

Dienogest, a Novel Synthetic Steroid, Overcomes Hormone-Dependent Cancer in a Different Manner than Progestins

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BACKGROUND. Dienogest is a synthetic progestational steroid that is used for contraception. It is being studied for the treatment of endometriosis, but its anticancer activity remains unknown. The authors investigated the anticancer effect of dienogest on hormone-dependent cancers.

METHODS. The authors used two cell lines derived from human endometrial carcinoma (HEC-88nu cells expressing estrogen receptors [ER] but not progesterone receptors [PR] and Ishikawa cells expressing both ER and PR) and a cell line derived from human breast carcinoma (MCF-7 cells expressing both ER and PR). The authors examined the in vivo antitumor activity and the antiuterotropic activity of dienogest in mice and compared it with the activity of several progestins.

RESULTS. At oral doses of 0.01–1 mg/kg/day, dienogest significantly suppressed the 17 β -estradiol benzoate (E₂)-dependent tumor growth of HEC-88nu cells, which were unresponsive to known progestins such as medroxyprogesterone acetate (MPA, 100 mg/kg/day, administered orally) and norethisterone (NES, 100 mg/kg/day, administered orally). The suppressive effect of dienogest on tumor growth was not diminished in the presence of excess MPA. Dienogest also suppressed the E₂-dependent tumor growth of both Ishikawa and MCF-7 cells, both of which responded to MPA. However, the minimum effective dose of dienogest (0.01–1 mg/kg/day) was much lower than that of MPA (100 mg/kg/day). In contrast, dienogest did not suppress the E₂-induced increase in uterine weight, whereas MPA and NES suppressed it significantly.

CONCLUSIONS. Dienogest showed potent anticancer activity against hormone-dependent cancers at doses at which progestins show no activity. *Cancer* 1997; 79:169–76. © 1997 American Cancer Society.

KEYWORDS: dienogest, endometrial carcinoma, breast carcinoma, mice.

Based on the hypothesis that the majority of endometrial and breast carcinomas proliferate in response to estrogenic stimulation,^{1,2} hormonal agents, such as progestins and antiestrogens, have been used to treat these tumors. However, the success rate of therapy with hormonal agents is not very high,^{3,4} and these agents often have serious adverse effects.^{1,4} Thus, new agents with a higher efficacy and fewer side effects would be highly desirable.

17 α -cyanomethyl-17 β -hydroxy-estra-4,9-dien-3-one (dienogest) (Fig. 1) is an orally administered synthetic steroid that shows progestational activity with little or no androgenic, estrogenic, antiestrogenic, or corticoid activities.⁵ Based on this hormonal profile, dienogest may cause fewer adverse effects than other hormonal agents. It is currently used in oral contraceptives and is being studied in Japan, Germany,⁶ and France as a possible treatment for endometriosis. However, its anticancer activity has not been explored. In the cur-

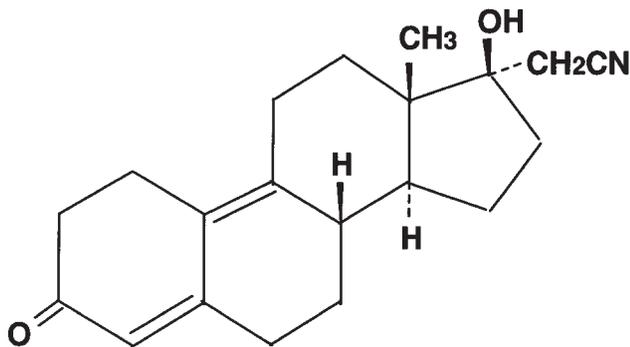


FIGURE 1. Chemical structure of dienogest (17 α -cyanomethyl-17 β -hydroxy-estra-4,9-dien-3-one) is shown.

rent study, we therefore investigated the anticancer action of dienogest and found a novel activity that differed from that of several known progestins.

MATERIALS AND METHODS

Animals

Female CB-17 SCID mice weighing 17–28 g (Clea Japan, Tokyo) were used. The animals were maintained in clean boxes (type FRP Bio-2000, Clea Japan) in a room controlled at 23 ± 2 °C and $55 \pm 15\%$ relative humidity with a 12-hour light/12-hour dark cycle. They were given a sterilized solid diet (gamma ray-irradiated CL-2, Clea Japan) and water ad libitum during the experiment.

In Vivo Studies with HEC-88nu Cells

HEC-88nu cells derived from a well-differentiated human endometrial carcinoma⁷ were injected subcutaneously (2×10^7 /mouse) into the posterior dorsolateral region of the animals to form tumors. The size of the lesions was measured according to the method of Aoki et al.⁸ In brief, the subcutaneous tumors were imaged noninvasively by ultrasonography using an echo camera (type SSD-650, Aloka, Tokyo) equipped with a 10 megahertz in-line sector scanner probe (type ASU-32WL-10, Aloka). The major axis (a), minor axis (b), and thickness (c) of each tumor were measured on the ultrasonic image (Fig. 2), and tumor volume was expressed as the product of a, b, and c in cubic millimeters (mm^3). The intraobserver coefficient of variation for tumor measurement by ultrasound was 2.2%. Measurement of the tumors was done once at each designated time. After the ability of the tumors to grow larger than 1000 mm^3 was confirmed, each tumor was extirpated and cut it into pieces that were transplanted subcutaneously into 52 other mice (40 mg/mouse). Tumor size was measured weekly; and when the volume reached 75 mm^3 or more, the host animals were ovariectomized under intraperitoneal



FIGURE 2. Representative ultrasonic image of a tumor in a mouse is shown. The tumor is clearly seen as a dark, subcutaneous area at magnification $\times 4$. Left: Cross-sectional image of the major axis. Right: Cross-sectional image of the minor axis.

(i.p.) anesthesia with pentobarbital sodium (50 mg/kg). Thereafter, 42 mice were treated with 17 β -estradiol benzoate (E_2) (0.5 mg/kg/day, i.p.) suspended in 0.9% sodium chloride (saline). The remaining 10 mice were injected with saline instead of E_2 . The E_2 -treated mice were simultaneously given dienogest (0.01, 0.1, and 1 mg/kg/day, administered orally [p.o.]; $n = 5$), medroxyprogesterone acetate (MPA) (100 mg/kg/day, p.o.; $n = 5$), norethisterone (NES) (100 mg/kg/day, p.o.; $n = 5$), 17 α -hydroxyprogesterone (OHP) (100 mg/kg/day, p.o.; $n = 5$), dienogest (1 mg/kg/day, p.o.) plus MPA (100 mg/kg/day, p.o.; $n = 5$), or the vehicle alone (0.5% carboxymethylcellulose sodium; $n = 7$). The 10 saline-treated mice also received dienogest (1 mg/kg/day, p.o.; $n = 5$) or vehicle ($n = 5$). All treatments were continued for 4 weeks. The day after the last dose, the mice were killed by exsanguination under ether anesthesia, and the tumor and uterus were removed from each animal. E_2 , MPA, NES, and OHP were purchased from Sigma (St. Louis, MO), and carboxymethylcellulose sodium was obtained from Tokyo Kasei (Tokyo). Dienogest was supplied by the Pharmaceutical Laboratory of Mochida Pharmaceutical Co., Ltd. (Shizuoka, Japan).

In Vivo Studies with Ishikawa and MCF-7 Cells

The Ishikawa cell line was derived from a well-differentiated human endometrial carcinoma.⁹ The MCF-7 cell line (derived from a human breast carcinoma¹⁰) was purchased from the American Type Culture Collection (Rockville, MD). Thirty mice were prepared with Ishikawa tumors and 35 mice with MCF-7 tumors and ovariectomized in the manner described previously. Twenty-five of the mice with Ishikawa tumors

received E_2 (0.5 mg/kg/day, i.p.), and the remaining 5 received saline instead of E_2 . The 25 E_2 -treated mice were simultaneously given dienogest (0.01, 0.1, and 1 mg/kg/day, p.o.; $n = 5$), MPA (100 mg/kg/day, p.o.; $n = 5$), or vehicle ($n = 5$) for 4 weeks. Tumor size was measured weekly. The day after the last dose, the mice were killed in the manner mentioned previously and their tumors were removed. The isolated tumors were fixed for 24 hours in ice-cold 70% ethanol and embedded in paraffin for histologic investigation (as will be described later in this article). The mice received an intravenous injection of 5-bromodeoxyuridine (BrdU; 10 mg/kg) in saline 30 minutes before sacrifice. Thirty of the mice with MCF-7 tumors received E_2 (0.5 mg/kg/day, i.p.), and the remaining 5 received saline instead of E_2 . Thirty of the E_2 -treated mice simultaneously received dienogest (0.001, 0.01, and 0.1 mg/kg/day, p.o.; $n = 5$), MPA (100 mg/kg/day, p.o.; $n = 5$), tamoxifen citrate (TAM) (10 mg/kg/day, p.o.; $n = 5$), or vehicle ($n = 5$) for 4 weeks. Tumor size was measured weekly. The day after the last treatment, the mice were killed in the same manner as mentioned previously and their tumors were removed. TAM was purchased from Sigma.

In Vitro Studies with HEC-88nu Cells

HEC-88nu cells in the logarithmic growth phase were suspended in phenol red-free minimal essential medium containing 10% fetal calf serum, treated with active carbon, and plated in triplicate into 24-well plates at a density of 10^5 cells per well. The cells were then cultured with dienogest or solvent in the presence of 10^{-8} mol/L 17β -estradiol for 7 days at 37 °C under 5% CO_2 -95% air, and the number of cells per culture were counted. We also measured the concentration of lactate dehydrogenase (LDH) in the culture medium to evaluate the cytotoxicity of the drug.¹¹

Histologic and Immunohistochemical Studies on Ishikawa Tumors

For morphologic examination, sections 4 μ m thick from blocks of Ishikawa tumors were stained with hematoxylin and eosin. For immunohistochemical examination, other sections were stained for BrdU, estrogen receptors (ER), or progesterone receptors (PR). Before ER and PR staining, deparaffinized sections were hydrated by autoclaving at 121 °C for 10 minutes. Mouse anti-BrdU (clone Bu20a, Dako, Santa Barbara, CA), antihuman ER (clone 1D5, Dako), and antihuman PR (clone 1A6, Novocastra, Newcastle, UK) monoclonal antibodies were used as primary antibodies, and biotin-conjugated rabbit antimouse immunoglobulins (Dako) were used as secondary antibodies. To identify nuclear antibody binding, we stained the sections by the peroxidase-conjugated streptavidin-bio-

tin complex method (LSAB kit, Dako), then performed light counterstaining with hematoxylin.

Statistics

Results are expressed as the mean \pm standard error. Dunnett's *t* test was used to analyze the differences among groups for statistical significance by the multiple comparison procedure.¹²

RESULTS

In Vivo Studies with HEC-88nu Cells

The effects of dienogest and other agents on the growth of HEC-88nu tumors in mice were investigated first. Tumor growth was significantly greater in the E_2 -plus-vehicle group than in the saline-plus-vehicle group (Fig. 3). In the E_2 -plus-dienogest groups, E_2 -induced tumor growth was significantly suppressed. In particular, dienogest at doses of 0.1 and 1 mg/kg/day significantly suppressed growth even after 1 week of treatment, and tumor size was comparable to that in the saline-plus-vehicle group throughout the 4-week experimental period (Fig. 3A). In contrast, MPA treatment failed to suppress E_2 -induced tumor growth. Likewise, NES and OHP also had no significant effect on tumor growth (Fig. 3B). The suppressive effect of dienogest (1 mg/kg/day) on E_2 -induced tumor growth was also significant in the presence of MPA (100 mg/kg/day) (Fig. 3C). In the E_2 -plus-vehicle group, uterine weight was significantly increased as compared with that in the saline-plus-vehicle group shown as E_2 -absent (Fig. 4). Dienogest had no significant effect on the E_2 -induced increase in uterine weight. In contrast, MPA and NES significantly suppressed the E_2 -induced increase in uterine weight, whereas OHP did not. The suppressive effect of MPA on uterine weight was not influenced by the addition of dienogest (1 mg/kg/day) (Fig. 4). In the saline-plus-dienogest group, there was no significant difference in tumor size or uterine weight as compared with the saline-plus-vehicle group (data not shown).

In Vivo Studies with Ishikawa and MCF-7 Cells

Next, the effect of the same agents on tumors derived from Ishikawa and MCF-7 cells was investigated. Ishikawa and MCF-7 are known to be MPA- and TAM-sensitive cell lines, respectively, and so they were selected to compare the anticancer actions of dienogest, MPA, and TAM. E_2 -induced stimulation of tumor growth and suppression of the growth by dienogest treatment were seen in tumors derived from both Ishikawa cells (Fig. 5) and MCF-7 cells (Fig. 6), as well as in HEC-88nu tumors. The suppressive effect of dienogest on the growth of MCF-7 tumors was also significant, even at the lowest dose tested (0.001 mg/kg/day), after 1, 2, and 3 weeks of treatment. MPA (100 mg/

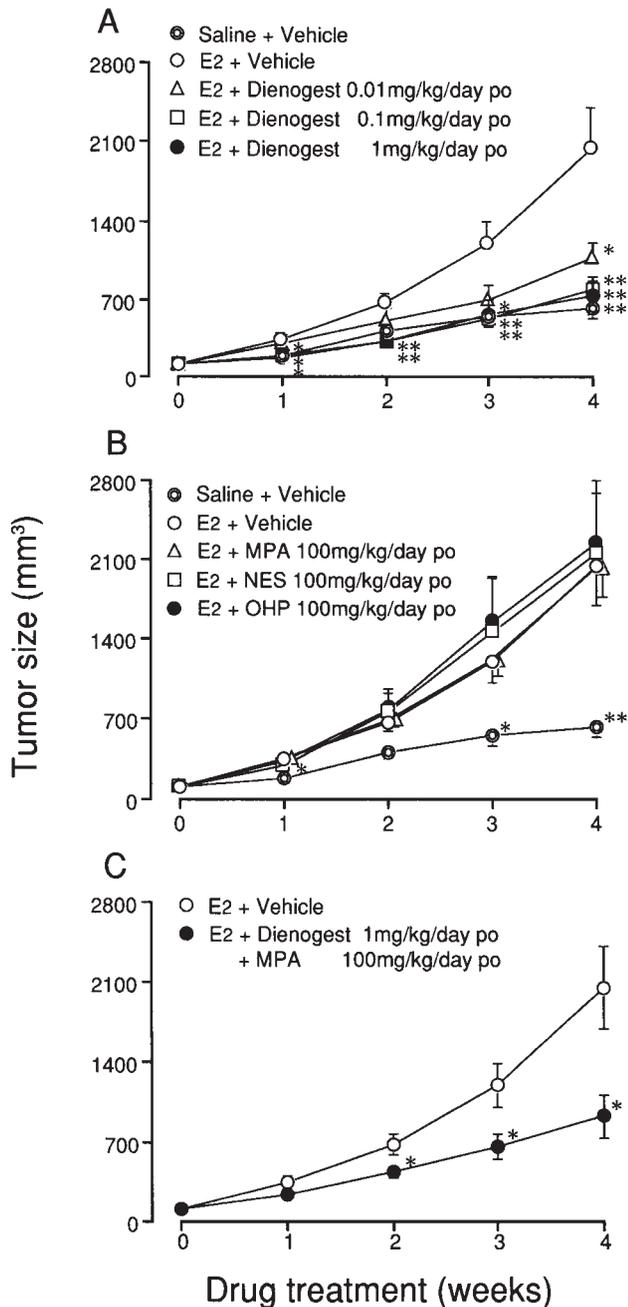


FIGURE 3. Effects are shown of various drugs on the growth of tumors of a human endometrial carcinoma cell line (HEC-88nu) in ovariectomized mice treated with 17 β -estradiol benzoate (E₂). (A) Effect of dienogest is shown. (B) Effects of medroxyprogesterone acetate (MPA), norethisterone (NES), and 17 α -hydroxyprogesterone (OHP) are shown. (C) Effect of dienogest plus MPA is shown. The saline-plus-vehicle and E₂-plus-vehicle groups show the same effects in each figure. E₂ was administered intraperitoneally at 0.5 mg/kg/day; other drugs were administered orally (po). Each point represents the mean \pm standard error (n = 5, except n = 7 in the E₂-plus-vehicle group). Data were analyzed by Dunnett's *t* test. Significant differences from the E₂-plus-vehicle group: **P* < 0.05, ***P* < 0.01.

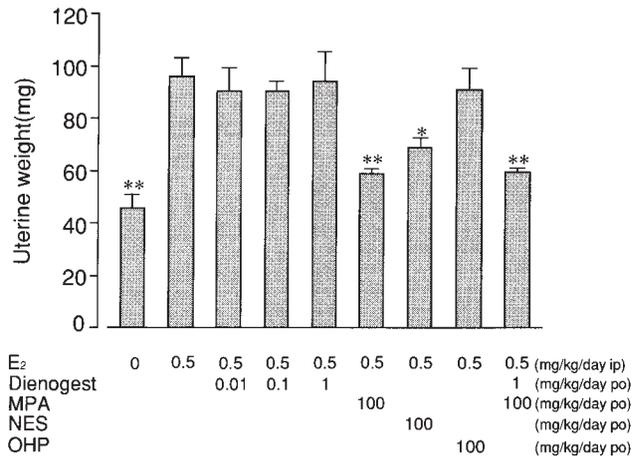


FIGURE 4. Effects are shown of dienogest, medroxyprogesterone acetate (MPA), norethisterone (NES), and 17 α -hydroxyprogesterone (OHP) on the uterine weight of ovariectomized mice with tumors of a human endometrial carcinoma cell line (HEC-88nu) treated with 17 β -estradiol benzoate (E₂). Each column represents the mean \pm standard error (n = 5–7; see footnote to Fig. 3). Data were analyzed by Dunnett's *t* test. In the figure, E₂ = E₂ plus vehicle. Significant differences from the E₂-plus-vehicle group: **P* < 0.05, ***P* < 0.01. ip: administered intraperitoneally; po: administered orally.

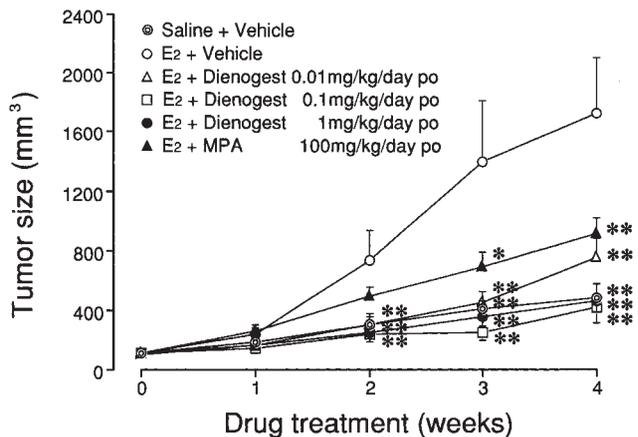


FIGURE 5. Effects are shown of dienogest and medroxyprogesterone acetate (MPA) on the growth of tumors of a human endometrial carcinoma cell line (Ishikawa) in ovariectomized mice treated with 17 β -estradiol benzoate (E₂). E₂ was administered intraperitoneally at 0.5 mg/kg/day; other drugs were administered orally (po). Each point represents the mean \pm standard error (n = 5). Data were analyzed by Dunnett's *t* test. Significant differences from the E₂-plus-vehicle group: **P* < 0.05, ***P* < 0.01.

kg/day) also significantly suppressed the E₂-induced growth of Ishikawa tumors. No significant difference in tumor size was observed between the MPA group and the dienogest (0.01 mg/kg/day) group (Fig. 5). Both MPA (100 mg/kg/day) and TAM (10 mg/kg/day)

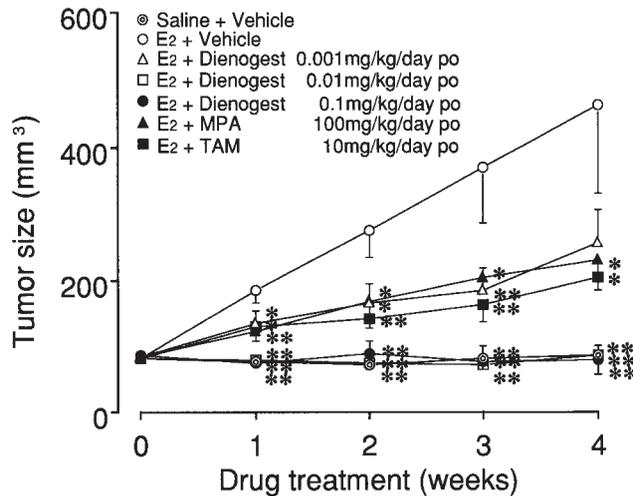


FIGURE 6. Effects are shown of dienogest, medroxyprogesterone acetate (MPA), and tamoxifen citrate (TAM) on the growth of tumors of a human breast carcinoma cell line (MCF-7) in ovariectomized mice treated with 17β -estradiol benzoate (E_2). E_2 was administered intraperitoneally at 0.5 mg/kg/day; other drugs were administered orally (po). Each point represents the mean \pm standard error ($n = 5$). Data were analyzed by Dunnett's t test. Significant differences from the E_2 -plus-vehicle group: * $P < 0.05$, ** $P < 0.01$.

TABLE 1
Effect of Dienogest in the Presence of 10^{-8} mol/L 17β -Estradiol on the Growth of HEC-88nu Cells

Drug	Concentration (mol/L)	No. of cells ^a ($\times 10^5$ /culture)	P value ^b
Control	—	3.9 ± 0.1 (100) ^c	
Dienogest	10^{-10}	3.7 ± 0.1 (95)	NS
	10^{-8}	3.4 ± 0.1 (87)	<0.01
	10^{-6}	2.1 ± 0.1 (54)	<0.01
	10^{-5}	0.8 ± 0.1 (21)	<0.01

NS: not significant.

^a Each value represents the mean \pm standard error ($n = 3$).

^b Data were analyzed by Dunnett's t test.

^c Values in parentheses represent percentages of the control value.

significantly suppressed the growth of MCF-7 tumors. Tumor size in the MPA and TAM groups was not significantly different from tumor size in the dienogest (0.001 mg/kg/day) group (Fig. 6).

In Vitro Studies with HEC-88nu Cells

To confirm that the action of dienogest was due to the drug itself and not due to other factors, such as metabolites, in vitro studies were also conducted. Dienogest inhibited the growth of HEC-88nu cells in a concentration-dependent manner (Table 1) and did

not increase the LDH concentration in the culture medium.

Histologic and Immunohistochemical Studies with Ishikawa Tumors

Because it was reported that Ishikawa cells possess both PR and ER,⁹ unlike HEC-88nu cells, we investigated the effect of dienogest on the histologic differentiation, receptor expression, and proliferation of Ishikawa tumors. In the E_2 -plus-vehicle group, glandular structures were evident (Fig. 7a). These structures were also seen in the E_2 -plus-dienogest group (Fig. 7b) but not in the E_2 -plus-MPA group, in which solid structures were prominent (Fig. 7c). Positive immunoreactivity of nuclei for BrdU, PR, and ER was evident in the E_2 -plus-vehicle group. Positive staining for BrdU was decreased in the E_2 -plus-dienogest group but not in the E_2 -plus-MPA group (Fig. 8). Positive staining for PR was almost absent in the E_2 -plus-MPA group, whereas no change was observed in the E_2 -plus-dienogest group (Fig. 9). An average of 93% of all nuclei were positive for ER in the E_2 -plus-vehicle group, and there was no significant difference in the other two groups.

CONCLUSIONS

In the current study, we demonstrated that dienogest had an anticancer action that was markedly different from that of well-known progestins.

First, the E_2 -stimulated tumor growth of HEC-88nu cells was not suppressed at all by typical progestins (MPA, NES, and OHP), a finding consistent with an earlier report.¹³ The only effective drug was dienogest. The HEC-88nu cell line does not express PR, although it is positive for ER.¹⁴ Our immunohistochemical study of HEC-88nu tumors also revealed them to be PR-negative (data not shown), which would explain the lack of a response to progestins. However, dienogest has been reported to have progestational activity and no other notable hormonal activities.⁵ To explain the difference in the anticancer actions of dienogest and the other progestins, we confirmed the existence of progestational activity in the mice with HEC-88nu tumors by measuring the uterine weight. MPA and NES suppressed the E_2 -induced increase in uterine weight, suggesting the presence of progestational activity (antiuterotrophic action) mediated via uterine PR.¹⁵ OHP has a PR binding affinity which is less than 1/1000 that of progesterone,¹⁶ and it failed to decrease uterine weight. Thus, OHP at a dose of 100 mg/kg was considered to show no progestational activity in this experimental system. Dienogest also had no effect on uterine weight. The antiuterotrophic action of dienogest in mice is reported to require a dose of 230 μ g per animal (approximately 10 mg/kg).¹⁷ Considered together, these data suggest that dienogest must have

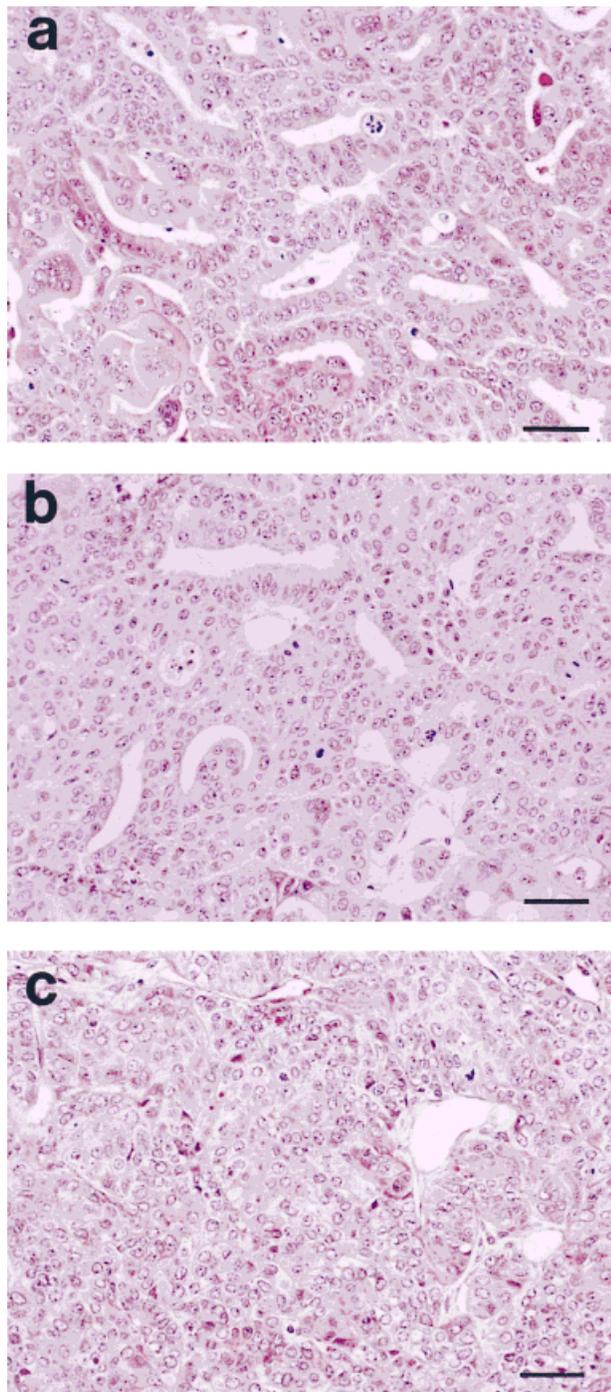


FIGURE 7. Representative histologic appearance of Ishikawa tumors from the E_2 -plus-vehicle group (a), the E_2 -plus-dienogest (0.1 mg/kg/day, orally) group (b), and E_2 -plus-medroxyprogesterone acetate (100 mg/kg/day, orally) group (c) (H & E, bar = 50 μ m).

unique activity in addition to its progestational activity and that this additional activity is manifested at doses lower than those with a progestational effect. Although an additional study using a PR antagonist such as RU486 might be preferable, this hypothesis was supported by the fact that the tumor-suppressive effect of dienogest

was not reduced in the presence of MPA. Dienogest has a lower binding affinity for human uterine cytosolic PR than MPA, NES, or progesterone.¹⁸ We also found that the binding affinity of dienogest for rabbit and rat uterine PR was lower than that of MPA (data not shown). Therefore, in the presence of excess MPA, the anticancer action of dienogest should be mediated by a pathway different from that involving PR.

Separation of the anticancer activity from the antiuterotrophic activity of dienogest was also observed in mice with Ishikawa and MCF-7 tumors. Dienogest showed no antiuterotrophic activity at tumor-suppressive doses, whereas MPA reduced uterine weight (data not shown). These results suggest that dienogest may be able to suppress the growth of a variety of endometrial and breast carcinoma cells through a mechanism different from that of progestins. In addition, dienogest inhibited the *in vitro* growth of HEC-88nu cells while showing no cytotoxicity. This finding suggests that inhibition of cell growth is a possible mechanism for the *in vivo* anticancer action of dienogest.

Histologic and immunohistochemical investigations confirmed the differences between dienogest and MPA with respect to anticancer activity. Dienogest, but not MPA, decreased the number of BrdU-labeled nuclei in Ishikawa tumors. Dienogest also decreased labeled nuclei in HEC-88nu tumors (data not shown). The glandular structure of the Ishikawa tumors was maintained in the dienogest group, whereas solid structures were prominent in MPA-treated tumors. A marked decrease in PR expression was observed in MPA-treated tumors but not in dienogest-treated tumors. Kauppila et al.¹⁹ have reported that both a higher grade of differentiation and higher levels of ER and PR are predictors of a favorable prognosis in patients with endometrial carcinoma. Thus, dienogest may improve the clinical outcome for some of these patients. On the other hand, it was reported that high-dose gestagen therapy is sometimes accompanied by loss of responsiveness of endometrial adenocarcinoma.²⁰ Solid, poorly differentiated subpopulations often appear in such cases and are unresponsive to progestins because of the lack of PR.²¹ Accordingly, tumor cell heterogeneity may have been responsible for the resistance of Ishikawa tumors to progestins in the current study.

Another notable finding in the current study was that the effective dose of dienogest for suppression of both Ishikawa and MCF-7 tumors was far lower than that of MPA or TAM, which should contribute to fewer adverse effects in clinical use.

Although the molecular basis of the anticancer action of dienogest is still under investigation, we conclude that this drug may be a useful therapeutic agent

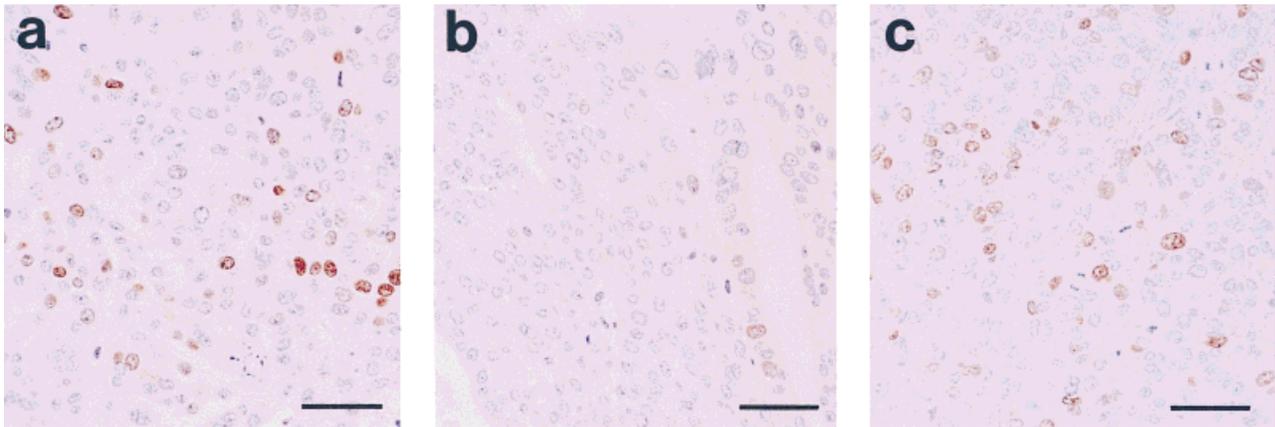


FIGURE 8. Representative histologic appearance of nuclei labeled with 5-bromodeoxyuridine in Ishikawa tumors from the E₂-plus-vehicle group (a), the E₂-plus-dienogest (0.1 mg/kg/day, orally) group (b), and the E₂-plus-medroxyprogesterone acetate (100 mg/kg/day, orally) group (c). Nuclei were counterstained with hematoxylin (bar = 50 μ m).

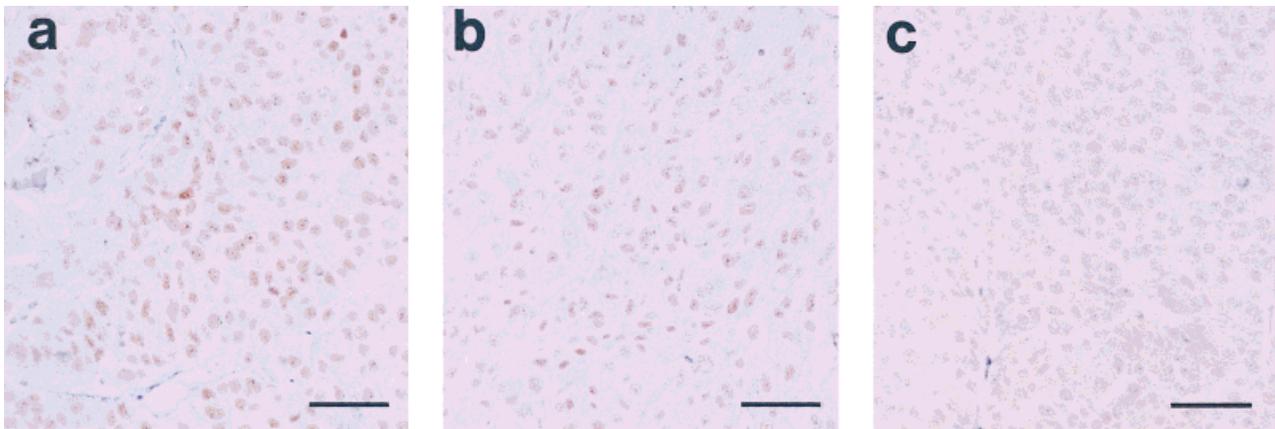


FIGURE 9. Representative histologic appearance of progesterone receptor-positive nuclei in Ishikawa tumors from the E₂-plus-vehicle group (a), the E₂-plus-dienogest (0.1 mg/kg/day, orally) group (b), and the E₂-plus-medroxyprogesterone acetate (100 mg/kg/day, orally) group (c). Nuclei were counterstained with hematoxylin (bar = 50 μ m).

not only for hormone-dependent PR-positive tumors but also for tumors lacking PR that have been more refractory to available therapy.

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