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Abstract

Growth hormone (GH) secreting pituitary adenomas are the main cause of acromegaly. Somatostatin analogs are the gold standard of medical therapy; however, resistance represents a big drawback in acromegaly management. We recently demonstrated that benzene (BZ) modifies the aggressiveness of GH-secreting rat pituitary adenoma cells (GH3), increasing GH secretion and altering the synthesis of molecules involved in the somatostatin signaling pathway. Based on these pieces of evidence, this study aimed to evaluate the effects of BZ on octreotide (OCT) efficacy in GH-secreting adenoma cells. In GH3 cells, BZ counteracted the anti-proliferative action of OCT. GH gene expression, unmodified by OCT, remained high in BZ-treated cells as well as after treatment with the association of both. GH secretion, reduced by OCT, was increased after treatment with BZ alone or when the pollutant was used with OCT. The combination of BZ and OCT greatly reduced the gene expression of ZAC1 and SSTR2; and this reduction was also present at a protein level. BZ caused an increase in the protein level of the transcription factor STAT3 and in its phosphorylated form. In the presence of BZ, OCT lost the ability to reduce the phosphorylated protein levels. Finally, in primary cultures of human pituitary adenoma cells, BZ caused an increase in GH secretion. OCT decreased GH secretion, but the addition of BZ reversed the OCT effect. In conclusion, our results suggest that BZ may have an important role in the resistance of pituitary adenomas to the pharmacological treatment with somatostatin analogs.

Keywords: pollution; benzene; GH; octreotide; pituitary adenoma.

Abbreviations: Abbreviations: AHR = aryl hydrocarbon receptor; AIP = aryl hydrocarbon receptor-interacting protein; BZ = benzene; DEHP = bis(2-ethylhexyl) phthalate; GH = growth hormone; GH3 = GH-secreting rat pituitary adenoma cells; MAPK = mitogen-activated protein kinase; OCT = octreotide; PTP = phosphotyrosine phosphatase; SS = somatostatin; SSA = somatostatin analogs; SSTR =

somatostatin receptor; STAT3 Signal transducer and activator of transcription 3; ZAC1 = zinc-finger protein.

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Introduction

Growth hormone (GH) secreting pituitary adenomas (10-12 % of pituitary secreting tumors) are the main cause of acromegaly, a rare but severe and complex endocrine disease resulting from increased circulating GH levels (Melmed 2015, Mehta & Lonser 2017).

The available therapeutic approaches for acromegaly consist in neurosurgery, radiotherapy and medical therapy whose gold standard is the treatment with somatostatin analogs (SSAs) (Melmed et al. 2018, Cuevas Ramos & Fleseriu, 2014, Paragliola et al. 2017). SSAs mimic the physiological somatostatin actions; they actually bind to somatostatin receptors (SSTRs) and induce a conformational change of SSTRs that leads to activation of an associated heterodimeric G-protein complex, that through the induction of various intracellular cascades, finally results in antiproliferative and antisecretory effects. Pathways and effects on cellular function may be different according to each specific SSTR subtype. Five different subtypes of SSTRs have been identified (SSTR1-5), each one being encoded by genes situated on different chromosomes (Patel 1999). Octreotide (OCT), the first SSA used in clinical practice, still widely used for acromegaly treatment, is an octapeptide with high binding affinity for SSTR2 and, to a lesser extent, for SSTR5. In general, SSTRs signaling inhibits adenyl cyclase activity and modulates potassium and calcium channels involved in hormone secretion; it affects phosphotyrosine phosphatase (PTP) and/or mitogen-activated protein kinase (MAPK) activity, involved in cell growth and differentiation. Moreover, PTP has been described to modulate the phosphorylation/activity of the signal transducer and activator of transcription 3 (STAT3) that is implicated in both GH synthesis as well as in cell growth, survival and differentiation.

Zinc-finger protein (ZAC1) is a transcription factor and coregulator that plays a key role in the signal transduction following SSTR2 activation (Theodoropoulou et al. 2010). It is involved in the development of normal pituitary and in tumorigenesis process; many studies have shown that its function is essential for SSA efficacy (Theodoropoulou et al. 2009, Chahal et al. 2012).

Unfortunately, some GH-secreting adenomas are resistant to octreotide therapy. Resistance to SSA, consisting in lack of hormonal normalization, increase in tumor size or tumor shrinkage less than 20%

compared with baseline tumor (Paragliola et al. 2017) and represents a big drawback in acromegaly management.

The molecular basis of different sensitivity of GH pituitary tumors to SSA has been widely investigated over the last years. The density of SSTRs with high affinity for SSA, mutations of genes encoding for SSTRs, desensitization of SSTRs for the uncoupling to the signaling cascade (Colao et al. 2011) have been identified as main factors underlying this phenomenon. Nevertheless, other elements may interfere with pharmacological response, such as other genetic mutations as well as epigenetic and environmental factors (Zhou et al. 2014, Dzobo et al. 2018).

Nowadays, pollution is a critical issue because of its adverse effects on human health. Benzene (BZ) is a very common environmental pollutant; its main sources are vehicle exhaust fuels, evaporation during handling and storage of petrol, industrial emissions and cigarette smoking.

Recent epidemiologic studies suggest an involvement of environmental pollution in pituitary adenoma outbreaks. As a consequence of Seveso accident in 1976 Pesatori and colleagues (2008) observed an increasing trend in pituitary adenomas incidence in areas characterized by high dioxin contamination. In the highly polluted area close to Messina (Italy) Cannavò et al. (2010) encountered a significantly higher prevalence of acromegaly respect to less polluted areas and to the general population. Patients living in highly polluted areas and carriers of genetic variants affecting normal functioning of the aryl hydrocarbon receptor (AHR) pathway (that is involved in xenobiotics cellular response) present larger pituitary tumors, more severe acromegaly symptoms, and are more frequently SSA resistance (Cannavò et al. 2016).

A link between epidemiological and molecular studies is provided by the in vitro study of Tapella et al. (2016) showing that pollutants such as BZ and DEHP (di-2-ethylhexyl phthalate) can modify normal rat pituitary cell proliferation and induce gene expression changes of AHR and other related genes.

Furthermore, our recent study (Fortunati et al. 2017) demonstrated that some common chemical pollutants are able to induce modifications in molecules related to GH secretion and somatostatin signaling pathway, focusing on their capacity of enhancing aggressiveness and modifying biological

behavior of pituitary tumors. Effects observed on ZAC1 and SSTR2 may imply an important role in sensitivity of pituitary adenomas to medical treatment. Based on these evidences, this study aimed to evaluate the effects of BZ on OCT efficacy in GH secreting adenoma cells, by investigating cell proliferation, GH expression and secretion, gene expression and protein level of molecules involved in somatostatin signaling pathway as well as phosphorylation status of STAT3.

Materials and Methods

Chemicals

Benzene (BZ) was purchased from Sigma-Aldrich Co. (St. Louis, MO) and was dissolved in DMEM-F12 medium added with dextran-charcoal stripped sera (12.8 μM). The experimental dose (130 pM) was within ranges observed in blood of exposed people (Hines at al. 2009, Yan et al. 2009, Hays et al. 2012, Chovancova et al. 2012) and as previously assessed in our recent study (Fortunati et al. 2017). Octreotide (OCT) was kindly provided by Novartis Pharma AG (Basel, Switzerland). Experimental doses (10 nM, 100 nM, and 1 μM) were within ranges in blood of treated patients as shown in pharmacokinetics studies (Tuvia et al. 2012).

Cell cultures

Rat pituitary adenoma GH3 cells, obtained from the American Type Culture Collection (ATCC), were maintained in DMEM-F12 culture medium (Lonza, Switzerland) supplemented with 2.5% fetal calf serum and 15% horse serum (Gibco, UK), at 37° C in a humidified atmosphere of 95% O_2 and 5% CO_2. Antibiotics (100 UI/ml penicillin and 100 μg/ml streptomycin) were added to growth media. The cells were routinely screened for mycoplasma contamination, and kept in serum-free medium for the 24 hours preceding the treatments with BZ and/or OCT.

Cell viability assay

Cell viability was assessed using Cell Proliferation Reagent WST-1, a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche Applied Science, Penzberg, Germany). The cells were seeded in 96-well plates at a density of 1 x 10^4 cells/well. After 72 hours the cells were treated with 10 nM - 1 μM OCT in the presence or absence of 130 pM BZ; untreated cells were used as control. At different time points (i.e. 0, 24, 48 and 72 hours) 10 μl of WST-1 was added to each well and after 1 hour incubation, absorbance at 450 nm was measured using a plate reader (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA). Four replicate wells were used to determine each data point.

Gene expression profile

Real Time PCR was used to evaluate the expression of GH, SSTR2, ZAC1, aryl hydrocarbon receptor interacting protein (AIP), and aryl hydrocarbon receptor (AHR). To assess gene expression profile, GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6 cells/flask, and then treated for 72h with 10-100 nM OCT in the presence or absence of 130 pM BZ.

Total RNA was extracted using TRIzol Reagent (Invitrogen Ltd, Paisley, UK), and 1 µg of the total RNA reverse-transcribed with iScript cDNA Synthesis Kit (BioRad Laboratories, Inc.), following manufacturer's protocol. Specific primers were designed using Beacon Designer 5.0 software; primers sequences are shown in Table 1.

Real-time PCR was performed using a BioRad MiIQ Detection System (BioRad Laboratories, Inc.) with SYBR green fluorophore. Reactions were performed in 15 µl volume, which included 7.5 µl IQ SYBR Green Supermix (BioRad Laboratories, Inc.), 0.3 µl each primer at 10 µM concentration, 1.90 µl RNAse-free distilled water, and 20 ng/5 µl of the previously reverse-transcribed cDNA template. For each primer set, the reaction was optimized using seven serial 5X dilutions of template cDNA obtained from cells in basal conditions (100, 20, 4, 0.8 and 0.16 ng). A melting curve analysis was performed following each run to ensure a single amplified product for each reaction. All reactions were carried out at least three times for each sample. Every gene expression level was normalized on the expression of three house-keeping genes (β-Actin, L13A and β-2-microglobulin) and expressed as relative expression fold *vs* untreated controls.

Western blot

GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6 cells/flask, and treated with 10-100 nM OCT in the presence or absence of 130 pM BZ. At different times after treatment, the cells were scraped from the flask in the presence of 1 ml lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, 10 mg/ml PMSF, 30 µl/ml aprotinin and 100 mM sodium orthovanadate). Cell lysates were incubated in ice for 30 min. At completion, tubes were centrifuged at 4 °C for 20 min at 15,000 × g. Clear supernatants were

stored at −80 °C until use. Proteins were separated on 10% SDS-PAGE for ZAC1 and SSTR2, transferred to PVDF and probed with the following antibodies: polyclonal anti-ZAC1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-SSTR2 (1:500 Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For the evaluation of STAT3 and its phosphorylated form pSTAT3, a monoclonal anti-PAN-STAT3 (1:1000, Cell Signaling Technology, Leiden, The Netherlands) and a polyclonal anti-Phospho-STAT3 (Tyr705) antibody (1:500, Cell Signaling Technology, Leiden The Netherlands) were used.

The membranes were then stripped and re-probed with a rabbit polyclonal anti-GAPDH antibody (1:10000, Sigma, Saint Louis, MO, USA) to check protein loading. Proteins were detected with Pierce Super Signal chemiluminescent substrate. The bands were photographed and analyzed using Kodak 1D Image Analysis software.

Growth hormone secretion

GH3 cells were seeded in cell culture flasks (25 cm^2) at a density of 1 x 10^6 cells/flask, and treated with 10-100 nM OCT in the presence or absence of 130 pM BZ. After a 72h exposure, cell medium was centrifuged and collected; secreted GH was measured in the medium using the Rat Growth Hormone ELISA Assay (KRC5311, Invitrogen Ltd, Paisley, UK), following manufacturer's instructions.

Primary culture establishment and GH-secretion

Pituitary adenoma samples were collected from patients that underwent surgery at the Division of Neurosurgery of Città della Salute e della Scienza University Hospital (Turin, Italy). Informed consent to the study was obtained from each patient, and the use of tissue samples was approved by "The Commitment for Human Specimen Utilization" of the Department of Medical Sciences, University of Turin. The study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Tissue samples were obtained by transsphenoidal surgery from 8 patients with GH-secreting pituitary adenomas. The diagnosis was established on the basis of clinical and biochemical characteristics of the patients and afterwards it was confirmed by the pathologist on the basis of both histology and

immunohistochemistry of the tumor sample. The tissues were collected under sterile conditions and processed immediately. Samples were washed three times in saline added with penicillin/streptomycin and nystatin.

Enzymatic digestion was performed using 0.35% collagenase (Sigma, Milano, Italy) at 37°C for 60 min. Cell suspensions were filtered through double layers of sterile gauze and washed twice with D-MEM-F12 plus 10% FCS. The cells were resuspended in complete medium added with 100 IU/ml penicillin and 100 μg/ml streptomycin, 50μg/ml gentamicin, 2.5μg/ml amphotericin B, 15% HS and 2.5% FCS. Cells were plated at 2 X 10^5 cells/well depending upon the abundance of the tumor specimen and after 72 hours treated with 100 nM OCT in the presence or absence of 130 pM BZ. Untreated cells were used as control. After 72 hours of treatment cell media were centrifuge and collected; GH secretion was evaluated using GH IRMA (IM1397, Beckman Coulter Inc., Chaska MN USA).

Statistical analysis

Data are expressed as mean ± SD, calculated on at least three independent experiments (N=3). The comparison between different treatments and control groups was performed with one-way ANOVA and the threshold of significance was calculated with the Bonferroni test. To analyze the effects of combination of BZ and OCT, the comparison between groups was performed with analysis of two-way ANOVA, and the threshold of significance was calculated with the Bonferroni test. Statistical significance was set at $p<0.05$.

Results

GH3 cells viability

OCT significantly reduced GH3 cells viability compared to control at 48 and 72 hours, but combined treatment with BZ abolished this effect. In particular, 48 hour treatment with 10 nM OCT decreased cell viability of 32.2%, while addition of BZ partially abolished OCT effect (18.7% reduction vs control) (Fig. 1A). 100 nM OCT reduced cell viability of 31% at 48 hours, and of 42.4% at 72 hours, addition of BZ to 100 nM OCT restored cell viability to control values at 48 hours and significantly reduced the antiproliferative OCT action of 20% at 72 hours (Fig. 1B). 1 µM OCT reduced cell viability of 26.8% at 48 hours and of 49.5% at 72 hours; treatment with BZ (Fig. 1C) decreased efficacy of 1 µM OCT treatment alone at 72 hours (reduction of 24.8% for 1 µM OCT plus BZ). BZ alone had no significant effect on cell viability.

GH gene expression and secretion

After 72 hour exposure, BZ induced GH gene expression (2.8 fold). An increased expression was also maintained when BZ was added to 10 and 100 nM OCT (1.6 fold vs OCT for both doses; 1.7 fold and 1.8 fold vs control respectively) (Fig.2A). As far as GH secretion is concerned, it was significantly reduced by 10 and 100 nM OCT (46.4%, and 24%, respectively) (Fig.2B) and significantly increased by BZ of 29.3%. A higher GH secretion (15% vs control and 114% vs 10 nM OCT), was also maintained with 10 nM OCT and BZ.

Somatostatin pathway: SSTR2, ZAC1 expression and production

Fig. 3A shows a reduction of ZAC1 gene expression by the combined treatment of both 10 and 100 nM OCT with BZ (vs OCT alone: 0.23 fold, and 0.24 fold, respectively; vs control: 0.20 fold, and 0.26 fold, respectively). BZ alone, as expected (Fortunati et al., 2017), did not modify ZAC1 gene expression. At protein level, western blot (Fig. 3B e 3C) revealed a reduction of ZAC1 after BZ exposure (0.7 fold), also present when cells were treated with 100 nM OCT plus BZ compared to 100 nM OCT alone (0.61 fold) and to control (0.56 fold). SSTR2 gene expression was highly decreased when BZ was added to OCT at both used concentrations in comparison with respective doses of drug alone (0.00015 fold for

10 nM OCT plus BZ, and 0.00048 fold for 100 nM OCT plus BZ; 0.38 fold for 10 nM OCT plus BZ vs control) (Fig. 3D). BZ reduced SSTR2 protein (0.8 fold) and significant effects were observed with combination of OCT and BZ in comparison with respective doses of OCT alone (0.72 fold 10 nM OCT plus BZ versus 10 nM OCT and 0.47 fold vs control; 0.25 fold 100 nM OCT versus 100 nM OCT, and 0.16 fold vs control). A reduction of SSTR2 protein was observed even when cells were treated with 10 e 100 nM OCT alone (0.64 fold, and 0.62 fold, respectively) (Fig. 3E and 3F). Gene expression of AIP and AhR were almost unmodified except for a slight reduction induced by 10 nM OCT plus BZ on AhR gene expression ($p < 0.05$) (Fig. 4).

STAT3 phosphorylation

Western blot (Fig. 5A) demonstrated that OCT statistically reduced STAT3 phosphorylation (Phospho-STAT3) at 10 nM (Fig. 5A and 5B) (0.8 fold) and at 100 nM (Fig. 5B) (0.18 fold). BZ increased Phospho-STAT3 (1.6 fold) and the addition of BZ to 10 or 100 nM OCT resulted in higher levels of Phospho-STAT3 compared to treatment with the drug alone (10 nM OCT plus BZ 5.25 fold, and 1.38 fold vs control; 100 nM OCT plus BZ 4.9 fold vs 100 nM OCT). BZ enhanced Pan-STAT3 protein (1.8 fold) (Fig. 5C), while 100 nM OCT reduced Pan-STAT3 (0.70 fold) as did 100 nM OCT plus BZ (0.71 fold).

GH secretion in primary culture cell medium

Fig.6 shows that BZ caused an increase in GH secretion of pituitary adenoma primary culture cells (38%). 100 nM OCT decreased GH secretion of 26.5 %, while addition of BZ reversed this effect and GH secretion resulted significantly higher compared to cells treated with 100 nM OCT alone (56%).

Discussion

For the last years, the interest on the effects of pollution on human health has been increasing and many studies demonstrated the relationship between pollutant exposure and the onset of several diseases. Many substances have been linked to endocrine tumor occurrence, but so far no study investigated the influence of pollution on the effectiveness of the pharmacological treatment of these diseases.

Based on our recent study (Fortunati et al. 2017), showing the ability of some common pollutants to modify the behavior of pituitary adenoma cells and the expression of molecules involved in the somatostatin signaling, this study aimed to evaluate the influence of benzene (BZ) on the somatostatin analogue octreotide (OCT) in the treatment of GH-secreting pituitary adenomas.

First, BZ reduced the efficacy of OCT in inhibiting GH3 cell proliferation. In this cell line model, BZ used alone had no effect on cell viability (Fortunati et al. 2017), but other studies conducted in normal rat pituitary primary cells showed an increased cell viability and proliferation induced by this chemical (Tapella et al. 2016), indicating that the effects can depend on cell type.

GH gene expression was not modified by OCT alone; exposure to BZ increased GH mRNA levels and this effect persisted also in cells treated with BZ and OCT. Even though data about the action of somatostatin on GH gene expression are not conclusive, it was suggested that somatostatin mainly acts on GH protein remodeling and secretion, lowering circulating GH levels, without inducing mRNA modifications (Eigler & Ben-Shlomo 2014).

Accordingly, our results revealed that OCT was very effective in lowering GH secretion in the medium; BZ was able to counteract this effect when the cells were treated with the lowest dose of OCT (10 nM). Also in human primary cultures OCT and BZ showed the same behavior. OCT significantly reduced GH secretion but the presence of BZ abolished this action.

SSTR2 and ZAC1 are important molecules involved in the somatostatin signaling. SSTR2 expression at cell membrane is essential for OCT action since SSTR2 is the receptor with the highest affinity for this drug. Low expression of SSTR2 results in a poor response to SSA treatment (Taboada et al. 2008, Ferone et al. 2008). BZ lowered SSTR2 protein level and the effect was very strong even in the presence of

OCT. A reduction of SSTR2 protein was evident also when the cells were treated with OCT alone. As a consequence of OCT binding SSTR2 reduction is likely to be due to receptor internalization. This phenomenon could account for the increased SSTR2 mRNA levels after OCT treatment, but reduced SSTR2 protein in cells treated with the combination of OCT and BZ.

ZAC1 controls cell proliferation and hormone synthesis and it is highly expressed in all hormone-producing cells of the pituitary. It exerts a pivotal role in SSA effects in pituitary cells (Theodoropoulou et al. 2009), and its loss of expression is frequently detected in pituitary adenomas (Theodoropoulou et al. 2010).

In our study, ZAC1 gene expression was not modified either by BZ or by OCT treatment, but it was reduced by the association of both compounds. At protein level, BZ induced a reduction of ZAC1 and this effect was present even when cells were treated with OCT at the same time. These results suggest that BZ may significantly impair this fundamental signaling pathway for OCT function.

Other studies reported an increase in ZAC1 gene expression after OCT exposure; nevertheless, they are hardly comparable to our data, because of the different experimental conditions (very short time of treatment and different drug concentration) (Theodoropoulou et al. 2006).

Some studies found a link between AIP and ZAC1 levels and suggested that this signaling pathway is important for SSA effects (Chahal et al. 2012). Actually, we could not observe any modification in AIP expression as well.

STAT3 is an important transcription factor and its dysregulated activation has been encountered in many types of cancers (Buettner et al. 2002, Bromberg et al. 2002). It is involved in cell survival and proliferation and in the pituitary it is also associated with GH synthesis (Zhou et al. 2016). Constitutively STAT3 activation has been also implicated in chemo-resistance (Real et al. 2002, Bharti et al. 2004, Boehm et al. 2008) and radio-resistance (Otero et al. 2006).

In our study we reported that OCT was able to reduce STAT3 phosphorylation accordingly to studies conducted in different types of tissues, as in retina explants (Mei et al. 2012). It was also reported that in GH4 pituitary adenoma cells OCT determined a reduction of STAT3 phosphorylation coupled with a

reduction in GH protein levels (Ezzat et al. 2017). This effect was mediated through activation of PTPs (e.g. SHP-1 or SHP-2) and it could be one of the pathways induced by OCT.

Exposure of cells to BZ, instead, increased STAT3 phosphorylation and counteracted OCT action. Diesel exhaust particles (Cao et al. 2007), tobacco smoke (Nagpal et al. 2002) and other carcinogens have been reported to activate STAT3., Other studies linked BZ to STAT3 gene expression as a consequence of epigenetic modifications (Yang et al. 2014) and alteration of its phosphorylation status was seen in plasma of BZ exposed people (Fenga et al. 2016). Therefore, STAT3 activation may have a key role in BZ pathogenic mechanism as well as in OCT response. It therefore deserves to be studied in detail.

Our results showed that BZ can interfere with pituitary adenoma response to SSA. Actually BZ reduced the action of octreotide on GH3 cells at many levels. BZ counteracted OCT capacity to inhibit cell proliferation and to reduce GH secretion. BZ modified molecular pathways involved in OCT mechanism of action; ZAC1 and SSTR2 gene expression and protein levels as well as the amount and phosphorylation status of STAT3 protein were affected by BZ exposure even in presence of OCT.

Taking together these results indicate that BZ is an environmental factor that could play an important role in pituitary adenoma responsiveness to SSA therapy. At present, only indirect evidence of the role of benzene exposure in SSA resistance in humans with GH-producing adenomas are available (Cannavò et al. 2016). Indeed, in 2016 Cannavò et al. observed more frequent SSA resistance in acromegalic patients living in highly polluted areas and carriers of AHR genetic variants. Therefore, our study can represent the rationale for investigating benzene levels and SSA responsiveness in humans treated with octreotide for GH-secreting adenomas. Overall, a better understanding of the role of BZ as well of other environmental pollutants in the resistance to pharmacological treatments will be achieved.

Declaration of interest: The authors declare no potential conflicts of interest.

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Figure legends

Figure 1 - Effects of OCT, BZ and OCT + BZ on GH3 cells proliferation. Cell viability after treatments with 10 nM (A) 100 nM (B) and 1 uM OCT (C), 130 pM BZ and their association for 24,48 and 72 h. Data are expressed as viability ratio vs T0 of three independent experiments (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Significance vs OCT treatment: $^{\#}$ $p<0.05$; $^{\#\#}$ $p<0.01$; $^{\#\#\#}$ $p<0.001$.

Figure 2 - Effects of OCT, BZ and OCT + BZ on GH in GH3 cells. mRNA expression of GH after treatments with 10 and 100 nM OCT, 130 pM BZ and their association for 72 h (A). Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N = 3). GH (ng/ml) secreted by GH3 cells after OCT, BZ and OCT + BZ exposure for 72 h (B) (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Significance vs OCT treatment: ° $p<0.05$; °° $p<0.01$; °°° $p<0.001$.

Figure 3 - Effects of OCT, BZ and OCT + BZ on ZAC1 and SSTR2. mRNA expression of ZAC1 (A) and SSTR2 (D) after treatments with 10 and 100 nM OCT, 130 pM BZ and their association 72 h. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as relative expression fold vs. control (N = 3). Western blot for ZAC1 (B) and SSTR2 (E), anti-GAPDH was used to confirm equal loading; the images show a typical experiment. Semiquantitative analysis of western blot results for ZAC1 (C) and SSTR2 (F) of three independent experiments (N=3). Significance vs Control: * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Significance vs OCT treatment: ° $p<0.05$; °° $p<0.01$; °°° $p<0.001$.

Figure 4 - Effects of OCT, BZ and OCT + BZ on AIP and AhR. mRNA expression of AIP and AhR after treatments with 10 and 100 nM OCT, 130 pM BZ and their association 72 h. Results are normalized for three different housekeeping genes (β-actin, β2μ and L13A) and are expressed as

relative expression fold vs. control (N = 3): * p<0.05; ** p<0.01; *** p<0.001. Significance vs OCT treatment: ° p<0.05; °° p<0.01; °°° p<0.001.

Figure 5 - Effects of OCT, BZ and OCT + BZ on Phosphorylated STAT3 protein (Phospho-STAT3) and Total STAT3 protein (Pan-STAT3). Western blot for and Pan-STAT3 (A), anti-GAPDH was used to confirm equal loading; the image shows a typical experiment. Semiquantitative analysis of western blot results for Phospho-STAT3 (B) and Pan-STAT3 (C) of three independent experiments (N=3). Significance vs Control: * p<0.05; ** p<0.01; *** p<0.001. Significance vs OCT treatment: ° p<0.05; °° p<0.01; °°° p<0.001.

Figure 6 - Effects of OCT, BZ and OCT + BZ on GH in pituitary adenoma primary cell cultures. GH (ng/ml) secreted by cells after OCT, BZ and OCT + BZ exposure for 72 h. Significance vs Control: * p<0.05; ** p<0.01; *** p<0.001. Significance vs OCT treatment: ° p<0.05; °° p<0.01; °°° p<0.001.

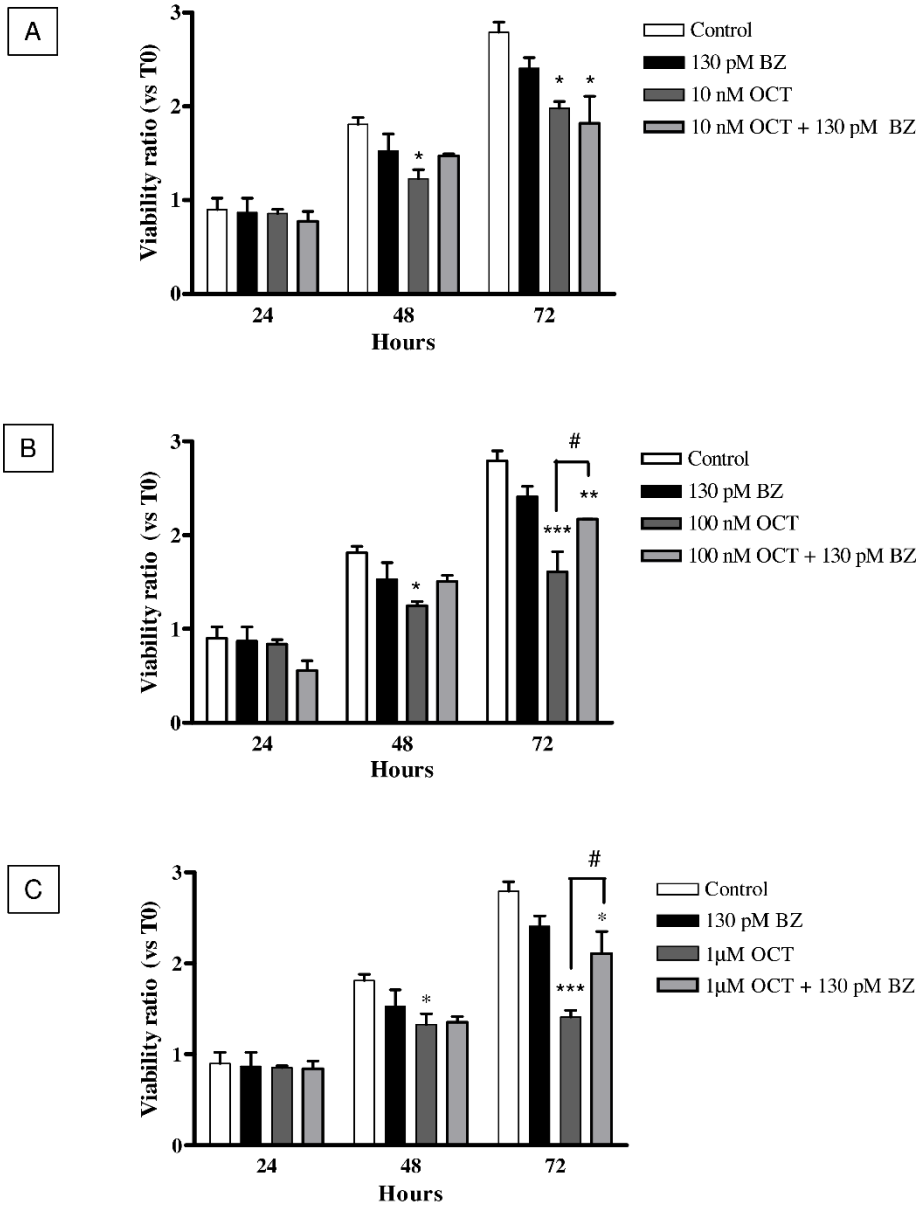

A
Viability ratio (vs T0)
Hours
24
48
72
Control
130 pM BZ
10 nM OCT
10 nM OCT + 130 pM BZ
B
100 nM OCT
100 nM OCT + 130 pM BZ
C
1µM OCT
1µM OCT + 130 pM BZ

Figure 1

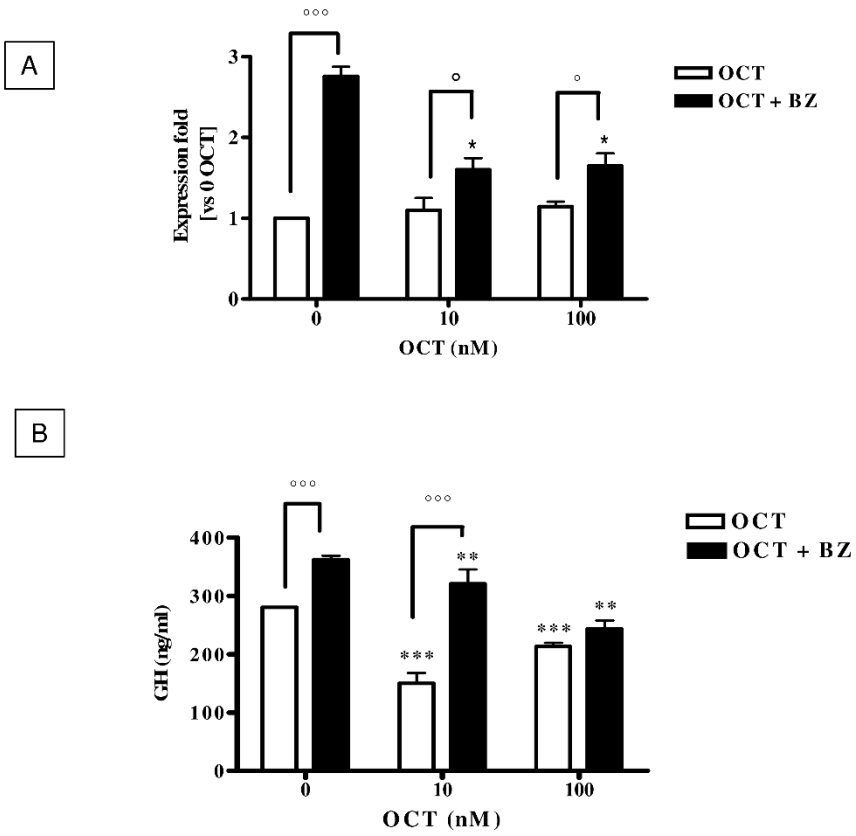

A
Expression fold
[vs 0 OCT]
OCT
OCT + BZ
OCT (nM)
B
GH (ng/ml)
OCT
OCT + BZ
OCT (nM)

Figure 2

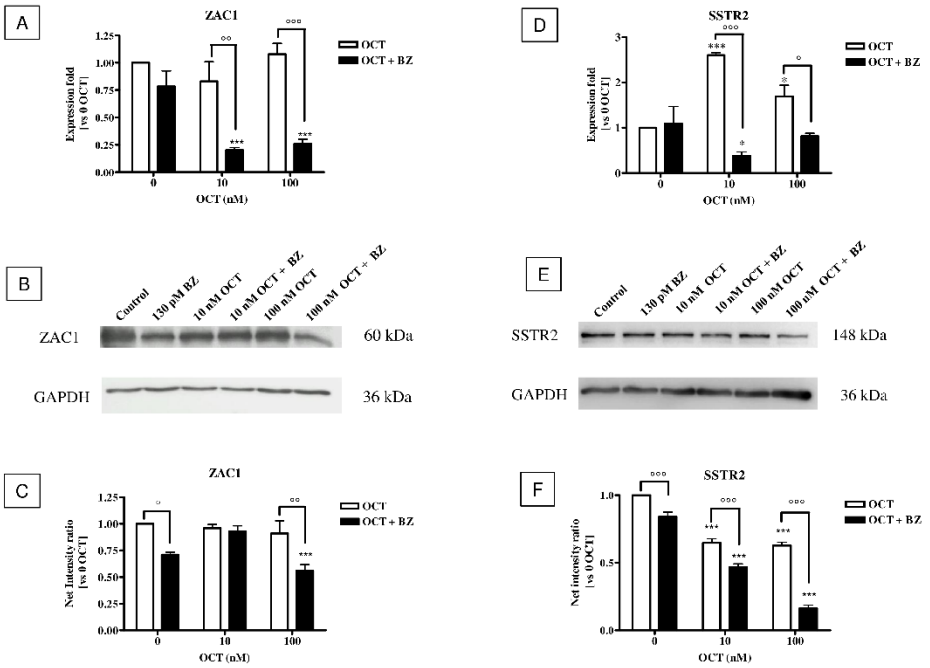


Figure 3

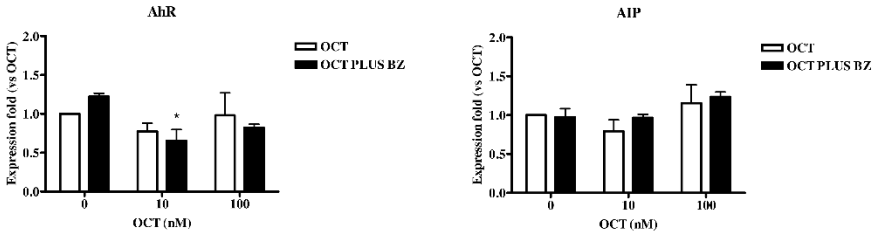
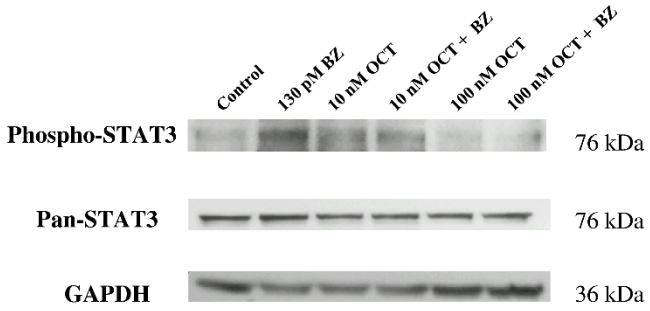
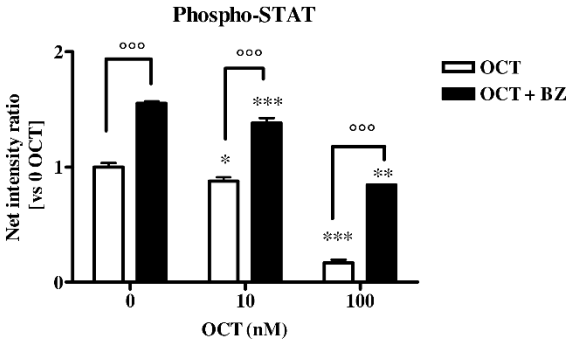


Figure 4

A


Control
130 pM BZ
10 nM OCT
10 nM OCT + BZ
100 nM OCT
100 nM OCT + BZ
Phospho-STAT3
76 kDa
Pan-STAT3
76 kDa
GAPDH
36 kDa

B


Phospho-STAT
Net intensity ratio [vs 0 OCT]
OCT
OCT + BZ
OCT (nM)
0
10
100
°°°
°°°
°°°
*
***
**
***

C

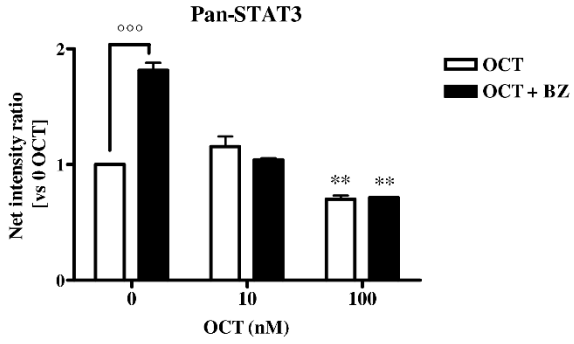

Pan-STAT3
Net intensity ratio [vs 0 OCT]
OCT
OCT + BZ
OCT (nM)
0
10
100
°°°
**
**

Figure 5

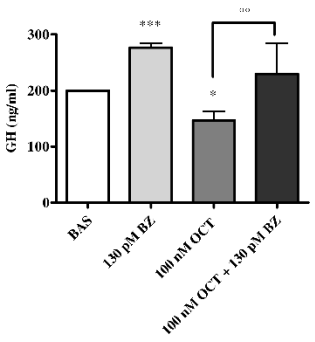


Figure 6

Table 1- Primers for real time PCR

Primers		Sequences (5'-3')
GH	sense	TCGCTTCTCGCTGCTGCTC
	antisense	TGCTTGAGGATCTGCCCAATACG
SSTR2	sense	TACTTCGTGGTGTGCGTGGTG
	antisense	CTTGGCGTAGCGGAGGATGAC
ZAC1	sense	GGCAAGAGGAGCGGAGAGC
	antisense	AATCAAGCAGGAACAACCACACG
AIP	sense	TCGTGCGTACCATGCGTGAG
	antisense	TGTTGCGGAGGCTCTTGGC
AhR	sense	ACCAGTGTAGAGCACAAGTCAGAG
	antisense	AGACGCATAGAAGACCAAGGCATC
β-actin	sense	CCACACCCGCCACCAGTTC
	antisense	GACCCATACCCACCATCACACC
β2–microglobulin	sense	TCTTTCTGGTGCTTGTCTCTCTGG
	antisense	CTATCTGAGGTGGGTGGAACTGAG
L13A	sense	AGGTGGTGGTTGTACGCTGTG
	antisense	GGTTGGTGTTCATCCGCTTTCG