IN VITRO ESTROGENIC/ANTI-ESTROGENIC EFFECTS OF CERTAIN FOOD ADDITIVES AND COSMETIC PRESERVATIVES

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Abstract

In the present study we evaluated the estrogenic/anti-estrogenic effects of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and butyl paraben (BP), compounds extensively used as antioxidants/conservatives in food, food packaging, cosmetics and pharmaceuticals, using the luciferase assay. BP and PG were found out to have dual effect. BHA was discovered to have only anti-estrogenic effects, while BHT was the only compound tested in the present studies that did not have any effect.

Rezumat

În prezentul studiu s-au evaluat efectele estrogenice/anti-estrogenice ale butilhidroxianisolului (BHA), butilhidroxitoluenului (BHT), propil galatului (PG) şi ale butil parabenului (BP) compuşi folosiţi ca antioxidanţi sau conservanţi în industria alimentară, a ambalajelor, cosmeticelor, produselor farmaceutice, folosind testul luciferazei. BP şi PG au prezentat efecte duale. BHA s-a dovedit a avea doar efect anti-estrogenic, în timp ce BHT a fost singurul compus testat care nu a prezentat niciun efect.

Keywords: BHA, BHT, PG, PB, luciferase assay.

Introduction

In recent years, concern has been expressed regarding the possible endocrine disrupting effects of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and butyl paraben (BP). Several *in vitro* assays have been performed to evaluate the potential of these antioxidants and preservatives to mimic or interfere with the effects of sex hormones.

Butylated hydroxyanisole (BHA) was considered to be a weak estrogenic compound, while butylated hydroxytoluene (BHT) was discovered to be less estrogenic than its' "chemical cousin", BHA, but was

also included on a list of nonestrogenic chemicals by other research groups [1, 6, 9, 12, 13, 14].

While for BHA and BHT there is a dilemma whether they are estrogenic, for propyl gallate (PG) the results are even more interesting, some studies suggesting that PG is an estrogenic compound, while others are presenting PG as an antagonist on the estrogen receptors [1, 13].

If the above three compounds generated controversy, when parabens were studied, they were reported as having estrogenic activity, the estrogenic potency increasing with the side-alkyl chain [4, 5, 8, 10, 11].

For analysing these *in vitro* estrogenic effects, different tests were developed: competitive ligand-ligand, cell proliferation, yeast two-hybrid and reporter gene assay.

The aim of this study was to characterize the potential estrogenic and/or anti-estrogenic effect of BHA, BHT, PG and butyl paraben (BP) using the luciferase assay, an assay that involves the compound binding to the estrogen receptor, ER_α or ER_β, followed by receptor-ligand dimerization, translocation to the nucleus, binding to estrogen-response-elements (ERE) in the DNA and transactivation of gene expression. The present work is a preliminary step in evaluating the endocrine disruptive effect of binary mixtures of 17-β-estradiol and the four tested compounds.

Materials and Methods

Chemicals and Reagents

BHA, BHT, PG, BP and 17β-estradiol (E_2, ≥ 98%) were purchased from Sigma Aldrich (Steinheim, Germany). All four compounds of interest were prepared as 1000µL stock solutions in dimethyl sulfoxide (DMSO, ≥ 99.5%, Riedel-de Haën, Seelze, Germany) at 510 mM, 500 mM, 1000 mM and 512 mM, respectively.

Stock solutions were subsequently diluted with DMSO to obtain serial concentrations of 0, 0.15, 0.5, 1.5, 5, 15, 30, 50, 150 and 300 mM for all four chemicals in aliquot volumes of 500 µL. These serial dilutions were then used to obtain the desired test concentration range for individual compounds.

E_2 was prepared as 1210 µL stock solution in DMSO at 100 mM, 1 mM and 10000 nM. Stock solutions were subsequently diluted with DMSO to obtain serial concentrations of 0, 0.005, 0.015, 0.05, 0.15, 0.5, 1.5, 5, 15, 50, 150, 300, 500, 1500, 5000 nM, in aliquot volumes of 500 µL. These serial dilutions were then used to obtain the desired test concentrations for the assays.

Resazurin, tricine, ethylenediamines tetraacetic acid (EDTA), dithiothreitol (DTT), and adenosine triphosphate (ATP) were purchased

from Sigma Aldrich. $(MgCO_3)_4Mg(OH)_2.5H_2O$ was obtained from Acros Organics (Geel, Belgium), 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) and Tris were from Fluka (Buchs, Switzerland) and luciferin (≥ 99%) was from Roth (Karlsruhe, Germany). All solvents and reagents were of analytical grade. RPMI1640 + GlutaMAX culture medium, trypsine and charcoal stripped fetal barrier serum (FBS) were purchased from Gibco (Paisley, UK). Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12) was purchased from Sigma Aldrich. Fetal Bovine Serum (FBS) was obtained from Sigma (Steinheim, Germany) and Dulbecco's Phosphate Buffered Saline (PBS) was obtained from Invitrogen.

Cell cultures

T47D-KBluc human breast cancer cells (estrogen receptor (ER_α and ER_β) positive) were obtained from the American Type Culture Collection (ATCC, USA). The cells were maintained in 75 cm^3 plastic flasks with RPMI1640 + GlutaMAX medium, supplemented with 10% FBS at 37ºC in an atmosphere of 5% CO_2, under saturating humidity. Dulbecco's Phosphate Buffered Saline was used to rinse the cells and trypsine was used to detach cells from plastics. Phenol red-free Dulbecco's Modified Eagle's Medium F-12 (DMEM F-12), containing 10% charcoal stripped FBS was used during the experiments.

Viability assay

Viability was assessed using a resazurin-based assay. The cells were seeded at $5x10^5$cells/mL (96 wells/plate) in RPMI1640+GlutaMAX medium. Following a 24 h incubation at 37ºC, 5% CO_2, the cells were rinsed with 200 µL PBS and then exposed to individual test compounds in increasing concentrations and mixtures compound-estradiol for 24 h. The experiments were performed in triplicate and included 2 controls in each plate (cells exposed only to culture medium and cells exposed to culture medium containing 0.2% DMSO) and blanks without cells containing 200 µL of a 100 µM resazurin solution in culture medium. Cell viability was evaluated by measuring the capacity of the cells to reduce resazurin, a non-fluorescent reagent, to resorufin, a fluorescent product. The cells were rinsed with 200 µL PBS and then incubated for 3 h at 37ºC, 5% CO_2 in the presence of a 200 µL resazurin solution 100 µM. The fluorescence was measured at $\lambda_{excitation}$=530/25; $\lambda_{emission}$=590/35, using a plate-reader (Synergy 2 Multi-Mode Microplate Reader, BioTek) and cell viability was expressed as a percentage of non-treated controls.

All experiments were repeated three times and in each plate the exposure for each concentration was done in triplicate.

Luciferase assay

The cells were seeded at $8x10^5$ cells/mL (96 wells/plate) in DMEM F-12. After a 24 h incubation at 37ºC, 5% CO_2, the cells were rinsed with 200 µL PBS and then exposed for 24 h to individual test compounds in increasing concentrations or binary mixtures of 30 pM E_2 and compounds. The assays for estrogenic activity included a positive control (E_2, 30 pM) and two negative controls (assay medium and assay medium with 0.2% DMSO respectively). The assays for anti-estrogenic activity included a positive control (E_2, 30 pM), two negative controls (assay medium and assay medium with 0.2% DMSO) and followed the same protocol, except that the cells were exposed to binary mixtures of E2 (30 pM) and BHA, BHT, PG or BP in increasing concentrations. After exposure to test chemicals, the cells were rinsed with 200 µL ½ PBS. Low salt buffer containing 10 mM Tris, 1.99 mM DTT and 2 mM EDTA was added at a volume of 30 µL per well. Following a 15-minute incubation at -20ºC, the plates were frozen at -80ºC for a minimum of 30 minutes to lyse the cells. Plates were thawed on ice and shaken for 30 minutes at room temperature. Luciferase activity was measured in 96-well plates in a luminometer (Synergy 2 Multi-Mode Microplate Reader, BioTek) with automatic injection of 100 µL luciferin FlashMix [0.47mM luciferin, 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 0.1 mM EDTA, 2 mM DTT and 5 mM ATP, pH 7.8] in each well. Light emission was extinguished with 50 µL NaOH 0.2 M to stop the reaction.

Data analysis and statistics

Relative luminescence units (RLUs) were corrected by substracting the mean response of control wells. To compare data, the maximum induction of luciferase, obtained at 30 pM E_2, was set as 100%. Data were expressed as mean percentages of the maximal signal observed for 30 pM estradiol.

To calculate the half-maximal efficient concentrations (EC_{50} for estrogenic effect) and half-maximal inhibitory concentrations (IC_{50} for anti-estrogenic effect) we used the four parameter Hill function. Graphing of the dose-response curves and nonlinear curve-fitting were carried out using SigmaPlot 12 software. The results were expressed in mean ± standard deviation (S.D.).

Results and Discussion

The range of concentrations at which the compounds were tested for endocrine disrupting effects are comparable to the ones reported in other *in vitro* assays [3, 4, 7, 9].

Cell viability after exposure to individual compounds

The solvent used during the experiments, namely DMSO 0.2% (v/v), did not induce any significant cytotoxicity.

There was no statistically significant decrease in the luminescence intensity in the case of cell exposure to BHT, PG and BP for concentrations between 0.3 – 100 μM. These results indicate that these compounds are not cytotoxic in concentrations between 0.3 and 100 μM. For BHA the tests were performed for concentration up to 200 μM.

Cell viability after exposure to a mixture of compound - estradiol (compound at increasing concentrations + E2 at 30 pM)

When analysing the viability data for mixtures compound-estradiol, it was noticed that none of them presented significant toxicity when compared to control.

A first observation regarding the viability is that when the toxicity of compound-estradiol (30 pM) mixture was tested, the E_2 turned out to protect the cells from the toxic effect of high concentrations of compounds. This cytoprotective effect was suggested in other research papers [2].

Estrogenic effect (individual exposure)

The cells were exposed for 24 hrs at BHA, BHT, PG and BP in the concentrations selected based on the viability data. Where it was possible, the EC_{50} value was calculated.

PG exhibited an estrogenic effect at concentrations of 30, 60 and 100 μM. Maximum effect was found at 30 μM and it represented 43.0% from E_2 max, while at 60 μM the signal was calculated to represent 37.9% from E_2 max. At 100 μM, the signal decreased at 23.7% from E_2 max. The cellular viability at 100 μM did not indicate any cytotoxicity; therefore the decreased signal is not a result of a toxic effect. In case of PG, only EC_{25} was calculated, $EC_{25} = 2.57$ μM.

BHA did not present any significant difference when compared to the data obtained in the wells where the cells were exposed to medium with DMSO.

The same way as with BHA, BHT also was found not to have estrogenic properties, while BP presented itself as an estrogenic compound with an EC_{50} value of 3.09 μM.

For BP, the maximum response was observed at 10 μM (124.4% from E_2 max) as seen in Figure 1.

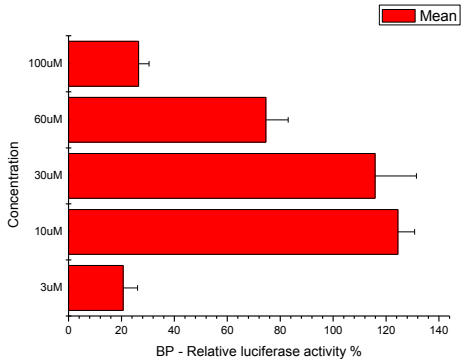

Figure 1.
Relative luciferase activity in case of exposure to BP

Anti-estrogenic effect

All four compounds were tested for anti-estrogenic properties. BHA, PG and BP were found to inhibit estradiol's activity, while BHT had no significant effect.

For BHA, PG and BP, the IC_{50} was calculated (BHA - IC_{50}= 170.91 µM, PG - IC_{50}= 45.03 µM, BP - IC_{50}= 59.82 µM). The potential of the selected compounds to decrease the luciferase induction produced by E_2 is presented in Figure 2-4.

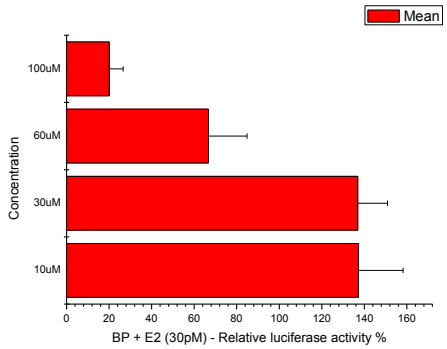

Figure 2.
Relative luciferase activity in case of exposure to BP + E_2

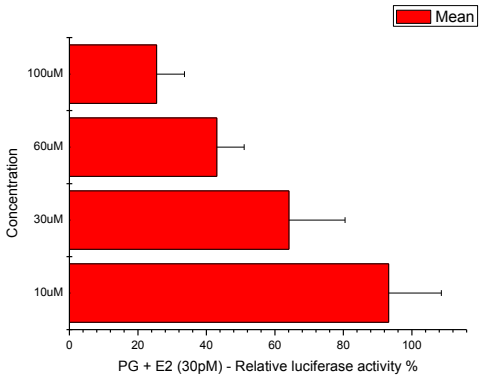

Figure 3.
Relative luciferase activity in case of exposure to PG + E_2

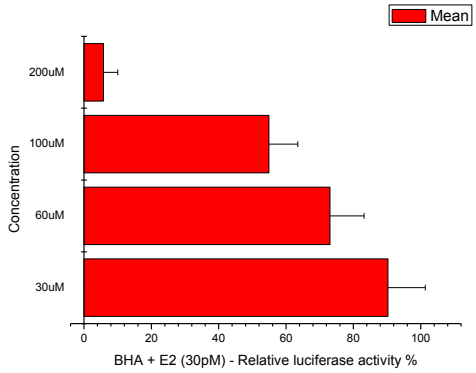

Figure 4.
Relative luciferase activity in case of exposure to BHA + E_2

BHA was reported in literature to have an estrogenic effect (when it was tested on U2-OS ER_α and ER_β and MCF-7 cell lines), but the present study using the same range of concentrations found no estrogenic activity for BHA [9, 12, 13].

Lowest effect concentration for BHA in ER_α in ter Veld et. Al, was 5.9 µM and 8.4 µM in ER_β, but the maximal effect obtained was not higher than 19%, (18.3% in case of ERα, 15.6% in case of ERβ) [13]. Also, the mixture BHA + E_2 was not found to have an additive effect, contrary to the results reported by ter Veld et al [13]. The IC_{50} calculated in the present study was higher than the concentrations tested by the above research group; this could explain the fact that the anti-estrogenic effect was not observed by ter Veld et al. Okubo et Kano in a relative binding affinity assay found BHA capable of competing for the ER_α and ER_β with E_2, showing IC_{50} values of 200 µM (for ER_α) and 900 µM (for ER_β) [9]. Our results on the

T47DK-Bluc cell line (expressing both ER receptors) sustain the anti-estrogenic activity with an IC_{50} of 170.91 μM.

BHT is the only compound tested in the present studies that did not have any effect. None of the concentrations used were able to give any signal when investigating the estrogenic effect, or significantly inhibit E_2 at 30pM. Wada et al. described BHT as an estrogenic compound using also a reporter gene assay (transfected embryonic kidney fibroblast 293T cell line), where an important luciferase activity (significant when compared to control) appeared at 50μM and over. They observed a statistically significant effect compared to control, but only slightly above 20% from E_2 max [14]. This concentration was included in our study, but our results did not confirm this kind of effect for BHT under the assay conditions. The results from the present study sustain the conclusion of Okubo and Kano, that BHT is a non-estrogenic compound [9].

In our study, PG exhibited an estrogenic effect but it represented only 43.0% (at 30μM) from the maximum induction showed by E_2. Also, when analysing the anti-estrogenic effect, the IC_{50} value of PG was calculated to be 45.03μM. So, in the same range of concentrations, PG could act as an agonist-antagonist; if alone, it could bind to the estrogen receptor and activate the gene expression, while in the presence of a more potent estrogen (E_2 in the present study) it might just block the estrogen receptors. Also, a second theory could be speculated. Due to the higher concentrations of PG in the tested mixtures (when compared to E_2), it could bind to more estrogen receptors than E_2. However, given its' lower transactivation potency compared to E_2, the measured signal would not be as significant as in the case of estradiol. E_2 has less receptors available, so the total signal E_2+PG would be smaller when compared to the signal induced by E_2 alone. The *in vitro* studies published by Adamasi et al. showed that PG is one of the strongest ligands among the xenochemicals that are known as ERα binders (at 54 nM), but without inducing any transactivation activity at the concentrations tested, suggesting that PG is a pure antagonist [1].

The present study supports the anti-estrogenic effect of PG, but the concentration needed to inhibit the E_2 effect (at 30 pM) is in the micromolar range, which means that for a concentration of 10^{-10} M E_2 it was needed a concentration of PG around 10^{-5}M. Even if the findings of Adamasi et al support the mentioned effect, their results (transactivation and binding studies) indicate PG as a more potent antagonist than it was found in our study, using T47DKB-luc cell line. According to Adamasi et al. the

estrogenic effect of 10^{-8}-10^{-7} M E_2 observed in a transactivation study was inhibited by a tenfold higher concentration of PG [1].

PG was also found by ter Veld et al. to have estrogenic effects in both ER_α and ER_β expressing cells; the potency in ER_α was found to be 109% from E_2 max, while in ER_β the potency was calculated to represent 60% from E_2 max [13]. Contrary to our results, ter Veld et al found the mixture PG and E_2 to be additive.

BP registered a maximal effect at 10 μM (124.4% from E_2 max) in the present research. This concentration gave also the maximal effect in the cell proliferation assay [4]. Above this concentration, there is still an estrogenic effect, but it decreases in intensity. As this cell line is not free of other endogenous steroid receptors, this should be considered when trying to explain the fact that the maximum response from BP is higher compared to E_2 max. Besides this, the superagonism could be explained by the BP potential modulation of the activity of several kinases involved in ERE (estrogen response elements) activation, but for this mechanism molecular, studies are needed, this being beyond the scope of this study [13].

Even if the latest reviews described parabens as compounds that gave full agonist response in whole cells at lower concentrations, our data show that in the same range of concentrations, BP can act either as agonist or antagonist [3]. To our knowledge, the anti-estrogenic effect of BP was not reported so far in the literature using the luciferase assay.

Conclusions

Most of the *in vitro* evaluations of the endocrine disruptive potential of the selected compounds reported in the literature involve proliferation, ligand binding or luciferase induction assays on MCF-7, respectively U2-OS cell lines. The present work offers additional information obtained on a different *in vitro* system, the T47DK-Bluc cell line, the reporter gene induction being recognised to reflect more accurately the interaction with the estrogen receptors.

BP was found to have an estrogenic effect, but also an anti-estrogenic effect at a concentration 6 times higher than the one that gave the maximal effect as an estrogen.

BHA was found to have only anti-estrogenic effects, while PG was discovered to have a dual effect.

The present results highlight the need for precaution regarding the population exposure to the selected compounds due to their potential negative impact on the endocrine system.

The impact of differences between the regulatory requirements included in the biowaiver guidance for *in vitro* testing on the dissolution profiles of mycophenolate mofetil has been evaluated, using several immediate release oral solid dosage forms containing mycophenolate mofetil. The experimental data indicated that the conclusion of similarity is not influenced by hydrodynamic parameters. The acidic stage didn't reveal any significant difference in release, due to the rapid disintegration of the formulations and to the high solubility of the drug. The acetate buffer media provided the most discriminatory conditions for the differences in the qualitative and quantitative compositions. Specific limitations in comparison of different concentrations in the non-sink media were reported.

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