Molecular Modulation of Estrogen-Induced Apoptosis by Synthetic Progestins in Hormone Replacement Therapy: An Insight into the Women’s Health Initiative Study

Elizabeth E. Sweeney, Ping Fan, and V. Craig Jordan

Abstract

Hormone replacement therapy (HRT) is widely used to manage menopausal symptoms in women and can be comprised of an estrogen alone or an estrogen combined with a progestin. The Women’s Health Initiative demonstrated in their randomized trials that estrogen alone HRT decreases the risk of breast cancer in postmenopausal women, whereas combined estrogen plus a progestin (medroxyprogesterone acetate, MPA) HRT increases this risk. Long-term estrogen-deprived MCF-7:5C cells were used to model the postmenopausal breast cancer cell environment. MPA is able to modify E2-induced apoptosis in MCF-7:5C cells. MPA, similar to dexamethasone, increases glucocorticoid receptor (GR) transcriptional activity, increases SGK1, a GR target gene, and can be blocked by RU486 (an antiglucocorticoid), suggesting that it functions through the GR. Norethindrone acetate (NETA), another progestin used in HRT, acts like an estrogen at high doses, upregulating estrogen receptor target genes and generating apoptosis in MCF-7:5C cells. The data suggest that women taking HRT comprising an estrogen plus MPA may have an increased risk of breast cancer due to MPA acting as a glucocorticoid and blunting E2-induced apoptosis in this environment. Therefore, perhaps other approved progestins (e.g., NETA) should be considered as alternatives to MPA. Cancer Res. 74(23), 7060–8. ©2014 AACR.
The central question to be addressed is whether a synthetic progestin, MPA, can modulate estrogen-induced apoptosis and cause breast cancer cell growth. Here, we demonstrate that different synthetic progestins have different pharmacologic actions that exert different selection pressures during long-term therapy in culture. We report for the first time that the glucocorticoid properties of MPA (17) are responsible for blunting the apoptotic actions of estrogen, resulting in the growth of a new breast cancer cell population that is better able to survive.

Materials and Methods

Cell culture
MCF-7:5C and MCF-7:WS8 cell lines were cultured in phenol red-free RPMI-1640 media supplemented with 10% charcoal-stripped fetal bovine serum. Media and treatments were replaced every 3 days. DNA fingerprinting patterns of the cell lines are consistent with the report by the ATCC (18). The MCF-7:5C cell line was chosen for its representation of the estrogen-deprived breast cancer cell and its ability to undergo estrogen-induced apoptosis; the MCF-7:WS8 cell line represents the estrogen-fueled breast cancer cell environment. Estradiol (E$_2$; Sigma-Aldrich), dexamethasone (Dex; Sigma-Aldrich), MPA (Sigma-Aldrich), norethindrone acetate (NETA; Sigma-Aldrich), R5020 (Sigma-Aldrich), RU486 (Sigma-Aldrich), 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich), ICI 182,780 (ICI; Sigma-Aldrich), and combinations were dissolved in ethanol and then in media. MPA and NETA were chosen as two representative progestins used in HRT.

Cell proliferation assay
MCF-7:5C cells were harvested after treatment with vehicle (0.1% ethanol), E$_2$ (10$^{-7}$ mol/L, 1 nmol/L), Dex (10$^{-6}$ mol/L, 1 μmol/L), MPA (10$^{-6}$ mol/L, 1 μmol/L), NETA (10$^{-6}$ mol/L, 1 μmol/L), R5020 (10$^{-6}$ mol/L, 1 μmol/L), RU486 (10$^{-6}$ mol/L, 1 μmol/L), 4-OHT (10$^{-6}$ mol/L, 1 μmol/L), or combinations, in triplicate, for specified time. Media and treatments were replaced every 3 days. DNA content was measured as using the Fluorescent DNA Quantitation Kit (Bio-Rad).

Immunoblotting
ERE (sc-544), GR (glucocorticoid receptor; sc-8892), and PR (progesterone receptor; sc-810) antibodies were purchased from Santa Cruz Biotechnology, PARP (#9532S) antibody from Cell Signaling Technology, and β-actin antibody (A5411) from Sigma-Aldrich. Proteins were harvested from cells in 10-cm dishes using cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II (Calbiochem). Bicinchoninic acid assay was used to quantify total protein content (Bio-Rad Laboratories). Protein (50 μg) was probed and visualized as previously described (18).

Annexin V analysis of apoptosis
Annexin V analysis of apoptosis was quantified by flow cytometry using the FITC Annexin V Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions. MCF-7:5C cells were treated, suspended in 1× binding buffer, and stained simultaneously with FITC-labeled Annexin V (FL1-H) and PI (FL2-H). Cells were analyzed using FACSort flow cytometer (Becton Dickinson).

Quantitative real-time RT-PCR
Cells were harvested from 6-well plates using TRizol, and RNA was isolated using the RNeasy Micro Kit (Qiagen). RNA was reverse transcribed using an Applied Biosystems kit. SYBR green (Applied Biosystems) was used for quantitative real-time PCR (RT-PCR) in triplicate in a 7900HT Fast Real-Time PCR system (Applied Biosystems).

GRE activity
Transient transfection assay was conducted using a dual-luciferase system (Promega). To determine GR transcriptional activity, cells were transfected in 24-well plates for 24 hours with a glucocorticoid response element (GRE)–regulated dual-luciferase reporter plasmid (gift from Dr. Anne Gompel, Université Paris Descartes, Paris, France) or an estrogen response element (ERE)–regulated dual-luciferase reporter plasmid (gift from Dr. Rebecca Riggins, Georgetown University, Washington, DC). Cells were treated with specified compounds for 24 hours following transfection, in triplicate. Cells were then harvested and processed for dual-luciferase reporter activity, in which firefly luciferase activity was normalized by Renilla luciferase activity.

Statistical analysis
Values reported are means ± SEM. Significant differences were found by the Student t test. P values <0.05 were considered statistically significant.

Results

MCF-7:5C growth patterns with HRT compounds
MCF-7:5C cells represent a stable cell line derived from parental MCF-7 cells by long-term estrogen deprivation, and are the relevant cells used in the following studies. They are estrogen receptor (ER)–positive, GR-positive, and PR-negative (Supplementary Fig. S1A). MCF-7:5C cells were treated for 8 days with a vehicle, R5020, Dex, MPA, or NETA (another progestin used in HRT; Fig. 1A). R5020 was used as a pure progestin-positive control and had no effect on growth. After 8 days, Dex and MPA caused a 28.7% and 21.6% decrease in MCF-7:5C cell DNA, respectively, compared with vehicle, E$_2$ alone, and E$_2$+Dex (Fig. 1C). Cells treated with combinations of E$_2$+MPA and E$_2$+NETA, and compared with vehicle, E$_2$ alone, and E$_2$+Dex (Fig. 1C). Cells treated with 1 nmol/L E$_2$ die during the first week of treatment, but Dex and MPA seem to reverse this effect; cells are able to...
grow. E2 + NETA trigger the same death response as E2 alone, suggesting NETA’s role as an estrogen. Also, NETA decreases MCF-7:5C ER levels similarly to the effect of E2 after 2 months treatment (Supplementary Fig. S1B). NETA caused the growth of MCF-7:WS8 cell and this was blocked by the 4-OHT or fulvestrant (Supplementary Fig. S3C).

**Dex and MPA block E2-induced apoptosis in MCF-7:5C**

MCF-7:5C cells underwent these same treatments for 72 hours, and Annexin V staining was measured by flow cytometry to indicate apoptosis (Fig. 2A). E2 caused 29.03 (±1.44)% of cells to undergo apoptosis. Dex was able to block this apoptosis as shown by E2 + Dex causing only 5.37 (±0.35)% of cells to stain for Annexin V. NETA and E2 + NETA show similar Annexin V staining as E2 alone; both treatments cause apoptosis (30.17 ± 0.65 and 33.23 ± 0.97%, respectively). Although MCF-7:5C cells are eventually able to grow under E2 + MPA treatment (Fig. 1C), MPA is not able to block initial E2-induced apoptosis at 72 hours; more time is required for MPA to exert its subtle long-term effect. When Poly (ADP-ribose) polymerase (PARP) cleavage is probed by Western blot analysis to indicate apoptosis, again we show that E2 alone, and in combination with MPA, causes apoptosis after 72 hour treatments. However, MPA can block E2-induced apoptosis after 6, 9, and 12 days of combination treatment, as shown by decreased cleaved PARP protein expression. This confirms the biologic effect already seen, as MCF-7:5C cells can grow in the presence of E2 + MPA (Fig. 2B).

After a 2-month treatment, microscopy photographs were taken to illustrate the dramatic effects Dex and MPA have on E2-treated cells. Cells treated with both E2 and E2 + NETA show a reduction in MCF-7:5C cell number. E2 + Dex and E2 + MPA treatments both allow MCF-7:5C cells to grow during the 2 months of treatment as demonstrated by increased cell number seen in the photographs (Fig. 2C).

**NETA functions as an estrogen at high concentration**

To illustrate estrogenic actions of NETA, MCF-7:5C cell DNA was measured after treatment with the drugs alone and in combination with 4-OHT, an antiestrogen. As a positive control, the data confirm that 4-OHT is able to reverse E2’s apoptotic action as shown by significantly increased DNA when MCF-7:5C cells are treated with combination compared with E2 alone. 4-OHT also has the ability to reverse NETA’s decrease in MCF-7:5C DNA, suggesting its role as an estrogen (Fig. 3A). Estrogen target gene mRNA expression was then measured in MCF-7:WS8 cells by RT-PCR. Whereas Dex and MPA generate no increase in estrogen target genes pS2 (Fig. 3B) or PR (Supplementary Fig. S4), NETA elicits significant upregulation of both pS2 (23.6- and 46.9-fold) and PR (30.6- and 81.0-fold) at the two higher concentrations (Fig. 3B and Supplementary Fig. S4). This result is similar to that of E2, which increases pS2 64.9-fold and PR 58.9-fold over vehicle (Fig. 3B and Supplementary Fig. S4) in MCF-7: WS8 cells after 24 hour treatment. Furthermore, when MCF-
7:5C cells are transiently transfected with an ERE–luciferase construct, NETA is able to generate 8.12-fold higher ERE activity than vehicle-treated cells (Fig. 3C). This increased ERE activity is blocked by ICI (Fig. 3C) and 4-OHT (Supplementary Fig. S5), suggesting its reliance on the ER. Similar ERE activity results were generated in MCF-7:WS8 cells (Supplementary Fig. S6).

MPA functions as a glucocorticoid through GR similarly to Dex
To classify MPA as a glucocorticoid similar to Dex, MCF-7:5C cell DNA was quantified after treatment with the drugs alone and in combination with RU486, an anti-glucocorticoid. Dex and MPA alone cause a reduction in MCF-7:5C cell DNA; however, RU486 can reverse this reduction when treated in combination (Fig. 4A). These data suggest that MPA could be working through the GR in these cells.

To further test glucocorticoid behavior of MPA, GR target gene SGK1 mRNA was quantified by RT-PCR. Dex and MPA dramatically increased SGK1 expression in a time-dependent manner (Fig. 4B). This upregulation was inhibited by at least 50% when RU486 was added in combination to treatment, suggesting a GR-mediated mechanism. Furthermore, GRE activity was detected by transiently transfecting MCF-7:5C cells with a GRE–luciferase reporter vector. The cells were then treated for 24 hours with a vehicle, RU486, R5020, Dex, MPA, or NETA for 2 months; magnification, ×10; exposure time, 1/1000 seconds.
Dex and MPA block E2-induced apoptosis-related genes similarly

To explore pathways involved in Dex and MPA blocking E2-induced apoptosis, RT-PCR was performed using primers for genes associated with E2-induced apoptosis. E2 triggers apoptosis in MCF-7:5C cells by increasing levels of TNFα, HMOX1, LTA, and LTB after 72 hours of treatment. Both Dex and MPA are able to significantly decrease upregulation of these genes in response to E2 when treated in combination (Fig. 5). This suggests that MPA can work in a similar way as Dex by blocking key genes necessary for E2 to initiate apoptosis in this setting.

Discussion

The hypothesis addressed in this study is whether a synthetic progestin in HRT that exhibits glucocorticoid activity can interfere with E2-induced apoptosis in breast cancer cells. The results of the WHI indicate an increase in the risk of breast cancer in women taking CEE + MPA (15); MPA was, therefore, expected to increase the growth of breast cancer cells in the presence of E2 in our well-studied cellular model, and was predicted to function as a glucocorticoid. To test this hypothesis, Dex was used as a known glucocorticoid-positive control that can block E2-induced apoptosis (Fig. 2A). This classification of Dex is consistent with reports in the literature that indicate glucocorticoids can prevent apoptosis in various contexts of cancer (19–21).

Reports in the literature demonstrate the potential of synthetic progestins to bind and activate other nuclear receptors than their own cognate receptors. NETA and other 19-nortestosterone derivatives have been shown to activate the ER and stimulate the growth of estrogen-responsive MCF-7 and T47D...
The estrogenic activity of NETA is confirmed in this work through its ability to increase cell replication (DNA) in MCF-7:WS8 cells (Supplementary Fig. S3C), to elevate estrogen target genes (Fig. 3B, Supplementary Fig. S4), generate apoptosis in MCF-7:5C cells (Fig. 2A), increase ER transcriptional activity (Fig. 3C, Supplementary Figs. S5 and S6), and has its action be blocked by 4-OHT and ICI (Fig. 3A and C). Notably, reports have indicated that the synthetic progestin, MPA can bind to and activate not only the PR, but also the GR in breast cancer (24). A more recent report shows that MPA has affinity for the GR and can compete with the natural glucocorticoid, cortisol, in the body (25). Others have recently demonstrated that MPA can increase glucocorticoid activity in MCF-7 cells (26).

The data presented in this study integrate the previously published findings and establish that MPA functions as a glucocorticoid in long-term estrogen-deprived breast cancer cells, blocking E2-induced apoptosis and allowing cells to grow (Fig. 1C). MPA cannot block E2-induced apoptosis as early as Dex (Fig. 2A); it requires at least 6 days to gain sufficient glucocorticoid activity (Fig. 2B and C). When MPA is able to inhibit E2-induced apoptosis, like Dex, it acts by preventing upregulation of important E2-induced apoptosis-related genes (Fig. 5). We have noted previously that Dex decreases the growth of MCF-7:5C cells and blocks E2-induced apoptosis (27). Dex blocks E2-stimulated growth of MCF-7:WS8 cells (Supplementary Fig. S7). MPA prevents E2-stimulated growth at high concentration (10^{-6} mol/L), with similar effect as the more potent Dex in wild-type MCF-7:WS8 cells (Supplementary Fig. S7). This inhibition is not an effect of the progestin action of MPA as R5020, a progestin with no glucocorticoid action has no effect on E2-stimulated MCF-7:WS8 growth (Supplementary Fig. S7A).
Although others have demonstrated MPA’s function as a glucocorticoid (24–26), it is illustrated here as a mechanistic explanation for the increased breast cancer risk observed in the CEE+MPA arm of the WHI clinical trials (15). By modeling the environment of postmenopausal breast cancer using long-term estrogen-deprived MCF-7:5C cells, we can predict clinical responses through laboratory experiments. In so doing, we propose a unifying hypothesis of the modulation of E2-induced apoptosis to explain the results of the WHI HRT trial in population of postmenopausal women over the age of 60 years (15).

Critical to the understanding of how these effects occur, studies describe the interplay between ER and GR, and how AP-1 can integrate their transcriptional responses (28). Recent reports (29) also show that the ER and GR can interact and cause differential activation by reconfiguring the chromatin structure at GRE or ERE sites in the DNA. Furthermore, it is shown that GR can inhibit ER transcriptional activity and ER-mediated proliferation in breast cancer (30). Consistently, Dex blocks E2-stimulated MCF-7:WS8 cell replication (Supplementary Fig. S7). We can speculate that when MPA binds to the GR, the complex binds to GREs in the DNA, which then affects the ability of ligand-bound ER to functionally transcribe ER target genes. In order for E2-induced apoptosis to occur, E2 binds to ER, resulting in transcription of genes (e.g., HMOX1, TNFα, etc.) necessary for apoptosis. When this process is inhibited, possibly by the chromatin remodeling actions of MPA-bound GR, apoptosis is decreased even in an estrogenic setting.

Figure 5. Dex and MPA block genes associated with E2-induced apoptosis. MCF-7:5C cells were treated with vehicle, 1 nmol/L E2, 1 μmol/L Dex, 1 μmol/L MPA, or combinations for 72 hours. RT-PCR was performed using primers for TNFα (A), HMOX1 (B), LTA (C), and LTB (D). 36B4 was used as an internal control. Means represent three samples in triplicate; ***, P < 0.001.
It is important to consider timing when considering the implications of this work; timing of HRT can make a dramatic difference in response to treatment (31, 32). MCF-7:5C cells represent long-term estrogent-deprived cells, we sought this is the biologic context required in the patient as well to replicate the patient population in the WHI CEE alone trial (15). Previous studies have examined how a ‘gap’ is needed after menopause to sufficiently deprive women’s nascent breast cancer cells of estrogen (33). Five to 10 years postmenopause is appropriate to ensure effective apoptosis when CEE is introduced to the woman (31). Without this delay, exogenous estrogen may stimulate breast cancer growth, resulting in increased breast cancer risk; in fact, studies show that breast cancer risk is greater when HRT is initiated closer to menopause (34).

The findings presented can have clinical impact as CEE+MPA is frequently taken by postmenopausal women to alleviate menopausal symptoms. Patients should perhaps be advised to choose a different approach with a selective ER modulator (SERM)/CEE combination (35), and also to delay HRT until they are appropriately past menopause. Because the synthetic progestin NETA can prevent endometrial cancer, and also cause breast cancer apoptosis like an estrogen, it appears to be a logical alternative to MPA as an alternative approved synthetic progestin that could be used in HRT.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: P. Fan, V.C. Jordan
Development of methodology: E. Sweeney, P. Fan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Sweeney, P. Fan, V.C. Jordan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Sweeney, P. Fan
Writing, review, and/or revision of the manuscript: E. Sweeney, V.C. Jordan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Fan
Study supervision: P. Fan, V.C. Jordan

Acknowledgments
The authors thank Dr. Rebecca Riggins (Georgetown University) for the ERE plasmid and for her help with the GRE plasmid preparation. The authors also thank Dr. Anne Gompel (Université Paris Descartes) for her gift of the GRE plasmid.

Grant Support
This work was supported by the Department of Defense Breast Program (award number W81XWH-06-1-0909) Center of Excellence, the Susan G. Komen for the Cure Foundation (award number SAC100009), and the Lombardi Comprehensive Cancer Center Support Grant (core grant NIH P30 CA051008). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 16, 2014; revised September 8, 2014; accepted September 24, 2014; published OnlineFirst October 10, 2014.

References


32. Jordan VC. Timing is key to avoid the bad and enhance the good of soy supplements. JNCI. In press.


Molecular Modulation of Estrogen-Induced Apoptosis by Synthetic Progestins in Hormone Replacement Therapy: An Insight into the Women’s Health Initiative Study

Elizabeth E. Sweeney, Ping Fan and V. Craig Jordan


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-1784

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/10/11/0008-5472.CAN-14-1784.DC1

Cited articles
This article cites 33 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/23/7060.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/74/23/7060.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.