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XR5944: A potent inhibitor of estrogen receptors

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Abstract

The anticancer drug XR5944 was originally developed as a topoisomerase inhibitor and was subsequently shown to be a transcription inhibitor. It has shown exceptional anticancer activity both *in vitro* and *in vivo* and was significantly more potent than traditional topoisomerase inhibitors. The solution structure of the XR5944/DNA complex recently obtained in our laboratory indicates that XR5944 bis-intercalates at the 5′-(TpG):(CpA) site of duplex DNA, which is found in the consensus DNA-binding site of estrogen receptor (ER). Thus, we tested the ability of XR5944 to inhibit ER activity both *in vitro* and in cultured cells. In electrophoretic mobility shift assays, it is seen that the DNA binding of recombinant ERα protein, as well as ER from nuclear extracts, is inhibited by XR5944 in a dose-dependent manner. In luciferase reporter assays, XR5944 inhibited the reporter gene expression from an estrogen response element–containing promoter but not from a basal promoter sequence that lacks any *cis*-acting elements. In contrast, the RNA polymerase inhibitor actinomycin D inhibits the transcription from both the above-mentioned promoters. The specificity of XR5944 activity is displayed by a separate reporter assay in which the transactivation of reporter gene expression by Sp1 proteins was not inhibited by XR5944. Collectively, these data suggest that XR5944 is capable of specifically inhibiting the binding of ER to its consensus DNA sequence and its subsequent activity. This represents a novel mechanism of ER inhibition, which may allow the development of agents capable of overcoming resistance to current antiestrogens. [Mol Cancer Ther 2007;6(1):213–9]

Introduction

In women, breast cancer is the most frequent type of cancer and represents the second leading cause of cancer death. Although there have been major advances in the treatment of breast cancer in the last 10 to 15 years, it remains a disease for which improved treatments are still urgently needed. Targeting signal transduction pathways in breast cancer chemotherapy has continued to develop as a compelling approach over time.

The link between human breast cancer and estrogen was first identified over a century ago and more recent epidemiologic data show clear associations of serum estrogen concentrations (1), use of estrogenic hormone replacement therapies (2, 3), and oral contraceptives (4) with an increased risk of breast cancer. Thus, antagonizing the biological effects of estrogens is an effective therapeutic strategy for women with breast cancer. Since the discovery of estrogen receptor (ER) as the modulator of estrogen action (5), ER has evolved to be one of the most effective targets for breast cancer therapy (6). Binding of estrogen to ER initiates a series of events that include dissociation of heat shock proteins and receptor dimerization. Such dimerization facilitates the binding of ER to specific estrogen response elements (ERE) that are located upstream of estrogen-regulated genes. Currently available methods for interfering with estrogen action are composed of ''antiestrogens'' that either compete with estrogens for binding to ER or reduce the circulating ER levels, and ''aromatase inhibitors'' that inhibit estrogen biosynthesis. The antiestrogen tamoxifen was the hormonal treatment of choice for ER-expressing breast cancers. Although it still remains so for premenopausal women, it is gradually being replaced by third-generation aromatase inhibitors in treatment of postmenopausal women with ER-expressing breast cancers (7). However, despite being the ideal treatment of choice for patients with ER-expressing breast cancer, these current ER-targeting therapies have a main limitation related with resistance. Half of the breast cancers expressing hormone receptors are inherently resistant to the current endocrine therapy (8), and most initially responsive breast tumors acquire resistance. Hence, there is an urgent need for new and effective agents that lack cross-resistance with existing treatments.

The anticancer drug XR5944 (Fig. 1A) is a bis-phenazine in phase I clinical trials (9). It originated from a rational drug design program aimed at developing potential topoisomerase inhibitors (10). The parent compounds of XR5944, phenazinecarboxamides, as well as closely related acridinecarboxamides, are both dual DNA topoisomerase I/II inhibitors (11–13). Although initial reports showed that XR5944 could bind strongly to DNA and that it may interfere with the normal function of topoisomerase I and II *in vitro* (14), recent studies have indicated that the primary mechanism of XR5944 action is independent of topoisomerases and, furthermore, that it is related with transcription inhibition (9, 15).

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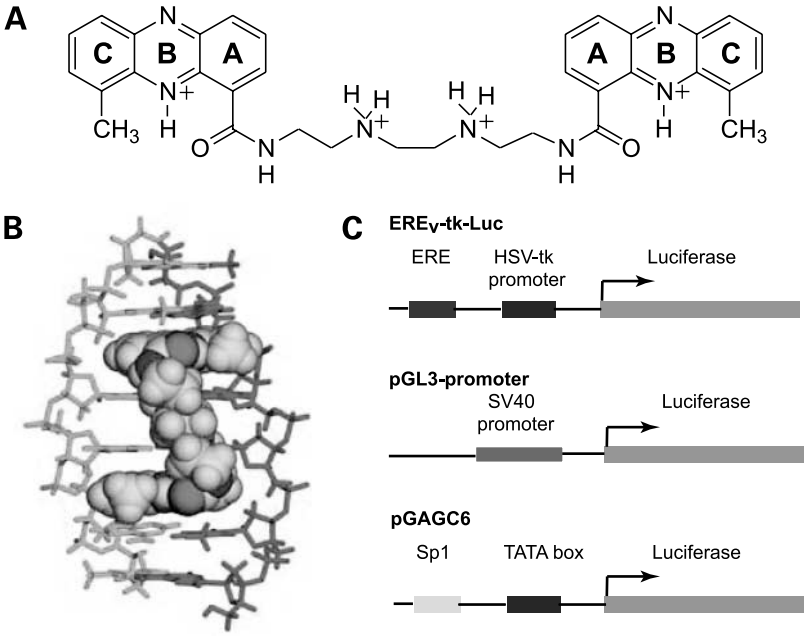

Figure 1. **A,** the chemical structure of XR5944 with nitrogen protonations as observed in the DNA-bound conformation. **B,** the XR5944-DNA complex structure. The CPK model of XR5944 shows the two phenazine rings intercalated with DNA parallel to the DNA base pairs and the amino alkyl linker lying in the major grove. **C,** schematic representations of the three reporter vectors used in this study. *Top,* the EREv-tk-Luc vector containing the vitellogenin A2 ERE and the thymidine kinase promoter upstream of the *luciferase* gene. *Middle,* the pGL3-promoter vector (Promega) containing the SV40 promoter sequence upstream of the *luciferase* gene. *Bottom,* the pGAGC6 plasmid containing multiple Sp1 binding sites and the adenovirus major late initiator TATA box upstream of the luciferase gene.

XR5944 has shown exceptional activity against human and murine tumor models both *in vitro* and *in vivo* and was significantly more potent than well-known topoisomerase inhibitors such as doxorubicin, topotecan, and TAS-103 (14). This potency translates well to *ex vivo* systems where XR5944 was 40- to 300-fold more potent than the other cytotoxic agents tested (16). Recent data show the *in vitro* and *in vivo* additive potency of XR5944 in combination with carboplatin or doxorubicin in non–small-cell lung carcinoma, and with 5-fluorouracil and irinotecan in colon cancer cell lines (17, 18).

Despite the progress in understanding the activity and function of XR5944, its precise mechanism of action is yet to be revealed. A major contribution to the understanding of the mechanism of action is the structure of the DNA-XR5944 complex recently determined by us (19). According to this structure, XR5944 has a novel DNA-binding mode using a DNA oligomer of sequence d(ATGCAT). XR5944 bis-intercalates at the 5′-(TpG):(CpA) site, with the carboxamide aminoalkyl linker lying in the major groove of the DNA (Fig. 1B). Such binding in the major groove is significant because most DNA-binding drugs bind in the minor groove, whereas transcription factors generally bind in the major groove. The DNA binding of XR5944 seems to be highly specific as shown by parallel studies of drug binding with other DNA sequences. The same dinucleotide sequence is found in the consensus binding site of the ER, which has a base sequence of GGTCAnnnTGACC with palindromic half sites (20, 21). Thus, we speculate that XR5944 can inhibit the DNA binding of ERs. Following up on previous findings that XR5944 inhibits DNA transcription (9) and that it inhibits DNA binding of activator protein 1 transcription factors *in vitro* to a consensus sequence that contains 5′-TG-3′ (19), we tested the ability of XR5944 to inhibit the DNA binding of ER and its transcriptional activation function. Our data show that XR5944 specifically inhibits the DNA binding, and the subsequent activity of ERs both *in vitro* and in cell systems. Furthermore, it is shown that the activity of XR5944 in inhibition of transcription is different from that of the RNA polymerase inhibitor-actinomycin D. Thus, XR5944 is likely to have a mechanism of action that has not been seen before with other clinically used ER inhibitors. Our data warrant the development of specific ER inhibitors that use a DNA-binding mode and a mechanism of action similar to XR5944, which may be useful in breast cancer therapeutics.

Materials and Methods

Cells and Reagents

MCF-7 cells were originally obtained from the American Type Culture Collection (Manassas, VA) and periodically tested negative for *Mycoplasma*. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin at 37°C and 5% CO_2 in air. XR5944 was provided by Xenova Ltd. (Slough, United Kingdom). The powder was dissolved in deionized water at a concentration of 10 mmol/L and stored at 4°C. Actinomycin D was purchased from Sigma (St. Louis, MO), dissolved at a concentration of 1 mg/mL in DMSO solution (Sigma), and stored at −20°C.

Electrophoretic Mobility Shift Assay

Binding of ER to the ERE was done using either MCF-7 nuclear extract (Active Motif, Carlsbad, CA) or recombinant ERα (Affinity BioReagents, Golden, CO). ER gel shift oligonucleotide of sequence 5′-GGATCTAGGTCACTGTGACCCCGGATC-3′ (the consensus half sites are underlined) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and ^{32}P 5′-end-labeled using T_4

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polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol). Approximately 8 μg nuclear extract or 0.3 μg ERα were mixed with indicated amounts of XR5944 and a 1× solution of Gel Shift Binding Buffer (Promega Corporation, Madison, WI) containing poly(deoxyinosinic-deoxycytidylic acid) in a total volume of 20 μL. The mixture was incubated at room temperature for 20 min and the binding reaction was initiated by adding ~40,000 cpm DNA per reaction. Following incubation for further 15 min, the samples were analyzed in a pre-electrophoresed 6% polyacrylamide native gel in 0.5× Tris-borate EDTA (Sigma). After 1 h of electrophoresis at 240 V, the gel was vacuum dried and subjected to autoradiography. The binding of ER and ERE was observed by Phosphorimaging analysis.

Plasmids

The plasmid ERE_v-tk-Luc, which was used to monitor the ER transactivation, contains the vitellogenin A2 ERE sequence from −336 to −310 inserted upstream of the herpes simplex virus thymidine kinase promoter and has been described previously (22, 23). The pGL3-promoter vector was purchased from Promega and contains an SV40 promoter of 202 bp upstream of the *luciferase* gene with no transcription factor binding sites. The plasmid pGAGC6, containing Sp1 binding sites upstream of the adenovirus TATA box, was a gift from Dr. A.S. Kraft (Medical University of South Carolina, Charleston, SC) and has been described previously (24).

Transient Transfection Assays

MCF-7 cells were plated at 2×10^5 to 3×10^5 per well in six-well plates. The following day, cells were transfected separately with 1 μg of ERE_v-tk-Luc plasmid, pGL3-promoter plasmid, or pGAGC6 plasmid per well using Fugene6 transfection reagent (Roche, Indianapolis, IN). Seven hours after transfection, indicated amounts of XR5944 or actinomycin D were added into the medium and incubated for a further 24 h. Cells were harvested in 50 μL of Reporter Lysis Buffer (Promega) per well. Following one freeze-thaw cycle, the protein concentration in each sample was quantitated using a Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). A volume of cellular extract corresponding to 20 μg of total protein per sample was used to measure the luminescence using the Luciferase Assay System (Promega). Variation in cell number and transfection efficiency between samples were compensated for by using a fixed amount of total protein and by carrying out each treatment in triplicate and repeating each experiment separately at least thrice.

Results

XR5944 Directly Inhibits the ER Binding to ERE *In vitro*

To examine the effect of XR5944 on ER binding to DNA, we first carried out electrophoretic mobility shift assay (EMSA) experiments using an MCF-7 cell nuclear extract and an ERE consensus oligonucleotide. MCF-7 is an ER-expressing cell line; therefore, the nuclear extract is a good source for observing the binding of ER in the presence of other cellular components. As shown in Fig. 2, incubation of radiolabeled oligonucleotide with nuclear extract resulted in two significant bands on a polyacrylamide gel subsequent to electrophoresis. Free DNA electrophoresed more rapidly compared with DNA bound to ER protein that has decreased mobility. The DNA binding of ER was significantly inhibited by XR5944 in a dose-dependent manner. Specifically, the ER DNA binding was clearly inhibited by XR5944 at 1.25 μmol/L concentration and was completely blocked by the drug at 25 μmol/L concentration (Fig. 2A). Whereas the EC_{50} for XR5944 in EMSA was ~1.25 μmol/L, the recorded IC_{50} value for this drug in cell culture systems is in the range of 0.4 to 4 nmol/L (14). This change in effective concentrations between *in vitro* (in test tube) and *in vivo* (cell culture) data is assumed to be due to the low sensitivity of *in vitro* assays and also due to the significantly different conditions between the two assays, such as incubation times with drug and incubation temperatures. Similar discrepancies between effective concentrations *in vitro* and *in vivo* have previously been seen with a number of cytotoxic drugs, e.g., topotecan, a topoisomerase I inhibitor that has an IC_{50} of 12 nmol/L in cells (25), but is used at 1 to 50 μmol/L concentrations in *in vitro* assays.

To confirm the inhibition of ER binding to DNA by XR5944, we carried out similar gel shift assays with recombinant ERα instead of the nuclear extract used above. Using 25 μmol/L XR5944, we show that the DNA binding of recombinant ERα in the presence of E2 is completely inhibited by the drug, similar to its inhibition of ER DNA binding from the nuclear extract (Fig. 2B).

XR5944 Inhibits ER-Mediated Transcription

The effect of XR5944 on transcriptional activity of ER in cell culture systems was determined using transient

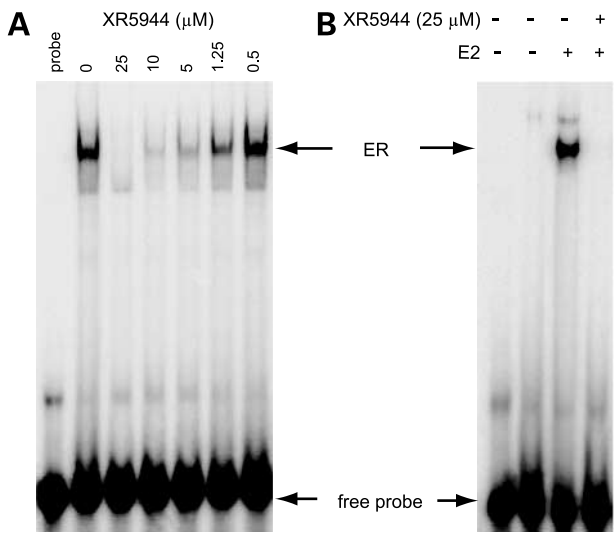

Figure 2. EMSAs of the ER binding to the ERE in the absence and presence of XR5944. **A**, XR5944 effect on the DNA binding of ERs from an MCF-7 nuclear extract. **B**, XR5944 effect on the DNA binding of recombinant ERα in the presence of E2. The bands of the free DNA probe and the ER-ERE complex are labeled. The concentrations of XR5944 used are as indicated. The phosphorimage shown is one of several repeats.

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transfection assays with a luciferase reporter plasmid construct containing an ERE inserted upstream of an herpes simplex virus thymidine kinase promoter (ERE_v-tk-Luc; Fig. 1C). These plasmids were transfected into MCF-7 cells that were subsequently treated with XR5944 at concentrations ranging from 0.75 to 10 nmol/L. The luciferase activity in each sample was measured to determine the level of ER transactivation. The samples were standardized by using a constant amount of total cell lysate protein for each measurement. Our data indicate that the transcriptional activity of ER is significantly inhibited by XR5944, even at 0.75 nmol/L concentration (Fig. 3A). This inhibition by XR5944 seems to be dose dependent, with the maximum inhibition observed at a concentration of 4 nmol/L drug. It seems that the observed inhibition of ER transactivation by XR5944 is not due to cell kill by the drug because no significant cell death is observed even in cultures treated with 10 nmol/L XR5944 for 24 h. In addition, the luciferase activity is measured using a constant amount of total protein of both control and treated samples, which normalizes the measurements to exclude cell death.

The same luciferase reporter system was used to test the activity of the universal transcription inhibitor actinomycin D as a positive control for transcription inhibition. Actinomycin D is an antibiotic that binds to DNA and inhibits RNA synthesis by interfering with RNA polymerase II activity (26, 27). Therefore, it is expected to inhibit the transcription from any transcriptionally active reporter construct, including the ERE_v-tk-Luc reporter used here. As expected, actinomycin D inhibits the transcription of the reporter gene at the standard concentrations (0.08–0.8 μmol/L) used in cellular assays (Fig. 3B).

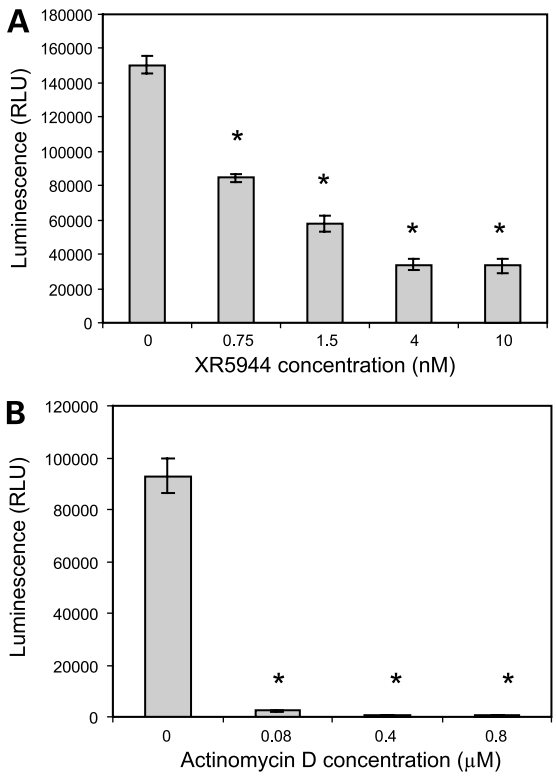

Figure 3. Effects of XR5944 (**A**) and actinomycin D (**B**) on ER transactivation in MCF-7 cells. Various concentrations of XR5944 and actinomycin D used are as indicated. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.01$, statistically significant difference.

The Effect of XR5944 Is Observed Only in the Inducible ER Transactivating Function but not in the Constitutive Promoter Activity

It was shown by our previously discussed data that XR5944 inhibits the transcriptional activity of the reporter vector containing the ERE element. This reporter plasmid also contains a basal herpes simplex virus thymidine kinase promoter in addition to the *cis*-acting ERE element. To verify that the inhibition of transactivation is due to the specific interference of ER binding to DNA by the drug, but not due to inhibition of basal promoter activity, we carried out similar transactivation assays with a reporter system (pGL3-promoter) containing only an SV40 basal promoter sequence but no specific *cis*-acting ERE elements (Fig. 1C). Unlike the ERE-containing luciferase reporter, the *luciferase* gene expression of this system was not significantly affected by XR5944 at the 0.75 to 10 nmol/L concentrations that it inhibited the transactivation of ERE-containing reporter (Fig. 4A). This result suggests that XR5944 specifically inhibit the activity of the ER transcription factor but not that of a basal promoter.

XR5944 Preferentially Inhibits Transcription Initiation

We have also carried out a similar study using the reporter containing the SV40 basal promoter sequence (pGL3-promoter) for actinomycin D. Actinomycin D inhibits RNA synthesis by interfering with the RNA elongation activity of RNA polymerase II (26, 27); therefore, it is expected to inhibit the transcription from the pGL3-promoter vector. As expected, actinomycin D was capable of inhibiting reporter gene expression from this vector (Fig. 4B). The fact that no such inhibition was observed with XR5944 (Fig. 4A) at the concentrations at which it inhibited ER activity suggests that, in the *luciferase* gene expression systems used, XR5944 does not act through inhibiting the RNA elongation or the basal promoter activities.

XR5944 Displays a Certain Degree of Specificity In Action

Our previous data (19) and the data presented here indicate that XR5944 inhibits the DNA binding of transcription factors activator protein 1 and ER, both of which contain 5′-TG-3′ in their consensus binding sites. To determine whether inhibition is specific to such transcription factors, whose consensus DNA binding sites contain 5′-TG-3′ motifs, we carried out similar reporter transactivation assays using the pGAGC6 plasmid (Fig. 1C) that contains tandem repeats of the consensus binding site (5′-GGGGCGGGGC-3′) for the Sp1 transcription factor. Sp1 binds to a DNA sequence containing multiple guanines and cytosines. Moreover, based on the preferred DNA-binding sequence of XR5944 revealed by structural information, the

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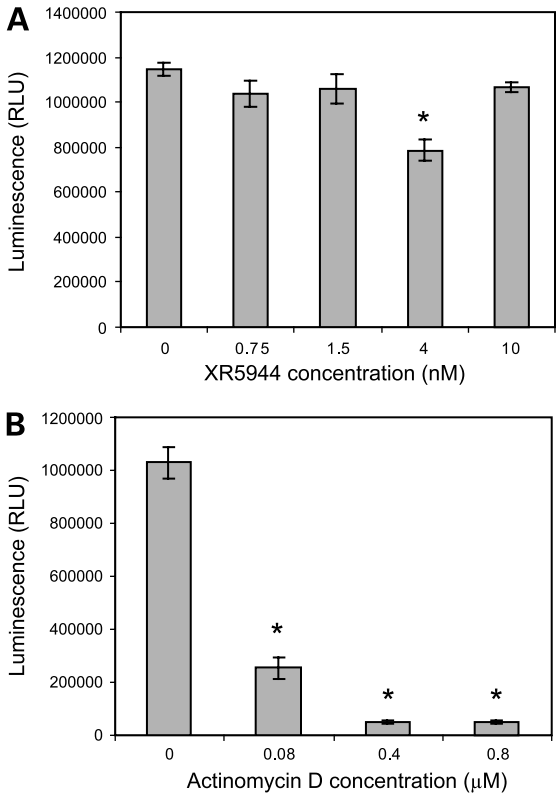

Figure 4. Effects of XR5944 (**A**) and actinomycin D (**B**) on *luciferase* reporter gene expression by the pGL3-promoter vector. This vector contains an SV40 minimal promoter upstream of the *luciferase* coding sequence. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.01$, statistically significant difference.

drug should have less affinity for the Sp1 binding site than for ER or activator protein 1 binding sites and therefore should inhibit the Sp1 activity to a lesser extent. Indeed, Sp1 transactivation is not significantly inhibited by XR5944 at the same concentrations at which it inhibits ER transactivation (Fig. 5A). Thus, the inhibition of transcription factor activity by XR5944 seems to be DNA sequence specific. In contrast, the RNA polymerase inhibitor actinomycin D still inhibits the transcription from this Sp1-containing promoter, as expected (Fig. 5B).

Discussion

Here, we have investigated the potential of XR5944 to inhibit the DNA binding and the subsequent transcriptional activity of the ER protein. Using *in vitro* and cell-based assays, we have shown that XR5944 specifically inhibits the DNA-binding and transactivation functions of ER. The potency of XR5944 in inhibiting the ER activity in cultured cells is reflected by its effective concentrations in luciferase reporter assays in which the drug is active at concentrations as low as 0.75 to 4 nmol/L following 24 h of incubation. It needs to be noted that these effective XR5944 concentrations observed by us in luciferase assays, and by Di Nicolantonio et al. (16) in *ex vivo* experiments, are significantly lower than the XR5944 concentrations used to obtain ER inhibition in EMSA. This difference in effective concentrations is likely due to the low sensitivity of *in vitro* EMSA and to the significantly different conditions between EMSA and cell culture–related assays, such as incubation times with drug and incubation temperatures. Similar discrepancies between effective concentrations *in vitro* and in cells have previously been seen with a number of cytotoxic drugs (25).

XR5944 does not seem to inhibit the basal promoter activity of reporter vectors, as shown by the assay with the vector containing an SV40 promoter. In contrast, the RNA polymerase inhibitor actinomycin D is capable of inhibiting the transcription from the same SV40 promoter-reporter vector. Hence, it is suggested that, unlike actinomycin D, XR5944 acts through inhibition of the DNA binding of transcription factors rather than through nonspecific inhibition of RNA elongation in these systems. It is of significance to note that XR5944 exhibits some degree of specificity in its action, as it does not inhibit the reporter gene transactivation by Sp1 transcription factors. Although the remarkable degree of cytotoxic potency exhibited by XR5944 may also suggest direct DNA damage by the drug,

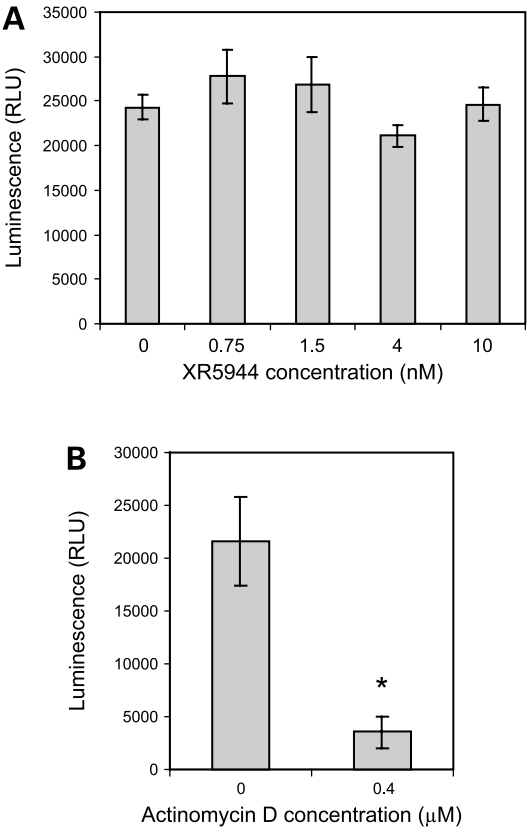

Figure 5. Effect of XR5944 on Sp1 transactivation in MCF-7 cells. The vector contains multiple Sp1 binding sites upstream of the adenovirus major late-initiator TATA box. *Columns,* mean of three independent experiments; *bars,* SE. *, $P < 0.01$, statistically significant difference.

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indirect evidences such as its effect being independent of p53, which is a major marker for DNA damage (15), and its inhibition of RNA synthesis occurring at a much lower concentration than inhibition of DNA synthesis (9), indicate that the primary action of XR55944 is likely to be related with its transcriptional inhibition. In addition, such potent inhibition of transcription factors has not been observed for other DNA-damaging agents, including DNA major groove binders.

The concept of ligand-DNA interactions with regard to hormones has been a subject of significant research and discussions (28–32). The ''ligand insertion hypothesis'' was a result of such observations, in which hormonal activation is hypothesized to involve a step in which the ligand is inserted between DNA base pairs, facilitated by receptor proteins (33, 34). A few estrogen agonists and antagonists have recently been designed *de novo* based on this hypothesis (35–37). This area of research, in which inhibition of ER binding to DNA is attempted by the use of DNA intercalators, is still in its early stages. It is thus quite significant that XR5944 has been shown to be capable of inhibiting the DNA binding of ER, both *in vitro* and *in vivo.* Together, these data render importance to XR5944 as a compound with an attractive mechanism of ER inhibition that may be used in the development of potential therapeutic agents for treatment of ER-expressing breast cancers.

Currently, endocrine therapy is the treatment of choice for patients with ER-expressing breast cancers. Only about half of metastatic breast cancers expressing ER respond to endocrine therapy, and, of these, only 50% reduction is seen in the development of recurrent disease (8). These statistics reflect the limitations of the use of endocrine therapy in treatment of ER-expressing breast cancers, where half of the cases seem to be *de novo* resistant to endocrine therapy and a significant proportion tend to develop acquired resistance. A major reason for the observed *de novo* or acquired resistance is likely to be the estrogen-independent receptor activation that continues to control the expression of estrogen-regulated genes. Such estrogen-independent receptor activation has been shown to occur as a result of various mechanisms, including phosphorylation of ER by different protein kinases (38, 39) and mutations in ER (40, 41). A significant feature of these resistance mechanisms is that they all still involve the DNA binding of ERs. Thus, a DNA intercalator that binds and occupies the ER binding site, and thereby inhibits the DNA binding of ER transcription factors, will be a useful breast cancer therapeutic agent that would overcome the resistance to the existing endocrine therapies.

In addition to the drug resistance, other disadvantages of endocrine therapy include the increased risks of endometrial cancer and thromboembolic disease seen with selective estrogen modulators like tamoxifen that act as estrogen agonists in bone, liver, and uterus (42, 43). Unlike tamoxifen, which binds to ER, drugs targeting ERE are less likely to display ER agonist activity and subsequent side effects. Moreover, this approach of targeting ERE may also be used in conjunction with other endocrine agents for management of ER-expressing breast cancers.

Previous data has suggested that while XR5944 may be a general inhibitor of transcription, it may also inhibit specific genes at low concentrations (9). We speculate that the specific transcription factor(s) inhibited by XR5944 in different cancer cells are likely to display a similar sequence specificity in their DNA binding. Our data presented here indicate that ER is specifically inhibited by XR5944 in MCF-7 breast cancer cells. Due to the high levels of ER activity observed, it is likely that ER is the major target for XR5944 action in these cells. Interestingly, it has been found that breast and gynecologic malignancies are very sensitive to XR5944 in *ex vivo* assays (16), possibly owing to the high levels of ER activity in these cancers.

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