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ESTROGEN RECEPTOR-DIRECTED RADIOTOXICITY WITH AUGER ELECTRON-EMITTING NUCLIDES: E-17α-[^{123}I] IODOVINYL-11β-METHOXYESTRADIOL AND CHO-ER CELLS

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ABSTRACT

The specific radiotoxicity of the Auger-electron-emitting nuclide, I-123, covalently attached to a steroidal estrogen, E-17α-[^{123}I] iodovinyl-11β-methoxyestradiol, [^{123}I]I-VME2, was studied in Chinese hamster ovary cell lines with or without estrogen receptor (ER) from a stable transfection with ER cDNA. Using the halodestannylation reaction, I-123-labeled estrogens have been synthesized with specific activities approaching the theoretical specific activity of I-123. When incubated for 1 h with suspended cells, [^{123}I]I-VME2 caused a dose-dependent, estrogen-inhibitable radiotoxicity in the cells expressing ER. Little effect was seen in the cells lacking the transfected ER. Autoradiography confirmed the nuclear localization of [^{123}I]I-VME2, but showed a heterogeneous distribution among cells, especially at lower ligand concentrations. Estimates suggest that the mean lethal dose for this ER-directed radiotoxicity is in the range of several hundred decays per cell,

compatible with its use in ER-containing cancers which usually contain thousands of ER per cell.

INTRODUCTION

Endocrine therapies have been used for the treatment of breast cancer for nearly a century (as reviewed in 1). At present there is increased specificity in the application of endocrine therapies due to the knowledge that responding cancers contain estrogen receptors (ER) (2), but not all ER-containing cancers respond. Certain ER-containing cancers, such as ovarian carcinoma, show little response to endocrine therapies (3) and even breast and endometrial cancers, which have higher response rates, often recur despite initial response. Part of the reason for this relates to the nature of current endocrine therapies, which are cytostatic rather than cytotoxic. Thus the treatment causes shrinkage of the tumor, due to the therapy-induced reduced proliferation rate, but does not actually kill the cancer cells.

Basic research over the last several decades has led to a better understanding of the role of steroid receptors in the regulation of growth and differentiated function in a variety of tissues (4). The estrogen receptor, a member of the steroid receptor family of nuclear, DNA binding proteins (5), stimulates growth of a number of normal and neoplastic tissues. After binding estrogen the receptor dimerizes (6) and in this form associates with the specific estrogen response elements in DNA, thereby initiating new or increased RNA synthesis leading to new proteins and cell proliferation. It has been recently recognized that some of the new proteins resulting from the estrogen-receptor interaction in the cell are growth factors (7). In fact, it is believed that constitutive production of these growth factors may be responsible for the estrogen independence of some of the ER-containing, endocrine-unresponsive cancers.

However, for the purposes of our research, the important characteristic of the family of steroid receptors, and particularly estrogen receptors, is their high affinity, non-covalent association with the specific response elements in the DNA. We therefore suggested that, in view of the cytotoxic effects of Auger-electron-emitting nuclides incorporated into DNA (8,9), Auger electrons emitted from DNA-associated, ER-bound radioligands should be effective in causing double-strand DNA breaks, and be specifically radiotoxic for ER positive cells and cancers (10). On the other hand, the evidence for

substantially reduced radiotoxicity for the Auger electron cascade process occurring outside the nucleus (11) would suggest that ER negative cells should be largely insensitive to radiotoxicity with ER-directed ligands bearing Auger-electron-emitting nuclides. Some time ago it was reported that ER-dependent radiotoxicity was observed using 16α-[^{125}I]iodoestradiol with cells in tissue culture (12). However, because of the long half-life of I-125 (60 days), and the short residence time of estrogens in ER-positive cells, it was necessary to freeze the cells for several half-lives to demonstrate the radiotoxicity, shown as reduced plating efficiency as a function of the time the cells were stored frozen. Clearly, with the numbers of estrogen receptors per cell (thousands) and the average residence time of estrogens (hours) it is unlikely that I-125 would be a feasible Auger-electron-emitting nuclide for such ER-directed therapy.

It is curious, therefore that an I-125-labeled antiestrogen, iodotamoxifen, has been reported to show specific radiotoxicity in the ER-containing cell line, MCF-7, without such cell freeze-storage (13,14). Initial studies compared the survival of ER-containing MCF-7 cells with ER-negative V79 cells in culture and showed a significantly greater sensitivity of MCF-7 cells to the radiotoxicity of [^{125}I]iodotamoxifen (13). However, the D_{37} value of 0.5 pCi/cell corresponded to more than 1 million molecules per cell, more than an order of magnitude greater than the ER content of MCF-7 cells, so it was clear that the uptake was not entirely ER-mediated. Furthermore, no data were available comparing the relative radiosensitivity of MCF-7 and V79 cells in general. A second study (14) used ethanol precipitation to assess the amount of [^{125}I]iodotamoxifen associated with protein or DNA. This result is difficult to interpret as the non-covalently ER-bound ligands are usually readily extracted from ER with ethanol, even from whole cells. However, since only a small fraction of the total cell associated I-125 remained after ethanol extraction, the survival curve of MCF-7 cells related to ethanol precipitated [^{125}I]iodotamoxifen was considerably closer to that seen with [^{125}I]iododeoxyuridine, incorporated into DNA. This was encouraging to the authors. However, a recent study (15) showed that a large portion of the radiotoxicity due to [^{125}I] iodotamoxifen could not be inhibited by co-incubation with either unlabeled estradiol (suppressing ER binding) or unlabeled tamoxifen (competing for the so-called antiestrogen sites), although the unlabeled tamoxifen was the more effective inhibitor of the two. Thus it is clear that most of the radiotoxicity of [^{125}I]iodotamoxifen was not mediated through the estrogen receptor, substantially decreasing the potential of this ligand for therapy of ER+ cancers.

To circumvent these problems, we decided to study an ER-binding ligand, 17α-iodovinyl-11β-methoxyestradiol, based on the steroidal estrogen, moxestrol, which has at least an order of magnitude higher affinity for ER than does tamoxifen. In addition, we employed the 13.2 h half-life nuclide, I-123, whose short half-life should allow direct assessment of radiotoxicity in tissue culture using two closely related cell lines. The cells used are Chinese hamster ovary cells (CHO), which are believed to lack ER, and a stable transfectant, CHO-ER (16), of the same cell line expressing ER introduced from ER cDNA of MCF-7 cells. Thus we could compare the same cell with or without ER, and study radiotoxicity in the presence or absence of excess unlabeled estrogen by direct colony assay. However, well into this study we discovered that the ER cDNA introduced in these cells contained a single base change from wild type ER (16,17), resulting in ER with a lower affinity for estrogen. Despite this complication, we can report here the estrogen-receptor-specific radiotoxicity of I-123-labeled estrogen in CHO-ER cells, demonstrating the feasibility of this new approach to therapy of ER-containing cancers with Auger-electron-emitting nuclides.

MATERIALS AND METHODS

Synthesis of E-17α-[^{123}I] iodovinyl-11β-methoxyestradiol, [^{123}I]I-VME2

One hundred μg E-17α-tributylstannylvinyl-11β-methoxyestradiol in 100 μl ethanol was added to the vial containing Na[^{123}I]I in 0.1 N NaOH (supplied by Nordion International, Inc, Kanata, Ontario), followed by 100 μl of a mixture of acetic acid:H_2O_2, 100:1, which had been preincubated at room temperature for 1 h. After mixing, the reaction was allowed to proceed at room temperature for 15 min, the mixture was drawn up into a syringe and its radioactivity determined in a Capintec dosemeter. The mixture was then injected onto a 10 mm by 250 mm Econosil 10 μ C_{18} reverse phase HPLC column, pre-equilibrated with 10% acetonitrile in water. The column was eluted with a 30-min gradient of 10-100% acetonitrile at 3 ml per min, under which conditions the [^{123}I]I-VME2 elutes between 27 and 28 min, while the tin precursor elutes at 37 min. The peak fractions were combined and evaporated to a small volume. Ether and water were added and mixed, the ether removed, and the aqueous portion re-extracted with additional ether which was combined with the first extract. The ether extracts were evaporated under reduced pressure and the [^{123}I]I-VME2 taken up in ethanol. The ethanol was evaporated under a stream of nitrogen at room temperature and

more ethanol added to azeotrope off any water absorbed by the ether. The [^{123}I]I-VME2 was then dissolved in 50% ethanol to maintain sterility.

Determination of Effective Specific Activity of [^{123}I]I-VME2

Six 100-µl portions of a 200,000 x g supernatant of a 1:10 homogenate of immature rat uteri in 10 mM Tris, 10 mM KCl, 1 mM EDTA, pH 7.4 buffer (TKE buffer) were placed in incubation tubes chilled in ice. Unlabeled estradiol (E2, 12.5 µl of 5 µM) was added to two of these tubes, TKE buffer (12.5 µl) was added to 3 other tubes and 12.5 µl of the antiestrogen receptor monoclonal antibody, H222 (18) was added to the final tube. Then 12.5 µl of what would be projected to be 50 nM [^{123}I]I-VME2, based on an anticipated specific activity of ~7.4 MBq/pmol (~200 µCi/pmol), was added to 4 of the tubes (two with buffer, one with H222, one with cold E2). To the other two tubes, 12.5 µl of a 50 nM tritiated estradiol solution (2.2 kBq/pmol, 60 nCi/pmol) was added. Each mixture was incubated for at least 1 h in ice, and added to a pellet of 125 µl of 1% charcoal (Norit A) + 0.1% Dextran T40 in TKE buffer (DCC) in an Eppendorf microtube. The DCC was resuspended with the mixtures, incubated for 10 min in ice to adsorb unbound ligands, and then centrifuged (Beckman microfuge) for 1 min at 13,000 rpm in the cold. One hundred µl aliquots of the DCC supernatants were layered on 10-30% sucrose gradients in either low salt (TKE) or high salt (TKE with 400 mM KCl) buffer. The tubes were placed in a Beckman SW 60 rotor and centrifuged for 15 h at 208,000 x g, 2°C. After sedimentation, the density gradients were fractionated from the bottom by displacement with paraffin oil and collected into glass tubes (I-123) or scintillation vials (H-3) for determination of radioactivity. The radioactivity for I-123 was corrected for decay to the time the gradients were layered or the *in vitro* incubations with cells began. The effective specific activity of [^{123}I]I-VME2 was calculated from the ratio of decay-corrected, cold estradiol inhibited, specifically-bound radioactivity of the 2 radioactive estrogens to the 8S receptor and the known specific activity of [^{3}H] estradiol:

$$\text{Sp.Act.}[^{123}\text{I}]\text{I-VME2} = (60)\left(\frac{\text{8S DPM } [^{123}\text{I}]\text{I-VME2 alone} - \text{8S DPM } [^{123}\text{I}]\text{I-VME2+excess E2}}{\text{8S DPM } [^{3}\text{H}]\text{E2 alone} - \text{8S DPM } [^{3}\text{H}]\text{E2+excess E2}}\right)$$

(using tritiated estradiol of 60 Ci/mmole). The result was confirmed by comparing the specific 8S DPM for [^{123}I]I-VME2 on low salt gradients with the specific DPM of the ER complex with [^{123}I]I-VME2 shifted downfield by the antibody in high salt gradients.

Radiotoxicity of [^{123}I]I-VME2 in CHO and CHO-ER Cells

Normal Chinese hamster ovary cells or those transfected with ER cDNA prepared from MCF-7 cell mRNA (16), growing in T150 flasks, were trypsinized, collected by centrifugation, suspended in medium (16) and the cell number counted with a hemocytometer. The cells were diluted to a concentration of 1.5 to 2 million cells per ml and incubated at 37°C with occasional mixing with various concentrations of [^{123}I]I-VME2 alone or together with an estimated 100-fold excess of unlabeled estradiol in medium. Similar incubations were also carried out with the same concentrations of unlabeled estradiol alone, sodium [^{123}I]iodide or medium alone. After incubation the cells were collected by centrifugation for 5 min at 500 x g at room temperature, washed twice by resuspending in medium and centrifugation, and finally resuspended in 1 ml of medium for cell counting, radioactivity determination, plating and autoradiography. For plating the cells were diluted to densities of 100 to 12,500 cells per 1 to 2 ml and plated in 6-well plates, usually with 6 replicates for each group. The cells were allowed to form colonies for 1 week, at which time the medium was removed, the wells washed with phosphate buffered saline, fixed with Bouin's fixative for 10 min, washed with water, stained with 0.45% crystal violet, washed with water to remove excess stain and air dried. The large colonies, greater than 50 cells per colony, were counted at the densities in which separate, individual colonies could be recognized. Cell survival was expressed as a percentage, based on the ratio of the number of colonies in an experimental group, divided by those in the medium controls. Aliquots of the initial radioactive incubation mixtures, as well as the washes and resuspended washed cells, were assayed to determine the initial concentrations of [^{123}I]I-VME2 in the incubations and the amount of the [^{123}I]I-VME2 retained by the washed cells after incubation. To establish the time-dependent retention of I-VME2 in cells after plating, an experiment was performed with plated cells exposed to [^{125}I]I-VME2. For this purpose CHO-ER cells were plated and, after attachment, exposed to [^{125}I]I-VME2 for 1 h at 25° or 37°C. The radioactive medium was replaced with standard medium, the cells incubated for varying times at 25° or 37°C, the medium removed and the radioactivity still remaining with the cells extracted with ethanol for assay in a γ counter.

Autoradiography

To determine the cellular distribution and subcellular localization of the [^{123}I]I-VME2 in washed CHO or CHO-ER cells following incubation in

suspension culture, 50 µl of the resuspended, washed cells were smeared on emulsion-coated slides. For this purpose ethanol washed slides were coated in a dark room by dipping in a solution of Kodak NTB3 emulsion diluted 1:1 in distilled water at 40°C, dried and stored at 2°C overnight. After distributing the cells on a slide with a glass rod in the dark room, the slide was immediately placed on dry ice and when a group of slides were finished they were transferred to a light-tight box that was stored at -20°C for two days. The images were developed by allowing the slides to warm to room temperature for 30 min, developing for 5 min in Kodak D-19, washing in water and fixing for 5 min in Kodak rapid fix A. The cells were then stained with hematoxylin and eosin, dehydrated, and mounted with Permount.

RESULTS

With limited numbers of ER per cell and a relatively short residence time of estrogens in ER-containing cells *in vivo*, it is important to maximize the number of molecules of the radiolabeled estrogen in each cell to deliver maximum radiotoxicity. It is therefore important to achieve very high specific activities for the I-123-labeled estrogens so that as high a proportion as possible of the estrogen molecules incorporated in each cell contain I-123. Fortunately, the iododestannylation reaction (19) provides an efficient, stereospecific, as well as rapid, method to incorporate I-123 into estrogens. Since even using mCi quantities of I-123 the mass of the iodoestrogen is too small to easily measure, conventional specific activity determinations, based on the ratio of radioactivity to UV absorption, are likely to be crude estimates. However, because of the high affinity of many I-123 labeled estrogens for the estrogen receptor, it is possible to use sedimentation analysis to accurately determine the specific activity by assessing specific binding to ER. As shown in Fig. 1, on low salt gradients [^{123}I]I-VME2 sediments with the 8S form of the receptor (Fig. 1B) at the same location as the standard estrogen, [^{3}H] estradiol (Fig. 1A). The specificity of this association is evident by the displacement of both of these radiolabeled estrogens from the receptor in the presence of excess unlabeled estradiol. The binding of [^{123}I]I-VME2 to ER is further confirmed by the recognition of the [^{123}I]I-VME2-ER complex by the ER antibody, H222 (Fig. 1C). In this case, the [^{123}I]I-VME2 associated with the ER 4S monomer, seen in high salt, sediments further downfield when incubated with H222 antibody. This is indicative of the ternary complex of ER antibody with the ER to which the [^{123}I]I-VME2 is non-covalently bound. By comparing the specifically inhibitable radioactivity of [^{123}I]I-VME2 (18,650,000 decay

corrected DPM) and that of [^{3}H]-estradiol (14,917 net DPM) bound to the 8S receptor, and using the known specific activity of [^{3}H]-estradiol (2.2 kBq/pmol, 60 Ci/mmol), one can calculate that the specific activity of [^{123}I]I-VME2 shown in Fig. 1 is approximately 2.78 MBq/pmole (75,000 Ci/mmole). This is about 31% of the theoretical specific activity of I-123. Recently, we have prepared [^{123}I]I-VME2 at specific activities as high as 8.47 MBq/pmol (229,000 Ci/mmol) and an I-123-labeled iodotriphenylethylene estrogen at essentially the theoretical specific activity of I-123.

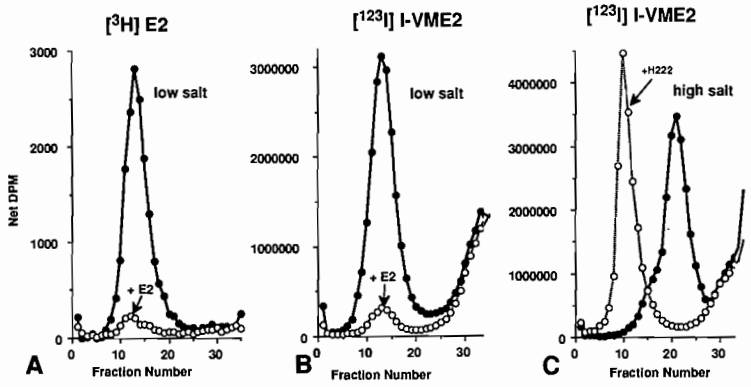

FIG. 1. Sedimentation analysis of [^{123}I]I-VME2. [^{3}H]E2 (A) or [^{123}I]I-VME2 (B,C) was added to aliquots of rat uterine cytosol without (closed circles) or with (A, B, open circles) approximately 100-fold excess of unlabeled E2, or with 1 μg/ml anti-ER antibody H222 (C, open circles). After incubation at 4°C, unlabeled ligands were removed by incubation with DCC, layered on low salt (A, B) or high salt (C) sucrose gradients and centrifuged for 15 h at 208,000 x g, 2°C. Gradients were collected from the bottom.

To show that [^{123}I]I-VME2 is specifically radiotoxic to ER-containing cells we incubated 1.2 nM [^{123}I]I-VME2 with both CHO-ER and CHO cells in suspension culture for one hour at 37°C. After washing, the cells were plated in multiwell dishes for colony assay. In this study we compared cells which had been incubated with: (1) [^{123}I]I-VME2 alone; (2) [^{123}I]I-VME2 in the presence of a 100-fold excess of unlabeled estradiol to inhibit binding of the radioiodoestrogen to ER; (3) unlabeled estradiol itself; and (4) medium alone. As seen in Fig. 2, the [^{123}I]I-VME2 was only radiotoxic to the CHO-ER cells. This concentration of [^{123}I]I-VME2 had little effect on the ER-negative CHO

cells. Furthermore, most, but not all of the radiotoxicity of the [^{123}I]I-VME2 could be prevented in the CHO-ER cells by co-incubation with excess unlabeled estradiol, and under the conditions used, the excess unlabeled estradiol alone had no effect on the survival of the cells. While the incubation with unlabeled estradiol reduced the amount of cell associated radioactivity, about 12% as much radioactivity remained with the washed CHO-ER cells incubated with [^{123}I]I-VME2 in the presence as in the absence of unlabeled E2. This shows that there is a significant amount of non-specific binding of the [^{123}I]I-VME2 to the CHO-ER cells when incubated in suspension culture. Interestingly, a similar amount of non-specific binding of [^{123}I]I-VME2 was seen with CHO cells incubated in the absence of unlabeled estradiol, but this residual [^{123}I]I-VME2 did not cause substantial cell killing (Fig. 2).

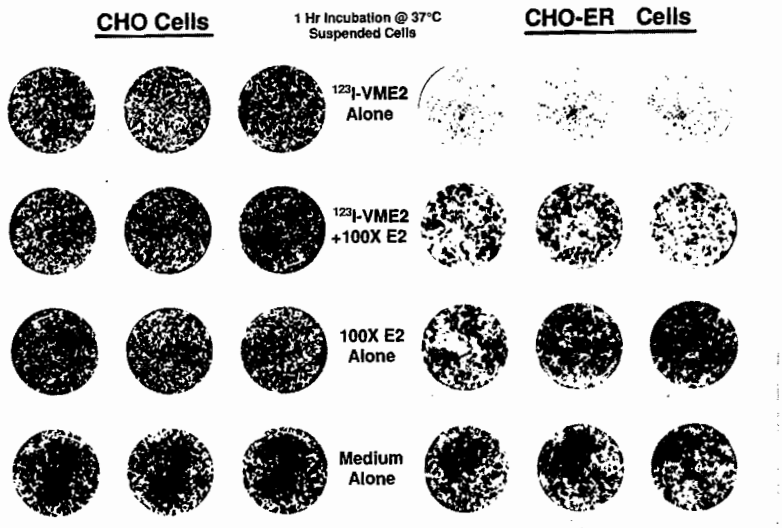

FIG. 2. Radiotoxicity of [^{123}I]I-VME2 in CHO and CHO-ER cells. Suspended cells were incubated with 1.2 nM [^{123}I]I-VME2 alone or along with 100-fold excess of unlabeled E2, the same concentration of estradiol alone or medium for 1 h at 37°C. After washing, the cells were plated in 6-well plates and allowed to form colonies which were fixed and stained with crystal violet.

The results shown in Fig. 2 indicated substantial cell killing, but the number of cells made colony counts of the control wells impossible. Colony assays at lower plating densities indicated that about 7% of the CHO-ER cells survived after incubation with [^{123}I]I-VME2 alone. Assessment of the

distribution of cell labeling by autoradiography, Fig. 3, provided a basis for understanding the radiotoxicity results. As seen in Fig. 3, there was a significant variation in the number of silver grains over various cells, ranging from what looked like thousands of silver grains densely covering some nuclei, to more moderate numbers of grains per cell to grain densities similar to the background. The autoradiograms confirmed the expected concentration of the labeled estrogen in the nuclei of the cells but unexpectedly indicated that between 5 and 10% of the cells had densities of silver grains which were similar to the background. For this experiment we harvested confluent cells. In subsequent experiments using cells harvested at lower cell densities we have seen a more uniformly high degree of cell labeling by autoradiography at the highest concentrations of [^{123}I]I-VME2 used.

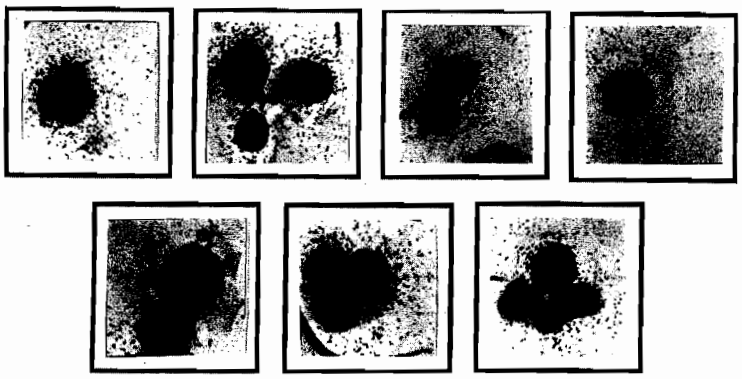

FIG. 3. Autoradiography of CHO-ER cells incubated with [^{123}I]I-VME2. CHO-ER cells incubated for 1 h with 1.2 nM [^{123}I]I-VME2 and washed to remove unbound ligand were spread on emulsion coated slides and processed after 2 days at -20°C. Panels show representative autoradiograms of various cells on the slide.

Further studies have shown that the radiotoxicity of [^{123}I]I-VME2 in CHO-ER cells is dose-dependent (Table I). Consistent with the results shown in Fig. 2, the effect of [^{123}I]I-VME2 on CHO cells was dramatically less than that on CHO-ER cells. In this experiment we found that at high concentrations of [^{123}I]I-VME2 there was some radiotoxicity of [^{123}I]I-VME2 even with the CHO cells (Table I). Clearly, this effect is small compared to the effect in the ER-positive CHO-ER cells. In recent experiments this difference in radiotoxicity of [^{123}I]I-VME2 to CHO-ER and CHO cells at nanomolar concentrations has

corresponded to more than 3 orders of magnitude. Sedimentation analyses on extracts of these CHO cells do not provide any evidence for extractable ER from the CHO cells. However, we can not completely rule out small amounts of a non-extractable estrogen binding component in the nucleus or elsewhere in the cells, although whole-cell assays of specific uptake of estrogens by these cells likewise do not provide definitive evidence for significant amounts of any specific estrogen binding components. In several experiments we have observed a small degree of radiotoxicity of [^{123}I]I-VME2 in CHO cells, which is largely prevented by co-incubation with excess unlabeled estradiol, consistent with a low concentration of an estrogen-specific binding component in the CHO cells. As seen in Table I, even higher concentrations of I-123 as sodium iodide have no radiotoxicity for the cells, indicating that the presence in the medium of similar concentrations of I-123 is not radiotoxic.

The protective effect of unlabeled estradiol is shown in Table II, along with analysis by autoradiography of the density range of silver grains over CHO-ER cells (as a function of concentration of [^{123}I]I-VME2 to which the cells

TABLE I

Radiotoxicity of I-123 Iodide and I-VME2 for CHO and CHO-ER Cells

Cell type	Group[a]	μCi/ml	nM	% Survival
CHO-ER	[^{123}I] I-VME2[b]	137	1.56	0.0 ± 0.0
		27.8	0.315	4.3 ± 1.8
		4.8	0.055	76.1 ± 8.0
		0.83	0.009	102.0 ± 7.3
	[^{123}I] I NaI	189		103.8 ± 11.2
CHO				
	[^{123}I] I-VME2[b]	145	1.64	62.7 ± 7.0
		29.5	0.33	95.6 ± 6.9
		4.96	0.056	108.1 ± 9.7

[a] Suspended cells were incubated 1 h, 37°C with the radioligand, the cells washed and plated in multiwell plates for colony assay.

[b] Specific Activity 3.23 MBq/pmol (88,000 Ci/mmol).

TABLE II
Relationship of Cell Labeling to Radiotoxicity[a]

[^{123}I] I-VME2[b] Concentration	unlabeled E2 conc Survival	Molecules/ Cell	Autoradiography (% of cells) high	mid	low	neg	%
0.69 nM		55,400	100	0	0	0	0.0
0.59 nM	170 nM	10,800	0	0	75	25	49 ± 25
0.20 nM		18,313	80	10	10	0	0.0
0.07 nM		5,000	0	60	30	10	70 ± 19

[a] Suspended CHO-ER cells were incubated for 1 h at 37°C with the [^{123}I] I-VME2, washed, and aliquots assayed for I-123, spread on emulsion coated slides for autoradiography and plated in multiwell plates for colony assay.

[b] Specific activity 8.48 MBq/pmol (229,000 Ci/mmol).

were exposed). At the highest concentration of [^{123}I]I-VME2, 0.69 nM, all the cells showed high density labeling, and the average number of [^{123}I]I-VME2 molecules per cell was about 55,000. When incubation with a similar concentration of [^{123}I]I-VME2 was carried out in the presence of a nearly 300-fold excess of unlabeled estradiol, the number of molecules per cell only dropped by about 80%, but now 25% of the cells showed no grain density above background, and the rest showed only low density labeling. When the concentration of [^{123}I]I-VME2 used was decreased 3-fold, the average number of molecules of [^{123}I]I-VME2 per cell dropped proportionately, but there was also a wider range of silver grain densities over the cells. Despite the change in the autoradiographic pattern, all the cells were significantly above the background level of silver grains and at low plating density all the cells were killed (indicating > 95% cell kill in this experiment). However, when the concentration was again decreased by a factor of 3, and the average number of molecules per cell dropped commensurately, there now were appreciable numbers of cells which were labeled at the low or background level, and about 70% of the cells survived. Thus, there is clearly a concentration dependence of the extent of cell killing due to [^{123}I]I-VME2 and concomitant with decreases in the average number of I-123 labeled estrogens per cell a change in the distribution of autoradiographic grain density over the cells. We have recently studied the fate of [^{125}I]I-VME2 initially taken up by CHO-ER cells and found that, in no doubt largely due to the decreased affinity of the mutated ER of these cells (17), even the [^{125}I]I-VME2 specifically bound is

rapidly lost from the cells at 37°C, with a mean residence time of only about an hour.

DISCUSSION

The high-LET type radiotoxicity associated with the Auger electron cascade process makes such electrons particularly attractive for cancer therapy due to the oxygen independence of their effects and their potential to deliver damage to key cellular components. However, the very short range of the electrons, a consequence of their generally low energy, has limited their applications. Obviously what is desirable is a reasonably specific mechanism to localize the Auger electron emitter in the nucleus of the target cells. The ideal route would be that of incorporation into the DNA, which has been shown to be very potent for causing multiple double-strand breaks in the DNA, and resultant efficient radiotoxicity in cells in culture (8,9). Although many cancers have greater rates of proliferation than many normal tissues, certain normal cells, such as the epithelium of the intestine, also may have very high proliferative rates so that systemic therapy with an Auger electron emitter incorporated simply as a function of the rate of cell proliferation of the tissue could be problematic.

We have been exploring the therapeutic potential of using a nuclear protein to deliver a non-covalently bound ligand with an Auger electron emitter to the vicinity of DNA. This approach is not without pitfalls, as a non-covalent association of the radiolabeled ligand will lead to eventual loss of the ligand from the cells, even assuming that one can concentrate it in the desired cells in the first place. Furthermore, there are few, if any, nuclear proteins believed to be specific to cancer cells. Because of the high concentration of estrogen receptors in some breast, endometrial and ovarian cancers and the recognition that ER are high affinity, DNA-binding proteins, ER-directed therapy with Auger-electron-emitting nuclides seemed to be a reasonable candidate for a trial of such therapy. While significant concentrations of ER are also found in the pituitary and normal tissues of the reproductive tract, the rate of replication in these tissues is lower in general than that of the cancers, and if necessary the reproductive organs could be removed (and often may have been already removed in older patients). In fact the removal of the pituitary was used for therapy of ER positive breast cancers in the past (1). However, before seriously considering such therapy for ER positive cancers in patients, it is important to understand the

characteristics of the potential radiotoxicity *in vitro* and in animal model systems. We therefore set out to determine whether it was possible to specifically kill cells in tissue culture in an ER-dependent manner, and to estimate the number of molecules per cell, or decays per cell, needed for such radiotoxicity. With such information in hand it would be more appropriate to study the uptake and retention of various potential radiolabeled estrogens in patients to determine the mean residence time as a function of route and dose to compare with the number of decays per cell needed, as established in the experimentally simpler *in vitro* studies.

The results here establish that the steroidal estrogen E-17α-[^{123}I]-iodovinyl-11β-methoxyestradiol specifically kills ER-containing cells in culture. Because of the high affinity association of estrogens with ER and the generally slow dissociation of estrogen already bound to ER, one would expect that short incubations of ER+ cells with the I-123-labeled estrogen should be sufficient. In fact, despite the unexpectedly rapid loss of [^{123}I]I-VME2 from the CHO-ER cells, a 1-hour incubation with the radiolabeled estrogen was sufficient to produce very effective cell killing of CHO-ER cells, as determined by colony assay. This radiotoxicity can be very substantially reduced by inclusion of a large excess of unlabeled estradiol in the incubations with the I-123-labeled estrogen, which also specifically reduces the I-123 uptake in ER-containing CHO-ER cells, but not in CHO cells in which the radiotoxicity of this I-123-labeled estrogen is several orders of magnitude lower. While the reasons that a more than 100-fold excess of unlabeled estradiol can not completely protect the ER positive cells from this radiotoxicity are not entirely clear, the large degree of protection clearly confirms the ER-mediation of the killing. In fact it appears that the lack of complete protection may be due to the presence of ER in the CHO-ER and may be specific to tissue culture experiments. After washing the cells, which would substantially reduce the levels of unlabeled estradiol, the non-specifically bound [^{123}I]I-VME2 would be free to exchange with ER-bound, unlabeled estradiol, now present in a smaller excess in the CHO-ER cells. However, in the CHO cells, with little or no ER present, there should be no mechanism for the non-specifically bound [^{123}I]I-VME2 to be directed to sites near the DNA, hence little radiotoxicity. Since the amount of [^{123}I]I-VME2 remaining associated with the washed CHO-ER cells incubated in the presence of excess unlabeled estradiol is 10-20% of that present in the absence of unlabeled estrogen (Table II), significant amounts of [^{123}I]I-VME2 remain associated with the cells. In this regard, it is of interest that as seen in Table II, the number of I-123 molecules per cell in the presence of unlabeled E2 is about double that seen following an

incubation with a lower concentration of [^{123}I]I-VME2 in the absence of unlabeled E2 that gave a 30% cell kill. Second, based on the relative affinities of estradiol and moxestrol (11β-methoxy estradiol), in which the higher affinity of moxestrol is reflected in a slower dissociation rate from ER, if the redistribution results in new occupancy of ER by [^{123}I]I-VME2, it may be preferentially retained by ER.

The results presented here are complicated by the lower affinity ER found to be present, probably as a cloning error, in the CHO-ER cell line. It has been determined that a single base change in the cDNA gives rise to a single amino acid change (glycine 400 to valine) in the ER, resulting in a lower affinity for estradiol (17). The lower affinity most certainly translates to a shorter residence time for estrogens in the cells. Our preliminary results indicate that the retention of I-VME2 at 37°C is substantially less than that at 25°C in CHO-ER cells and the actual average residence time for I-VME2 in these cells at 37°C is less than an hour. This loss could also reflect a greater rate of metabolism of the ligand at the higher temperatures as well as the processing of the receptor but since it is less dramatic with MCF-7 cells which have wild type receptor it is likely that the lower affinity of the CHO-ER receptor is the cause. Because of this rapid loss of bound ligand, our calculations indicate that only 5 to 6% of the I-123 initially bound to the CHO-ER cells will decay while still associated with these cells at 37°C. While this complicates the model, it indicates the substantial potential for even more effective killing of cells which contain the higher affinity, wild type ER. Studies are currently underway in our laboratory with MCF-7 cells containing apparently normal ER. Nevertheless, the comparison of CHO and CHO-ER cells, which should display similar radiosensitivities, helps to establish the ER-dependency of the killing. Such does not appear to be the case for the studies previously reported (13-15) using [^{125}I] iodotamoxifen with MCF-7 cells. In those studies the number of molecules per cell calculated from the cell associated I-125 at the mean lethal number of molecules is about 1.3 million, at least 10-30 times greater than the total number of ER molecules present in the MCF-7 cell. Hence it was likely, based on this data alone, that ER was not required for the effect. Furthermore, as recently shown, a substantial amount of the toxicity could be inhibited by unlabeled antiestrogen, which was not inhibited by cold estrogen, indicative of mediation of the non-ER antiestrogen specific binding sites. Such sites, found in many cells and at substantial concentrations in some normal tissues, would not constitute a good targeting mechanism to approach ER-containing cancers. This is supported by the diverse distribution of radiolabeled antiestrogens in animals. Finally, if one calculates the number

of decays which could occur during the average 6-12 hour residence time of ligands for ER *in vivo*, it becomes clear that using the 60-day half-life nuclide, I-125, with an ER-directed ligand is not a practical *in vivo* approach to therapy based on the Auger electron cascade process.

Our autoradiographic studies suggest that the cellular content of ER in a population of cells may not be uniform. This may reflect changes in ER expression as a function of cell cycle or growth conditions, as suggested by the differences we have observed with cells harvested at different densities, as well as a normal variation of ER content among cells in the same population. When incubated with nanomolar concentrations of radioiodoestrogen, cells harvested at less than 50% confluence show a uniform, high autoradiographic grain density. However, the same cells incubated with lower concentrations of the same iodoestrogen demonstrate more variability in grain density, consistent with a variation in ER content within a uniformly growing population.

Such differences in ER content would be expected to have important implications for therapy, as the uptake and retention of estrogen by a cell will depend on the concentration of the estrogen and of ER. It is generally recognized that there is heterogeneity of ER expression within breast cancers. This can be clearly shown by immunohistochemical staining for ER using specific monoclonal anti ER antibodies (20). Nonetheless, it is found that most ER positive breast cancers initially respond to endocrine therapy despite such heterogeneity of ER distribution. This can be understood in light of evidence for a paracrine mechanism of growth stimulation. There is increasing evidence now for estrogen-dependent production of peptide growth factors in ER-containing breast cancer cells, at least *in vitro* (7). Such products, released by ER+ cells, could stimulate the growth of nearby ER negative cells as well. However, by the mechanism described, if the ER-containing cells were eliminated, any ER negative cells dependent on the growth factors produced by the ER positive cells would be expected to stop proliferating.

Considerable challenges remain to bring estrogen receptor-directed therapy to the clinics. Clearly the delivery of sufficient I-123-labeled estrogen to the ER positive cancer cells, without causing excessive radiotoxicity to the non-cancerous ER positive cells, or nonspecific radiotoxicity to the patient, will be a challenge. Nonetheless, the first critical step is to demonstrate the radiotoxicity of an Auger-emitter/estrogen complex specific to ER-containing

cells, which we have presented here. Based on the results presented in this paper, and the estimated mean retention time of I-VME2 in the CHO-ER cells of less than one hour, it can be calculated that the mean lethal number of decays of I-123 is in the range of 300 decays per cell. Especially with the higher affinity, wild type ER expected to be present in most human cancers, numbers of cancer-cell-bound radioiodoestrogens necessary to localize more than the mean lethal number of the radiolabeled ligands in these cells would seem to be reachable *in vivo*, in view of the many thousands of ER present per cell in some cancers. It is of theoretical significance that the preliminary estimates of mean lethal number of I-123 decays for an I-123-labeled estrogen in these studies is similar in magnitude to the estimates of mean lethal decays of I-123 incorporated into the DNA of V79 cells, *i.e.* 277 decays (21). These results are consistent with an earlier report by Sundell-Bergman and Johanson (22) which indicated that the efficiency of inducing double strand DNA breaks with the thyroid hormone [^{125}I] triiodothyronine, mediated by the T3 receptor, also a member of the steroid receptor superfamily (5), was as high as that due to [^{125}I] iododeoxyuridine incorporated into DNA. This would suggest that the radiolabeled estrogen must indeed be held in close proximity to the DNA by its association with the estrogen receptor. It also suggests that the probable envelopment of the estrogen within the receptor protein does not substantially reduce the DNA-lytic potential of the Auger electrons. Thus the therapeutic challenge will be to deliver the radiolabeled estrogen as specifically as possible to the ER+ cancer, borne by an estrogen which maximizes its cellular retention. Although it would be wonderful to be able to specifically kill all the cancer cells without otherwise harming the patient, current cytotoxic therapies generally have significant side effects. Clearly factors such as the biological half-life of the ligand and its rate of metabolism will also be of importance to the radiotherapeutic potential of such compounds. Furthermore, animal studies will have to clarify that the even small potential of such radiolabeled estrogens to kill ER negative cells *in vitro* will not be a problem *in vivo*. To be able to deliver a therapeutic dose to the cancer with only minimal side effects to the patient would be a contribution to cancer therapy. The next step will be to identify the best ER-binding carrier, based on cell and animal distribution studies, to be able to proceed to tests of the radiotoxicity in tumors in animals and to begin patient studies. However, we feel that the specificity and dosimetry of the reported results in cells in culture provide some cause for optimism for the substantial process that remains before this new type of therapy can be applied in patients.

CONCLUSION

These studies demonstrate that it is possible to prepare very high specific activity I-123-labeled estrogens, approaching the specific activity of I-123, using the stereospecific halodestannylation reaction with commercially available I-123 sodium iodide. They also show that the I-123 Auger electron cascade targeted to the cell nucleus via covalent attachment to an estrogen which has high affinity to the estrogen receptor can effectively and specifically kill ER positive cells in a dose-dependent, estrogen-inhibitable manner following only a short exposure of the cells to the radiolabeled estrogen in suspension culture. Cells lacking ER show little damage by such treatment, and, as expected, even quite substantial amounts of I-123 iodide are without significant effect in the cells. Estimates of the mean lethal number of I-123 decays for the radiolabeled estrogen are in the same range as previous reports of the radiotoxicity of I-123 incorporated into DNA, consistent with the delivery of the I-123 attached to the estrogen in close proximity to DNA.

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DISCUSSION

Van den Abbeele, A. D. What is the stability of this compound *in vivo* or in plasma at 37°C?

DeSombre, E. R. The ^{123}I-VME2 does not bind to transport protein in the blood and is rapidly cleared from the body. However, the cleavage of the iodine carbon bond is not a major process as indicated by the relatively low thyroid uptake. This is likely due to the stability of the vinyl carbon-iodine bond.

Harapanhalli, R. S. In the radiolabeling method described by you "halodestannylation" do you see any *cis-trans* isomerizations?

DeSombre, E. R. The halodestannylation reaction is particularly advantageous because it occurs with complete retention of the steric configuration of the precursor tributyl compound. For our purposes, to prepare ligands with short half-life nuclides it is also convenient since it occurs rapidly, often complete in 10-15 min at room temperature.

Johanson, K. J. Have you studied whether the chromosomal damages appear at any specific sites of the chromosomes? What is the range of the number of estrogen receptors per cell in the human body?

DeSombre, E. R. 1) We are interested in studying specific damage of chromosomes as some estrogen regulated genes have been located on specific chromosomes. We have not yet been able to carry out these studies. 2) The numbers of estrogen receptors in breast cancers range from an average of possibly 500 per cell for a low ER positive tumor to more than 10,000 for an ER rich cancer.

Laster, B. H. Have you ever attempted your binding assays at 4°C?

DeSombre, E. R. Not yet.

Martin, R. In the case of the diphenyl compound, how stable is the vinyl iodide *in vivo*?

DeSombre, E. R. We do not have any detailed data on the effect of the somewhat bulky phenyl groups or the stability of the vinyl iodide *in vivo*. Our general impression is that the stability of the triphenyl vinyl iodide is not appreciably different than that of the steroidal vinyl iodides.