2-Methoxyestradiol, a Promising Anticancer Agent

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Estrogens occurring naturally in the body are metabolized to catecholestrogens (2- and 4-hydroxyestradiol) by the cytochrome P450 enzymes. 2-Hydroxy catecholestrogens are further metabolized by catechol-*O*-methyltransferase to 2-methoxyestradiol, which is known to be protective against tumor formation. 2-Methoxyestradiol exhibits potent apoptotic activity against rapidly growing tumor cells. It also possesses antiangiogenic activity through a direct apoptotic effect on endothelial cells. Other molecular mechanisms, including microtubule stabilization by inhibition of the colchicine-binding site, have been reported. The exact mechanism of action of 2-methoxyestradiol is still unclear, but it has been shown to be effective in preventing tumor growth in a variety of cell lines. 2-Methoxyestradiol also possesses cardioprotective activity by inhibiting vascular smooth muscle cell growth in arteries. It has a lower binding affinity for estrogen receptor α compared with that of estradiol, and its affinity for estrogen receptor β is even lower than that of estrogen receptor α, thus it has minimal estrogenic activity. 2-Methoxyestradiol is distinct because of its inability to engage estrogen receptors as an agonist, and its unique antiproliferative and apoptotic activities are mediated independently of estrogen receptors α and β. A phase I clinical trial of 2-methoxyestradiol 200, 400, 600, 800, and 1000 mg/day in 15 patients with breast cancer showed significant reduction in bone pain and analgesic intake in some patients, with no significant adverse effects. Another phase I study of 2-methoxyestradiol 200–1000 mg/day in combination with docetaxel 35 mg/m^2/week for 4–6 weeks performed in 15 patients with advanced refractory metastatic breast cancer showed no serious drug-related adverse effects. A phase II randomized, double-blind trial of 2-methoxyestradiol 400 and 1200 mg/day in 33 patients with hormone-refractory prostate cancer showed that it was well tolerated and showed prostate specific antigen stabilizations and declines. We have started a phase I clinical trial to explore dosages greater than 1000 mg/day.

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Conclusions

Estrogens are produced and secreted mainly by the ovaries and partly by the adrenals in the female body. Androgens are common precursors of estrogens. Androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase.[1, 2] Testosterone is rapidly converted to estradiol by demethylation at the C-19 position followed by aromatization, which can be performed by both aromatase and 17β-hydroxysteroid dehydrogenase.[3] Other than estradiol, the ovary secretes estrone, which serves as a source for estradiol. 17β-Hydroxysteroid dehydrogenase type 1 primarily catalyzes the reduction of estrone to estradiol,[4] whereas 17β-hydroxysteroid dehydrogenase type 2 primarily catalyzes the conversion of estradiol to estrone.[4]

The other pathway that plays an important role in estrogen biosynthesis is the peripheral conversion of C-19 precursors (e.g., androgens) in the adipose tissue to free estrogens in obese women and men.[5, 6] Estriol is considered to be a peripheral metabolite of estradiol and estrone and is considered a detoxification or inactivation product since it is less active than estrogen. 2-Methoxyestradiol is a metabolite of estradiol-17β, which is produced by sequential hydroxylation and *O*-methylation of estradiol at the 2 position.

Metabolism of Estrogens

The metabolism of endogenous estradiol primarily involves cytochrome P450 (CYP)-dependent hydroxylations at C-2, -4, or -16, yielding either 2- or 4-hydroxyestradiol estrogens, or 16α-hydroxyestradiol. The 2-hydroxylation of estradiol and estrone, catalyzed by CYP1A2 and CYP3A enzymes,[7–9] is the major metabolic pathway in the liver[10] compared with the 4-hydroxylation, which is catalyzed by the CYP3A enzyme. Catechol-*O*-methyltransferase (COMT) catalyzes the *O*-methylation of catecholestrogens[11, 12] by transferring a methyl group from the cofactor S-adenosyl methionine to the 2-OH or 4-OH groups and forms 2-methoxyestradiol and 4-methoxyestradiol. This enzyme is ubiquitous, found in many tissues including the uterus,[13] liver, kidney, breast, lymphocytes, and erythrocytes. Another important function of COMT is the *O*-methylation of endogenous catecholamine and catechol drugs such as levodopa and methyldopa.[14, 15] The 4-OH estradiol undergoes metabolic oxidation-reduction cycling to generate free radicals such as superoxides, and the chemically reactive estrogen semiquinone-quinone intermediates may damage DNA and other cellular components, induce cell transformation, and initiate tumorigenesis.[16, 17] The quinones-semiquinones can form conjugates with glutathione, catalyzed by glutathione S-transferase, or be reduced to catechol estrogens by quinone reductase. DNA adducts can result from these reactive metabolites if the inactivating conjugative pathways are incomplete or absent. The 4-OH estradiol has been associated with kidney cancer in Syrian male hamsters.[18]

The role of 16α-hydroxyestradiol in carcinogenesis has not been clearly established. It is produced in abundance during pregnancy, and women who have been pregnant have a reduced risk of breast cancer. Results of two epidemiologic studies suggest that the amount of 16α-hydroxylation is greater in women with breast cancer[19] and in women with a high familial risk of breast cancer[20] than in control subjects. However, a third study found no elevation of 16α-hydroxylation in patients with breast cancer compared with that in control subjects.[21]

Steroid sulfation is another important biotransformation step involved in the regulation of tissue-specific estrogen levels. All the hydroxylated metabolites mentioned above undergo hydroxylation by means of the estrogen sulfotransferase SULT1A1, a cytosolic enzyme that also sulfates estradiol and estrone. Estrone sulfatase, a membrane-bound enzyme, is responsible for the desulfation of estrone sulfate and exhibits highest activity in the liver.[22] Thus, the sulfated form of estrogen serves as the biologic "storage form" of the steroid, and desulfation regenerates the biologically active form. Therefore, functionally significant genetic polymorphisms within these sulfotransferases and sulfatases may be a noteworthy risk factor in the development of estrogen-dependent cancers.

Results of previous studies indicate that the uridine 5′-diphosphate–glucuronosyltransferase (UGT)1A1, 1A3, and 2B family of isoenzymes catalyze catechol estrogen glucuronidation.[23, 24] When the glucuronidation kinetics of expressed human UGT enzymes with catechol estrogens was tested, it was found that UGT2B6(Y) reacted with a higher efficiency toward 4-hydroxy-estrogenic catechols, whereas UGT1A1 and UGT1A3 showed higher activities toward 2-hydroxyestrogens.[25] Deconjugation of estrogen glucuronides also occurs and may be an important source of both primary estrogens and

their catechol metabolites in male Syrian hamster kidney.[26] Figure 1 provides an overview of the metabolism of estrogens.

Antitumor Activity of 2-Methoxyestradiol

The antitumor activity of 2-methoxyestradiol is thought to be due to the following components: antiproliferative and antimetastatic activity, and antiangiogenic activity.

Antiproliferative and Antimetastatic Activity

2-Methoxyestradiol inhibits the growth of pancreatic cancer cell lines (PaTu 8902, 8988t, and 8988s) by 50–90% in a dose- and time-dependent fashion.[27] Also, studies in an in vivo murine lung metastasis model showed that 2-methoxyestradiol inhibited the number of lung colonies by 60% as compared with that of controls.[27]

Xenograft experiments found that 2-methoxyestradiol targets both the tumor and the tumor vasculature. Oral administration of 2-methoxyestradiol was effective at reducing the rate of xenograft tumor growth and tumor vascularization in mice in the absence of significant toxicity. After 2 weeks of treatment with 100 mg/kg/day, the tumor growth of MethA sarcoma and B16 melanoma cells was inhibited by 66% and 88%, respectively.[28] A concomitant reduction in tumor vascularization was observed as well.[28] Also interesting is that the antitumor activity is not estrogen receptor dependent.[29] Estrogen receptor–negative human breast cancer line MDA-MB-435 was found to be sensitive to 2-methoxyestradiol treatment. 2-Methoxyestradiol has 500- and 3200-fold lower affinity than that of estradiol for estrogen receptors α and β, respectively, and the antiproliferative activities of 2-methoxyestradiol do not require estrogen

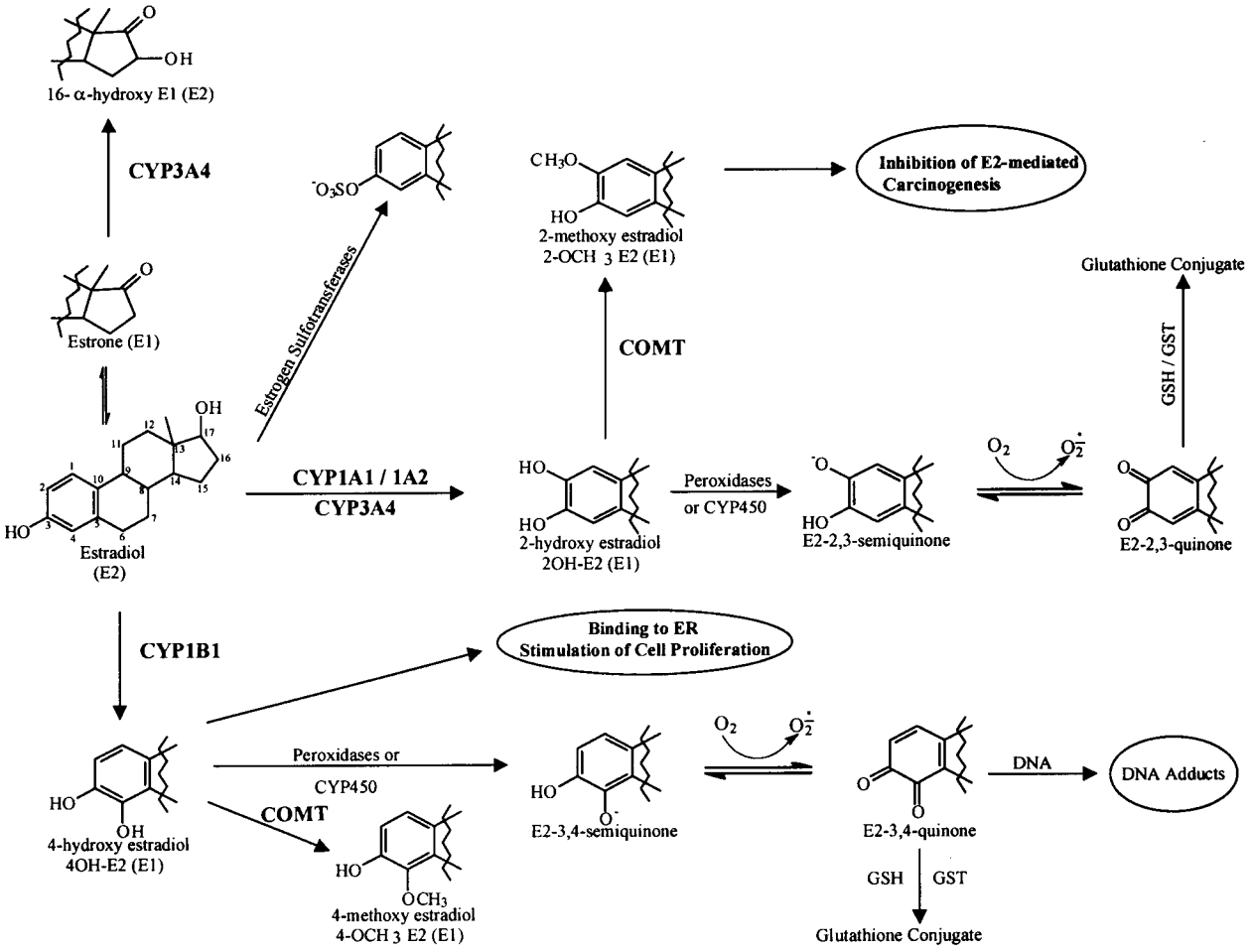

Figure 1. Biotransformation scheme for estrogen metabolism and the role of various metabolites in estrogen-induced carcinogenesis. E1 = estrone; E2 = estradiol; CYP = cytochrome P450; COMT = catechol-*O*-methyltransferase; GSH = glutathione; GST = glutathione S-transferase; ER = estrogen receptor.

receptors and are not influenced by estrogen receptor antagonists or agonists.[30] Table 1 shows a comprehensive list of the cell lines that are inhibited by 2-methoxyestradiol.

Antiangiogenic Activity

Angiogenesis has been shown to be a critical step in the proliferation of cancers that are larger than 1 mm^3.[42, 43] 2-Methoxyestradiol reduced tumor vasculature in mice injected subcutaneously with MethA sarcoma and B16 melanoma cells and treated orally with 2-methoxyestradiol.[28, 29] In vivo antiangiogenic activity of 2-methoxyestradiol has been demonstrated in the corneal micropocket[29] and chick chorioallantoic model systems.[41] In the corneal micropocket, neovascularization induced by vascular endothelial growth factor and basic fibroblast growth factor was reduced by 54% and 39%, respectively. In the chorioallantoic model, 2 µmol/L of 2-methoxyestradiol exposure for 3 days completely prevented basic fibroblast growth factor–induced angiogenesis. In vitro, 2-methoxyestradiol was found to inhibit the neovascularization developing from the rat aortic ring assay.[44, 45] 2-Methoxyestradiol appears to affect the angiogenesis cascade at various steps. It also blocks the tubule formation, as well as the invasion through the collagen matrix.[1] In a human neuroblastoma xenograft model in mice, 2-methoxyestradiol was found to reduce tumor growth significantly after 14 days of treatment and showed clear antiangiogenic and apoptotic effects.[46]

No difference in the number of microvessels (CD 31 staining) within MIA PaCa (pancreatic cancer) pulmonary metastases was noted in those animals treated with 2-methoxyestradiol 1 mg/day versus controls.[27] Hence, ambiguity exists with regard to the exact mechanism of antiangiogenic properties of 2-methoxyestradiol. Although 2-methoxyestradiol has been labeled as an antiangiogenic, endothelial cells are not necessarily more sensitive to its antiproliferative effects. The antiangiogenic and apoptotic activities of 2-methoxyestradiol vary with cell type and the regulatory microenvironment.[47] For some murine tumor models, administration of oral 2-methoxyestradiol reduces the rate of tumor growth without signs of toxicity, with a concomitant reduction in tumor vascularization.[28] Thus, sufficient data exist to establish that 2-methoxyestradiol effectively inhibits angiogenesis, but it is still difficult to isolate the exact mechanism of action.

Table 1. Cell Lines Inhibited by 2-Methoxyestradiol

Cell Type	Inhibitory Concentrations (µmol/L)
Human tumor	
Lung (HOP-62)[31]	0.7
Lung (H460)[32]	5.0
Lung (A549)[32]	5.0
Colon (HCT-116)[32]	0.47
Central nervous system (SH-SY5Y)[31]	1.3
Central nervous system (SF-539)[31]	0.32
Melanoma (UACC-62)[31]	0.36
Ovarian (OVCAR-3)[31]	0.21
Renal (SN12-C)[31]	0.95
Prostate (DU-145)[31]	1.8
Breast (MDA-MB-435)[29, 33]	0.08–0.61
Breast (MDA 231)[33]	1.03
Breast (MCF-7)[33]	0.45
Lymphoblast (Jurkat)[34]	0.3
Lymphoblast (TK6)[35]	1–2
Lymphoblast (WTK1)[35]	1–2
Human nontumor	
Skin fibroblast (HFK2)[28]	2.0
Human endothelial	
HUVEC[33]	0.45
Nonhuman tumor	
Lung (Lewis lung, murine)[33]	1.68
Melanoma (B16BL6, murine)[33]	0.4
Melanoma (B16F10, murine)[33]	0.3
Endothelial (EOMA, murine)[33]	0.89
Endothelial(H5V, murine)[36]	1.0
Nonhuman nontumor	
Lung (V79, hamster)[37]	3
Ovarian (granulose, porcine)[38]	3
Smooth muscle (aorta, rabbit)[39]1	
Adipocytes (murine)[40]	1.7
Nonhuman endothelial	
Brain capillary (bovine)[29]	0.19–0.49
Pulmonary artery (bovine)[41]	0.5

Adapted from reference 33.

Mechanisms of Action

The above-mentioned antitumor activities of 2-methoxyestradiol result from the following mechanisms of action: apoptotic activity, microtubule activity, and production of superoxides.

Apoptotic Activity

2-Methoxyestradiol appears to induce caspase-3 activation and causes apoptosis in gastric carcinoma cell lines (SC-M1 and NUGC-3) through the caspase activation cascade, resulting in G_2-M cell cycle arrest.[35] When these gastric carcinoma cell lines were treated with 2-methoxyestradiol for 24 hours, the result was a reduction of cell viability in a dose-dependent

and a time-dependent fashion. Moreover, the caspase cascade induced by 2-methoxyestradiol, which is believed to be the major mechanism causing apoptosis, begins with the activation of caspase-8 followed by caspase-3 and eventually induces DNA fragmentation. 2-Methoxyestradiol–induced apoptosis requires caspase activation, and the sequential activation of caspase-8, -9, and -3 is consistent with the triggering of apoptosis through the membrane-bound receptor.[48] Flow cytometry established 2-methoxyestradiol–induced G_2-M cell cycle arrest.

In some studies, the amount of apoptosis detected was correlated with the antiproliferative activity of 2-methoxyestradiol. Apoptotic cell death induced by 2-methoxyestradiol was shown to be p53 mediated.[32] Increased functional activity of p53 has been observed after treatment with 2-methoxyestradiol. The number of metastatic lung colonies was found to be synergistically reduced in mice treated with 2-methoxyestradiol 50 mg/kg/day in combination with adenoviral p53.[49] However, several other reports indicate that the induction of apoptosis by 2-methoxyestradiol may be p53 independent.[27, 50] 2-Methoxyestradiol treatment results in upregulation of death receptor 5 expression both in vitro and in vivo.[48] Moreover, blocking death receptor signaling by expression of dominant-negative FADD severely attenuates the ability of 2-methoxyestradiol to induce apoptosis.

Microtubule Activity

A clear increase in the mitotic figures has been demonstrated in cells treated with 2-methoxyestradiol during the metaphase.[34, 37] In endothelial cells, 2-methoxyestradiol caused selective disruption of microtubules as opposed to other cytoskeletal structures.[50] Also, micromolar amounts caused disruption of the microtubular network in nonsynchronized Chinese hamster V79 cells[37] and multipolar and irregular spindles in synchronized MCF-7 cells. On the contrary, 2-methoxyestradiol was found to inhibit aromatase activity, which is a characteristic observed with drugs that stabilize tubulin and not with agents that inhibit tubulin polymerization.[51]

2-Methoxyestradiol, in superstoichiometric concentrations, inhibits the nucleation and propagation phases of tubulin assembly but does not affect the reaction extent.[52] Similarly, in substoichiometric concentrations, 2-methoxyestradiol completely inhibits polymerization. 2-Methoxyestradiol inhibits the rate, but not the degree, of polymerization and depolymerization of tubulin induced by other agents. The inhibition of colchicines binding to tubulin was found to be competitive in nature (inhibitory constant 22 µmol/L). Thus, it is speculated that 2-methoxyestradiol binds to the colchicine site of tubulin and, depending on the reaction conditions, either inhibits assembly or seems to be incorporated into a polymer with altered stability properties.[52]

Production of Superoxides

Two contradictory reports have been published on this subject. In the first report,[53] the authors reported that 2-methoxyestradiol and 2-hydroxyestradiol inhibit both copper-zinc and manganese superoxide dismutases and are toxic to human leukemia cells. In the other report,[54] the authors showed that when human leukemia HL-60 cells were incubated in a medium containing 2-methoxyestradiol, cells showed 53% decrease in aconitase activity (aconitases are very sensitive to superoxide-mediated inactivation) compared with that of controls. Paraquat (a superoxide-generating oxidation-reduction cycling agent) decreased aconitase activity in HL-60 cells by 67%. The authors of this second study showed that although 2-methoxyestradiol supports the increased production of superoxides, it does not produce that effect by inhibiting superoxide dismutase.

Cardioprotective Effects

It has been known for years that estradiol may protect premenopausal women against coronary artery disease.[50] Recent studies hint at the possibility of these cardioprotective effects being independent of the estrogen receptor status.[55, 56] 2-Hydroxyestradiol and 2-methoxyestradiol are two metabolites of estrogen that possess little or no affinity for estrogen receptors. Recent studies also indicate that these two metabolites, 2-hydroxyestradiol and 2-methoxyestradiol, are more potent than estradiol in preventing vascular smooth muscle cell growth.[4]

When the local metabolism of estradiol to methoxyestradiols was tested in human vascular smooth muscle cells,[57] the authors reported that methoxyestradiols and their precursors, hydroxyestradiols, are more potent than estradiol in inhibiting vascular smooth muscle cell growth, that the inhibitory effects of estradiol on vascular smooth muscle cell growth are enhanced by CYP

inducers, and that the inhibitory effects of estradiol both in the presence and absence of CYP inducers are abolished by CYP and COMT inhibitors.

Estrogenic Activity

2-Methoxyestradiol has 500- and 3200-fold lower affinity than that of estradiol for estrogen receptors α and β, respectively.[30] When administered continuously at 1 µg/hour to oophorectomized rats, 2-methoxyestradiol was ineffective in sustaining uterine growth and failed to induce estrus. In contrast, 2-hydroxyestrone, 4-methoxyestrone, and estradiol had a strong uterotropic activity.[27] With long-term administration, 2-methoxyestradiol did not affect the seminal vesicle weights, whereas estradiol significantly reduced the seminal vesicle weights in male mice. Similarly, no tumors were reported in male Syrian hamsters receiving long-term exposure to 2-methoxyestradiol compared with estrogens, which caused the induction of renal tumors.

Estradiol, 4-hydroxyestradiol, and 2-hydroxyestradiol were found to sustain tumor growth of an estrogen-dependent hamster kidney tumor cell line, H-301 cells.[28] Also, MCF-7, which is an estrogen-dependent cell line, showed sustained proliferation of these cells at a low concentration of estradiol (0.1 nmol/L), whereas concentrations up to 10 µmol/L of 2-methoxyestradiol were not able to sustain the proliferation of these cells.[58]

However, high doses of 2-methoxyestradiol administered orally to rats daily for 14 and 28 days and to dogs treated for 28 days showed estrogenic activity.[58] It is speculated that this estrogenic activity may be due to the formation of 2-hydroxyestradiol and possibly from other not yet identified metabolites of 2-methoxyestradiol.

Clinical Experience

There are numerous 2-methoxyestradiol clinical trials under way in the United States. A clinical trial being conducted at Indiana University (Indianapolis) has reported no grade 4 toxicity and only minor grade 3 toxicity, which could be attributed to disease progression,[59] after the administration of 200, 400, 600, 800, and 1000 mg/day to a total of 15 patients. The elimination half-life of the drug was reported to be 10 hours. Some patients showed clinically significant reduction in bone pain and analgesic intake while receiving 2-methoxyestradiol. All patients receiving 1000 mg (three patients) experienced hot flashes.

In a phase II multicenter, randomized, double-blind trial of two dosages (400 and 1200 mg/day) of 2-methoxyestradiol in 33 patients with hormone-refractory prostate cancer, 2-methoxyestradiol was very well tolerated and prostate specific antigen stabilization and declines were observed.[60] In a small number of patients, grades 2 and 3 liver function abnormalities were observed, but liver function returned to normal rapidly after discontinuation of 2-methoxyestradiol. Analysis of growth factor data indicated that there was an initial increase in the levels of plasma vascular endothelial growth factor and basic fibroblast growth factor from baseline to month 1, followed by a decrease of 40% and 55%, respectively, from month 1 to month 3 on study.

A phase I study of 2-methoxyestradiol plus docetaxel in 15 patients with metastatic breast cancer was performed at Indiana University.[61] Docetaxel at 35 mg/m^2 was administered weekly for 4 of 6 weeks, and 2-methoxyestradiol 200-1000 mg/day was given orally once/day for 28 days followed by a 13-day observation period in cycle one, then continuously thereafter. After a maximum of six cycles of combined therapy, responding or stable patients continued to receive 2-methoxyestradiol alone until progression. There was no grade 4 toxicity. Grade 3 fatigue, diarrhea, and hand-foot syndrome occurred in five patients, four patients, and 1 patient, respectively. Three patients had grade 3 aminotransferase elevations that returned to normal with continued treatment. 2-Methoxyestradiol did not alter docetaxel clearance or dose-normalized area under the curve. Extensive metabolism to 2-methoxyestrone was observed. 2-Methoxyestradiol trough and peak levels were not altered by concurrent docetaxel administration.

A phase I clinical trial is under way at the National Cancer Institute (Bethesda, MD) in which 2-methoxyestradiol is being evaluated in patients with histologically confirmed solid tumors. One of the objectives of the study is to assess changes in apoptosis on biopsy specimens of endothelial cells or tumor cells by using a terminal deoxynucleotidyl transferase–mediated deoxy uridine triphosphate nick end labeling (TUNEL) assay. The rat aorta model for assessing angiogenesis activity will be used as a bioassay to determine angiogenesis inhibition with patient plasma. Other objectives of the

study include measuring changes in specific circulating growth factors (tumor necrosis factor α, tumor growth factor β, basic fibroblast growth factor, and vascular endothelial growth factor), alterations in the expression of these growth factors and/or receptors in biopsy tissues, and changes in microvessel counts through immunostaining for factor VIII and/or CD34.

Conclusions

2-Methoxyestradiol is a physiologically occurring steroid that shows considerable promise as an antitumor agent. Oral administration in animals has shown little or no toxicity at therapeutic effective doses. 2-Methoxyestradiol does not display considerable estrogenic activity at clinically efficacious doses and does not seem to promote carcinogenesis. Moreover, it has been found to be orally active at inhibiting tumor growth in preclinical models.

2-Methoxyestradiol has been administered in patients with metastatic breast cancers and prostate cancers and has shown therapeutic potential. It is metabolized mainly to the 2-methoxyestrone form. No serious toxicities have been reported; although hot flashes, fatigue, diarrhea, and reversible liver enzyme elevations have occurred.

The exact mechanism by which 2-methoxyestradiol inhibits tumor cell growth is still not completely clear, and several possible molecular mechanisms and targets have been suggested. Nevertheless, we can conclude from all the studies that 2-methoxyestradiol shows considerable promise as a therapeutic alternative in various solid tumors.

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