RESEARCH

Suppression of lymphoma growth by the xenoestrogens bisphenol A and genistein

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Abstract

Well-defined physiological functions of estrogens are mediated via nuclear estrogen receptors α (ESR1) and β (ESR2). With regard to hematological malignancies, expression of ESR2 has been found in both B and T cell lymphomas. In addition to endogenous estrogens or selective ESR2 agonists, ESR2 signaling may be affected by both environmental synthetic estrogen-mimicking compounds and dietary phytoestrogens. In the present study, we demonstrate that oral exposure with either the synthetic compound bisphenol A (BPA) or the dietary phytoestrogen genistein reduced the growth of grafted murine T cell (EG7) and human B cell (Granta-519 mantle cell) lymphomas which both express ESR2. Suppression of lymphoma growth was due to reduced proliferation (BPA and genistein) and induction of apoptosis (genistein). Inhibition of lymphoma growth was seen at a BPA dose of 50 µg/kg body weight (BW)/day considered to be safe human exposure dose or a genistein dose of 1 mg/kg BW/day orally, which is reached in soy-rich diets. Thus, our study indicates that the environmental xenoestrogens BPA and genistein have anti-proliferative effects on ESR2-expressing lymphomas. Our data suggest that phytoestrogens may be considered as a dietary supplement for lymphoma patients and possibly for prevention of lymphoid malignancies.

Key Words

- lymphoma
- estrogen receptor β
- bisphenol A
- genistein
- xenografts

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Introduction

Many physiological processes, such as reproduction, growth and development, are regulated by estrogens. Furthermore, disruption of estrogens signaling has been shown to be involved in a variety of adverse health effects in several diseases including carcinogenesis (1). The functions of estrogens are mainly mediated by two distinct nuclear estrogen receptors, α (ESR1) and β (ESR2) (2). While ESR1 is generally considered to elicit pro-proliferative effects, especially on cells of the reproductive tissues thereby promoting a pro-carcinogenic activity, the effects of ESR2 appear to be more complex since ESR2 e.g. shows anti-proliferative and pro-apoptotic activities on breast and prostate tumor cells (3, 4), but can decrease sensitivity of medulloblastoma cells to cytotoxic chemotherapeutics (5). With regard to the immune system, ESR2 appears to be the main ESR expressed in peripheral blood leukocytes, tonsils and spleen (6). This also seems to be the case in lymphomas (7, 8).

Several epidemiological studies have shown gender differences with regard to both incidence and prognosis of most lymphomas and leukemias, with men demonstrating higher incidence, worse prognosis and poorer survival (9, 10). For example, in diffuse large B cell lymphoma (DLBCL), it was shown that the poorer survival rate in males vs females was mainly seen before women entered the menopause (11). In addition, an association of postmenopausal hormone therapy and number of pregnancies with a decreased risk of non-Hodgkin's lymphomas (NHLs) has previously been reported (12, 13). These epidemiological data suggest a protective effect

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of estrogens on lymphoma incidence and progression. Despite this, lymphomas are not generally considered as endocrine-related malignancies.

In addition to the endogenous estrogenic ligands, estrogen signaling may be influenced by environmental synthetic or natural (dietary) xenoestrogens, so-called endocrine-disrupting chemicals (EDCs). An industrial EDC broadly found in the environment is bisphenol A (BPA). This compound is one of the most common industrial chemicals produced worldwide and is used primarily in the production of polycarbonate plastics and epoxy resins but is also found in several other products, thereby making human exposure to it common (14). BPA shows estrogenic activity in transcriptional activation assays and binds to both ESR1 and ESR2, with a 10-fold higher preference for ESR2 in comparison to ESR1 (15). With regard to carcinogenesis, BPA exposure has been linked to the development of estrogen-dependent malignancies, such as breast and prostate cancers (16, 17).

The United States Environmental Protection Agency and the European Food Safety Authority consider 50 µg/kg BW/day to be a safe human exposure dose (18). However, toxicological studies have demonstrated that doses of BPA, which are well below the reference dose of 50 µg/kg BW/day, may cause morphological and functional changes in the male and female genital tract and mammary glands and abnormalities of the reproductive system (19, 20). Moreover, a nonmonotonic dose response to BPA was reported in e.g. LNCaP prostate cancer and MCF-7 breast cancer cells, where BPA induced maximal proliferation at 1 nM and a lower response at higher concentrations, indicating a complexity of BPA-mediated effects (21, 22).

Natural dietary xenoestrogens, such as isoflavones, abundantly found in soy-rich diet also demonstrate ESR-binding activities with genistein being one of the most potent so-called phytoestrogens (23). With regard to ESR-binding activities, genistein binds to both ESR1 and ESR2 and activates these receptors but like BPA has some selectivity for ESR2 (24). Moreover, genistein has been shown to stimulate the expression of ESR2 in T74D cells (25). Interestingly, several epidemiological studies have reported that consumption of genistein and other isoflavones is associated with decreased incidence of estrogen-dependent tumors, such as breast and prostate cancers (26, 27). The suppressive effects of genistein in the case of prostate carcinogenesis have been suggested to be related to the stimulation of ESR2 expression and suppression of tumor cell proliferation in line with an 'anti-proliferative' effect of ESR2 (28).

Previous results from our group have provided experimental support for an estrogen-dependent regulation of lymphoma growth *in vivo*. For example, we demonstrated that male mice, grafted with murine T cell lymphoma cells, developed larger tumors compared to female mice, a difference that was abolished following ovariectomy, suggesting estrogen-dependent inhibition of lymphoma progression *in vivo* (29). In line with this, inhibition of estrogen synthesis by administrating aromatase inhibitors enhanced lymphoma progression in both female and male mice (30). Importantly, we have also shown that treatment of mice with selective ESR2 agonists inhibits growth of both grafted murine T cell and human B cell lymphomas (7, 29, 31). Moreover, activation of ESR2 by selective ESR2 agonists was found to suppress angiogenesis and lymphangiogenesis in the lymphomas as well as dissemination of grafted lymphoma cells (7). No effect was seen when analyzing lymphoma growth following treatment with the selective ESR1 agonist propylpyrazole *tris*phenol (29).

The role of xenoestrogens on lymphomas is not well established. Thus, in the present study, we aimed to investigate the effects of the ESR-modulating environmental synthetic compound BPA and dietary isoflavone genistein, respectively, on the growth of mouse and human lymphomas *in vivo* following oral exposure in doses to which humans may be exposed.

Materials and methods

Mice, cell lines and estrogen receptor modulating compounds

C57Bl/6J male mice (8–10 weeks of age) were purchased from Charles River (Lille Skensved, Denmark). Immunodeficient NOD/SCID/IL2γ^{null} (NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ) male mice (8–10 weeks of age) (32) were bred at the Animal Facility, Campus Flemingsberg, Karolinska Institutet, Stockholm, Sweden. Mice were kept on soy-free diet starting from 2 weeks before the experiments.

Mouse T lymphoma cell line EG7 and human mantle cell lymphoma (MCL) cell line Granta-519 and Burkitt's lymphoma cell line Daudi were maintained as previously described (7). Granta-519 and Daudi cell lines were authenticated to be correct using STR profiling at National Genomic Infrastructure Core Facility at SciLifeLab, Uppsala. The cell lines were also tested Mycoplasma free by the MycoAlert Mycoplasma Detection Kit from Lonza

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(Basel, Switzerland). Bisphenol A was purchased from Sigma-Aldrich. Genistein and the selective ESR2 agonist diarylpropionitrile (DPN) were obtained from Tocris Bioscience (Ellisville, MO, USA).

Quantitative real-time polymerase chain reaction (qPCR)

Isolation of RNA, cDNA synthesis and qPCR were performed as described previously (7). For the qPCR, the following specific forward and reverse primers were used (*ESR2*: forward – CCGATGCTTTGGTTTGGGTG; reverse – GAGCAGATGTTCCATGCCCT; *36B4* (for normalization): forward – TTCTCGCTTCCTGGAGGGTG; reverse – GACAA GGCCAGGACTCGTTT).

Ki67 staining, TUNEL assay and *in vivo* experiments

Ki67 staining and TUNEL assay and quantification of the results were performed on lymphoma tissue as previously described (7).

Surgical castration of C57BL6J mice was performed as previously described (30). Animal care, surgical castration and treatments were performed in accordance with the guidelines of Karolinska Institutet and all animal experiments were performed in accordance with ethical committee approval (permit number S61-14). During all the experiments, the mice were fed a soy-free diet.

For the lymphoma induction experiments, C57BL6J or NOD/SCID/IL2γ^{null} mice were grafted subcutaneously with mouse EG7 or human Granta-519 lymphoma cell lines respectively, using 3×10^5 EG7 cells or 15×10^6 Granta-519 cells per mouse in sterile PBS (total volume injected – 100 µL per animal). Starting from the day when subcutaneous lymphomas became palpable (approximately 50–100 mm^3), the mice were treated *per os* once daily with 50, 1 or 0.02 µg BPA/kg BW in 10% ethanol/90% rapeseed oil or 10, 1 or 0.1 mg genistein/kg BW in 10% ethanol/90% rapeseed oil or vehicle. Alternatively, the mice were treated with DPN (3 mg/kg BW) in 10% ethanol/90% rapeseed oil or vehicle alone administrated subcutaneously. The size of lymphomas was measured every day during the period of the experiment, and the tumor volume (TV) was calculated using the following formula: TV (mm^3) = 0.5 × length (mm) × width^2 (mm^2). The *in vivo* experiments were repeated three times with reproducible results.

Statistical analysis

The Student's *t*-test for the statistical analysis was performed using GraphPad Prism 5. Significance was considered when $P<0.05$.

Results

Oral treatment of mice with the xenoestrogens BPA and genistein, respectively, reduces murine EG7 lymphoma growth *in vivo*

To study if oral exposure to BPA affects the growth of lymphomas *in vivo*, male C57Bl6 mice were surgically castrated to minimize the potential effects of endogenous estrogens, which are produced from androgens in males through aromatization, and subcutaneously engrafted with murine EG7 lymphoma T cells and treated orally once a day with 50, 1 or 0.02 µg BPA/kg BW. We have previously shown that EG7 lymphoma cells express ESR2 but hardly any ESR1 (29). The growth of EG7 lymphoma tumors was significantly inhibited by BPA treatment with a dose of 50 µg/kg BW/day (Fig. 1A). However, no significant growth was observed by oral exposure to 1 or 0.2 µg BPA/kg BW/day (Fig. 1A and B). To analyze the effect of dietary isoflavonoids on lymphoma growth, surgically castrated C57Bl6 mice were engrafted with EG7 lymphoma cells and treated orally once a day with 10, 1 or 0.1 mg genistein/kg BW. The growth of EG7 lymphoma tumors was significantly suppressed by genistein both at a dose of 10 and 1 mg/kg BW/day (Fig. 2). No effect was seen by 0.1 mg/kg BW/day (data not shown).

The xenoestrogens BPA and genistein suppress proliferation of mouse EG7 lymphoma cells *in vivo* but differently affect apoptosis

To investigate the mechanism of the reduced TV by the treatment with BPA or genistein, cell proliferation and apoptosis were analyzed in the above lymphoma samples by Ki67 staining and TUNEL assay, respectively. Treatment with either 50 µg/kg BW/day of BPA or 10 mg/kg BW/day of genistein significantly reduced the expression of Ki67, demonstrating a reduction of lymphoma cell proliferation by these compounds (Fig. 3A). In addition, apoptosis was significantly increased in the grafted lymphomas treated with genistein (Fig. 3B). However, treatment with BPA did not show a significant effect on apoptosis (Fig. 3B).

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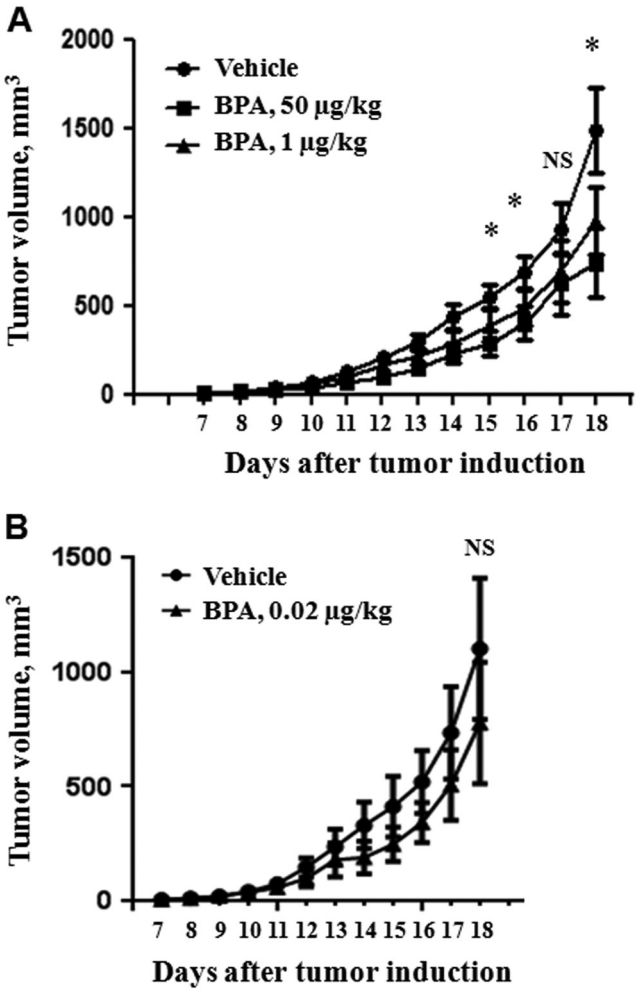

Figure 1
Inhibitory effect of oral BPA administration on murine lymphoma growth *in vivo*. Male C57/Bl6J mice were surgically castrated and subcutaneously grafted with 3×10^5 ESR2-positive murine EG7 lymphoma T cells. Starting from day 7 after tumor cell grafting (when the subcutaneous tumors became palpable), mice were treated orally once daily with BPA, 50 or 1 or 0.02 µg/kg BW or vehicle alone. The groups consisted of six mice treated with (A) vehicle (●), BPA, 50 µg/kg BW (■) and BPA, 1 µg/kg BW (▲) or (B) vehicle (●) and BPA, 0.02 µg/kg BW (▲). For vehicle vs BPA 50 µg/kg BW: $*P<0.05$. No significant difference (NS) was seen between vehicle and 1 or 0.02 µg/kg BW at any day of treatment. The results depicted in figure A and B, respectively, originate from experiments performed separately.

BPA and genistein suppress human lymphoma growth *in vivo*

In order to investigate whether BPA and genistein also affect the growth of other lymphomas, including human B cell lymphomas, we grafted immunocompromised NOD/SCID/IL2γ^{null} mice with the human MCL Granta-519, a B cell lymphoma cell line, which likewise the murine EG7 T cell lymphoma cells express ESR2 (7). Treatments with either BPA (50 µg/kg BW/day) or genistein (10 mg/kg BW/day) orally showed significant suppression of Granta-519 lymphoma tumor growth *in vivo* (Fig. 4). These results revealed that the inhibitory effect of BPA and genistein, respectively, on lymphoma is not restricted to one lymphoma type and is seen both for murine and human lymphomas expressing ESR2.

To evaluate whether the inhibition of lymphoma growth observed might correlate with ESR2 expression in lymphoma cells, we tested the effects of BPA and genistein on the growth of Daudi lymphoma cells which express low *ESR2* mRNA compared to Granta-519 cells (and no *ESR1*, data not shown) (Supplementary Fig. 1, see section on supplementary data given at the end of this article) by grafting these cells to NOD/SCID/IL2γ^{null} mice. The growth of Daudi lymphomas was not affected by treatment with 10 mg/kg BW/day genistein, 50 µg/kg BW day BPA or 3 mg/kg BW/day of the selective ESR2 agonist DPN (Supplementary Fig. 2) in contrast to Granta-519 lymphomas (see above) or to other lymphoma cells which express ESR2 (7, 8).

Discussion

Our results show an inhibitory effect of the environmental compounds BPA and genistein on ESR2-expressing mouse and human lymphoma growth *in vivo* following oral exposure. Both compounds significantly suppressed lymphoma cell proliferation as demonstrated by Ki67 expression analysis. In our study, genistein and the ESR2 agonists DPN inhibited the growth of both the murine EG7 and the human MCL Granta-519 lymphoma with high expression of *ESR2* mRNA. Furthermore, our previous studies have shown that the selective ESR2 agonist DPN suppressed the growth of Burkitt's lymphoma Raji and Ramos cells with high ESR2 expression (7, 33). Noticeably, neither genistein nor DPN significantly altered the expression of *ESR2* mRNA in either the murine EG7 or the human MCL Granta-519 tumors, respectively (data not shown).

In line with our results, genistein was suggested to have a chemotherapy-potentiating effect by enhancing the anti-proliferative activity of a combination of cytostatic drugs on DLBCL cells *in vitro* and in a xenograft mouse model of DLBCL (34). These observations together with the results presented in the current report are in line with previous studies showing that genistein interacts with and activates ESR2 (15, 28) and that ESR2 activation by synthetic selective ESR2 agonists leads to suppression of lymphoma progression in mouse xenografts (7, 8, 29).

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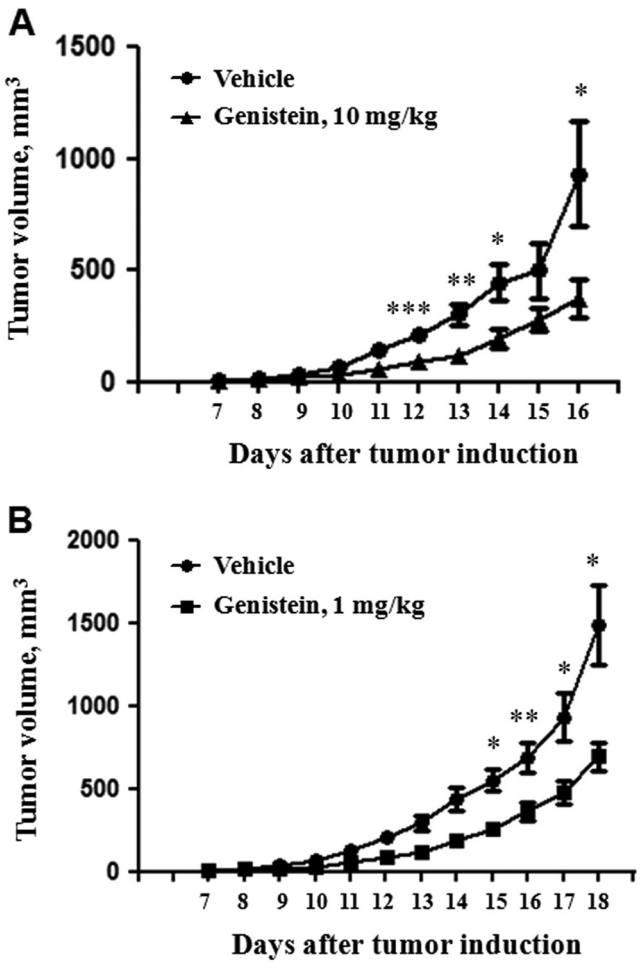

Figure 2
Inhibitory effect of oral genistein administration on murine lymphoma growth *in vivo*. Male C57/Bl6J mice were surgically castrated and subcutaneously grafted with 3×10^5 ESR2-positive murine EG7 lymphoma cells. Starting from day 7 after tumor cell grafting, mice were treated orally once a day with genistein, 10 or 1 mg/kg BW or vehicle. Each groups consisted of six mice treated with (A) vehicle (●) and genistein, 10 mg/kg (▲); or (B) vehicle (●) and genistein, 1 mg/kg (■). For vehicle vs genistein: *$P<0.05$, **$P<0.01$, ***$P<0.001$. The results depicted in figure (A) and (B), respectively, originate from experiments performed separately.

The involvement of ESR2 in the response is also supported by the inability of genistein to suppress lymphoma growth in the lymphoma expressing very low levels of ESR2 (Supplementary Figs 1 and 2). However, since genistein may interfere with other signaling pathways as well, we cannot with full certainty conclude that other signaling pathways are not involved.

The oral dose of genistein required to observe a significant suppressive effect on lymphoma growth was found to be ≥1 mg/kg BW/day. The dose of 1 mg genistein/kg BW/day corresponds to a daily soy intake of approximately 70 g/day or less, and this intake is reached when consuming a typical Japanese diet (35). The lower dose of genistein, 0.1 mg/kg BW/day, which more corresponds to a soy intake of 7 g/day as seen in a general Western diet (36), did not affect the growth of lymphomas *in vivo*.

Our previous studies have demonstrated inhibition of lymphoma tumor growth in a ligand-dependent way *in viv*o of lymphomas endogenously expressing ESR2 following treatment with selective ESR2 agonists (7, 8, 29). Ligand-mediated suppression of tumor growth by selective ESR2 agonists has also been seen in *in vivo* models for other malignancies expressing endogenous ESR2, e.g. mesothelioma and metastasis of melanoma cells (37, 38). In addition, the selective ESR2 agonist LY500307 was recently shown to inhibit metastasis of lung tumor cells in a mouse model via the recruitment of neutrophils (38). However, the impact of environmental xenoestrogens following oral exposure in these systems is less well established.

With regard to lymphoma incidence and phytoestrogen exposure, very little is known. However, an epidemiological study performed in Japan found a significant association of moderate (27–51 g/day) and high (>51 g/day) soy intake with a decreased risk of NHLs in women (39). A similar inverse association between soy intake and NHL incidence was found in men in a Swedish study (40). As to the effects of genistein on

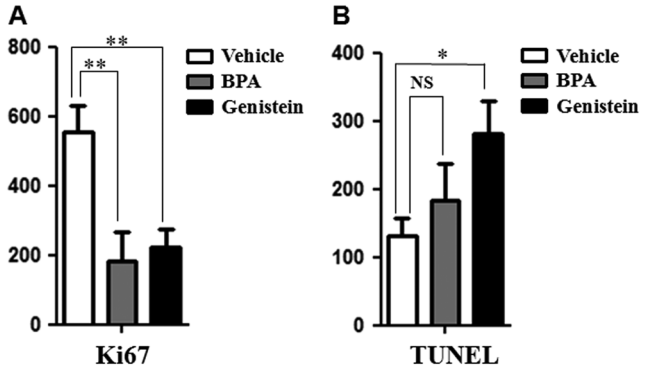

Figure 3
Oral administration with the exogenous xenoestrogens BPA and genistein suppresses the EG7 lymphoma growth *in vivo* via inhibition of proliferation and induction of apoptosis. (A) Ki67 expression as analyzed by immunohistochemistry in sectioned lymphomas from mice treated with BPA (50 μg/kg BW/day) or genistein (10 mg/kg BW/day) compared to vehicle-treated mice. (B) The level of apoptosis as determined by TUNEL assay of mice treated orally with BPA (50 μg/kg BW/day) or genistein (10 mg/kg BW/day) were compared to vehicle-treated mice. The results of Ki67 and TUNEL stainings are presented as the number of positive cells per field at ×200 magnification (eight analyzed fields per each tumor). *$P<0.05$, **$P<0.01$ in treated vs control mice. The tumors for the analysis were taken from six mice in each treatment group.

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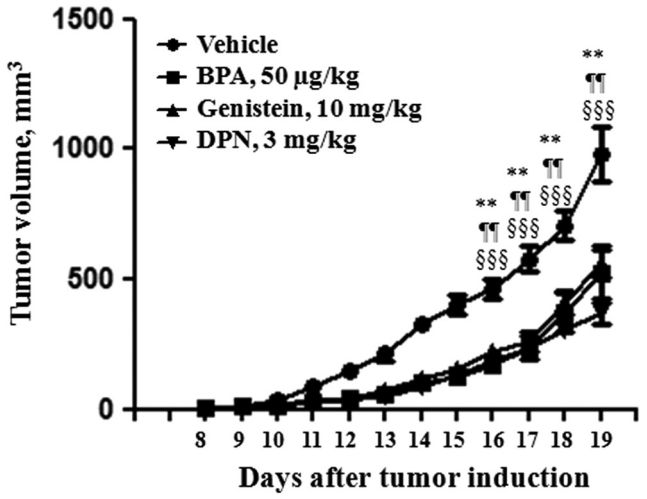

Figure 4
Effects of the exogenous ESR-modulating compounds BPA and genistein on the growth of ESR2-positive human Granta-519 B lymphoma cells *in vivo*. Male NOD/SCID/ IL2γ^{null} mice were subcutaneously grafted with 15×10^6 ESR2-positive human mantle lymphoma cells Granta-519. Starting from day 8 after tumor cell grafting, mice were treated orally once a day with BPA (50 µg/kg BW) or genistein (10 mg/kg BW) or once a day subcutaneously with the ESR2 selective agonist DPN (3 mg/kg BW) or vehicle alone. Each group consisted of five mice treated with vehicle (●), BPA (■), genistein (▲) or DPN (▼). For vehicle vs BPA: $^{**}P<0.01$; vehicle vs genistein: $^{¶¶}P<0.01$; vehicle vs DPN: $^{§§§}P<0.001$.

malignant lymphoid cell types, earlier *in vitro* studies have demonstrated suppression of proliferation and induction of apoptosis by genistein in human acute T lymphoblastic leukemia Jurkat and B lymphoma Ramos cells *in vitro* (41).

Our results showed that the BPA at the approved safe exposure dose of 50 µg/kg BW/day inhibited both human and mouse lymphoma growth. To our knowledge, this is the first report of a suppressive effect of BPA on lymphoma growth *in vivo*. Considering that the 50 µg/kg BW/day exposure dose corresponds to the upper level considered as a safe human exposure dose according to federal authorities, this clear response on estrogen signaling on lymphoma growth is noteworthy. Thus, notwithstanding the potential negative effects on the reproductive and other systems, the exposure to BPA and related compounds at 'safe' doses may have anti-proliferative effects on ESR2-expressing malignancies.

The inability of BPA, in contrast to genistein, to significantly increase lymphoma cell apoptosis at concentrations where both BPA and genistein inhibited lymphoma cell proliferation, may indicate that the two xenoestrogens harbor partially different properties when it comes to inhibition of lymphoma growth. Notably, lower BPA daily exposure doses of 1 µg or 0.02 µg/kg BW did not affect the growth of lymphoid tumors. The lack of effects at these low doses of BPA in comparison to the 50 µg/kg BW/day exposure argues against a nonmonotonic dose response curve, which has been proposed to exist for BPA according to several *in vitro* studies (22, 42, 43, 44). With regard to the nonmonotonic effects of BPA on tumor cells *in vivo*, low, but not high doses of BPA were shown to significantly affect mammary tumorigenesis in a mice model (45). Since it has been suggested that the nonmonotonic dose response seen at low levels of BPA exposure might be mediated by a non-ESR mechanism (taking into account a response in some tissues at BPA concentrations far below a significant ESR binding (46)), the requirement for a higher BPA dose to suppress lymphoma growth would support that the effects seen by BPA on lymphoma growth is mediated by ESR2. A lack of effect of 50 µg BPA/kg BW/day on lymphoma tumor growth in mice grafted with Daudi lymphoma cells with very low *ESR2* mRNA expression supports this conclusion (Supplementary Fig. 2).

Although our results focus on the impact of xenoestrogens on lymphoma progression, this might be extrapolated to lymphoma incidence considering that epidemiological data suggest that estrogens like phytoestrogens with an ability to bind and activate ESR2 to protect against development of malignancy in other cells/tissues expressing ESR2 (39, 40). Furthermore, a lower lymphoma incidence in females vs males and an implicated protective effect of hormone replacement therapy and pregnancy on lymphoma incidence support that estrogens also can protect against lymphoma initiation (10, 12). Combined with the notion that ESR2 is the main ESR expressed in leukocytes and that lymphomas are the malignancies which express the highest levels of *ESR2* mRNA (Supplementary Fig. 3), these data indicate that higher doses of genistein corresponding to a high soy intake may be considered potentially protective against lymphoma development. Supporting such a conclusion is that an epidemiological study has shown a significant association of moderate and high soy intake with a decreased risk of NHL in women (39). However, a definitive conclusion this to be the case requires experimental support.

In conclusion, both the environmental synthetic and natural dietary xenoestrogens BPA and genistein, respectively, show suppressive effects on mouse and human lymphoma growth *in vivo* in a murine model. Our results indicate that despite the potential carcinogenic activity and negative effects on the reproductive system, BPA may have anti-proliferative effects on human lymphomas. Our findings also suggest that foods rich in

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isoflavones may be considered to be included in the diet for lymphoma patients and possibly for prevention of lymphoid malignancies.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/EC-18-0459.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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