agonist and VUF as
223 HRH4 agonist, all at a concentration 10^{-5} M, that we have previously used (Medina et al. 2011; Mondillo et al.
224 2005; Pagotto et al. 2012). As can be seen in Figure 1, C and D, there were no differences in steroid production
225 under any treatment condition with respect to control values.

226 StAR is a protein that mediates the rate-limiting step in steroid hormone biosynthesis (Stocco and Clark 1996). As
227 we already showed that HA diminishes the levels of StAR protein in Leydig cells (Mondillo et al, 2009), we
228 studied the effect of HA on StAR expression in adrenocortical cells.

229 Y1 and H295R cells were incubated for different times in the absence or presence of 10^{-5} M HA, and in the
230 presence of 1mUI/ml ACTH or 0.5 mM db-cAMP, for each cell line, respectively. The HA concentration (10^{-5} M)
231 was the same that we previously used for treatment of MA-10 Leydig cells, showing a marked reduction in db-
232 cAMP-stimulated StAR protein expression (Mondillo et al. 2009). In contrast with Leydig cells, but in
233 concordance with results observed for adrenal steroidogenesis, HA did not modify the StAR protein expression at
234 any time with respect to controls neither for Y1 cells (Figure 2A) nor for H295R (Figure 2B). The progressive

235 increase of StAR expression in both cell lines treated with their respective stimuli is coincident with previous
236 reports from our group (Piotrkowski et al. 2009) and others (Manna, et al. 2009).
237
238 **5.2. Effect of HA on proliferation in Y1 and H295R adrenocortical cells**
239 In order to evaluate the effect of HA on cellular proliferation, we performed [3H]-Thymidine incorporation assay,
240 Different results were obtained with both cell lines; HA did not modify Y1 cell proliferation (Figure 3A), but a
241 concentration-dependent inhibition was observed on H295R cell proliferation (Figure 3B) with a maximal effect
242 at 10^{-5} M HA (32.6 % inhibition respect to the control). Complete medium (5 % FCS) was used as positive
243 control.
244 To assess which HA receptor subtype/s could be involved, cell proliferation was studied in the presence of
245 specific HA agonists, all at a concentration of 10^{-5} M. As previously described, in the presence of HA, the
246 treatment with agonists did not modify [3H]-Thymidine incorporation in Y1 cells (Figure 3C). On the contrary, in
247 H295R cells, the HRH1 agonist, FMPH, inhibited the proliferation in a similar extent to that observed in the
248 presence of 10^{-5} M HA (35.7 %) (Figure 3D).
249 To confirm the HA-mediated inhibitory effect through HRH1 receptor on cellular proliferation, H295R cells were
250 preincubated for 30 minutes with 10^{-8} M pyrilamine (specific antagonist for HRH1 receptor). Cells were then
251 incubated with HA or FMPH (10^{-5} M) and [3H]-Thymidine incorporation was determined. Figure 4A shows that
252 the treatment with pyrilamine reversed the inhibitory effects of HA and FMPH on cellular proliferation, but had
253 no effect when incubated alone. These results were corroborated using the MTT assay as an alternative method to
254 measure proliferation (Figure 4B).
255 To exclude a non-specific toxic effect of the tested compounds over H295R cells, cell viability was evaluated
256 using PI staining for the detection of non-viable cells by flow cytometry. As no differences in cell viability were
257 found between treatments at the evaluated time (data not shown) a toxic effect of HA was discarded.
258
259 **5.3. Characterization of HRH1 in H295R adrenocortical cells**

260 Since HA inhibited H295R cells proliferation through HRH1 receptor, we considered to further characterize this
261 receptor subtype in the cell line. A saturation binding assay was performed using [3H]-Pyrilamine as specific
262 ligand (Figure 5B). The non linear regression fitted best a one-site model, suggesting the presence of a single
263 class of sites for HRH1 receptor in H295R cells with a KD value of 124.4 ± 15.8 nM (95% CI = 93.52 to 155.2)
264 and a Bmax of 4.0 ± 0.2 fmol/mg protein (95% CI = 3.7 to 4.4). The same assay for Y1 cells was done for
265 comparison (Figure 5A). The binding to intact Y1 cells also fitted best a one-site model and a single class of sites
266 with a KD value of 21.8 ± 8.3 nM (95% CI = 4.8 to 38.8) and a Bmax of 1.2 ± 0.2 fmol/mg protein (95% CI =
267 0.8 to 1.5).
268
269 **5.4. Signaling pathway of HA anti-proliferative effect in H295R cell line**
270 In order to evaluate the signaling pathway activated by HRH1 receptor in H295R cells, cAMP an InsPn were
271 measured in cells incubated with HA and FMPH, the specific HRH1 agonist. Forskolin and NaF were used as
272 positive controls for each second messenger, respectively.
273 Cyclic AMP levels were not modified by neither HA nor FMPH, while both of these compounds produced a two
274 fold increase in the total InsPn content of H295R cells above the basal level (Figure 6, A and B, respectively).
275 Furthermore, blockage of PLC by using the specific inhibitor U73122 in the presence of FMPH prevented the
276 decrease in [3H]-Thymidine incorporation observed with HRH1 agonist alone, whereas the U-73343, a no-
277 functional inhibitor analog of U73122, was not able to block FMPH-induced anti-proliferative effect (Figure 6 C).
278
279 **5.5. Effect of HA on apoptosis and cell cycle control of H295R adrenocortical cells**
280 In order to determine whether growth inhibitory effect of HA on H295R cells affected apoptosis, cells were
281 cultured with HA or FMPH (10^{-5} M) for 24 and 72 h and apoptosis was evaluated by Tunel assay using flow
282 cytometry. As shown in Figure 7A, apoptotic levels were not different between treatments and control, whereas
283 cells treated with Doxorubucin (an apoptosis inducer) significantly increased the proportion of apoptotic cells in a
284 concentration-dependent manner. For simplicity, Figure 7 only shows 24 h-treatment. Similar results were
285 obtained for 72 h incubation.

286 In order to confirm the above results, presence of activated caspase-3 (an apoptosis marker) was evaluated by
287 Western Blot using protein extracts from H295R cells incubated with HA or FMPH (10^{-5} M) at different times (0,
288 6,18 ,24 ,48 and 72 h). Bands corresponding to cleaved forms of caspase-3 (corresponding to 17 and 11 molecular
289 weight) were not detected by immunoblot at any time analyzed (Figure 7B).
290 The effect of HA on cell cycle progression was next examined. H295R cells were treated with 10^{-5} M HA or 10^{-5}
291 M FMPH for 24 h and cell cycle distribution was analyzed using flow cytometry and PI staining (Figure 8A).
292 Figure 8B shows a significant increase in the percentage of cells in G2/M phase when treated with both HA and
293 FMPH (in % of cells: C = 4.6 ± 0.8; HA = 8.75 ± 0.9; FMPH = 10.9 ± 1.5), with a concomitant decrease in the
294 proportion of cells in S phase (in % of cells: C = 43.0 ± 4.9; HA = 28.6 ± 1.6; FMPH = 31.2 ± 2.11).
295

296 **5.6. Expression of HDC enzyme and endogenous content of HA in the H295R cell line**

297 In an attempt to find a possible explanation for the differential effects of HA on the proliferation of H295R *versus*
298 Y1 cells, and considering the well documented correlation between HDC expression and cell proliferation in
299 several experimental models (Falus et al. 2011), we aimed at comparing the expression levels of HDC enzyme in
300 both cell lines by Western blot analysis. As depicted in Figure 9A, the active form of HDC enzyme (53-55
301 molecular weight) is expressed at significantly higher levels in Y1 compared to H295R cells. Stomach was used
302 as positive control. Coinciding, the endogenous content of HA revealed by immunocytochemistry and quantified
303 as described in Materials and Methods, was higher in Y1 cells (Figure 9B, upper and lower panel).
304 As an approach to demonstrating more directly the role of HDC and HA content on Y1 and H295R cell
305 proliferation, we evaluated the effect of the catechin EGCG, known to potently inhibit HDC activity, as was
306 recently shown by us (Pagotto et al. 2012) and others (Nitta, et al. 2007; Ruiz-Perez, et al. 2012), on the
307 proliferation of Y1 cells. As can be seen in Figure 10, EGCG inhibited Y1 cell proliferation in a concentration
308 dependent manner. EGCG concentrations higher than 4 x 10^{-5} M were toxic.
309

310 **6. Discussion**

311 The existence of a functional histaminergic system in the testis of different species has been previously
312 demonstrated by us (Mondillo et al., 2005, 2007, 2009; Pagotto et al., 2012) and others (Albrecht, et al. 2005;
313 Khan and Rai 2007; Mayerhofer et al. 1989; Pap, et al. 2002). Particularly, we have reported that low
314 concentrations of HA (10^{-9} M) stimulate Leydig cell steroidogenesis and higher concentrations (10^{-5} M) inhibit
315 (Mondillo et al. 2005). In order to assess whether the effects of HA on the steroids synthesis could be extrapolated
316 to other steroidogenic tissues, we studied the direct action of the amine and its agonists on steroidogenesis in Y1
317 and H295R adrenocortical cells, two well-documented cell lines for the study of adrenal cortex function.
318 Considering that adrenal steroids and regulation of steroidogenesis vary among species, as H295R cells come
319 from human origin and Y1 is a murine cell line, we evaluated the production of major steroids for each
320 cell line and the biosynthetic rate-limiting step enzyme StAR. On this respect, no significant effect was
321 found on steroid production or enzyme associated expression in any of the evaluated conditions.
322 These results agree with previous works in bovine co-cultures of adrenal medulla and cortex cells, in which it is
323 suggested an indirect effect of HA through the HRH1 present in adrenal medulla, so inducing release of
324 neuropeptides that would act on adrenocortical cells, regulating the secretion of cortisol (Ehrhart-Bornstein, et al.
325 2000; Ehrhart-Bornstein, et al. 1998; Yoshida et al. 1997). In our study, we have used adrenocortical cell lines
326 excluding contamination with chromaffin cells. Then, according to our observations in Y1 and H295R cells, HA
327 would not be able to directly regulate steroid synthesis of adrenocortical cells, showing that the modulatory effect
328 observed in Leydig cells can not be extrapolated to all steroidogenic systems.
329 Regarding the ability of HA to regulate adrenocortical cell proliferation, results were different between tumor cell
330 lines evaluated. Y1 murine line did not respond to treatment with HA, whereas the H295R human cells reduced
331 growth to about 60% of control with 10^{-5} M HA. This effect was reproduced by FMPH, specific agonist for
332 HRH1 subtype receptor.

333 In bovine adrenal gland, expression of HRH1 has been reported in medulla and cortex with different expression
334 level and affinity, both being higher in medulla (Chang, et al. 1979; Yamashita, et al. 1991). Herein, the presence
335 of a functional HRH1 was reported in the human adrenocortical carcinoma cell line H295R.
336 The differential effect of HA on H295R and Y1 cells proliferation could be explained, at least in part, if
337 considering that Y1 cells showed higher expression levels of HDC enzyme and endogenous HA content than
338 H295R cells. To test this hypothesis, HDC enzyme from Y1 cells was inhibited and proliferation was measured.
339 In fact, inhibition of proliferation was observed suggesting that higher HA content in Y1 cells avoids the
340 inhibition observed in H295R cells in the presence on exogenous HA. The endogenous HA content sustained over
341 time could have triggered the internalization of its receptors, as reported in other systems, canceling
342 responsiveness to HA (Hishinuma, et al. 2010; Miyoshi, et al. 2006). The lower number of HRH1 in Y1 compared
343 to H295R cells, calculated by Scatchard analysis (1.2 vs 4.0 fmol/mg protein, respectively), supports this
344 hypothesis.
345 There is growing evidence that HA can negatively modulate cell proliferation in diverse systems through the
346 activation of different subtype receptors, for example, HRH1 (Valencia, et al. 2001), HRH2 (Cricco et al. 2006),
347 HRH3 (Francis et al. 2009) and by HRH4 (Meng et al. 2011).
348 Particularly in humans, the influence of HA on adrenocortical cells had already been suggested by Szabó et al
349 (Szabo et al. 2009), who compared histamine-related gene expression in normal and tumoral adrenal cortex
350 tissues. They found not only differential expression patterns for HA receptor subtypes in ACC but also a
351 reduction in HDC expression level and HA content, compared with normal tissues. These observations are in
352 agreement with the results presented here in which the addition of HA was able to inhibit proliferation in H295R
353 cells.
354 As it was previously mentioned, HA-mediated growth inhibition in H295R cells was carried out by the activation
355 of HRH1, with an increase in InsPn, suggesting that in adrenocarcinoma cells, activation of HRH1 would be
356 associated to the classic signaling pathway involving a phospholipase C (PLC). The reversion of the HA-
357 antiproliferative effect in the presence of specific PLC- inhibitor U-73122 confirmed these results.

358 A similar HA-anti-proliferative signaling mechanism was described for prostate cancer cell line DU-145
359 (Valencia et al. 2001) as well as CHO cells stably transfected with HRH1, where HA activated a PLC, leading to
360 an inhibition of proliferation through a mechanism mediated by GTPasa, Rac and c-Jun-kinase (Notcovich, et al.
361 2010). It is known that Angiotensin II stimulates aldosterone production in H295R cells through AT1 receptor
362 coupled to PLC increasing the production of InsPn (Rainey et al. 2004). Although HA inhibited H295R cell
363 proliferation by increasing InsPn without activating aldosterone production, it would be possible that HA
364 stimulates NOS enzyme activity (via Ca^{2+}) blocking steroidogenesis as we have previously described in MA-10
365 Leydig cells (Mondillo et al. 2009) and it has been observed in other steroidogenic systems (Ducsay and Myers
366 2011). Regarding this, it has been demonstrated that NOS can inhibit L-type calcium channel (Wang et al. 2008),
367 which is necessary for AII mediated steroidogenesis. Supposing HA induced NOS in H295R cells, the entry
368 of calcium through the L-channel would be blocked thus preventing aldosterone synthesis, without affecting
369 proliferation pathway. Nevertheless, an activation of other kinase signaling pathways by other HA receptors, with
370 an antagonizing effect, can not be discarded.
371 The present work demonstrates that treatment with HA or FMPH, the HRH1 agonist, is capable of inhibiting
372 cellular proliferation of human adrenocortical tumor cells *in vitro* without inducing apoptosis, as the Tunel and
373 caspase-3 immunoblot assays confirmed. In addition, treatment with HA or FMPH, induced a cell cycle arrest of
374 H295R cell line in G2/M phase. Transition between cell cycle phases is a process that relays on the formation of
375 cyclin- cyclin dependent kinase complexes as well as their interaction with specific inhibitors.
376 Several proteins have been associated with the entry control to G2/M phase (Smits and Medema 2001). In this
377 regard, in H295R cells it has been described a G2/M phase arrest induced by combinatory treatment with mitotane
378 and ionizing radiations. These agents act by attenuating the DNA repair mechanisms and keeping high levels of
379 cyclin B1/cdc2 complexes (Cerquetti, et al. 2010). It is likely that at least some of these events participate in the
380 G2/M phase arrest induced by HA. Further studies must be conducted in order to confirm this hypothesis.
381 Currently, non-surgical treatments for human ACC are scarce and based on ionizing radiation in association with
382 high doses of adrenalytic drugs, bringing about toxic side effects that limit its usefulness (Maluf, et al. 2011). Our
383 results suggest that HA would exert a cytostatic effect on H295R cells, arresting cell growth in a DNA damaging

384 sensitive phase (G2/M), without inducing death. Future studies must be done in order to evaluate if these features

385 could make HA a good candidate for new ACC therapies.

386

387 **7 Declaration of interest**

388 The authors have nothing to disclose. **T**here is no conflict of interest that could be perceived as prejudicing

389 the impartiality of the research reported

390

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394

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399

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535 **13. Figure legends**

536 **Figure 1: Effect of HA and its specific agonists on adrenocortical steroidogenesis.** The murine cell line Y1
537 and the human cell line H295R, were incubated with increasing concentration of HA (A, B) or specific agonists
538 for each HA subtype receptors, known as FMPH (HRH1), AMTH (HRH2), IMET (HRH3) and VUF (HRH4) at a
539 concentration 10^{-5} M (C, D), for 24 h under basal and stimulated steroidogenesis. After incubation period, media
540 were recovered and the main steroidogenic product for each cell line was quantified. (A, C) Progesterone
541 produced by Y1 cells. (B, D) Cortisol produced by H295R cells. Bars represent the mean ± SEM of at least three
542 independent experiments.

543

544 **Figure 2: Effect of HA on StAR protein expression.** Y1 and H295R adrenocortical cells were incubated in the
545 presence or absence of 10^{-5} M HA under stimulated steroidogenesis, for different times, as described in Materials
546 and Methods. After incubation, proteins were extracted and the expression of StAR protein was analyzed by
547 Western Blot. Data were normalized to internal control β-tubulin. (A, B) Representative Western Blot of StAR
548 protein in Y1 and H295R cells, respectively. (C, D) Quantitation of StAR protein levels by scanning densitometry
549 in Y1 and H295R cells, respectively. Each bar shows the mean ± SEM of three independent experiments
550 performed with triplicate samples. Different letters above the bars indicate that the groups differ significantly at
551 least at $P < 0.05$.

552

553 **Figure 3: Effects of HA and its specific agonists on adrenocortical cell proliferation.** The murine cell line Y1
554 and the human cell line H295R, were incubated with increasing concentration of HA (A, B respectively) or
555 specific agonists for each HA subtype receptors: FMPH (H1), AMTH (H2), IMET (H3) and VUF (H4) (C,D
556 respectively), at a concentration of 10^{-5}M, for 24 h. Cells were labeled with a pulse of [3H]-Thymidine during the
557 last 12 h of incubation and the radioactivity incorporated into DNA was measured as described in Materials and
558 Methods. Data are expressed as proliferation percentage respect to the control (cells incubated without HA;
559 dashed line). Bars represent the mean ± SEM of at least three independent experiments. *, $P < 0.1$ *vs* control, **,
560 $P < 0.01$ *vs* control; ***, $P < 0.001$ *vs* control.

561 **Figure 4: Effects of HRH1 antagonist Pyrilamine on HA-mediated H295R cell proliferation.** H295R cells
562 were preincubated with 10^{-8} M Pyrilamine and after 30 minutes HA, FMPH or medium were added to the culture
563 and incubated for 24 h. (A) Cell proliferation was measured by [3H]-Thymidine incorporation into DNA as
564 described in Materials and Methods. (B) Cell proliferation was measured by MTT assay. Cells were incubated
565 with 0.5mg/ml MTT and OD was recorded at 570 nm. Cell number was calculated using a linear relation between
566 OD values and cell number. Data are expressed as proliferation percentage respect to the control (cells incubated
567 without HA; dashed line). Bars represent the mean ± SEM of at least three independent experiments. *, $P < 0.05$
568 *vs* control; **, $P < 0.01$ *vs* control.
569
570 **Figure 5: Binding assay for HRH1 receptor in adrenocortical cell lines.** Saturation binding assays were done
571 in intact Y1 cells (A) and H295R cells (B) using [3H]-Pyrilamine as specific HRH1 ligand. Saturation analysis
572 revealed a single and saturable binding site in both cell lines. Insets shows Scatchard plot of [3H]-Pyrilamine
573 specific binding. Shown is a typical result of experiment replicated three times, with data representing mean of
574 duplicate determinations for each cell line. Bars = SEM.
575
576 **Figure 6: Signaling pathway of HA anti-proliferative effect in H295R.** (A) Intracellular cAMP production.
577 Cells were incubated for 20 minutes with 10^{-5} M HA, 10^{-5} M FMPH or 5 x 10^{-4} M forskolin (Forsk), used as
578 positive control. cAMP levels were measured by radioimmunoassay. (B) Total [3H]-Inositol phosphates
579 accumulation. Cells preincubated with [3H]-Myo-inositol were treated with 10^{-5} M HA, 10^{-5} M FMPH or 10^{-3} M
580 NaF, used as positive control. Total [3H]-Inositol phosphates were quantified by recovered radioactivity, as
581 described in Material and Methods. Bars represent mean ± SEM of at least three independent experiments.
582 Different letters above the bars indicate that the groups differ significantly at least at $P < 0.01$. (C) Involvement of
583 phospholipase C on H295R cell proliferation. H295R cells were incubated with the specific PLC inhibitor U-
584 73122 or its no functional analog U-73343 in presence of FMPH for 24 h and proliferation was determined by
585 DNA incorporation of [3H]-Thymidine during the last 12 h of incubation, as described in Materials and Methods.

586 Data are expressed as proliferation percentage respect to the control (cells incubated without HA; dashed line).
587 Bars represent the mean ± SEM of at least three independent experiments. *, $P < 0.05$ *vs* control.
588
589 **Figure 7: Effect of HA and FMPH on H295R cell apoptosis.** (A) Evaluation of apoptosis by Tunel assay.
590 H295R cells were incubated with HA or FMPH, both at a concentration of 10^{-5} M for 24 h, processed by Tunel
591 reaction and analyzed by flow cytometry as described in Material and Methods. Doxorubicin was used as positive
592 control for apoptosis. Bars represent the mean ± SEM of three independent experiments. **, $P < 0.01$ *vs* control;
593 ***, $P < 0.001$ *vs* control (B) Evaluation of caspase-3 activation by Western blot. H295R cells were incubated
594 with HA or FMPH (both at a concentration 10^{-5} M) at 0, 6, 18, 24, 48, and 72 h. Proteins were extracted and
595 subjected to SDS-PAGE electrophoresis. Caspase-3 was detected using specific antibody in both forms, inactive
596 precursor (molecular weight 32) and active subunits (molecular weight 17 and 11). Active subunits of caspase-3
597 were not detected even at 72 h of treatment with HA nor FMPH in H295R cells. EDS- treated MA-10 cells were
598 used as control for anti caspase-3 antibody.
599
600 **Figure 8: Effect of HA and FMPH on H295R cell cycle progression.** H295R cells were incubated with HA or
601 FMPH, both at 10^{-5} M for 24 h. After incubation cells were fixed, permeabilized and stained with propidum
602 iodide as described in Material and Methods. DNA content was analyzed by flow cytometry. (A) Histogram of
603 DNA content for each treatment, from representative experiments. (B) H295R cell percentage distribution in
604 G1/G0, G2/M and S cell cycle phases from all experiments. Bars represent the mean ± SEM of three independent
605 experiments. *, $P < 0.05$ *vs* control.
606
607 **Figure 9: HDC protein expression and endogenous HA content in adrenocortical cell lines.** Y1 and H295R
608 cells were lysed and subjected to Western blot analysis for the detection of HDC protein, as described in Materials
609 and Methods. Data were normalized to internal control β-tubulin. (A, upper panel) Representative Western blot of
610 HDC protein. Rat stomach was used as positive control. (A, lower panel) Quantitation of protein levels by
611 scanning densitometry. Each bar shows the mean ± SEM of three independent experiments performed with

612 triplicate samples. Different letters above the bars indicate that the groups differ significantly at least at $P < 0.05$.
613 (B, upper panel) Immunocytochemical staining of endogenous HA content in Y1 and H295R cell lines. As a
614 negative control, the primary antibody was replaced with normal rabbit serum in PBS. Scale bar = 50 µm. (B,
615 lower panel) Quantitation of HA content by scanning densitometry in arbitrary units (AR). Each bar shows the
616 mean ± SEM of three independent experiments, five hundred cells for each cell line were analyzed. Different
617 letters above bars indicate that the groups differ significantly at least at $P< 0.05$.
618
619 **Figure 10: Effect of inhibition of endogenous HDC on Y1 cell proliferation.** The murine cell line Y1 was
620 incubated with increasing concentration of EGCE, an inhibitor of HDC for 24 h. Cells were labeled with a pulse
621 of [3H]-Thymidine during the last 12 h of incubation and the radioactivity incorporated into DNA was measured
622 as described in Materials and Methods. Data are expressed as proliferation percentage respect to the control (cells
623 incubated without EGCE; dashed line). Bars represent the mean ± SEM of at least three independent experiments.
624 *, $P < 0.1$ *vs* control; ***, $P < 0.001$ *vs* control.
625

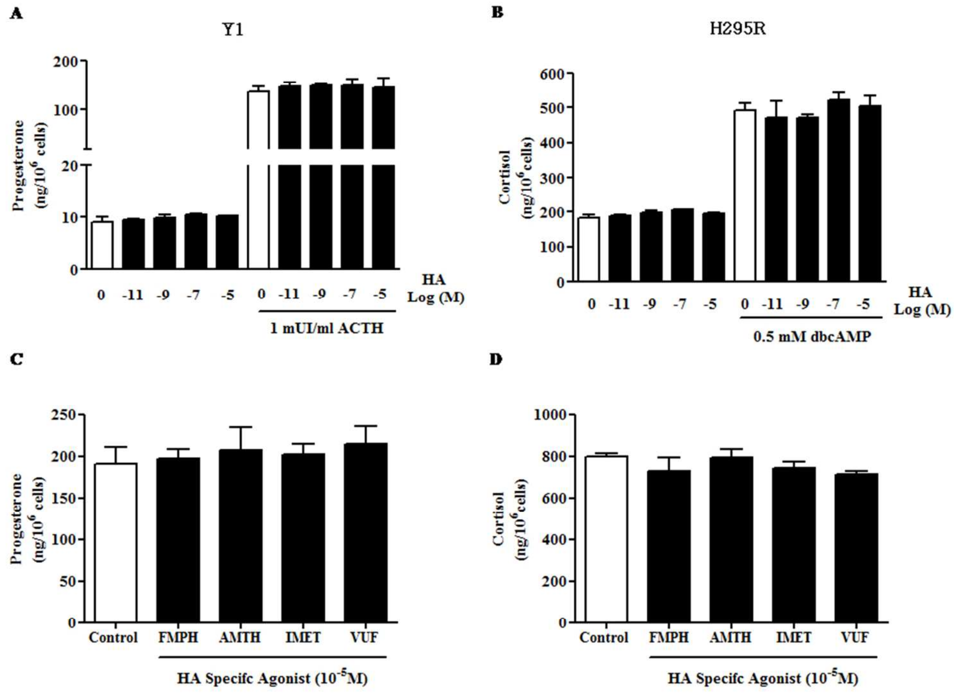


Figure 1-Y1 and H295R cell steroidogenesis
254x190mm (96 x 96 DPI)

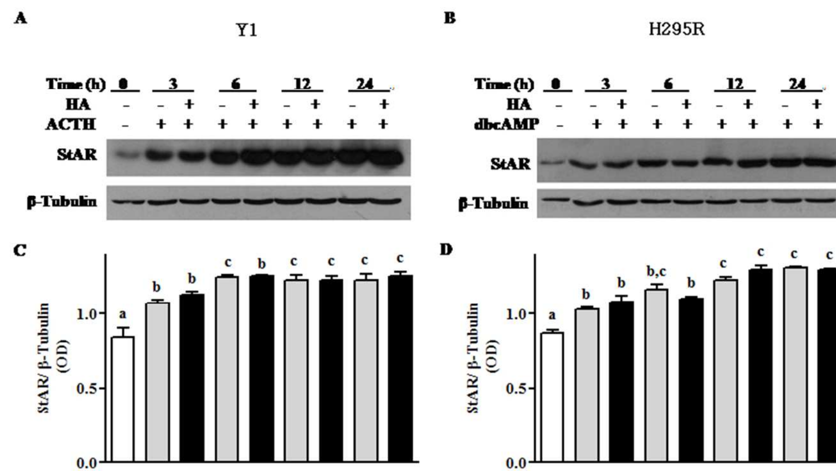


Figure 2-HA and Y1 and H295R cell StAR expression
254x190mm (96 x 96 DPI)

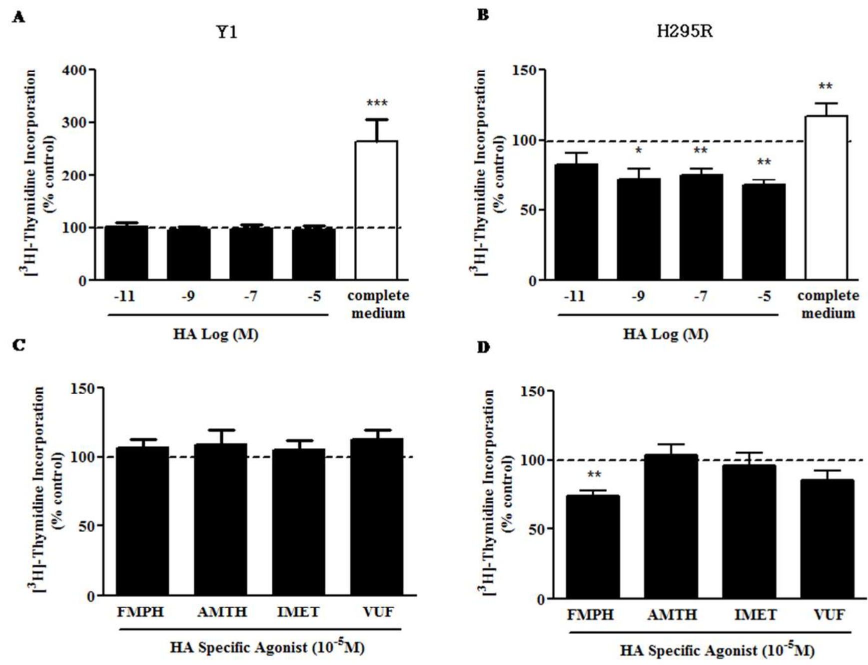


Figure 3-HA and Y1 and H295R cell proliferation
254x190mm (96 x 96 DPI)

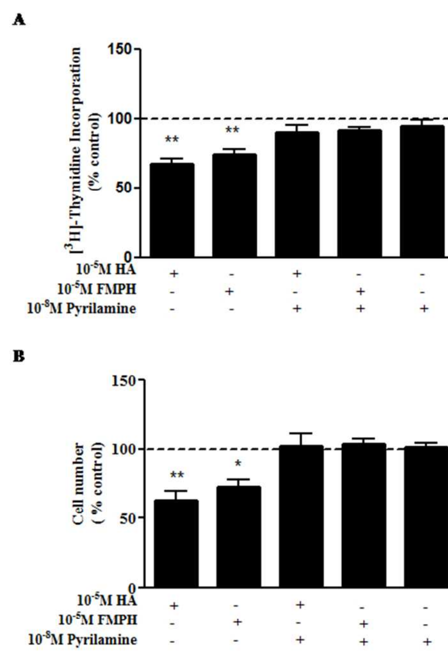


Fig 4-Pyrilamine and H295R cell proliferation
254x190mm (96 x 96 DPI)

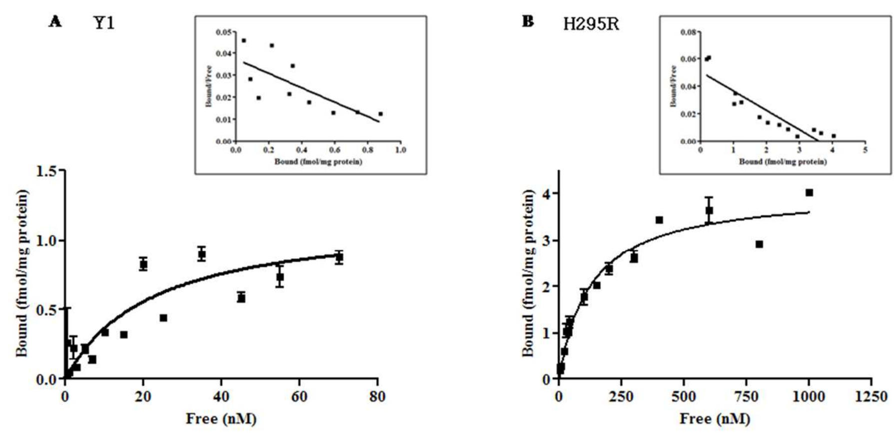


Figure 5-Y1 and H295R cell binding
254x190mm (96 x 96 DPI)

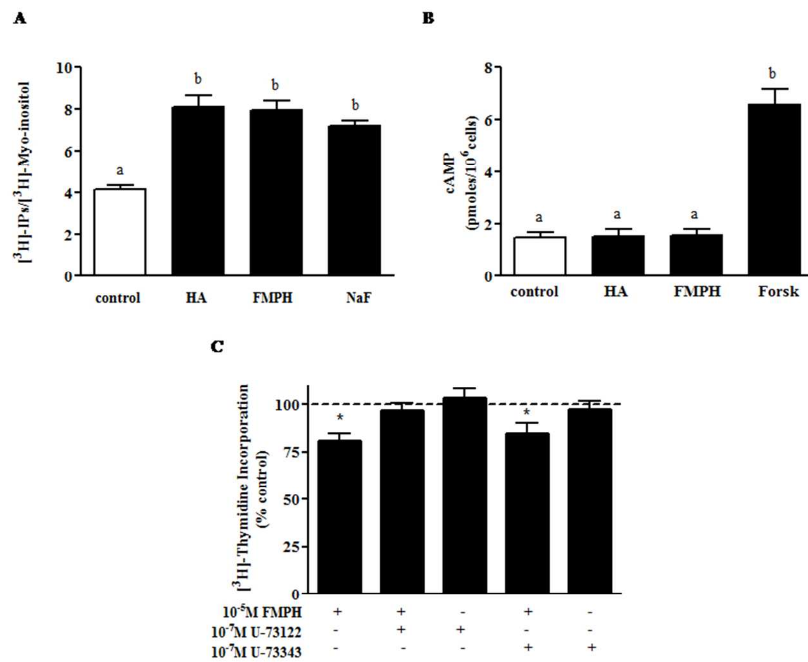


Figure 6-cAMP and IPs in H295R cells
254x190mm (96 x 96 DPI)

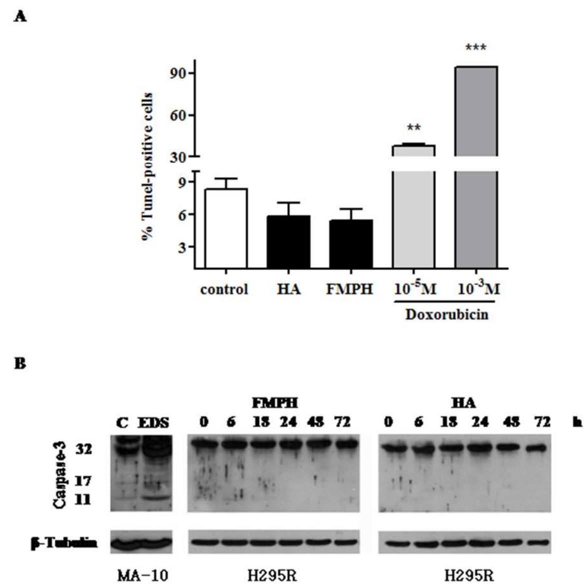


Fig 7-H295R cell apoptosis
254x190mm (96 x 96 DPI)

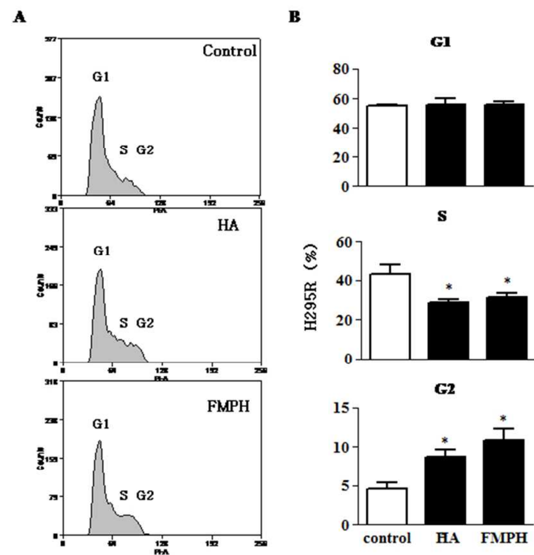
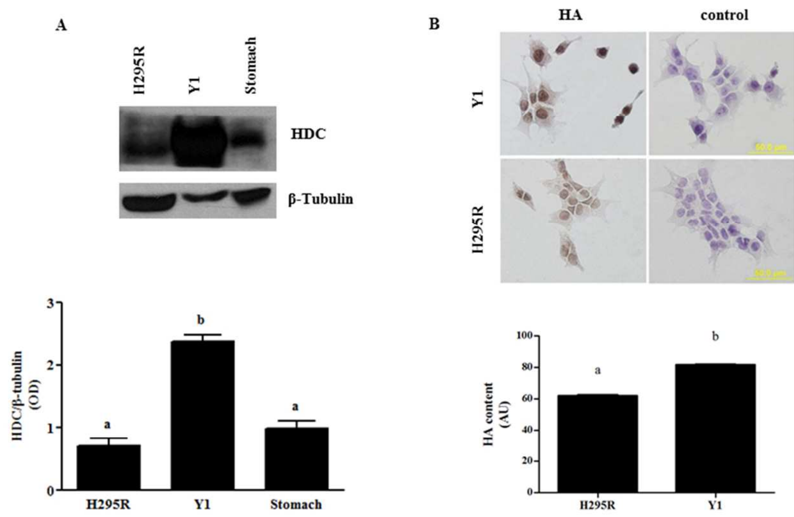
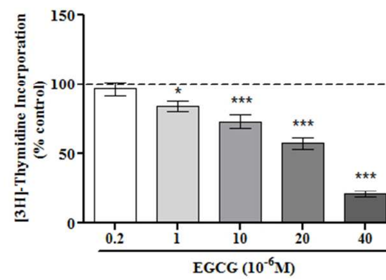


Figure 8-H295R cell cycle
254x190mm (96 x 96 DPI)



HDC activity and HA content in Y1 and H295R cells
254x190mm (96 x 96 DPI)



Proliferation of Y1 cells in presence of HDC inhibitor
254x190mm (96 x 96 DPI)