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2-Fluoroestradiol

Separation of Estrogenicity from Carcinogenicity

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SUMMARY

Estrogenic and carcinogenic activity are shown to be separable properties. 2-Fluoroestradiol, a modified estrogen, did *not* induce renal clear-cell carcinoma in male Syrian hamsters despite its estrogenic potency, which is comparable to that of estradiol. 4-Fluoroestradiol, also a potent estrogen, did induce renal clear-cell carcinoma in this animal model, but more slowly than estradiol. 2,4-Dideuterioestradiol was found to be as estrogenic and also as carcinogenic as estradiol itself.

Prolonged treatment of experimental animals with exogenous estrogens has been reported (1, 2) to induce tumors of the testes, cervix and uterus, lymphoid tissues, mammary tissue, adrenal cortex, kidney, and anterior pituitary gland. The exact mechanism of tumor induction by these hormones is unknown. It has been proposed (3, 4), however, that the ring-A hydroxylated estrogens, the primary products of estradiol metabolism (5), although almost devoid of estrogenic activity, could be responsible for the carcinogenic action of estradiol. These metabolites, 2- and 4-hydroxyestradiol and 2- and 4-hydroxyestrone, are the major biotransformatory products of estradiol in most species (5) and are known to bind covalently to protein or peptides (6–8) and to DNA (9) *in vitro*. Such binding to protein can be suppressed by prior methylation of the catechol using *S*-adenosylmethionine-*o*-catechol-*O*-methyltransferase, i.e., conversion to a less reactive methoxyestradiol or methoxyestrone (10).

If metabolic activation to a catechol is indeed a necessary step in the chain of events resulting in tumors, then interference with metabolic oxidations should inhibit carcinogenesis. This concept was tried successfully by Li and Li (11), who were able to inhibit diethylstilbestrol-induced renal carcinoma in Syrian hamsters by chronic administration of inhibitors of microsomal cytochrome P-450 enzymes. Another approach to prevention of carcinogenesis would be the use of estrogens modified to prevent oxidation to carcinogenic catechol estrogens. Biotransformation reactions can indeed be influenced by appropriate chemical substituents (12). The metabolic pathways of caffeine, antipyrene, methsuximide (13), and cyclophosphamide (14) were altered by replacing hydrogen with deuterium at defined sites of the drug molecule. Furthermore, replacement of hydrogen with fluorine in positions prone to oxidation has been successfully used (15) to make modified ecdysone insect hormones which were not metabolically deactivated when administered to insects.

Deuterium or fluorine as the metabolism-inhibiting substituent is an appropriate choice for modifying estrogens, since either element can be substituted for hydrogen with little or no change in the conformation of the molecule. This ensures recognition of the modified estrogens by hormone receptors and, therefore, their retained estrogenic activity.

To test the possible prevention of estrogen-induced carcinogenesis, 2-fluoroestradiol, 4-fluoroestradiol, and 2,4-dideuterioestradiol were synthesized according to published procedures (16, 17) and their biological activities were tested. 2,4-Dideuterioestradiol (after trimethylsilylation) was analyzed by gas chromatography/mass spectrometry and found to be chromatographically pure. The mass spectrum was identical with that of the trimethylsilyl derivative of estradiol except that masses were shifted by 2 a.m.u. The following isotopic purity was observed: 92% of 2,4-dideuterioestradiol, 6% of monodeuterioestradiol, and 2% of estradiol. The purity of the two fluorinated estradiols and their structural identities were checked by mass spectrometry. The spectrum of 4-fluoroestradiol was found to be identical with that of an authentic sample obtained from the Drug Synthesis and Chemistry Branch, National Institutes of Health (Bethesda, Md.). The spectrum of 2-fluoroestradiol was that of a pure compound and was characteristic of the structural features of this material. Each of the fluorinated estradiols was prepared in a seven-step synthesis. The purity and the structural identity of each of the precursor compounds were also checked by mass spectrometric analysis. At the end of the biological experiments, when the hamsters were killed, the remains of the estrogen implants were excised, and the contents were extracted

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into methanol and analyzed by gas chromatography/mass spectrometry (after trimethylsilylation). The analysis confirmed the composition of the pellet material: the corresponding fluoroestradiol and cholesterol.

The estrogenicity of the modified estrogens was measured by using two different methods. The most common method, increase in uterine wet weight in ovariectomized, immature rats upon administration of estrogen, showed that the fluorinated estrogens are approximately as estrogenic as estradiol (Table 1). Furthermore, the weights of both testes of the male Syrian hamsters, which were used in the *in vivo* carcinogenicity assay, were measured on the 224th day after s.c. implantation of 25-mg pellets of estrogen. The weights were measured in grams and expressed as percentage of total body weight. Treatment of animals with modified estrogens or with estradiol resulted in the shrinking of the testes to approximately 10% of the weights of testes in untreated hamsters. The estrogenicity measurements carried out in our laboratory compare well with experiments reported by Heiman *et al.* (18) and Katzenellenbogen *et al.* (19). These authors reported the estrogen receptor binding affinity of the fluorinated estrogens. When expressed as ratios of association constants ($K_{a\ \text{compound}}/K_{a\ \text{estradiol}}$) × 100, the binding affinity of 2-fluoroestradiol was found to be 86; that of 4-fluoroestradiol was 128. In this procedure, the binding affinity of estradiol was defined as 100. These data confirmed the reported findings that 4-fluoroestradiol was slightly more estrogenic, and 2-fluoroestradiol slightly less estrogenic, than estradiol. In more recent experiments, Pfeiffer *et al.* (20) measured the estrogen receptor affinity of 2- and 4-fluoroestradiol in cytosol of the hypothalamus-preoptic area, the pituitary, and the uterus. The fluorinated estradiols were found to have high receptor affinity. Furthermore, the biological responses to the fluorinated estrogens (such as elicitation of luteinizing hormone surges, of proceptivity, and of lordosis behavior) in rats were comparable with those elicited by estradiol (21, 22).

TABLE 1

Estrogenic activity of modified estrogens

Compound	Male hamsters, testis wt[a]	Female rats, uterine wet wt[b]
	% total body wt	*mg*
2-Fluoroestradiol	0.18–0.25	92 ± 20
4-Fluoroestradiol	0.18–0.28	118 ± 19
2,4-Dideuterioestradiol	0.19–0.24	nd
Estradiol	0.12–0.26	114 ± 20
Control	1.85–2.06	33 ± 6

[a] Data shown were recorded from the same animals used in the carcinogenicity testing (Table 2). The estrogen treatment of these animals is described in Footnote *a*, Table 2. Two hundred and twenty-four days after treatment with estrogens began, hamsters were weighed and killed; both testes were excised and their weights were measured in grams. Data presented are the ranges of testis weights from four animals in each group.

[b] Female Sprague-Dawley rats, 3–4 weeks of age, were ovariectomized. Four days after ovariectomy the animals were separated into four groups of equal group weight (17 animals/group, average weight 90 ± 1 g/animal), and estrogen treatment was begun. Each animal received an s.c. injection of estrogen (3.5 μg of estrogen in 0.7 ml of saline per animal) once daily for 3 days. Saline solutions were prepared by dissolving the estrogen in ethanol and diluting this solution with saline until a mixture of 5% ethanol/95% saline was obtained. The control group received only saline. On the 4th day, the animals were killed, and the uteri were excised, freed of luminal fluid, and weighed. Data are averages of uterine wet weights ± standard deviations.

The carcinogenicity of the modified estrogens was measured in male Syrian hamsters *in vivo*. This tumor model was well described by Kirkman (23), and was later investigated by McGregor *et al.* (24), by Sirbasku and Kirkland (25), and by this laboratory.[1] Estrogen, and estrogen alone, causes renal clear-cell carcinoma in 100% of the male animal population when exposed to s.c. implants of this hormone (23). This kidney tumor is estrogen-induced and estrogen-dependent for development and growth (23–25). Failure to resupply estrogen every 3 months or surgical removal of the implants resulted (23, 24) in tumor regression within a few weeks. Similarly, a tumor cell line derived from the Syrian hamster renal clear-cell carcinoma was found (4, 25) to be strongly estrogen-dependent *in vivo*, showing 90% tumor regression 10 days after removal of the source of estrogen. The incidence of spontaneous kidney tumor in Syrian hamsters without estrogen treatment is very low (23).

Male hamsters (15–20 animals/group) received implants of estrogen (Table 2) and were resupplied with pellets of the same composition approximately 3 months later. Two hundred and twenty-four days after the beginning of the experiment, four animals per group were killed and their kidneys were examined histologically for the occurrence of renal clear-cell carcinoma. Whereas all of the animals treated with estradiol or with 2,4-dideuterioestradiol had developed visible renal tumors, none of the animals treated with 2-fluoroestradiol showed evidence of kidney tumor induction (Table 2). Histologically, kidneys from this group were indistinguishable from kidneys of totally untreated animals. Among the four animals treated with 4-fluoroestradiol, one had small tumors and the kidneys of two animals were judged to be preneoplastic.

Similar results were obtained when, 279 days after the initial pelleting with estrogens, a second group of male hamsters was examined for renal clear cell carcinoma. Again, all animals treated with estradiol (the positive control in this experiment) had renal tumors. All animals treated with 2,4-dideuterioestradiol showed large, multiple tumor nodules on their kidneys. Therefore, all animals treated with this deuterated estrogen were killed at this time, since the carcinogenic activity of this compound was not different from that of estradiol itself. None of the five animals treated with 2-fluoroestradiol showed any kidney neoplasms. Three of six animals with 4-fluoroestradiol implants showed renal clear-cell carcinoma. Again, these results were confirmed by histological examination.

The remaining animals were killed 345 days after the initial s.c. implantation of estrogen pellets and their kidneys were excised and examined for the occurrence of renal clear-cell carcinoma. By visual inspection, none of the animals had visible tumors; i.e., not even animals treated with estradiol showed renal neoplasms. The histological examination of these kidneys revealed small,

[1] J. G. Liehr, unpublished results.

TABLE 2

In vivo carcinogenicity of modified estrogens

Treatment[a] (no. of animals)	No. of animals lost or dead[b]	No. of animals with tumors[c] (no. of animals examined) on various days after s.c. implantation of estrogen		
		224 days	279 days	345 days
Estradiol (18)	5	4 (4)	5 (5)	0 (4)
2,4-Dideuterioestradiol (20)	3	4 (4)	13 (13)	—
2-Fluoroestradiol (15)	3	0 (4)	0 (5)	0 (3)
4-Fluoroestradiol (15)	2	1 (4)	3 (6)	3 (3)
Control (10)	0	0 (3)	0 (3)	0 (4)

[a] Male Syrian hamsters, 3–4 weeks of age (Harlan/Sprague-Dawley, Madison, Wisc.), were given a 25-mg s.c. implant consisting of 10% cholesterol and 90% estrogen. A second 25-mg pellet of the same composition was implanted 106 days after the initial estrogen treatment. A lack of fluorinated estrogens prevented repelleting of the animals approximately 6 months after the initial estrogen treatment. Estrogen treatment was carried out according to the procedure of Kirkman (23). Control animals were left untreated.

[b] Number of animals lost or dead from various causes during the course of the experiment. Tumors appear approximately 6 months after pelleting with estrogen (23, 25). Therefore, this group included all hamsters found dead during the first 6 months of the experiment, since tumors were not expected and not evident in the kidneys of these animals. Hamsters found dead after 6 months were also included in this group, if their kidneys could not be recovered. Two dead hamsters with recoverable kidneys were included in the nearest examination group at 279 days (one animal treated with 2,4-dideuterioestradiol with tumorous kidneys and one animal treated with 4-fluoroestradiol without renal carcinoma).

[c] The hamsters were divided into three groups. The first group was examined 224 days, the second 279 days, and the third 345 days after the initial implantation of estrogen. The animals were decapitated, and the kidneys were excised and inspected visually for the occurrence of renal clear-cell carcinoma. Sections were prepared of all kidneys and studied histologically. The results of the histological examinations are tabulated.

regressed tumors in kidneys of animals treated with 4-fluoroestradiol. This last group of hamsters did not receive reimplants and so had been without estrogen treatment for approximately 7½ months; therefore any existing tumors probably had regressed, and went undetected in the histological studies. Kidney carcinomas in 4-fluoroestradiol-treated hamsters did not regress completely, probably because of the longer-lasting estrogenic activity of this modified estrogen. Since in this third group none of the estradiol-treated hamsters (the positive control in this experiment) showed evidence of carcinomas, the significance of the results obtained 345 days after the beginning of the experiment remains very questionable. In the evaluation of the carcinogenic activity of fluorinated estrogens, the data obtained at this third time point are not useful owing to the lack of a positive control and the long time period (7½ months) between the last implantation of estrogens and the day of sacrifice.

The clear and unquestionable results from the first two kidney examinations described above establish 2-fluoroestradiol as highly estrogenic yet noncarcinogenic in the animal model used. 4-Fluoroestradiol was carcinogenic with a longer induction period for renal tumors than was established for estradiol. The separation of estrogenic from carcinogenic action, reported for the modified estrogen above, shows that these properties are not intrinsically associated with each other but depend on the chemical and structural characteristics of the hormone molecule. The noncarcinogenic estrogens may be used as a tool with which to study the promotion of estrogen-dependent tumors separately from the tumor-inducing properties of estrogens.

The mechanism of action of the noncarcinogenic estrogens is yet unknown. Since the C—F bond is among the strongest bonds known to organic chemists (26), it is suspected that prevention of tumorigenesis is due to the inability of oxidizing enzymes to cleave the C—F bond on fluoroestradiols and the resulting inability to form catechols. Pfeiffer *et al.* (20) and Krey *et al.* (22) tested the fluorinated estradiols for catechol formation by using rat liver microsomes and a methylation system (catechol-*O*-methyltransferase, [^{3}H]*S*-adenosylmethionine) to convert newly synthesized catechol estrogens to stable [^{3}H] methyl ethers. Considering that the enzyme preparation contained primarily 2-hydroxylases, the extremely low rate of catechol estrogen formation seen with 2-fluoroestradiol was taken as evidence for only 4-hydroxylation. The rate of catechol formation from 4-fluoroestradiol, which was still less than that seen for estradiol itself, was assumed to reflect only 2-hydroxylation.

The carcinogenic activity of the estrogens tested closely reflects their ability to be converted to catechol estrogens. The extremely low rate of conversion of 2-fluoroestradiol to catechol estrogen (20, 22) probably corresponds to the diminished carcinogenic activity of this modified estrogen. 4-Fluoroestradiol, which was carcinogenic but with a longer induction period than found for estradiol, was converted to catechols with a rate between that found for 2-fluoroestradiol and estradiol.

In analogous experiments, fluorination of polycyclic aromatic hydrocarbons at specific sites of the ring system was shown (27, 28) to result in a reduction of tumorigenicity and of dihydrodiol formation.

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