The antagonist properties of Bazedoxifene after acute treatment are shifted to stimulatory action after chronic exposure in the liver but not in the uterus.

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Abstract

A promising alternative to conventional hormone therapy for postmenopausal symptoms is treatment combining Bazedoxifene (BZA), a third-generation selective estrogen receptor modulator (SERM), and conjugated equine estrogen (CE). This combination is also known as a tissue-selective estrogen complex (TSEC). Understanding the tissue-specific actions of SERMs and the TSEC remains a major challenge to try to predict their clinical effects.

The aim of this study was to compare acute *versus* chronic treatment with BZA, CE or CE+BZA in two major targets of estrogens, the uterus and the liver. In these two tissues, acute treatment with CE, but not with BZA, induced similar gene expression change than the most important endogenous estrogen, 17-β estradiol (E2). Acute induction of gene expression by E2 or by CE was antagonized by the addition of BZA. Concomitantly, BZA alone or in combination with E2 or CE induced a partial degradation of ERα protein after acute exposure. In uterus, chronic treatment of BZA alone had no impact on tissue weight gain or on epithelial cell proliferation, and also antagonized CE-effect in uterus, thereby mimicking the acute effect. By contrast, in the liver, chronic BZA and CE+BZA elicited agonistic transcriptional effects similar to those of CE alone. In addition, at variance to BZA acute effect, no change in ERα protein abundance was observed after chronic treatment in this tissue.

These experimental *in vivo* data highlight a new aspect of the time-dependent tissue–specific action of BZA or TSEC, *i.e.* they can act acutely as antagonists but become agonists after chronic treatment. This shift was observed in liver tissue, but not in proliferative sex target such as the uterus.

1. **Introduction**

The decline of estrogen levels after menopause is associated with bone loss, hot flushes and vulvar–vaginal atrophy, affecting sexual function, relationships, and quality of life. Estrogen–progestin therapy was previously considered as the standard for managing moderate to severely bothersome symptoms associated with menopause but this drug combination is now controversial due to the increased risk of breast cancer and thromboembolism (Anderson et al., 2004, Chlebowski et al., 2003, Rossouw et al., 2002). The combination of conjugated estrogen (CE) with Bazedoxifene (BZA) is known as the tissue-selective estrogen complex (TSEC). It was designed to minimize the undesirable effects of hormone therapy on breast tissue yet allow the beneficial effects of estrogen on other estrogen-target tissues, thus suppressing climacteric symptoms and preventing osteoporosis (Mirkin and Komm, 2013). This drug combination was developed by Pfizer and has been marketed in the US for two years as Duavee® (CE, 0.45 mg / BZA, 20 mg). It has also been approved by the European Medicines Agency (EMA) (Komm and Mirkin, 2013). The efficacy and safety of CE+BZA in postmenopausal women, and the impact of this drug combination on quality of life was evaluated through a series of five pivotal phase 3 randomized, double-blinded, multicentered, active and/or placebo-controlled studies, called the Selective estrogens, Menopause And Response to Therapy (SMART) trials (Kagan et al., 2010, Mirkin and Komm, 2013, Pickar et al., 2009, Pinkerton et al., 2009, Umland et al., 2016). From these trials, CE+BZA was found to be associated with significant benefits such as a reduction in the frequency and severity of vasomotor symptoms, the prevention of bone loss, improved sleep and better menopause-specific quality of life, whilst also providing a reasonable level of protection against endometrial hyperplasia. Additionally, CE+BZA exhibited promising effects on breast tissue since it did not increase mammographic breast density (Goldberg and Fidler, 2015, Lobo et al., 2009, Pinkerton et al., 2014, Pinkerton et al., 2010, Pinkerton et al., 2009, Ronkin et al.,

2005, Stovall et al., 2011). The SMART trials also suggested that CE+BZA treatment was associated with similar rates of coronary heart disease, stroke, venous thromboembolism and menorrhea as placebo-treated patients (Skouby et al., 2015), although definitive conclusions cannot yet be made on these aspects due to the total number of women enrolled in these trials and the trial durations.

The molecular mechanisms underlying TSEC action are far from fully understood. CE is a mixture of different natural estrogens and BZA is one of a new generation of selective estrogen receptor modulators (SERM) that demonstrate selective binding to the estrogen receptors (ER) over other members of the nuclear receptors family (Alio Del Barrio et al., 2017). SERMs are thought to be ER agonists or antagonists depending on the tissue involved. Contrary to the action of 17-β–estradiol (E2) or CE that allow ER to recruit coactivators, leading to an enhanced transcription of target genes, SERM is reported to rather provoke the mobilization of corepressors to the ERs (Shang and Brown, 2002). This differential recruitment of ER coregulators by estrogens *versus* SERMs can be attributed, at least in part, to the different ER conformational changes induced by the binding of these ligands (Alio Del Barrio et al., 2017). In addition, TSEC was shown to cause ERα degradation *via* the ubiquitin proteasome system in the breast and uterus, which was at least in part responsible for the suppression of ERα-mediated transcription (Han et al., 2016). However, this ERα modulation appears to be cell- and tissue-specific since BZA treatment was able to exert an estrogen-mimetic action on bone (Palacios et al., 2015) and on the metabolism contributing to beneficial glucidic and lipidic effects (Barrera et al., 2014, Kim et al., 2014).

Thus, the consequences of the co-administration of estrogens and SERM at the molecular, cellular and whole body levels are undoubtedly highly complex. Here, our goal was to explore the tissue-specific effects of BZA combined with CE *in vivo* in mice comparing in two major targets of estrogens, the uterus and the liver (Ahlbory-Dieker et al., 2009, Boverhof et al.,

2004, Gao et al., 2008, Gordon et al., 2014, Handgraaf et al., 2013, Kim et al., 2014, Kobayashi et al., 2013, Palierne et al., 2016, Pedram et al., 2013). Our results indicate that the effects of BZA treatment varies according to the duration of treatment (acute *versus* chronic administration) and the tissue, with the responses observed in the liver being dramatically different from those observed in the uterus.

2. Materials and Methods

2.1. Mice

Female C57BL/6J mice were purchased from Charles River Laboratories. All procedures were performed in accordance with the principles established by the National Institute of Medical Research and were approved by the local Ethical Committee of Animal Care. Mice were anesthetized by intraperitoneal injection of ketamine (25 mg/kg) and xylazine (10 mg/kg) and ovariectomized at 4 weeks of age. To study the chronic effects of CE, BZA and BZA+CE, 2 weeks after ovariectomy mice were implanted with subcutaneous osmotic minipumps (Alzet; Alza, Palo Alto, CA) that released CE (3 mg/kg/day) or BZA (10 mg/kg/day) alone or in combination diluted in a solution of hydroxypropyl-β-cyclodextrin – with a 0.63 degree of substitution - in HEPES buffer for a 3 week period. For acute treatment, ovariectomized mice were orally administered with vehicle (PBS containing 0.5% methylcellulose, 2% Tween 80 and 5% DMSO), CE (3 or 10 mg/kg) or E2 (1 mg/kg) alone or in combination with BZA (10 mg/kg). These dose have been chosen based on previous studies (Kim et al., 2014, Barrera et al., 2014, Della Torre et al., 2011, Fontana et al., 2014, Naqvi et al., 2014, Oliva et al., 2013, Peano et al., 2009, Song et al., 2012) (Supplementary Table 1) and in particular on those realized by Peano *et al.* in which uterus weight was used as final endpoint to determine the minimal dose of CE (i.e 3 mg/kg/day) inducing uterine hypertrophy and that of BZA (i.e 10 mg/kg/day) antagonizing this effect.

2.2. Histological analysis

Paraffin-embedded transverse sections (4 µm) from formalin-fixed uterine specimens was stained as previously described (Abot et al., 2013) with anti–Ki-67 (RM-9106; Thermo-scientific) and anti-ERα antibodies (MC-20, Santa Cruz Biotechnology, Santa Cruz, California). Sections were examined after numerization using a NanoZoomer Digital Pathology®. To examine the proliferative effects of each treatment, the ratio of Ki-67–

positive epithelial cell/total cell number was evaluated from two microscopic fields of measurement at x20 magnification for each uterine section.

2.3. Analysis of mRNA levels by qPCR

Tissues were homogenized using a Precellys tissue homogenizer (Bertin Technology, Cedex, France), and total RNA from tissues was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). A total of 500 ng to 1 µg (depending on the tissue) was reverse transcribed for 10 minutes at 25°C and 2 hours at 37°C in a 20 µL final volume using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Villebon sur Yvette, France). We evaluated genes expression levels in liver by high throughput qPCR, using 6.5 ng cDNA from each sample in 96.96 Dynamic Arrays analyzed in the microfluidic BioMark system (Fluidigm Corporation, CA, USA). For gene expression in uterus, qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) on a StepOne instrument (Applied Biosystems). Primers (Supplementary Table 2) were validated by testing PCR efficiency using standard curves ($95\% \leq \text{efficiency} \leq 105\%$). Gene expression was quantified using the comparative C_T method. Genes' functional annotations were retrieved from different databases. Kegg and Reactome were obtained under the webgestalt platform (http://www.webgestalt.org/option.php) and wikipathways directly from their website (http://www.wikipathways.org/). Genes with no clear annotations were then manually curated in the Biosystem database from the ncbi gene portal (https://www.ncbi.nlm.nih.gov/gene). Genes involved in the regulation of metabolic pathway in at least one of these databases are highlighted in yellow in the supplementary tables of results (Supplementary Tables 3 to 5).

2.4. Statistical analysis

Results are expressed as means ± SEM. Statistical analyses were performed using graph pad. 1-way ANOVA was used to determine significance. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3. RESULTS

3.1. Similar inhibitory action of BZA after acute treatment in the uterus and the liver.

To determine the relative effects of TSEC after acute treatment (4 hours), we first used 2 doses of CE (3 or 10 mg/kg) and the expression profile of selected genes known to be regulated by E2 was evaluated in the uterus (Abot et al., 2013). We found that acute CE treatment (3 mg/kg) induced a weak transcriptional response whereas a higher dose of CE (10 mg/kg) was able to induce gene expression changes to a similar extent as E2 (1 mg/kg) (Figure 1A). The addition of BZA to E2 or to CE significantly attenuated these effects, while BZA alone had no effect. Analysis of protein levels by Western blot indicated that BZA alone or in combination with E2 or CE markedly down-regulated the amounts of ERα protein (Figures 1B and 1C). In contrast, none of these treatments had a significant impact of on ERα mRNA levels (Figure 1D).

Then, we analyzed the transcriptional response of liver to these acute treatments (Tables 3 and 4) analyzing a set of genes described to be regulated by estrogens or selective ER modulators exposure in the liver (Palierne et al., 2016, Boverhof et al., 2004, Gordon et al., 2014, Kim et al., 2014, Pedram et al., 2013, Ahlbory-Dieker et al., 2009, Kobayashi et al., 2013). As observed in the uterus, a dose of 3 mg/kg CE was less efficient than 10 mg/kg CE to induce an optimal transcriptional response. Only around 40% of the genes induced by 10 mg/kg CE or 1 mg/kg E2 were induced by 3 mg/kg CE (Figures 2A and 2B). Co-administration of BZA strongly attenuated the transcriptional induction observed with E2 (Figure 2A and Supplementary Table 3) or CE (Figure 2B and Supplementary Table 4). Acute BZA treatment, both alone and when combined with estrogens, elicited a clear-cut down-regulation of ERα at the protein level (Figures 3A and 3B), but not at the level of mRNA (Figure 3C).

Altogether, these results demonstrated that acute BZA treatment is able to exert similar antagonistic action on the transcriptional modulations induced by E2 or CE in both uterus and liver. Concomitantly, we observed reduced amounts of ERα protein in response to BZA in these two tissues, an effect that occurred in a post-transcriptional manner as no differences in ERα mRNA expression were observed following drug treatment.

3.2. Chronic BZA treatment exerts inhibitory action on uterus and stimulatory effect on liver.

To determine the relative effects of TSEC after a chronic treatment of the animals, 4-week-old ovariectomized female mice were treated with placebo, BZA (10 mg/kg/day), CE (3 mg/kg/day) or CE+BZA for 3 weeks, as previously described (Barrera et al., 2014, Della Torre et al., 2011, Fontana et al., 2014, Kim et al., 2014, Naqvi et al., 2014, Oliva et al., 2013, Peano et al., 2009, Song et al., 2012) (Supplementary Table 1). Analysis of the uterine wet weight confirmed that BZA antagonized the uterotrophic effect of CE (Figure 4A). Accordingly, epithelial proliferation was significantly decreased in CE+BZA-treated mice compared to CE-treated mice, as indicated by the immunohistochemical detection of the Ki-67 antigen (Figures 4B and 4C). After chronic exposure, large changes in uterine cellular populations induced by CE treatment did not allow for a relevant comparison of protein expression in tissue homogenates by Western blotting. Thus, ERα protein levels were evaluated by immunohistochemistry (Figure 4D) which is essentially qualitative rather than quantitative, and no obvious effect of BZA or CE+BZA on ERα abundance was apparent. Altogether, the analysis of different parameters including uterine weight and epithelial proliferation clearly shows that BZA antagonizes the action of CE and E2 in the uterus.

No difference in body weight was observed between ovariectomized untreated mice (mean = 22.4 ± 2.1 g, n = 20), CE-treated mice (23.8 ± 0.5 g, n = 16), BZA-treated mice (21.8 ± 1.6 g,

n = 23) and CE+BZA-treated mice (21.4 ± 1.2 g, n = 22), showing that CE, BZA or CE+BZA administrated subcutaneously for 3 weeks had no detectable impact on weight gain. Although some genes were found to be regulated by either CE or BZA, the majority of the genes studied were regulated by both CE and BZA (Figure 5 and Supplementary Table 5). Overall, chronic BZA and CE+BZA elicited agonistic effects similar to those of CE alone (Figure 5). For example, whereas BZA antagonizes CE-induced transcriptional activation of gene such as p21, Trim2, Psen2, Pgep1 or Inf2 after acute exposure (Supplementary Table 4), these genes are induced by CE+BZA treatment after chronic exposure (Supplementary Table 5A). Genes described to be involved in the regulation of metabolic pathways do not correspond to a specific regulatory profile in response to estrogens or BZA and are found in all the analyzed cases (i,e regulated by estrogens only, BZA only or after combination of BZA + CE) (Supplementary Table 5). In addition, chronic CE administration increased ERα protein and mRNA abundance in the liver, whereas BZA alone or CE+BZA had no effect compared to vehicle (Figures 6). Altogether, these data highlight a new aspect of the action of BZA on liver tissue, where it can have antagonistic effects after acute treatment and agonistic effects after chronic exposure.

4. **DISCUSSION**

The approval of Duavee® CE (0.45mg)/BZA(20mg) was based on Selective Estrogen, Menopause, and Response to Therapy (SMART) trials that aimed to evaluate CE/BZA's safety and efficacy in the management of moderate-to-severe vasomotor symptoms, bone mineral density, endometrial hyperplasia, moderate-to-severe vulvar/vaginal atrophy, and overall safety (Kagan et al., 2010, Mirkin and Komm, 2013, Pickar et al., 2009, Pinkerton et al., 2009, Umland et al., 2016). In parallel, preclinical studies in animal models reported beneficial effect on metabolic parameters (Barrera et al., 2014, Kim et al., 2014) and have

shown that, when combined with CE, BZA attenuates CE-induced uterine and mammary gland cell proliferation (Kim et al., 2014, Barrera et al., 2014, Peano et al., 2009, Song et al., 2012). However, assessing the relative effects of estrogens or SERMS *in vivo* in rodents is complicated by the fact that contrary to human, mice do not express the sex hormone binding globulin (SHBG) which modulates the bioactivity of sex steroids by limiting their diffusion into target tissues. Based on the uterine weight as endpoint, dose response study determinates CE (3mg/kg/day) and BZA (10 mg/kg/day) as reference doses (minimum fully effective agonist and antagonist doses respectively) for combination studies with SERMS (Peano et al., 2009). Our results confirm that BZA (10 mg) antagonizes CE (3 mg) on uterus weight and proliferative response after chronic treatment in uterus and also show that BZA inhibits the CE-mediated transcriptional gene regulations (10 mg) after acute treatment extending this particular mechanism of action of BZA on sex targets. Furthermore, in cellular models of breast cancer, BZA was shown to function as a pure ERα antagonist and effectively inhibited the growth of both tamoxifen-sensitive and tamoxifen-resistant breast tumor xenografts (Wardell et al., 2013). The antiestrogens that are currently available fall into two general classes, acting as selective estrogen receptor modulators (SERMs) and/or selective estrogen receptor down-regulators (SERD). We found that, after acute treatment, BZA led to a decrease in ERα protein levels in both uterus and liver without impacting ERα mRNA levels. Indeed, BZA has been described to induce a unique conformational change in ERα that results in its proteosomal degradation, although this property was shown to be dispensable for its antagonistic activity (Wardell et al., 2013). ERα immunodetection generated similar signals in tissues from untreated, CE-, BZA- and CE+BZA-treated ovariectomized mice, suggesting that BZA behaves as a SERM rather than a SERD after chronic treatment. Interestingly, a very specific profile of BZA was previously reported in biochemical and cell-based assays, as its combination with CE displayed different properties from those observed following the

combination of CE with other SERMs (Berrodin et al., 2009, Chang et al., 2010, Wardell et al., 2012). Indeed, *in vitro*, whereas raloxifene and lasofoxifene completely inhibited the CE-mediated recruitment of all cofactor peptides by ER, BZA inhibited the CE-mediated recruitment of only some of the evaluated peptides. Furthermore, in MCF-7 breast cancer cells, the combination of BZA with CE did not totally abrogate the transcriptional response of a subset of CE-responsive genes which were, in contrast, completely antagonized by raloxifene and lasofoxifene. Interestingly, the CE-regulated genes antagonized by all three SERMs were involved in cell cycle regulation and cell-to-cell signaling (Berrodin et al., 2009, Chang et al., 2010). Subsequent studies of differential gene regulation by TSEC, estrogen or the SERM alone highlighted the importance of the promoter context on the activity of these ER ligands (Wardell et al., 2012). However, these studies only questioned the short term action of CE, BZA or TSEC using *in vitro* models.

Here, we used an *in vivo* model to show that BZA acts as an antagonist after acute treatment in the liver, but displays agonistic effects in the liver following chronic exposure. This underlines the importance of the time- and tissue–specific action of BZA, which induces a very similar action to that of CE or E2 following chronic treatment. Our transcriptional analyses also revealed a group of genes for which CE and BZA cooperatively regulated their expression under chronic treatment. This means that the effect of CE+BZA cannot be simply extrapolated from its short-term action and suggests that additional pathways facilitating combined agonist/antagonist action must exist. It was previously proposed that, in addition to the competitive inhibition of BZA on CE, BZA and CE could also cooperatively activate gene transcription with one ER monomer bound to CE and the other one occupied by BZA (Liu et al., 2013). High-throughput sequencing of chromatin immunoprecipitation experiments (ChIP-seq) performed on livers from mice subjected to acute treatment with E2 led us to recently show that the ERα cistrome (all of the ER binding sites in the genome) in mouse

liver has unique properties (Palierne et al., 2016). In order to acquire novel insights into the specific mechanisms involved in the shift of action of BZA from antagonism to agonism, it would therefore be of major interest to compare the liver cistrome of ERα in response to acute and chronic treatment with CE, BZA and CE+BZA. These experiments would question: *i*) whether ERα bound to CE or BZA targets different/specific chromatin regions, which would explain the difference in gene regulation; *ii*) whether these cistromes vary according to the acute or chronic administration of these hormones, in relation to differences in gene regulation; and *iii*) whether the specific properties of the E2-bound ERα cistrome are conserved using these treatments. Interestingly, the cistrome of ERα bound to 4-hydroxytamoxifen (OHT), another SERM, has been determined in MCF-7 cells and was found to be comparable with that of E2-bound ERα (with 60-75% similarity) (Hurtado et al., 2011), although this SERM had no agonist activity on gene transcription. It was therefore proposed that, in these cells, OHT-bound ERα is able to target the same sequences on chromatin (although with less efficiency than E2-bound ERα) but as an inactive complex or repressive form since OHT-bound ERα is able to recruit corepressors (Shang and Brown, 2002). Hence, the shift of the action of BZA from antagonism to agonism may have a limited impact on the cistrome of ERα and instead reflect a corepressor to coactivator exchange. The mass spectrometry analysis of proteins associated with ER on chromatin (ChIP-MS) when bound to these different ligands (Mohammed et al., 2013) would also generate important knowledge to further decipher the mechanisms of action of TSEC.

Finally, Mauvais-Jarvis and coworkers recently reported that BZA and TSEC exhibits estrogen-mimetic action with regard to glucose and energy homeostasis (Kim et al., 2014). Interestingly, they reported that lipid oxidation and increased energy expenditure promoted by CE, BZA and TSEC involved different pathways as revealed by different target gene pattern in the liver for these ERα ligands. Thus, the shift reported here from antagonist to agonist

activities of BZA in the liver, and potentially in other tissues, have undoubtedly contributed to the metabolic benefit of BZA alone or in TSEC that mimic those provided by estrogens in female mice on high fat diet.

In conclusion, these observations provide novel insights into the mechanisms underlying BZA action which can not only act as an agonist or antagonist in a tissue-specific fashion, but can also, in a given tissue such as the liver, be acutely antagonistic and chronically agonistic. This could help to explain, among other reasons, the reported beneficial effects of TSEC such as its favorable effects on glucidic and lipidic metabolism (Barrera et al., 2014, Kim et al., 2014).

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478 **Figure legends**

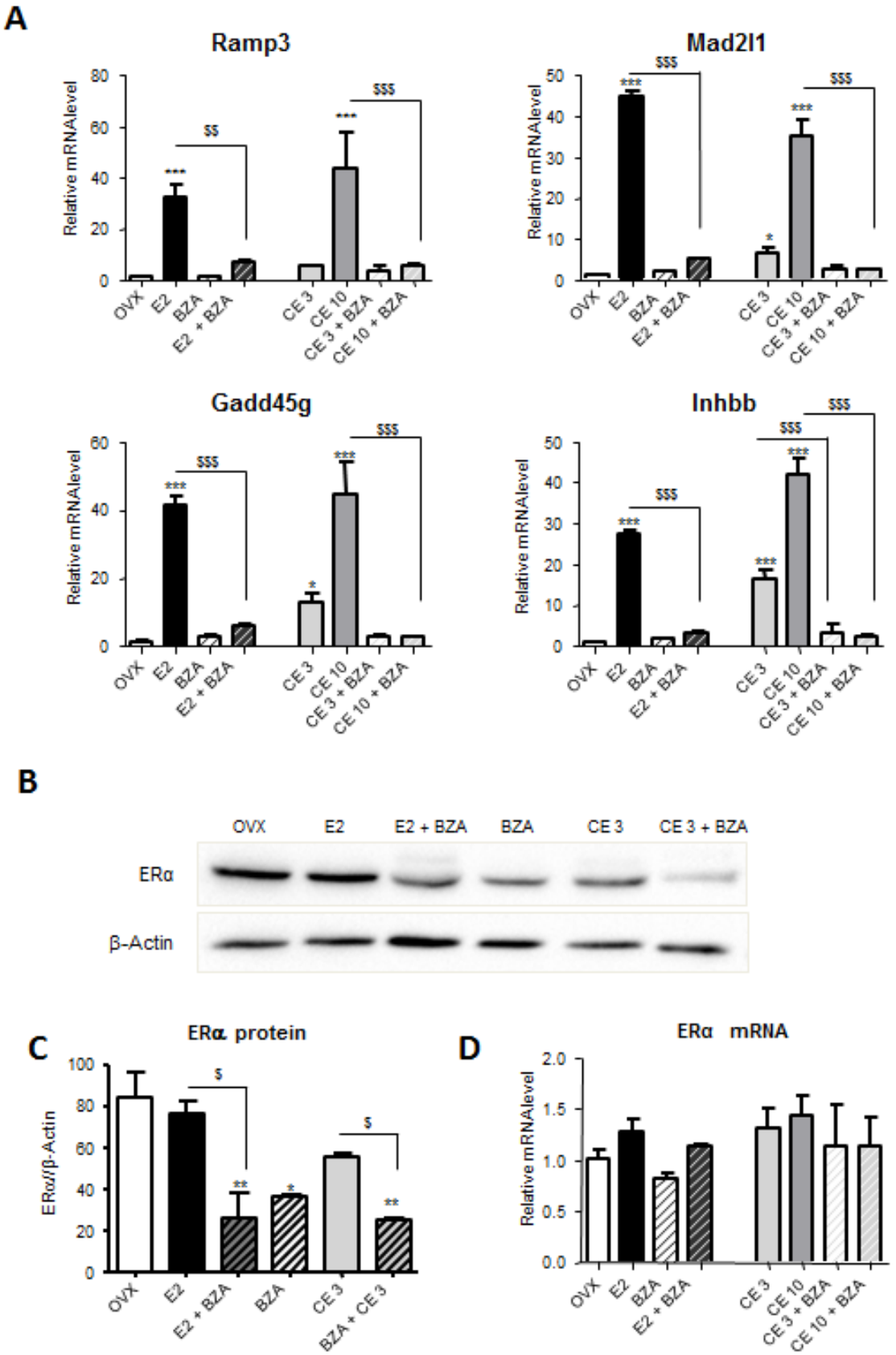

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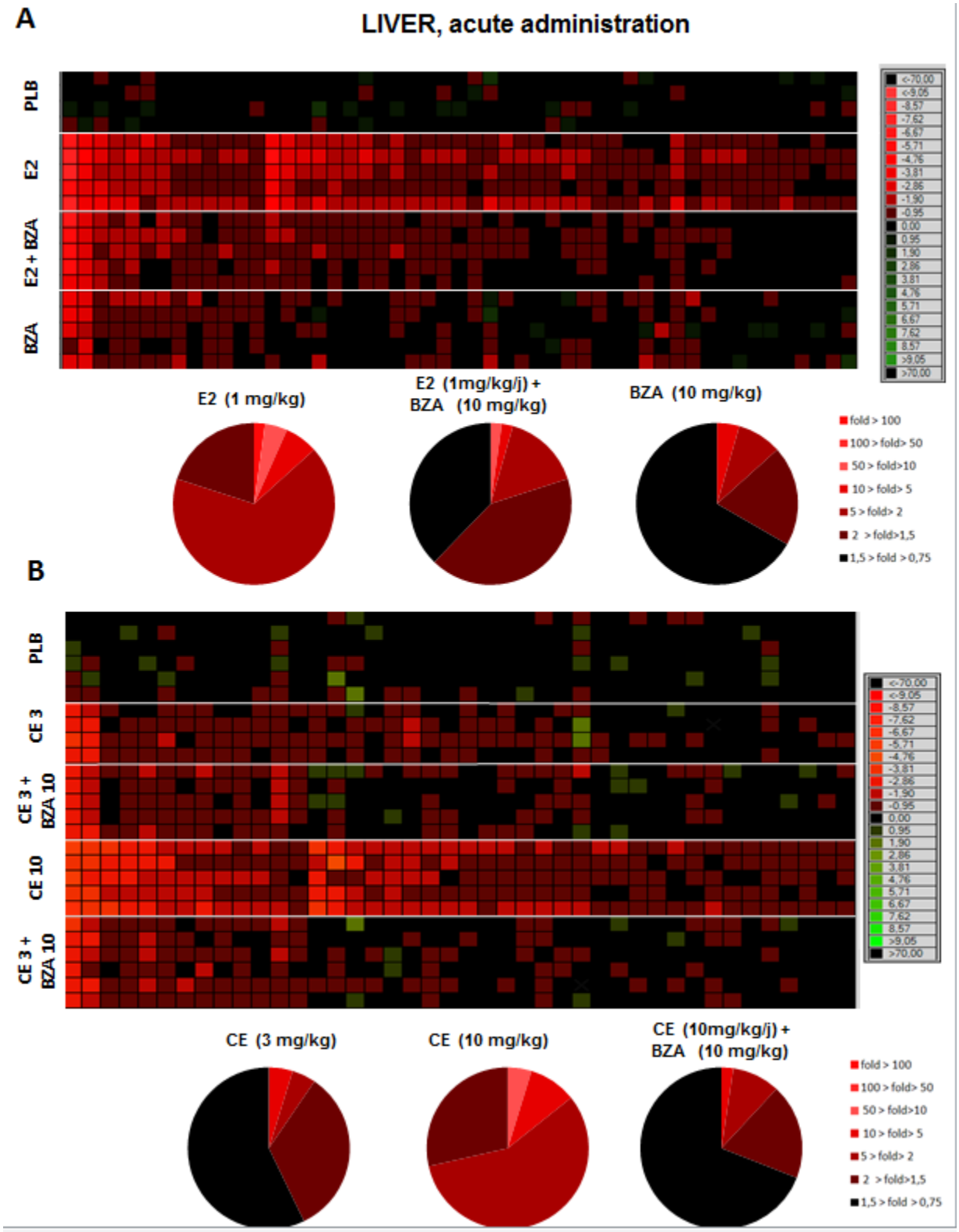
Figure 1: Effect of acute administration of E2 and CE alone or in combination with BZA on uterus

Four-week-old ovariectomized mice received an oral administration of vehicle (PBS containing 0.5% methylcellulose, 2% Tween 80 and 5% DMSO), 17-β estradiol (E2, 1 mg/kg), conjugated equine estrogen (CE, 3 or 10 mg/kg) alone or in combination with Bazedoxifene (BZA, 10 mg/kg). **(A)** Quantification of mRNA levels of the indicated genes, **(B)** representativeWestern blot and **(C)** quantification of ERα protein levels and **(D)** quantification of ERα mRNA levels in the uteri from these mice. Results are expressed as means ± SEM. To test the respective roles of each treatment, one-way ANOVA and a Bonferroni's multiple comparison test were performed.



491

492 **Figure 2: Effect of acute administration of E2 and CE alone or in combination with BZA**

493 **on transcriptional regulation in the liver**

Four-week-old ovariectomized mice received an oral administration of vehicle (PBS containing 0.5% methylcellulose, 2% Tween 80 and 5% DMSO), 17-β estradiol (E2, 1 mg/kg), conjugated equine estrogen (CE, 3 or 10 mg/kg) alone or in combination with Bazedoxifene (BZA, 10 mg/kg). **(A)** Data obtained from 96.96 Dynamic Arrays were used to generate a cluster diagram of the significant gene expression changes. Each vertical line represents a single gene. Each horizontal line represents an individual sample. **(B)** Pie chart displaying the distribution of significantly induced genes according to the value of the fold change. Genes that were up-regulated at least 1.5 fold relative to placebo are in red and the color intensity indicates the degree of variation in expression. Genes whose expression was not affected by treatments appear in black.

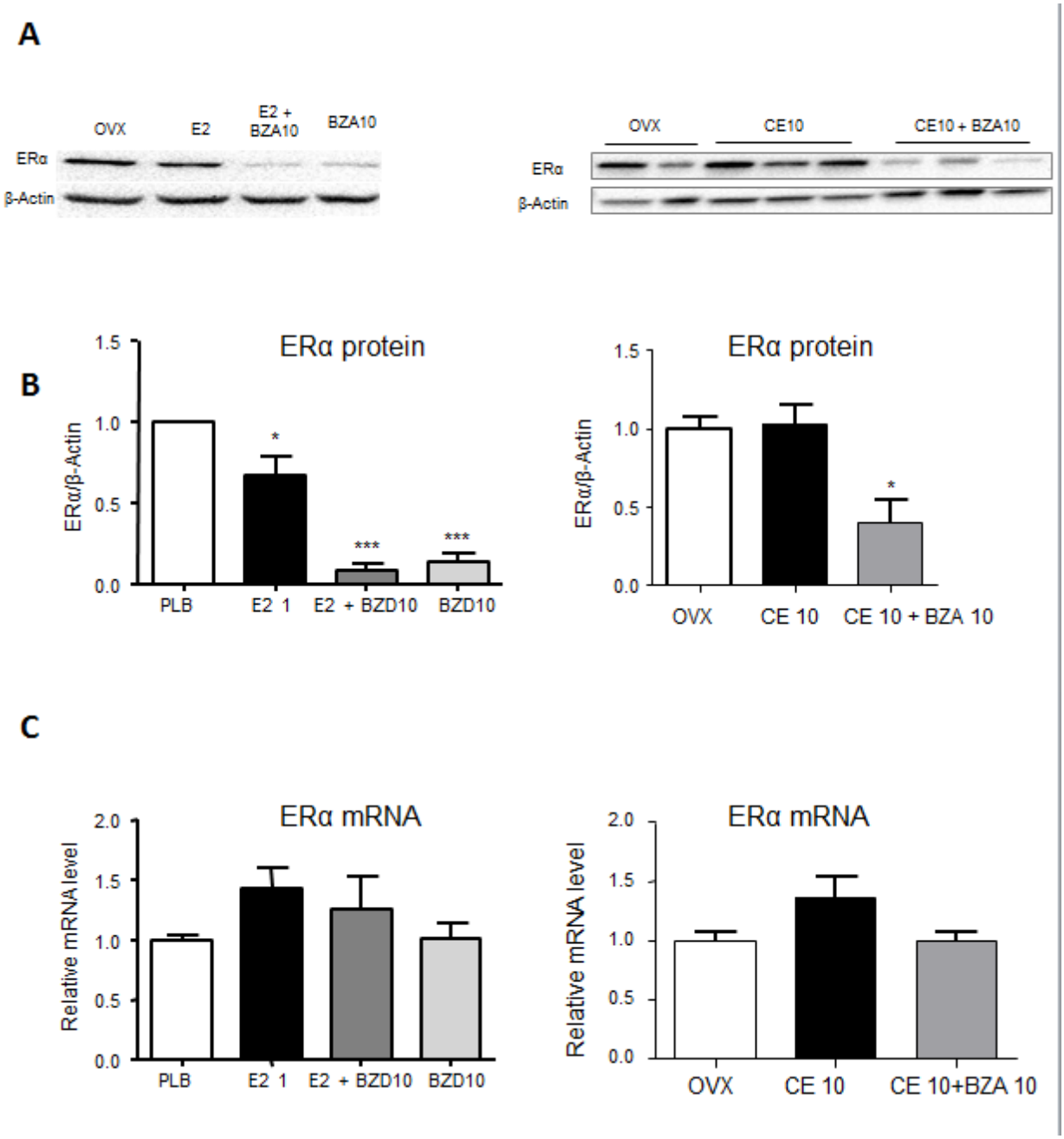
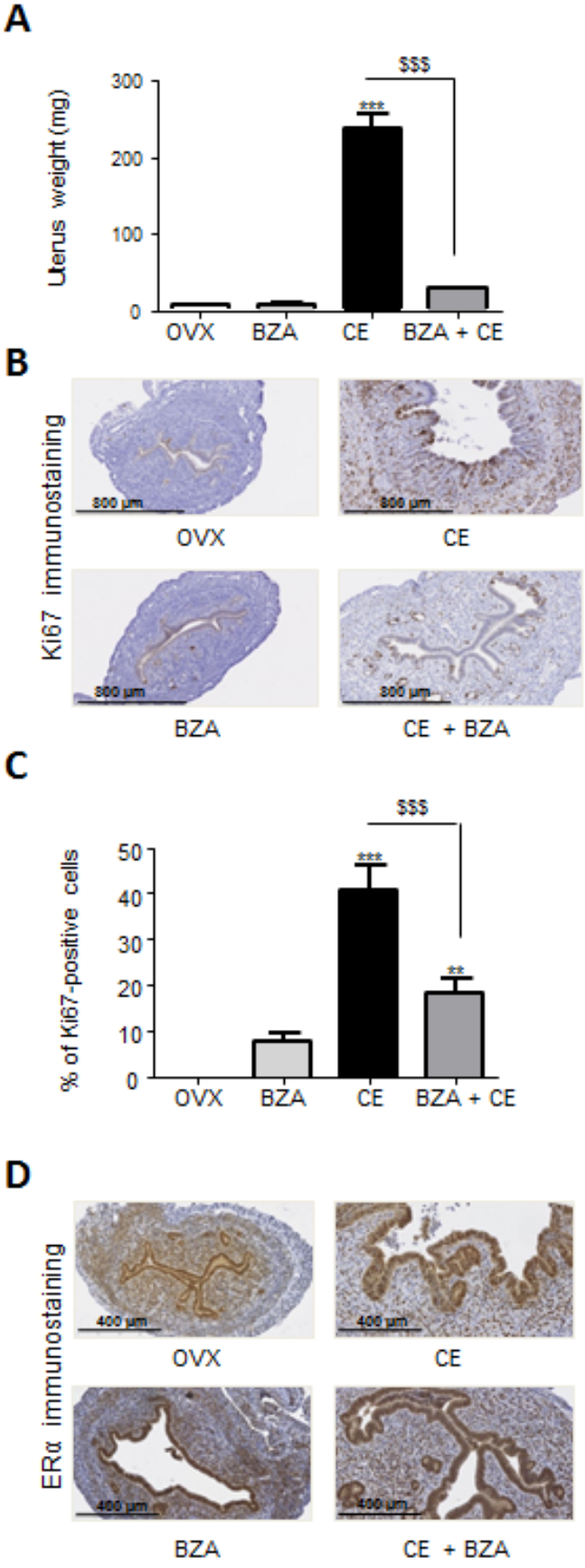


Figure 3: Effect of acute administration of E2 and CE alone or in combination with BZA on ERα expression in the liver

Four-week-old ovariectomized mice received an oral administration of vehicle (PBS containing 0.5% methylcellulose, 2% Tween 80 and 5% DMSO), 17-β estradiol (E2, 1 mg/kg), conjugated equine estrogen (CE, 10 mg/kg) alone or in combination with Bazedoxifene (BZA, 10 mg/kg). **(A)** Representative Western blots of ERα protein levels and (**B**) quantification of ERα protein expression. **(C)** Quantification of ERα mRNA levels by

qPCR. Results are expressed as means ± SEM. To compare the effects of each treatment, one-way ANOVA and a Bonferroni's multiple comparison test were performed (treatment *versus* placebo: *** $p<0.001$).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BZA
CE
BZA + CE
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800 µm
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BZA
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OVX
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ERα immunostaining
400 µm
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BZA
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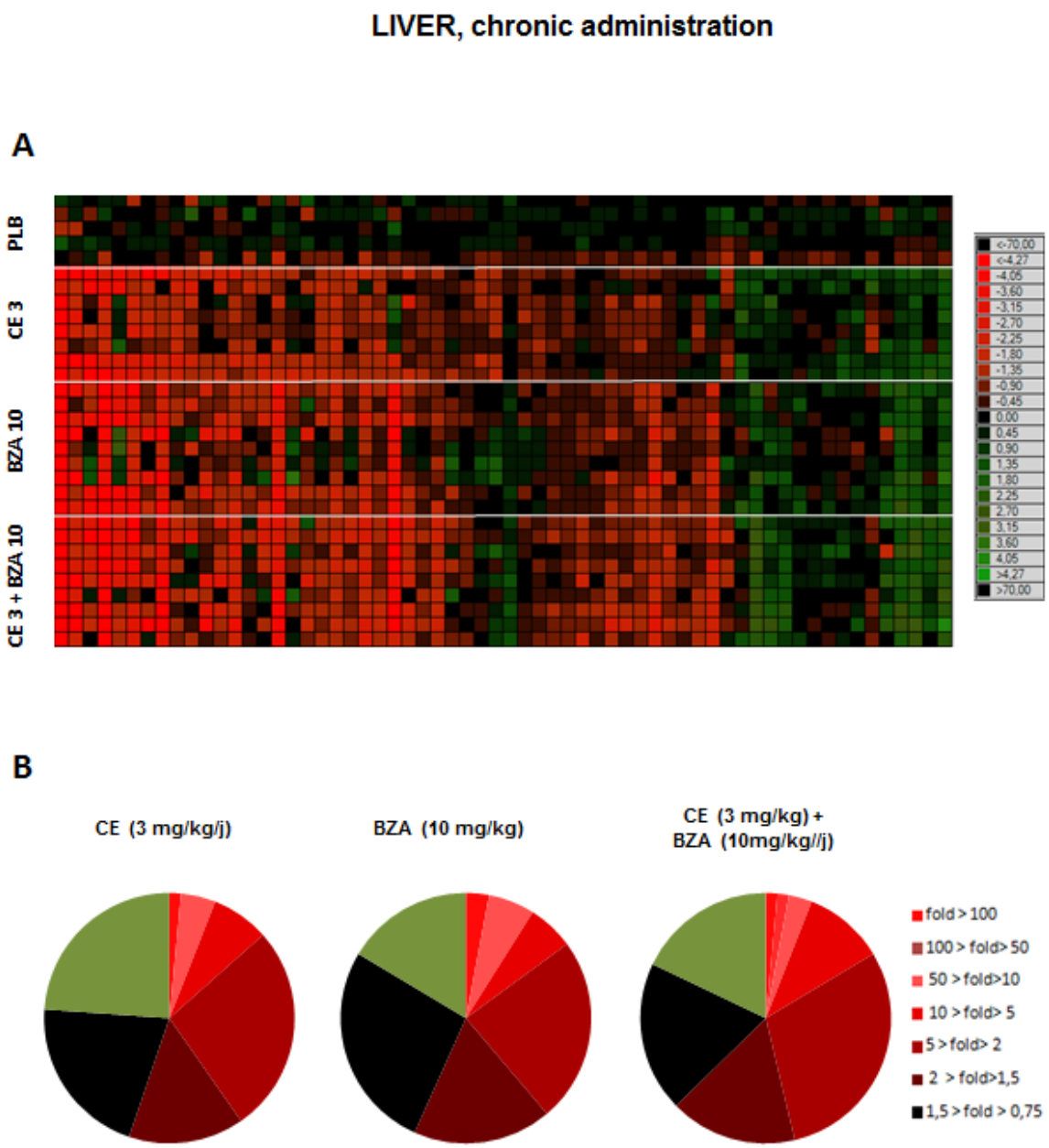
Figure 4: Effect of chronic administration of E2 and CE alone or in combination with BZA on uterus

Four-week-old ovariectomized C57Bl/6J mice were treated with BZA (10 mg/kg/day), CE (3 mg/kg/day) alone or in combination (BZA+CE) during 3 weeks. **(A)** Uterus weight, **(B)** representative Ki-67 immunostaining picture **(C)** and quantification in transverse uterus sections (scale bar = 800 μm). Results are expressed as means ± SEM. To test the respective roles of each treatment, one-way ANOVA and a Bonferroni's multiple comparison test were performed. **(D)** Detection of ERα by immunochemistry in transverse uterus sections (scale bar =400μm).



526

527 **Figure 5: Effect Effect of chronic administration of E2 and CE alone or in combination**
528 **with BZA on transcriptional regulation in the liver**

529 Four-week-old ovariectomized C57Bl/6J mice were chronically treated (3 weeks) with BZA
530 (10 mg/kg/day), CE (3 mg/kg/day) alone or in combination (BZA+CE). Gene regulation was
531 then analyzed using extracted liver RNA by 96.96 Dynamic Arrays. **(A)** Data obtained from
532 96.96 Dynamic Arrays were used to generate a cluster diagram of the significant gene

533 expression changes. Each vertical line represents a single gene. Each horizontal line
534 represents an individual sample. **(B)** Pie chart displaying the distribution of significantly
535 induced genes according to the value of the fold change. Genes that were up-regulated at least
536 1.5 fold relative to placebo are in red, whereas down-regulated genes are in green. The color
537 intensity indicates the degree of variation in expression. Genes whose expression was not
538 affected by treatments appear in black.
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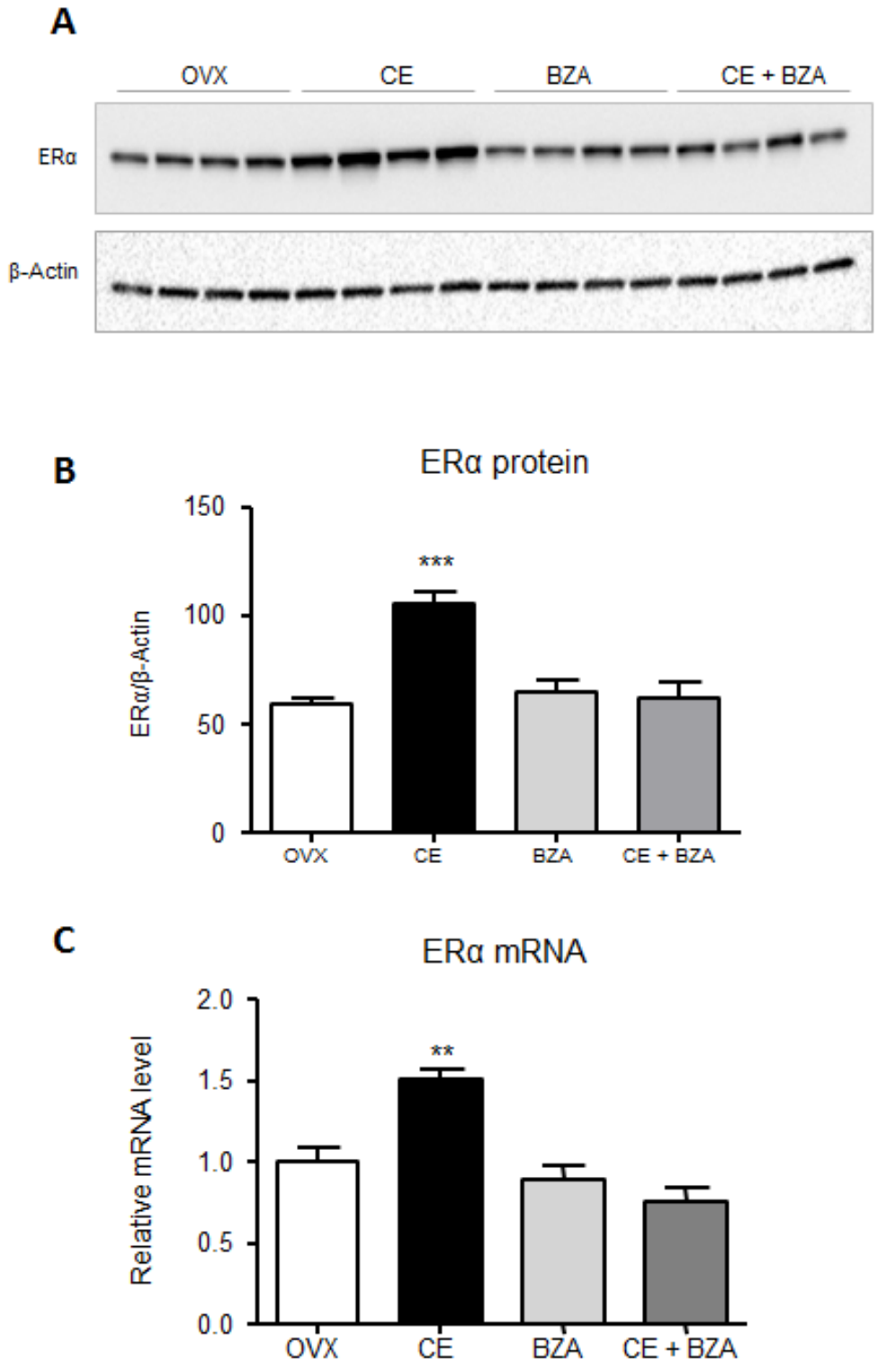

Figure 6: Effect of chronic administration of E2 and CE alone or in combination with BZA on ERα expression in the liver

Four-week-old ovariectomized C57Bl/6J mice were chronically treated (3 weeks) with BZA (10 mg/kg/day), CE (3 mg/kg/day) alone or in combination (BZA+CE). **(A)** Representative Western blots of ERα protein levels and (**B**) quantification of ERα protein expression. **(C)** Quantification of ERα mRNA levels by qPCR. Results are expressed as means ± SEM. To compare the effects of each treatment, one-way ANOVA and a Bonferroni's multiple comparison test were performed (treatment *versus* placebo: *** $p<0.001$).