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Disruption of Gene Expression and Induction of Apoptosis in Prostate Cancer Cells by a DNA-Damaging Agent Tethered to an Androgen Receptor Ligand

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Summary

The goal of our work was the design of DNA-damaging agents that disrupt both DNA repair and signaling pathways in prostate tumor cells. A DNA alkylator (*N,N-bis*-2-chloroethyl aniline) was linked to a steroid ligand (17β-hyroxy-estra-$\Delta^{4(5),9(10)}$-3-one) to produce a complex molecule (11β-dichloro) that forms DNA adducts that bind the androgen receptor (AR). We speculated that DNA adducts in an AR-DNA adduct complex would be camouflaged from DNA repair proteins that would otherwise remove the adducts in prostate cancer cells. Furthermore, transcription dependent on the AR would be antagonized by AR redistribution to sites distant from AR-driven promoters. The anticancer potential of 11β-dichloro was demonstrated against prostate cancer cells in vitro and in vivo. 11β-dichloro induces a unique pattern of gene disruption, induces apoptosis in apoptosis-resistant cells, and shows promising anticancer activity in animals.

Introduction

Cytotoxic agents that act by covalent modification of DNA were the first modern anticancer chemotherapeutics and remain major components of combination chemotherapy regimens [1, 2]. In combination with drugs that act by other mechanisms, alkylating antitumor drugs have produced impressive and even curative responses in the treatment of some cancers (e.g., cisplatin in testicular cancer) [3]. Frequently, however, tumors are found to have inherent resistance to these compounds or to develop resistance during the course of treatment. The rapid evolution of resistance makes it imperative to develop new agents that can defeat the molecular barriers responsible for clinical failure.

The strategy of combining several agents that can increase the vulnerability of certain cancers to alkylating drugs is one approach to overcoming resistance. Sometimes, however, drug-drug interactions or overlapping side-effect profiles make multidrug regimens problematic.

Another strategy is the design of alkylating compounds with novel mechanisms that disrupt multiple biochemical pathways responsible for tumor growth and survival. We report the design, synthesis, and biological activities of a multifunctional compound based on an original concept that sought to incorporate several mechanisms of action into a single anticancer agent. Our idea was to produce a compound that could form covalent DNA adducts that have high affinity for a protein essential for the growth and survival of tumor cells. Protein-adduct complexes formed in these cells would not only camouflage the DNA adduct, making it difficult to repair, but also prevent the protein from performing its role in cell growth and/or survival. Figure 1A illustrates these mechanisms showing alternative fates of DNA adducts formed by the reactive "warhead" portion. On the left, exposed DNA adducts that are not bound to proteins through the protein recognition (or "ligand") domain can be detected and efficiently excised by repair enzymes, limiting potential genotoxic effects. On the right, however, the "ligand domain" (e.g., a steroid) forms a complex with its cognate protein in target cells, camouflaging adducts and interfering with both the repair process and the normal biological function of the protein bound to the adduct. Cells that express proteins that bind to the ligand domain will be more vulnerable to the toxic effects of the synthetic toxins.

The concept that one could use a custom-designed DNA-damaging agent to hijack a nuclear transcription factor required for cancer-cell growth was introduced by the design and synthesis of compounds that produce DNA adducts to which the estrogen receptor (ER) binds with high affinity [4–6]. Breast cancer cells that express high levels of the ER show greater sensitivity toward these compounds. The current paper describes the design, chemical synthesis, and biological activities of compounds that have been designed to form DNA adducts that bind to the androgen receptor (AR), a transcription factor expressed at high levels in many prostate cancers [7]. AR expression is found in primary prostate cancer and is frequently observed in both hormone-sensitive and hormone-refractory forms of the disease [8]. There is currently no effective long-term treatment for the advanced hormone-refractory stage of the disease. Mutations in the AR, along with aberrant expression of coactivator proteins, can allow transcriptional activation in response to antiandrogens and endogenous hormones, leading to cancer progression and therapeutic resistance [9, 10]. The premise of sequestering the AR at sites of DNA adducts was that repair of the genetic damage that is camouflaged in the receptor-adduct complex would be impeded, as would the function (or functions) of the AR responsible for tumor growth.

We report the synthesis and activities of a bifunctional compound composed of *N,N-bis*-(2-chloroethyl)-aniline linked to an 11β-substituted estra$\Delta^{4(5),9(10)}$-3-one. This new compound, 11β-dichloro (Figure 1B), is capable of producing DNA adducts that interact with the AR. To investigate the mechanism of action of this compound, we prepared an unreactive analog that could not form DNA adducts (11β-dimethoxy) (Figure 1B). Investigation of the biological responses of LNCaP cells to our 11β compounds revealed biochemical changes

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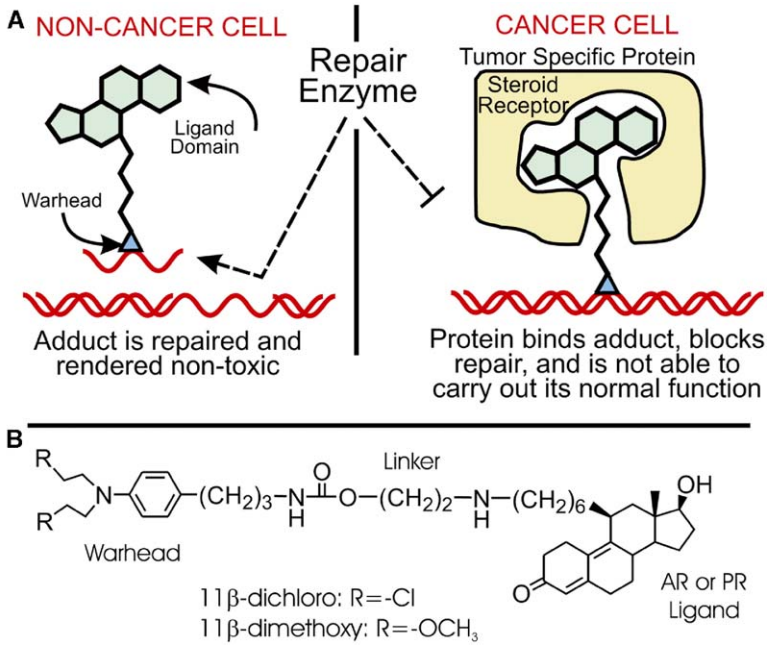

Figure 1. Scheme Illustrating the Mechanism of Selective Toxicity by Compounds that Form DNA Adducts that Attract Proteins in Cancer Cells

(A) 11β-dichloro was designed to have the ability to covalently modify DNA and still retain the ability to bind the protein with which the protein recognition domain was designed to interact. In noncancer cells, which don't express the protein of interest at high levels, adducts are repaired by DNA repair enzymes. In cancer cells, in which the protein of interest is overexpressed, binding of the protein to adducts blocks adduct repair and prevents the protein of interest from carrying out its normal function in the cell.

(B) Structure of 11β compounds. 11β-dichloro forms DNA adducts with affinity for the AR and PR. 11β-dimethoxy does not form DNA adducts but does have affinity for the AR and PR.

that are of interest with regard to the antitumor effects we observed in animal models.

Results

Synthesis of Bifunctional 11β-Substituted Estradien-3-One Compounds

The design of the androgen ligand portion of the 11β compounds is based on the reported antiandrogenic activity of molecules containing 11β-substituted 17β-hydroxy-estra-$\Delta^{4(5),9(11)}$-3-one [11]. The general synthetic strategy for linking the 11β-substituted estradien-3-one to *N,N-bis*-(2-chloroethylaminophenyl)-propylamine is shown in Figure 2. Epoxide **1** was prepared by epoxidation of the known compound 3,3-ethylenedioxy-17β-hydroxyestra-5(10),9(11)-diene by a published procedure [12]. The alkyl chain was then introduced at the 11β position by conjugate opening of the allylic epoxide (**1**) by the Grignard reagent prepared from the alkyl bromide in the presence of copper(I) [13]. Steroid alcohol (**2**) was converted to the bromide and allowed to react with a protected ethanolamine to give the alcohol (**3**), which was converted to a carbonate intermediate with p-nitrophenyl chloroformate. The carbonate was then coupled to *N,N-bis*-(2-chloroethylaminophenyl)-propylamine, and the final product, 11β-dichloro (**4**), was obtained by removal of protecting groups under acidic conditions. Details of some of these procedures have been published [5] and are contained in Supplemental Data. An unreactive analog of 11β-dichloro, in which the two chlorine atoms of the 2-chloroethyl groups were substituted by methoxy groups (11β-dimethoxy) (Figure 1B), was prepared by reflux of 4-(*N,N-bis*-2-chloroethylaminophenyl)-propylamine with $NaOCH_3$ producing 4-(*N,N-bis*-2-methoxyethylaminophenyl)-propylamine, which was then substituted for its chloro analog in the synthesis as described for 11β-dichloro.

11β-Dichloro Forms DNA Adducts that Interact with the Androgen Receptor and the Progesterone Receptor

Key to the biological activities of our bifunctional molecules is their ability to covalently modify DNA, forming adducts that bind to the androgen receptor (AR). A radiolabeled, self-complementary deoxyoligonucleotide, 5′[^{32}P]-d(ATTATTGGCCAATAAT)-3′, was incubated either with the 11β-dichloro compound or with the unreactive 11β-dimethoxy analog in order to determine the ability

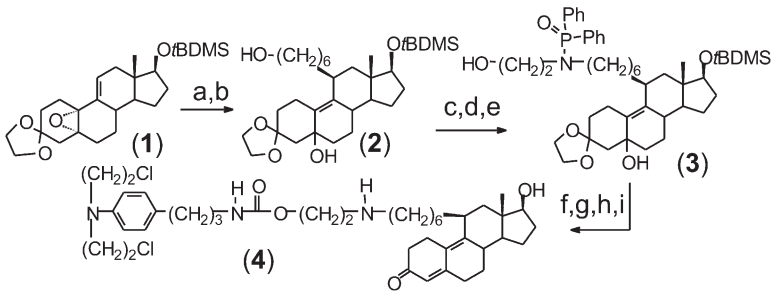

Figure 2. Synthetic Scheme for Synthesis of the 11β-dichloro Compound

(a) *t*BDMSO$(CH_2)_6$MgBr, CuBr•Me_2S, THF, –20°C; (b) t$BuNH_4F$, THF, room temperature (rt); (c) CH_3SO_2Cl, DIEA, THF, 0°C; (d) LiBr, DMF, 45°C; (e) $Ph_2P(O)NH(CH_2)_2O$*t*BDMS, Bu_4NBr, NaH, benzene, 65°C; (f) *t*$BuNH_4F$, THF, rt; (g) *p*-nitrophenylchloroformate, DIEA, TFH, rt; (h) 4-(*N,N-bis*-2-chloroethylaminophenyl)-proplyamine, DIEA, THF, 75°C; (i) HCl, THF, rt.

Table 1. Relative Binding Affinities of 11β-Compounds and 11β-DNA Adducts for the Androgen Receptor and Progesterone Receptor

Receptor	11β-Dichloro	11β-Dimethoxy	11β-Modified DNA
AR	11.3% (9.7–13.2)	18.1% (15.5–21.1)	0.22% (0.12–0.43)
PR	4.2% (3.5–4.8)	3.6% (3.1–4.3)	0.04% (0.02–0.08)

RBA: relative binding affinity of test compound compared with R1881 (RBA = 100%) for AR or progesterone (RBA = 100%) for PR as determined by competitive binding assay with [^{3}H]-R1881 (AR) or [^{3}H]-progesterone (PR); values in parentheses represent the 95% confidence interval for calculated RBAs.

of the compounds to covalently modify DNA. The location of modified bases as well as the extent of modification were determined by piperidine cleavage followed by gel electrophoresis [5]. As expected, the 11β-dichloro formed covalent products that resulted in fragmentation of the deoxyoligonucleotide at either guanine residue upon incubation with piperidine (data not shown). Approximately 75% of the oligonucleotide treated for 4 hr with 125 uM 11β-dichloro was found to be piperidine labile. Under the same conditions, the 11β-dimethoxy did not produce any piperidine-labile sites.

The relative affinities of the 11β compounds for the AR and the progesterone receptor (PR) were determined by competitive binding assays. The results of these assays are shown in Table 1. The 11β-dichloro compound was found to have a relative binding affinity (RBA) of approximately 11% for the AR in whole-cell LNCaP extracts when compared with the synthetic androgen [^{3}H]-R1881. Because the affinity of R1881 for the LNCaP AR is about twice that of a natural ligand, dihydrotestosterone (DHT) [14], the RBA of 11% implies that the affinity of 11β-dichloro is approximately 20% that of a natural ligand. Comparing 11β-dichloro with [^{3}H]-progesterone, we found that 11β-dichloro had a much lower affinity for the PR, with an RBA of 4%. Substitution of chlorine atoms by methoxy groups in the 11β-dimethoxy analog did not influence receptor binding. The RBAs of the 11β-dimethoxy compound for the AR and PR were similar to those of 11β-dichloro (Table 1).

We also estimated the RBA for the AR of 11β-DNA adducts formed by incubation of the self-complementary deoxyoligonucleotide 5′-d(ATTATTGGCCAATAAT)-3′ with the 11β-dichloro compound. Addition of 11β-modified DNAs—but not unmodified DNAs—resulted in decreased amounts of [^{3}H]-R1881 bound to the AR in cell-free extracts from LNCaP cells. Based on the molar concentration of DNA adducts added, we estimate an RBA of 0.2% for the covalently linked ligand when compared to R1881 (Table 1).

11β Compounds Induce Growth Arrest and Apoptosis in LNCaP Cells

The biological effects of the 11β-dichloro and 11β-dimethoxy compounds were examined in AR-positive LNCaP prostate cancer cells in culture. LNCaP cells exposed to 11β-dichloro at concentrations >5 μM underwent rapid morphological changes associated with apoptosis. Figure 3 shows the physical appearance of LNCaP cells left untreated (Figure 3A), after a 6 hr exposure to 20 μM chlorambucil (Figure 3B), after 6 hr treatment with 10 μM 11β-dichloro (Figure 3C), and after 6 hr treatment with 10 μM 11β-dimethoxy (Figure 3D). We selected chlorambucil for comparison because it contains the identical *N,N-bis*-(2-chloroethyl)-aniline moiety as does 11β-dichloro and would therefore be expected to modify covalently the same atoms in DNA [15]. Despite their chemical similarities, treatment with 11β-dichloro resulted in striking cytoplasmic contraction and detachment from the culture dish within 6 hr, whereas treatment with chlorambucil did not. Treatment of LNCaP cells with 11β-dimethoxy produced a transient and less-dramatic change in cell morphology. The cells remained attached to the surface of the culture dish and recovered to their pretreatment morphology by 24 hr.

To determine whether an apoptotic program was activated by 11β-dichloro, we examined the status of several apoptotic markers in the treated cells. Annexin V staining was used as an early marker of changes in membrane phospholipids that are associated with cells undergoing apoptosis [16]. LNCaP cells treated for various lengths of time with 11β compounds or chlorambucil were stained with the vital stain 7-amino-actinomy-

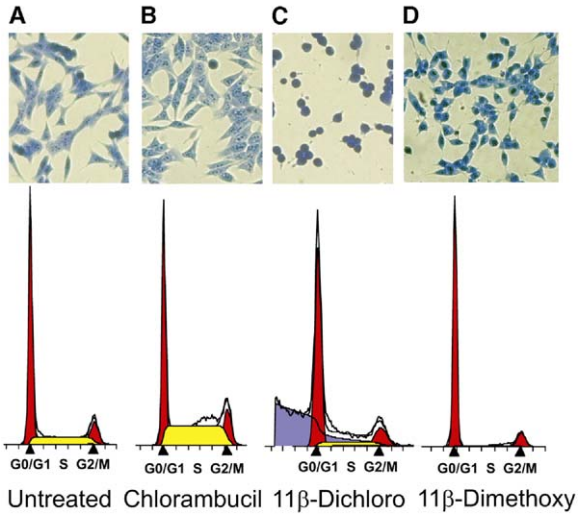

Figure 3. Images of LNCaP Cell Morphology and Cell-Cycle Analysis of LNCaP Cells Treated with 11β Compounds

Top: LNCaP cells after 6 hr treatment with 11β compounds (10 μM) or the anticancer drug chlorambucil (20 μM). Cells in exponential growth phase were treated for 6 hr, fixed, and stained with Giemsa. (A) Vehicle-treated LNCaP cells. (B) Cells exposed to chlorambucil showed no effect on cellular shape. (C) Cells treated with 11β-dichloro showed dramatic contraction and detachment. (D) Cells treated with the unreactive 11β-dimethoxy showed slight contraction, which was reversed by 24 hr (not shown). Bottom: cell-cycle analysis of LNCaP cells treated with indicated compounds for 17 hr.

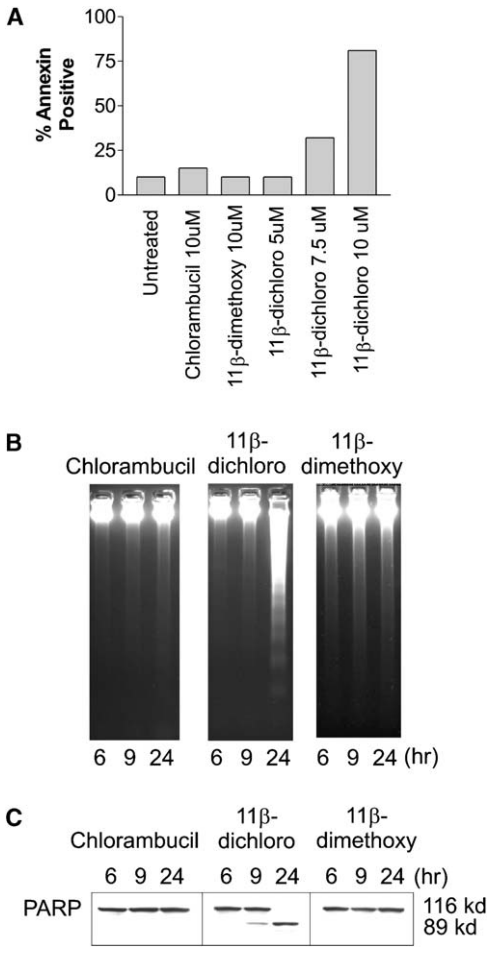

Figure 4. LNCaP Cells Undergo Apoptosis Upon Treatment with 11β-dichloro

(A) Annexin V staining of LNCaP cells after treatment for 15 hr with indicated compounds. Cells treated with >5 μM 11β-dichloro showed evidence of increased Annexin V staining.

(B) Agarose gel electrophoresis of DNA isolated from LNCaP cells after 24 hr exposure to chlorambucil (20 μM), 11β-dichloro (10 μM), or 11β-dimethoxy (10 μM). DNA fragmentation occurs in cells treated with 11β-dichloro.

(C) Western blot analysis of cellular extracts probed with antibodies against full-length and cleaved PARP showed that treatment with 11β-dichloro (10 μM) led to cleavage of PARP within 9 hr. No cleavage was seen in cells treated with either chlorambucil (20 μM) or 11β-dimethoxy (10 μM).

cin D (7-AAD) as well as a phycoerytherin-labeled antibody to Annexin V. Analysis of stained cells by flow cytometry revealed a progression of cells from those that were initially Annexin-positive to a population that stained with both Annexin and 7-AAD, indicating a loss of membrane integrity. These changes were not observed in LNCaP cells treated with either 11β-dimethoxy or chlorambucil (Figure 4A). Analysis of DNA isolated from treated cells revealed the internucleosomal fragmentation and laddering pattern of genomic DNA that is consistent with the activation of an apoptotic endonuclease [17] only in LNCaP cells treated with 11β-dichloro (Figure 4B). DNA that was isolated from cells treated with 11β-dimethoxy or chlorambucil remained at high molecular weight. Furthermore, cleavage of poly-ADP ribose polymerase (PARP), another apoptotic marker [18], was also seen in LNCaP cells treated with 11β-dichloro but not in cells treated with 11β-dimethoxy or chlorambucil (Figure 4C). These data indicate that 11β-dichloro is clearly effective in overcoming the roadblocks in LNCaP cells that prevent the conventional DNA damaging agent chlorambucil from initiating apoptosis.

Although it did not cause apoptosis, the unreactive 11β-dimethoxy compound did have an effect on cell growth. Analysis of the cell-cycle distribution of LNCaP cells treated with 11β-dimethoxy indicated that the cells arrested in the G1 phase of the cell cycle (Figure 3D). Similar analyses revealed a sub-G1 apoptotic fraction in LNCaP cells treated with 11β-dichloro (Figure 3C), whereas cells treated with chlorambucil were primarily blocked in S phase (Figure 3B).

The results described above led us to investigate the expression of the G1 cell-cycle checkpoint proteins, specifically the cyclin-dependent kinase (CDK) inhibitors p21 and p27, to determine their involvement in the growth arrest and apoptotic responses of LNCaP cells treated with the 11β compounds. Figure 5A shows the levels of these two CDK inhibitors in LNCaP cells exposed for up to 15 hr with 11β-dichloro, 11β-dimethoxy, or chlorambucil. The levels of p21 were affected by all three compounds. Amounts of p21 initially decreased in cells treated with either of the 11β compounds. This initial reduction was followed by either recovery of p21 protein to a basal level in cells treated with 11β-dimethoxy (6–9 hr) or an increase by several fold in cells treated by 11β-dichloro (15 hr). In response to chlorambucil, the amount of p21 showed a constant increase throughout the 15 hr period. Thus, p21 levels were increased at 15 hr in cells treated with either compound that was capable of forming DNA adducts but not in those treated with the unreactive 11β-dimethoxy. The expression level of p27 was unchanged by treatment with chlorambucil. Both of the 11β compounds, however, increased the level of p27 expression.

Given that our compound design was based on the tethering of two distinct chemical entities—a ligand binding portion similar in structure to the PR and AR antagonist mifepristone (RU486) and a DNA-damaging moiety similar to the nitrogen mustard chlorambucil—we were interested in learning if the rapid changes we observed in the levels of the two CDK inhibitors could be reproduced by treatment with RU486 alone or in combination with DNA damage caused by chlorambucil. Figure 5B clearly illustrates the need for the two entities to be linked. As previously determined, chlorambucil increased expression levels of p21. However, treatment with chlorambucil alone, RU486 alone, or a combination of the two did not affect the levels of p27 in cells during exposures up to 15 hr. In addition, cell-cycle analysis of LNCaP cells treated with the combination of RU486 and chlorambucil did not reveal any evidence of nuclear fragmentation (cells with a sub-G1 DNA content) suggesting that—similar to treatment with chlorambucil alone—this combination was not capable of inducing apoptosis (data not shown).

The levels of p27 can be regulated by the protein's

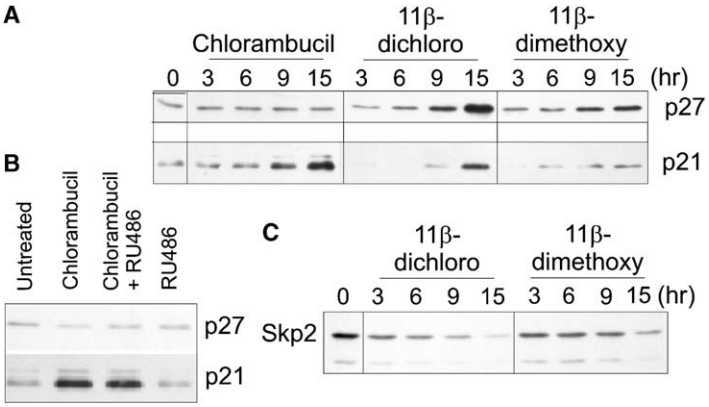

Figure 5. Immunoblot Analysis of Cell-Cycle Checkpoint Proteins in LNCaP Cells

(A) Levels of $p21^{CIP1}$ and $p27^{KIP1}$ in extracts from LNCaP cells that were treated with chlorambucil (20 μM), 11β-dichloro (10 μM), or 11β-dimethoxy (10 μM) for up to 15 hr.

(B) Levels of $p21^{CIP1}$ and $p27^{KIP1}$ in extracts of LNCaP cells treated for 15 hr with chlorambucil (10 μM), RU486 (10 μM), or both (each at 10 μM).

(C) Levels of Skp2 in extracts of LNCaP cells treated under the same conditions as in (A).

ubiquitin-dependent degradation through the ubiquitin E3 ligase SCF^{SKP2} [19, 20]. Therefore, it was of interest to probe the effects of 11β-dichloro and its dimethoxy analog on the expression levels of Skp2, the F box protein component of the SCF ubiquitin ligase complex. Figure 5C shows the effects of both compounds on Skp2 levels in LNCaP cells treated for up to 15 hr. Treatment with either of the 11β compounds resulted in decreased expression of Skp2. The decreases in Skp2 expression correlate well with the relative levels of increase in the expression of p27 in LNCaP cells treated with the 11β compounds. The reciprocal pattern of expression of these proteins shown in Figures 5A and 5C is consistent with the view that Skp2 is directly involved in the increased expression of p27 in LNCaP cells treated with these compounds.

11β-Dicholoro Inhibits Growth of LNCaP Cells in a Mouse Xenograft Model of Human Cancer

The sensitivity of LNCaP cells in culture to 11β-dichloro led us to investigate its antitumor activity against LNCaP cells growing as xenografts in nude mice. Preliminary toxicology studies in nontumor-bearing mice found that a consecutive 5-day schedule of 30 mg/kg was well tolerated by the animals as evidenced by minimal weight loss and lack of elevated liver transaminase levels (data not shown). Tumor-bearing animals were treated on a schedule of seven consecutive weekly 5-day cycles with a daily dose of 30 mg/kg administered by intraperitoneal injection. Control animals received vehicle only. As shown in Figure 6, this regimen resulted in a 90% inhibition of tumor growth when assessed on the final day of the study (mean tumor volume [treated] versus mean tumor volume [control] on day 45; $p < 0.0001$). During the 45-day period, the treatment group experienced a mean weight loss of 9.7% compared with the vehicle-treated group. These data demonstrate that at tolerable doses, 11β-dichloro inhibits the growth of LNCaP prostate cancer cells in vivo. The tumoristatic effect that we observed in this model may be due to the relatively small initial size of the tumors (5 mm diameter) at the initiation of treatment with 11β-dichloro. We are investigating the responses of larger, more established tumors to determine whether the cytotoxic responses observed in vitro occur in xenografts resulting in the reduction in tumor size.

Discussion

In this report, we examined the effects of a new, rationally designed, multifunctional compound on the growth of LNCaP prostate cancer cells in culture and in a xenograft animal model. We programmed our compound to produce DNA adducts that would be able to form complexes with a protein that was aberrantly expressed in tumor cells. The new compound (11β-dichloro) comprises a steroid moiety with high affinity for the androgen receptor tethered to a reactive *N,N-bis*-(2-chloroethyl)-aniline. We found that 11β-dichloro causes increased expression of the cell-cycle checkpoint proteins p27 and p21 along with the rapid induction of apoptosis. Because 11β contains a DNA alkylator, one could argue that the DNA damage response initiates the observed cellular effects; we did not observe, however, upregulation of p27 or apoptosis with free chlorambucil. Neither did we observe apoptosis with an unreactive 11β analog that induced p27 and growth arrest at the G1 stage of the cell cycle. The checkpoint and apoptotic responses in LNCaP cells distinguish the

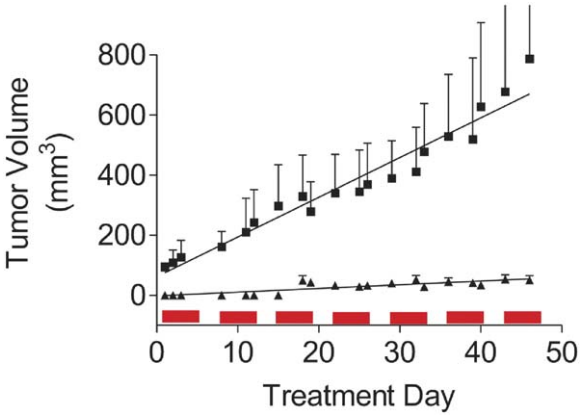

Figure 6. Response of LNCaP Androgen-Dependent Xenografts to 11β-Dichloro

Bars at bottom each represent five consecutive daily doses of 30 mg/kg. Triangles represent tumor volume in mice treated with 11β-dichloro; squares represent tumor volume in control mice treated with vehicle only. (Average tumor volume ± s.d.; n = 5)

11β-dichloro compound from conventional alkylating antitumor drugs.

There are examples in the literature of molecules in which steroid receptor ligands have been combined with chemically reactive genotoxins [21–25]. In most cases, the steroid portion of such molecules is designed to function as a delivery agent and the linker is programmed to hydrolyze and release the active toxin in target cells that express the cognate receptor. In contrast, the linker in our compounds is designed to possess chemical stability as well as to resist hydrolysis by proteases and esterases. Our experiments demonstrate that this manner of linking the two active pharmacophores in 11β-dichloro resulted in cellular responses distinct from those of the independent compounds.

The results reported here expand on earlier work in which we prepared a group of compounds with the ability to form DNA adducts that can bind the estrogen receptor and selectively kill ER-positive breast cancer cells [4, 5]. Based on the structural similarity of the AR to other steroid receptor proteins [26], we incorporated the molecular features and dimensions that proved successful in the development of our initial ER binding bifunctional compounds into the design of new compounds that have good affinity for the AR. Previous studies established the importance of an alkanyl chain of at least six carbons for binding of modified ligands to the ER. We based our attachment of a six-carbon alkyl chain at the 11β position of 17-hydroxyestra$\Delta^{4(5),9(11)}$-3-one on the reported affinity of the antiprogestin drug RU486 for the AR [27]. The remaining features of the new compound directed at prostate cancer cells were adopted from our previous work [4]. Biophysical characterization of the new molecule found that it had good affinity for the AR of LNCaP cells compared to the natural ligand dihydrotestosterone. The LNCaP cell line has a mutation at residue 877 of the AR that alters its affinity for androgen agonists and antagonists [28, 29]. This mutation has been found in patients with advanced, androgen-independent metastatic prostate cancer [30]. It may be necessary to alter the ligand portion of the 11β molecule to increase its affinity for other genetic variants of the AR that occur in prostate malignancies.

The 11β-dichloro compound formed reactive intermediates that produced covalent adducts in DNA. The relative affinity for the AR of these DNA-bound ligands was lower than that of the unreacted 11β-dichloro compound. This may be because unlike the free compound, the 11β-modified DNA contains a variety of structures that may have different steric requirements for binding to the AR. Additional investigations are required to determine if a range of affinities for the AR exists for various 11β-DNA adducts and their relevance to our intended mechanisms of action.

Our studies explored the relationship between the formation of DNA adducts and the ability of 11β-dichloro to cause cell-cycle arrest and apoptosis. Through chemical substitution, we prepared an unreactive analog of 11β-dichloro that was incapable of forming DNA adducts. We demonstrated that the unreactive analog (11β-dimethoxy) was not able to induce apoptosis, although it was able to block LNCaP cells in the G1 phase of the cell cycle, at least temporarily. The results with the control unreactive derivative of 11β-dichloro shed light on the mechanisms of action independent of DNA damage that contribute to the rapid and dramatic response of LNCaP cells to 11β-dichloro.

Direct interaction of the 11β-dimethoxy with the AR in the LNCaP cell line provides one possible explanation for its ability to induce G1 cell-cycle arrest through increased expression of p27, the CDK inhibitor that acts to inhibit G1 cyclin/CDK complexes [31]. The effects of androgens on the proliferation of LNCaP cells are well characterized. Low concentrations of androgens such as DHT stimulate growth, whereas high concentrations are inhibitory [32, 33]. Treatment of growing LNCaP cells with 100 nM DHT has been shown to result in accumulation of p27 and growth arrest within 24 hr [34]. In addition, p27 accumulation during androgen-induced growth arrest has been linked to decreased expression of the Skp2 subunit of the ubiquitin E3 ligase SCF^{Skp2} [20]. The direct binding of Skp2 to the cyclin kinase subunit CKS1 directs the ubiquitination and subsequent proteolysis of p27 [35, 36]. We have uncovered a similar reciprocal relationship between decreased expression of Skp2 and accumulation of p27 in LNCaP cells treated with the 11β-dichloro compound. In the case of 11β-dichloro, however, the cells do not arrest in G1 but instead, rapidly undergo apoptosis. This latter property, of course, is of value in an anticancer agent.

During the cell cycle, downregulation of the Skp2 protein is mediated by its APC^{cdh1}-mediated ubiquitination and destruction [37]. It has been reported that Cdh1 can be activated by some forms of DNA damage [38]. This possibility suggests that 11β-dichloro may be particularly effective in decreasing Skp2 expression because of its effect on both the AR and DNA-damage response pathways that can control Skp2 expression.

The association of the AR with 11β-DNA adducts would be expected to antagonize the transcription of genes from AR promoter sequences. Androgen ablation as well as therapy with androgen antagonists that prevent AR-mediated gene expression are effective means to control prostate cancer in its early stages. These strategies fail, however, when mutations activate the AR directly or result in its activation by former antagonists that stimulate cell growth. Sequestration of the AR by 11β-DNA adducts would be expected to overcome both of these resistance mechanisms.

We also observed the increased expression of another CDK inhibitor, p21, in LNCaP cells treated with either chlorambucil or 11β-dichloro but not with the unreactive 11β-dimethoxy analog. p21 plays an essential role in growth arrest after DNA damage [39]. Our findings suggest that p21 expression is modulated by DNA damage produced by the reactive compounds, most likely through activation of the p53 pathway. LNCaP cells express wild-type, functional p53 [40], which is a major regulator of p21 transcription in response to DNA damage [41]. There are several reports indicating that increased expression of p21 protects prostate cancer cells, as well as other cell types, against apoptosis induced by a variety of anticancer agents [39, 42]. These findings suggest significance to the different patterns of p21 induction that we observed in LNCaP cells

treated with chlorambucil or 11β-dichloro. Although chlorambucil produced a rapid increase in the level of p21, the 11β-dichloro compound initially decreased levels of p21 in LNCaP cells. Because the initial reduction of p21 levels also occurred with the unreactive 11β-dimethoxy analog, other features of our compounds, unrelated to DNA damage, are likely involved in this response.

The capacity of p21 to halt cell-cycle progression through inhibition of CDKs, as well as through its interaction with the proliferating cell nuclear antigen (PCNA), which stops DNA synthesis [43], provides an explanation for the accumulation in S phase and the survival of LNCaP cells after treatment with chlorambucil. In contrast, we did not observe an increase in the number of LNCaP cells in S phase during treatment with 11β-dichloro. Early elimination and delayed increase in the expression of p21 in cells treated with 11β-dichloro may disable a key checkpoint that normally arrests cells in S phase to allow for the repair of DNA damage, thus sensitizing the cells to apoptosis. Further investigation is required to identify the mechanism (or mechanisms) underlying the initial reduction of p21 in 11β-treated cells and the role this reduction plays in the cytotoxic effects of our compounds.

The mustard class of anticancer agents includes the first drugs used in modern chemotherapy as well as many that are used daily in the clinic today. The development of new DNA-damaging anticancer drugs, however, has not been the subject of intense research in recent years. Discovery of new anticancer drugs has increasingly focused on targeting pathognomonic changes in cancer cells. The objective of this work was to create multifunctional compounds that combine the modern "targeted" approach with the more traditional methods that are directed against the broad target of DNA replication, upon which tumor cells depend. Selectivity and efficacy of these compounds may not depend on absolute restriction of a single target in a malignant cell but, rather, from the decreased ability of malignant cells, as compared with their normal counterparts, to cope with perturbations in multiple pathways.

Significance

We report the induction of growth inhibition, apoptosis, and antitumor activity by a unique bifunctional conjugate that comprises a DNA alkylator and a steroid ligand for the androgen receptor. The new molecule (11β-dichloro) was designed to target several biochemical pathways upon which prostate tumor cells depend for growth and resistance to existing anticancer drugs. Unlike previous steroid-alkylator combinations, the conjugate molecule has a chemically stabile linkage that confers the ability to form covalent DNA adducts that have affinity for the androgen receptor (AR). We propose that creation of AR-DNA adduct complexes interferes with DNA repair and disrupts AR-mediated transcription and signaling. Evidence of a unique mechanism of action was obtained by investigation of the cytotoxic and biochemical effects of the new conjugate in prostate cancer cells. We observed inhibition of cell growth and rapid induction of apoptosis in LNCaP cells that are resistant to other chemotherapeutics that act through damage to DNA. Cells treated with 11β-dichloro showed a specific pattern of activation of cell-cycle checkpoint proteins that was not observed with other DNA-damaging agents or with a combination of the steroid and alkylator portions of the molecule. Importantly, 11β-dichloro blocked the growth of LNCaP xenograft tumors in immunocompromised mice. Our results suggest that stable conjugates that can form adduct-protein complexes in DNA have unique cytotoxic mechanisms that may be useful in treating cancers.

Experimental Procedures

Chemical Synthesis

The bifunctional compounds used in these studies were chemically synthesized in our laboratory. Details of the synthetic steps and characterization of the final compounds by NRM and mass spectrometry are contained in Supplemental Data.

Reaction of 11β Compounds with DNA

The self-complementary oligonucleotide 5′-d(ATTATTGGCCAATAAT)-3′ was obtained from IDT DNA (Coralville, IA) and was purified by denaturing PAGE. The oligonucleotide was 5′ end labeled with [γ-^{32}P]ATP and allowed to react with test compounds at 37°C for 4 hr. The adducted oligonucleotide was treated with 1 M piperidine for 1 hr at 90°C, and fragments were resolved by denaturing PAGE to determine sites of modification. Reaction products were visualized and quantified by PhosphorImager analysis. The calculated percent cleavage is the proportion of radioactivity in the fragments divided by the total and represents the extent of covalent modification by the test compound.

Relative Affinity of 11β-Compounds for Steroid Receptors

The relative binding affinities (RBA) of 11β compounds for the AR and PR were assessed by a competitive binding assay. Whole-cell extracts prepared from LNCaP and T47D cells were used as sources of the AR and PR, respectively. RBAs were determined by addition of increasing amounts of unlabeled test compounds to cell extracts in the presence of radiolabeled ligands ([^{3}H]-R1881, 83.5 Ci/mmol, or [^{3}H]-progesterone 103.0 Ci/mmol; NEN, Boston, MA). The amount of radiolabeled ligand that remained bound to protein after removal of free ligand by adsorption to dextran-charcoal was determined by scintillation counting [4].

Relative Affinity of 11β-DNA Adducts for the AR and PR

We used an identical competitive binding assay to investigate the ability of 11β-DNA adducts to bind to the AR and PR. In this case, we used as competitor the covalently modified 16-mer deoxyoligonucleotide prepared as described above. After reaction with 11β-dichloro, unreacted compound was removed from the modified 16-mer via three consecutive ethanol precipitations. We confirmed the absence of unreacted 11β-dichloro and estimated the concentration of covalent adducts in the DNA by conducting a parallel experiment with [^{14}C]-11β (synthesized in our laboratory; details to be published). Increasing amounts of modified or unreacted DNA were added to cell extracts in the presence of radiolabeled ligands. After incubation, unbound ligand was removed and the amount remaining bound to the receptor determined as described above.

Cell Culture

Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The LNCaP cell line was maintained in RPMI 1640 supplemented with 2.5 mg/ml glucose, 10% fetal bovine serum (FBS; Hyclone, Salt Lake City, UT), 2 mM glutamax, 1 mM sodium pyruvate, and 100 mM HEPES. The T47D line was maintained in MEM-α medium containing 10% FBS (Hyclone, Logan, UT), 0.1 mM nonessential amino acids, 100 mM HEPES, 2 μg/ml bovine insulin, and 1 ng/ml human epidermal growth factor (Invitrogen, Carlsbad, CA). Cells were grown in a humidified 5% CO_2/air atmo-

sphere at 37°C. For studies of cell morphology, LNCaP cells were grown on 13 mm diameter Nunc Thermanox cover slips coated with poly-L-lysine (Invitrogen). At indicated time after treatment, cells were washed twice in PBS, fixed in methanol, air dried, and stained with Giemsa.

Cell-Cycle Analysis

Cells in exponential growth were treated with test compounds dissolved in DMSO. At the indicated times, drug-containing media was removed and detached cells were collected by centrifugation. Attached cells were harvested by trypsinization, pooled with recovered detached cells, and washed once in PBS. Cells were fixed in 70% ethanol and stored at 4°C. For flow cytometry, cells were resuspended in 0.5 ml of a PBS solution containing 0.1% Triton X-100, 0.2 mg/ml DNase-free RNase, and 0.02 mg/ml propidium iodide (Sigma, St. Louis, MO). Cells were analyzed with a Becton Dickinson FACScan flow cytometer with Cell Quest software (MIT Flow Cytometry Core Facility). Data were analyzed with ModFitLT 2.0 software.

Annexin V Staining and Analysis

LNCaP cells in exponential growth were treated with test compounds as described for cell-cycle analysis. At indicated times, cells were trypsinized, washed with PBS, and stained with Annexin V-PE and 7-amino-actinomycin D according to manufacturer's protocols (BD Pharmigen, San Diego, CA). Stained cells were analyzed by flow cytometery.

DNA Isolation and Gel Electrophoresis

Adherent cells were scraped directly into growth media and collected along with any detached cells by centrifugation at 0°C. Cells were lysed in a solution containing 50 mM Tris (pH 8.0), 100 μM EDTA, 0.5 mg/ml proteinase K, and 0.5% sodium lauryl sulfate. After incubation at 50°C for 3 hr, the lysates were extracted once with phenol chloroform, and nucleic acids were precipitated with ethanol and dissolved in TE (pH 7.5). RNA was digested with DNase-free RNase (Roche Biochemicals, Indianapolis, IN), and the solution was extracted once again with phenol chloroform. DNA was then isolated by ethanol precipitation, and the quantity recovered determined by O.D. 260 nm. Equal amounts of DNA from each sample were loaded onto a 1.5% agarose gel containing 0.1 μg/ml ethidium bromide and resolved by electrophoresis. DNA was visualized with a UV transilluminator.

Immunoblot Analysis

After exposure to various compounds for indicated times, LNCaP cells were harvested in medium by scraping, washed once in PBS, and suspended in 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.5% Na-deoxychloate, 1 mM Na_3VO_4, 1 mM NaF, and protease inhibitor cocktail (P8340; Sigma, St. Louis, MO) at 0°C. The cell lysate was centrifuged at 14,000 × g for 10 min, and supernatants were collected for analysis. Protein concentrations were determined by the Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA). Lysates were combined with SDS-PAGE sample buffer (0.3 M Tris [pH 6.8], 2% SDS, 1% 2-mercaptoethanol, 10% glycerol), and equal amounts of protein were resolved by SDS-PAGE, followed by transfer to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (0.1% Tween 20, 10 mM Tris [pH 7.4], 150 mM NaCl) and probed with antibody against the protein of interest. Antibody complexes formed with horseradish peroxidase-conjugated secondary antibodies were visualized by chemiluminescence (Supersignal West; Pierce, Rockford, IL). Antibodies were as follows: PARP (06-557; Upstate Biotechnology, Lake Placid, NY), p27 Kip1 (2552; Cell Signaling Technologies, Beverly, MA), p21 (sc-397; Santa Cruz Biotechnology, Santa Cruz, CA), and p45 Skp2 (32-3300; Zymed Laboratotries, South San Francisco, CA).

Animal Studies

4- to 6-week-old NIH Swiss nu/nu athymic male mice (25 gm) were obtained from the National Cancer Institute-Frederick Cancer Center (Frederick, MD). Experiments were carried out under guidelines of the MIT Animal Care Committee. Animals were injected subcutaneously in the right flank with 5×10^6 LNCaP cells suspended in a solution of 50% PBS/50% Matrigel (Collaborative Research, Bedford, MA). Therapy commenced when a palpable tumor of approximately 4 × 4 mm formed (n = 5 per treatment group). The 11β-dichloro compound was dissolved in a vehicle composed of cremophor EL, saline, and ethanol (43:30:27). Tumor dimensions were measured with vernier calipers. Tumor volumes were calculated with the formula: $\pi/6 \times$ larger diameter × (smaller diameter)^2. Statistical analyses were performed with a paired t test. At the end of the study period, animals were euthanized with CO_2. At the time of sacrifice, blood samples were taken from several animals in each group for a complete blood count, along with serum chemistry and liver function analyses. A complete necropsy was also performed, including histopathology on two animals from each group.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/12/7/779/DC1/.

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