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A Rationally Designed Genotoxin that Selectively Destroys Estrogen Receptor-Positive Breast Cancer Cells

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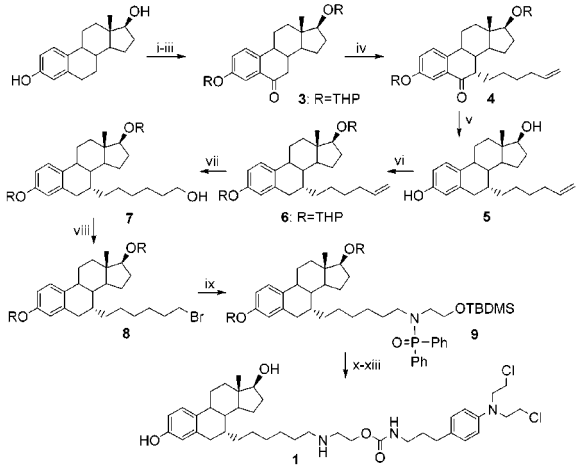
The persistence of genetic damage produced by alkylating agents[1] as well as the antagonism of essential biochemical processes such as transcription can have lethal consequences for malignant cells.[2] Both mechanisms have been identified in studies to uncover the reasons for the efficacy of cisplatin in the treatment of several cancers.[2a,3] We describe a synthetic strategy to create bifunctional molecules that produce DNA adducts capable of binding the estrogen receptor (ER), which is aberrantly expressed in many breast cancer cells.[4] It is speculated that DNA adducts that form complexes with the ER will be poorly repaired in these cells because they are camouflaged from detection by DNA repair enzymes. Consequently, the DNA lesions persist. Furthermore, the DNA adducts would be expected to act as "molecular decoys" capable of displacing the ER from its natural targets and antagonizing its role in malignant growth. In healthy cells, where the abundance of the ER is minimal, no such ER-DNA adduct complexes will be present, and the cell should survive.[5]

In this report we describe the design and synthesis of compound **1**, a bifunctional agent that can form covalent DNA adducts capable of binding the ER with high affinity and specificity. We show that **1** has selective toxicity toward ER+ breast cancer cells compared to ER− cells in vitro.

Compound **1** consists of a bis-chloroethyl aniline mustard as the DNA alkylating unit tethered to estradiol, the natural ligand for the ER. The site of substitution of estradiol in **1** was based on reports that relatively large alkyl groups can be attached at the 7α position with retention of high affinity for the ER.[6] The synthetic strategy for **1** is shown in Scheme 1. Compound **7**, a key compound in the synthesis, was prepared by a modification of a published strategy.[7] Briefly, **3** was functionalized with a 6-carbon chain at the 7-position in α-stereochemistry to provide the alkenyl steroid **4**. Efficient reduction of the 6-oxo group in **4** was achieved with $Et_3SiH/BF_3 \cdot Et_2O$; however, this treatment also caused the loss of 3,17-tetrahydropyranoxy (THP) groups producing diol **5**. The 3,17-OHs of **5** were reprotected with THP groups to afford **6**, followed by oxidation of the alkene at the terminus of the linker to provide alcohol **7**. Steroid alcohol **7** was converted to bromide **8**, which was subsequently allowed to react with a protected ethanolamine to give **9**. Compound **9** was desilylated with tetrabutylammonium fluoride (TBAF) and converted to a carbonate intermediate with *p*-nitrophenyl chloroformate. The carbonate was coupled to (*N*,*N*-bis-2-chloroethylaminophenyl)propylamine that was prepared from chlorambucil via the Curtius reaction.[8] Deprotection of the product in HCl/methanol furnished **1**.

The affinity of **1** for the ER was first determined. Using a competitive binding assay[9] with [^{3}H]-17β estradiol, compound **1** was found to have a relative binding affinity (RBA) for the calf uterine ER of 30; RBA of estradiol = 100.

Scheme 1 [a]



[a] Conditions: (i) DHP, pyridinium *p*-toluenesulfonate, reflux, 92%; (ii) *n*-BuLi, KO*t*Bu, $B(OMe)_3$, H_2O_2; (iii) PCC, 59%; (iv) 6-iodohexene, KO*t*Bu, Et_3B, 47%; (v) $CH_3COCl/MeOH$, Et_3SiH, $BF_3.Et_2O$, 74%; (vi) DHP, pyridinium *p*-toluenesulfonate, 77%; (vii) $BH_3.THF$, KOH/H_2O_2, 66%; (viii) methane sulfonyl chloride, LiBr, 86%; (ix) $Ph_2P(O)NH\text{-}CH_2\text{-}CH_2\text{-}O$*t*BDMS, NaH, catalyst tetra-*n*-butylammonium bromide, 73%; (x) TBAF, 73%; (xi) *p*-nitrophenylchloroformate, DIEA; (xii) 4-(*N*,*N*-bis-2-chloroethylamino-phenyl)-propylamine, DIEA; (xiii) H+, 60%.

Next, the ability of **1** to modify DNA covalently was investigated. Plasmid DNA was incubated with 100 μM [^{14}C]-**1**[10] at 37 °C for up to 6 h. After unbound **1** was removed by phenol-$CHCl_3$ extraction and ethanol precipitation, the radioactivity associated with DNA was measured. The amount of radioactivity bound to DNA increased at a constant rate over the 6-h period indicating the formation of covalently bound **1** (see Supporting Information). On the basis of previous studies on DNA alkylation by nitrogen mustards,[11] it is likely that covalent adducts of **1** are formed primarily at the N*7* atom of guanines. To investigate the identity of the covalent adducts, **1**-modified DNA was treated with 0.1 N HCl, the major product from the digested DNA was isolated by reversed-phase HPLC and analyzed by full scan electrospray mass spectrometry to yield a prominent molecular ion signal at M + H^+/z 813.5051. This mass is consistent with a chemical structure in which one ethylene arm of the mustard of **1** is attached to guanine and the other arm contains a −OH substituted for the Cl atom.[12]

The affinity of DNA adducts of **1** for the ER was investigated using an Electrophoretic Mobility Shift Assay (EMSA). Substrates for this assay were prepared by reaction of 5′[^{32}P]-labeled self-complementary oligonucleotide 5′-d(ATTATTGGCCAATAAT) with **1** for 4 h at 37 °C. To assess quantitatively the level of covalent modification under these conditions, the DNA was treated with piperidine (1 M, 90 °C, 1 h) and the products were separated on a denaturing 20% polyacrylamide gel (Figure 1A). This analysis

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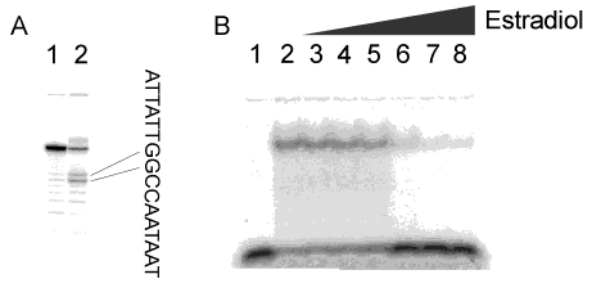

Figure 1. (A) Piperidine treatment of self-complementary deoxy oligonucleotide 5′-d(AATATTGGCCAATATT) treated with **1**. Lane 1: untreated oligonucleotide, Lane 2: oligonucleotide + 200 μM **1**. (B) Retarded mobility of oligonucleotide-**1** in the presence of ER-LBD illustrated by EMSA, and disappearance of the retarded band by competition with estradiol. Lane 1: untreated oligonucleotide + ER-LBD, Lane 2: oligonucleotide modified by 200 μM **1** + ER-LBD, Lanes 3−8: modified oligonucleotide + ER-LBD + 10−300 nM estradiol.

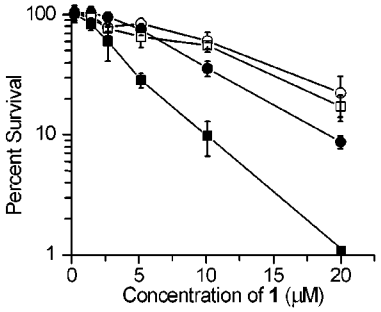

Figure 2. Survival of MCF-7 (ER+) (■, □) and MD-MB231 (ER-) (●, ○) cells treated with **1** (closed symbols) or chlorambucil (open symbols).

found that 50% of the 16-mer oligo was cleaved, forming two products identical with those produced by the Maxam−Gilbert sequencing reaction for guanine (data not shown). EMSA was performed by combining the modified [^{32}P]-labeled 16-mer with a 30 kD fragment of the ER containing the ligand binding site for estradiol (ER-LBD).[13] Analysis on a 4% nondenaturing polyacrylamide gel (0.5X TBE) revealed that approximately 50% of the 16-mer formed a slowly migrating band (Figure 1B; lane 2). The amount of retarded band corresponded closely with the level of modification of the 16-mer as revealed by piperidine treatment. Furthermore, no slowly migrating band was seen with unmodified 16-mer (Figure 1B, lane 1) or with 16-mer modified with the nitrogen mustard chlorambucil (see Supporting Information). To examine the identity of the complex, increasing amounts of 17β-estradiol were added to the mixture containing the ER-LBD and **1**-modified 16-mer. As shown in Figure 1B (lanes 3−8), addition of 17β-estradiol resulted in disruption of the ER-LBD-DNA complex. These in vitro results indicated that ER-LBD selectively forms a complex with **1**-modified oligo, and raises the strong possibility of formation of similar complexes in cells.

Clonal survival assays were performed in ER+ and ER− breast cancer cell lines to determine if ER status affected the sensitivity of cells to the toxicity of **1**. To reveal clearly the effect of DNA adducts, cells were exposed to **1** for 2 h after which fresh drug-free medium was added to the cell cultures. This procedure minimized the possibility that unreacted **1** could function as a receptor antagonist and thereby inhibit growth or survival. Under these conditions **1** was found to possess a significantly lower EC_{50} in the ER+ cell line MCF-7 (EC_{50} = 3.5 μM) as compared to the ER− cell line MDA-MB231 (EC_{50} = 9.2 μM) (Figure 2). In contrast to these results, chlorambucil under the same conditions did not show any difference in EC_{50} between these two cell lines (Figure 2).

The fact that ER status affected the EC_{50} of **1**, but not of chlorambucil, is consistent with the role of the ER as an effector of selective toxicity of **1**. Covalent **1**-guanine adducts have been identified in DNA isolated from treated cells (unpublished). Therefore, **1** is stable to cell culture conditions and can form DNA adducts in cells identical with those formed in vitro. We are currently investigating the role of **1**-DNA adducts in vivo to determine if they are less efficiently repaired in ER+ cells and to evaluate whether the association of the ER with DNA adducts of **1** is directly responsible for the greater sensitivity of MCF-7 cells.

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Supporting Information Available: Procedures for the synthesis of **1**, hER-LBD expression and purification, RBA experiments, DNA-damaging studies, gel shift experiments and toxicity experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) ^{14}C]-**1** was obtained by reacting the carbonate intermediate with 4-(*N*,*N*-bis-2-chloroethylaminophenyl)-[1-^{14}C] propylamine in step xii in Scheme 1; additional details are given in the Supporting Information.

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(12) DNA modified with d_4-**1** (deuterium labeled **1**) and analyzed using the same conditions for hydrolysis, HPLC, and mass spectrometry revealed a molecular ion signal at M + H$^+$/z 817.5. This result is consistent with that of d_4-**1** attached to guanine as described. The preparation of d_4-**1** is described in the Supporting Information.

(13) The 30 kD ER-LBD was obtained by expression of a gene fragment containing amino acids 282 to 595 of the human estrogen receptor in *Sf*21 cells using the baculovirus expression system (see Supporting Information).

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