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13. ABSTRACT *(Maximum 200 Words)*

The purpose of our research is to develop better chemotherapeutic drugs for the treatment of prostate cancers. We have used chemical synthetic methods to create bifunctional compounds consisting of a ligand for the androgen receptor linked to a reactive alkylating group that can produce covalent damage in cellular DNA. It is proposed that such damage would persist in tumor cells that express the androgen receptor (AR) because the DNA lesions would be masked by their association with the AR. Initial work prepared chemically modified non-steroid and steroid derivatives that were tested for their affinity for the AR. This work led to the identification of structures that when attached to a linker molecule still retained good affinity for the AR. Subsequently, a number of bifunctional compounds were constructed and tested in biochemical assays and in prostate cancer cells in culture. We have identified a lead compound containing an 11β-substituted steroid linked to an aniline mustard. This compound damaged DNA and retained good affinity for the AR. We discovered, however, that when added to prostate cancer cells in culture its AR binding activity is lost. We have prepared radiolabled compounds to study their metabolic fate both in cell culture and in animal models.

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Introduction:

The objective of our research is the development of selective genotoxins that can provide more effective treatments for prostate cancers. Our proposal described the chemical synthesis and biological evaluation of bifunctional molecules that produced DNA damage that persists in tumor cells leading to greater toxicity and potentially a higher therapeutic index. We have employed chemical synthetic methods to prepare molecules in which a group capable of alkylating DNA is tethered to a ligand for the androgen receptor (AR). In principle the alkylating group of such bifunctional molecules will form covalent DNA adducts that have high affinity for the AR. Adducts engaged in tight complexes with the AR protein will be concealed from proteins that remove and repair such damage to cellular DNA, resulting in greater cytotoxic and therapeutic response in prostate tumors that express high levels of the AR. We employed a stepwise process to construct the target bifunctional molecules. The first objective of our research was to identify small organic molecules that can be linked to an alkyl chain and function as ligands for the AR. Initially, we prepared a variety of small organic molecules that were potential ligands for the AR. A competitive binding assay was used to identify candidates with high affinity for the receptor. These molecules were modified by addition of a six carbon alkyl chain at various positions and again screened for affinity for the AR. We thus identified a molecular core structure and the position of its modification that produced that ligand binding portion that was incorporated into the bifunctional compound by addition of a nitrogen mustard. The toxic effects and therapeutic potential of these new compounds were then evaluated in cell culture models of prostate cancers. Our lead compound showed a small increase in specific toxicity toward cells that express the AR. This led us to investigate its interaction with the AR in vivo producing evidence of metabolic inactivation. We subsequently synthesized radiolabeled derivatives of this compound and are currently studying its metabolic fate. Several structural variants have also been made that are less susceptible to metabolism and have shown a greater affinity for the AR. The funding period expired before the toxicological characteristics of these compounds could be investigated in any more detail.

Body:

Task 1: *Identify small organic compounds that have high affinity for the AR and discover means by which they can be linked to reactive "warheads," while maintaining good affinity for the AR.*

Our synthetic work initially focused on the modification of non-steroid molecules. Our attempts to link an aromatic nitrogen mustard to arylthiohydantoins that reportedly have high affinity for the androgen receptor (AR) were unsuccessful. We found that the AR binding of these molecules was quite sensitive to structural modifications (c.f. 1999 Annual Report). Similarly, synthetic work that evaluated a series of phthalimide and benzamide compounds for AR affinity did not identify any potential ligands that had high affinity for the receptor. (Structures of the evaluated compounds are shown in Figure 2 of the September 1999 Annual Report.)

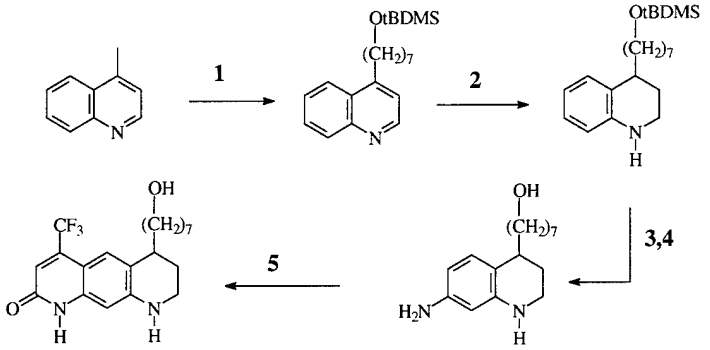

Figure 1 Synthesis of C4-substituted 1,2-dihydropyridono[5,6-*g*]quinolone: 1. LDA, Br-$(CH_2)_6$-O*t*BDMS, THF, -78°C; 2. $NiCl_2$-$6H_2O$, $NaBH_4$, MeOH; 3. HNO_3, H_2SO_4, -10°C; 4. $NiCl_2$-$6H_2O$, $NaBH_4$, MeOH; 5. CF_3-$COCH_2COOC_2H_5$, $ZnCl_2$, EtOH, reflux.

During the last year we have investigated several 1,2-dihydropyridono[5,6-*g*]quinolones, another group of non-steroid compounds that reportedly can have high affinity for the AR. These compounds were synthesized by modification of published procedures. Based on reported structure-activity studies positions C4 and N9 were chosen as potential sites for alkyl linker substitution. The synthetic routes to the C4 and N9-substituted compounds are shown in Figures 2 and 3. We chose initially to attach a hexyl-6-ol group to at these position. We then evaluated the affinity of each compound for the AR using an *in vitro* competitive binding assay to determine the effect of the hexyl substitution.

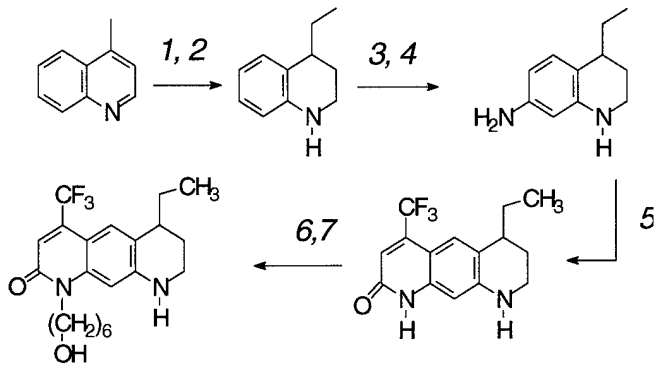

Figure 2 Synthesis of N9-substituted 1,2-dihydropyridono[5,6-*g*]quinolone: 1. Iodomethane, LDA, THF -78°C; 2. $NiCl_2.6H_2O$, $NaBH_4$, MeOH, 0°C→rt; 3. HNO_3, H_2SO_4, -10°C; 4. $NiCl_2.6H_2O$, $NaBH_4$, MeOH, 0°C→rt; 5. ethyl-4,4,4-trifluoroacetoacetate, $ZnCl_2$, EtOH, reflux; 6. NaH, $Br(CH_2)_6O$*t*BDMS; 7. *t*BAF, THF.

The results of the competitive binding experiments for these compounds are shown in Figure 3. Compared to the synthetic androgen R1881, the substituted dihydropyridono[5,6-

g]quinolones had relative binding affinities[1] (RBA) of 0.3 for the 4-substituted and 0.8 for the 9-substituted compound. Thus, modification at the N9 position is more favorable for receptor binding. The RBAs of these compounds are low compared to R1881, however, this synthetic androgen has a greater affinity for the AR than does the natural ligand dihydrotestosterone (DHT). Compared to DHT the RBAs of the 4- and 9-substituted compound are estimated to be two to three times greater than those relative to R1881.

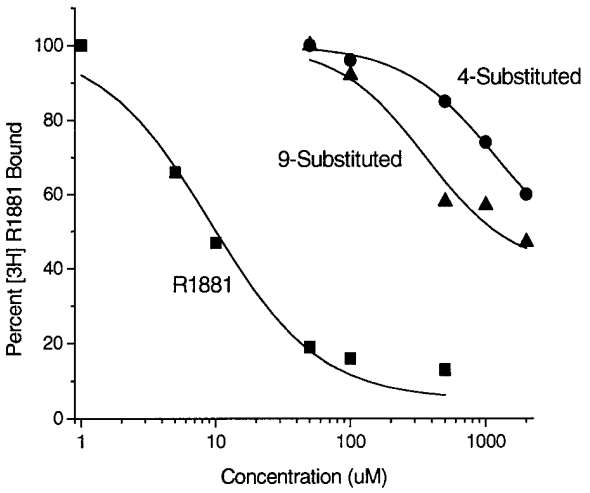

Figure 3 Competitive binding of 4- and 9-substituted 1,2-dihydropyridono[5,6-g]quinolones for the LNCaP androgen receptor.

We consider the affinities of the present C4- and N9-substituted compounds too low to justify continued effort at their incorporation into bifunctional compounds containing the aniline mustard. However, we will continue to investigate the effects of other linking groups and alternate positions of substitution on the affinity of the 1,2-dihydropyridono[5,6-*g*]quinolones for the AR. One advantage of these compounds is that they are synthetically more accessible than the 11β-estradiens (see below). The synthetic procedures for the construction of these compounds are quite adaptable for producing structural variants. Thus, in the future we will be able to explore the structure-activity relationship for AR affinity of a large family of these compounds. We hope that this will lead to structures that have high affinity for the AR and can be linked to the nitrogen mustard or other alkylating group with retention of AR binding.

The second strategy that we took to develop the desired bifunctional compounds was based on modifications to steroidal androgens. We have linked several androgenic steroids with aniline mustards and tested their affinities for the AR in competitive binding assays. As reported previously, this work initially focused on the synthesis of dihydrotestosterone (DHT) compounds substituted at the 7α position. DHT compounds substituted with simple alkyl groups of up to six carbon atoms showed good binding to the AR. However, when we attached other groups (e.g. amino, carbamate) to the alkyl chain, affinity for the AR was lost (See September 1999 report). Bifunctional molecules consisting of the 7α DHT linked to an aniline mustard were found to have little if any affinity for the AR. Consequently, our synthetic work on these compounds was abandoned.

We have obtained the most promising results to date with 11β-substituted steroid compounds.

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[1] Relative Binding Affinity (RBA) is the ratio of the concentrations of unlabeled R1881 and the test compound that results on a 50% decrease in [3H] R1881 bound multiplied by 100.

We found that large groups attached at the 11β position resulted in retention of the molecule's interaction with the AR. For example, substitution of an N,N-dimethyl-6-aminohexyl group at the 11β position of estra-Δ4(5),9(10)-3,17β-diol produced a compound with good affinity for the AR (see Figure 4). These encouraging results led us to synthesize the 11β-linked mustard compound via the synthetic route shown in Figure 5. The steps described in this figure provide a general route for the preparation of bifunctional compounds consisting of 11β-substituted dienones linked to an aniline mustard.

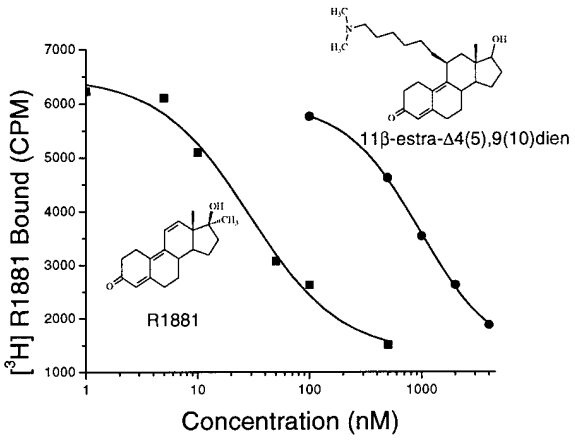

Figure 4 Competitive binding of R1881 and 11β-N,N-dimethly-1-hexyl-estra-D4(5),9(10)dien for the LNCaP androgen receptor.

Competitive binding experiments that utilized extracts from LNCaP cells as a source of the AR revealed that the bifunctional 11β-substituted mustard compound had good affinity for the AR. These data are shown in Figure 6. Relative to R1881 – which has a higher affinity for the AR than the natural ligand dihydrotestosterone – the new compound has an RBA of 5. While this is not outstanding, it is similar to the affinities of lead compounds that were identified in the parallel project to develop similar compounds that interact with the estrogen receptor. Therefore, we investigated the cytotoxic effects of this compound toward prostate cancer cells in culture (Task 3).

From a synthetic view point our original route for the preparation of 11β-substituted estradien compounds (Figure 5) has not facilitated the synthesis of structural variants that would allow us to evaluate structure-activity relationships and thereby optimize the biological activities of this compound. If funding were continued we would have modified the synthetic procedure to enable the preparation of structural analogs.

We have also prepared enough of the 11β-(Δ4,5;9,10 estra)C6NC2-mustard compound to perform

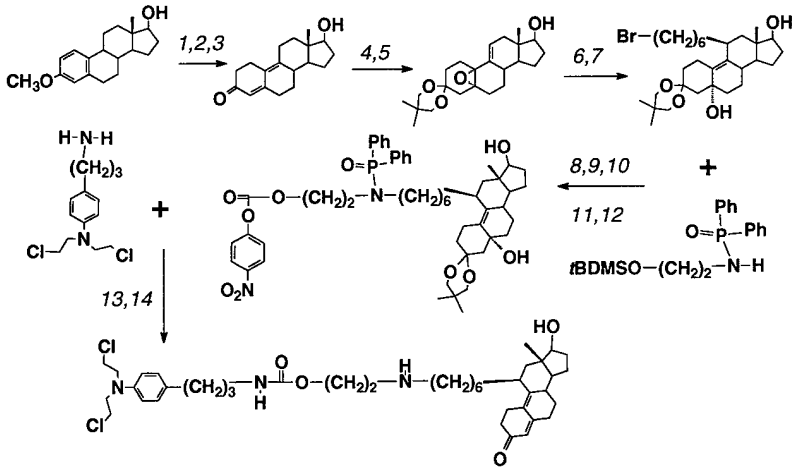

Figure 5 Synthesis of 11β-substituted-(Δ4,5;9,10 estra)-C6NC2-mustard: **1**. Li, NH_3/THF; **2**. $(COOH)_2$, H_2O; **3**. Br_2, Pyridine; **4**. Neopentylglycol, $CH(OCH_3)_3$, pTSA; **5**. H_2O_2, $(CCl_3)_2O$, Pyridine/CH_2Cl_2; **6**. BrMg-$(CH_2)_6$-O*t*BDMS, CuCl, THF; **7**. *t*BAF/THF; **8**. CH_3SO_2Cl, iPr_2NEt, THF; **9**. LiBr, DMF, 60oC; **10**. NaH, DMF; **11**. *t*BAF, THF; **12**. pNO_2Phenylchloroformate, iPr_2NEt, CH_2Cl_2; **13**. iPr_2EtN, THF; **14**. HCl, THF.

initial toxicity studies in mice. Our initial synthesis produced 30 mg of compound which was adequate for both competitive binding studies *in vitro* and investigation of cytotoxic effects in cell culture. The next step in evaluation of the clinical potential of this compound is the assessment of its acute and chronic toxicity in an animal model. Such studies in mice typically require 0.5 to 1.0 gm of compound. By increasing the scale of the reactions shown in Figure 5 and improving yields at key steps we have produced 550 mg of the 11β-(Δ4,5;9,10 estra)C6NC2-mustard compound. We shall try to obtain funding to initiate toxicity studies in mice, which will be complemented by investigation of pharmacokinetics in this species (see Task 4).

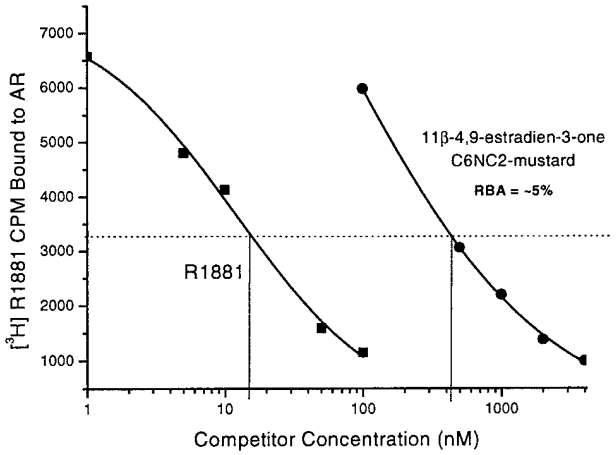

Figure 6 Competitive binding of R1881 and 11β-(Δ4,5;9,10 estra)-C6NC2-mustard for the androgen receptor in LNCaP cell extracts.

Another synthetic project on which we have made considerable progress is the preparation of 17β-OH-Δ4,5;9,10 estradien-3-one compounds that have a 17α methyl substitutent. These compounds are desirable because they reportedly have increased affinity for the AR compared to the 17α-H compound. In addition, the methyl group at the 17α position prevents oxidation of the 17β-OH group. At this point we have produced gram quantities of the key intermediate for this synthesis; 17α-methyl-17β-OH-Δ4,5;9,10 estradien-3-one. Competitive binding studies with R1881 found that the 17α-methyl-substituted compound has an RBA of 83 while the RBA of the unsubstituted (17α-H) compound is 45. Thus, we are optimistic that these ligands, when coupled to the aniline mustard will show increased affinity for the AR and greater selectivity for killing AR-expressing prostate cancer cells.

Task 2: *Construct libraries of bifunctional molecules containing both the AR ligand and the warhead and identify those compounds that can form DNA adducts with high affinity for the AR.*

Progress on this task has been incorporated into the report on Task 1 (see above).

Task 3: *Determine whether compounds identified in Aim 2 show enhanced toxicity in prostate cancer cells that express the AR.*

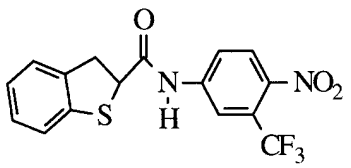

Figure 7 Benzamide compound.

Evaluation of the biological activities of the benzamide and phthalimide series of compounds was described in the September 1999 Annual Report. The 16 compounds that were tested had relatively low

affinities for the AR. One compound, however, at low uM concentrations inhibited the growth of several prostate cancer cell lines. This effect was independent of AR status.

The bifunctional 11β-substituted compound that showed good affinity for the AR was tested for their cytotoxic effects toward AR+ LNCaP cells as well as several other prostate cancer cell lines that did not express the AR including DU145 and PC3 (Figure 7). Although at the highest dose LNCaP cells appear to be more sensitive, the results of these toxicity experiments did not indicate a significant amount of selective toxicity. We investigated whether, in fact, the 11β estradien compound bound to the AR in LNCaP cells in culture using a whole cell competitive binding assay. We found a much lower affinity for the AR than had been evidenced using cell extracts. A possible explanation for these results is the conversion of the estradien to lower affinity metabolites. LNCaP cells have been reported to posses all the major enzymes involved in testosterone metabolism including 17β-hydroxysteroid-oxidoreductase (17β-HSOR), which oxidizes the 17β-hydroxy group of androgens producing 17-keto compounds that have lower affinity for the AR (25). A solution to this problem is the substitution of the 17α position with a methyl or ethynyl group thereby blocking the oxidation of the hydroxyl group at this carbon. We have made significant progress in the synthesis of these 17α-substituted compounds (see above).

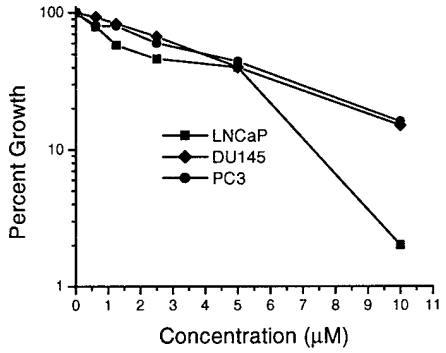

Figure 8 Inhibition of growth of prostate cancer cell lines by 11β-substituted-(Δ4,5;9,10 estra)-C6NC2-mustard.

Task 4: *Investigate the biochemical mechanism(s) responsible for selective toxicity.*

During the last year we have designed experiments to investigate the reasons why the 11β compound did not show better selective toxicity toward AR+ prostate cancer cells. Given the contradictory findings in competitive binding assays performed using cell extracts and intact cells in culture we speculated that our compounds may be undergoing metabolic transformation to inactive species. To facilitate investigation of this hypothesis we prepared a radiolabeled version of the 11β androgen-mustard compound. Figure 7 shows the synthetic scheme that we used to incorporate a [^{14}C] atom into *p*-3-aminopropyl(N,N 2-chloroethyl) aniline (**3**) that was subsequently used in the synthetic procedure described in Figure 5 above. The labeled compound was added in the last part of the synthetic procedure just prior to removal of the protecting groups. The[^{14}C]-labeled androgen-mustard has a specific activity (SA) of 53 mCi/mM which is adequate for metabolism studies.

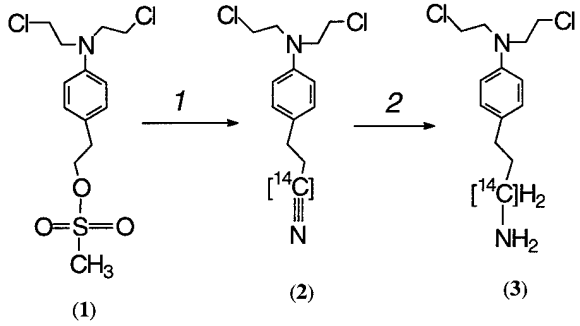

Figure 9 Synthesis of radiolabeled *p*-3-aminopropyl(N,N-2-chloroethly)aniline: 1. K[^{14}C]N, 18-Crown-6, DMSO, 50°C; 2. LAH, THF. 80°C.

In addition to metabolism studies in cell cultures the radiolabeled compound will be used to examine the pharmacokinetics of the 11β compound in animal models. The results of these investigations will tell us if these compounds have good bioavailability as well as their distribution and metabolic fate *in vivo*. The results from these studies should give us a firm basis for deciding whether to proceed in testing this compound in a mouse xenograft model of human prostate cancer.

As a final note, we deeply appreciate the support of the Army for this 2.5 year project. It is always exciting to begin work in a new area. We, of course, are disappointed that we did not submit a competitive competing renewal application, as we would like to bring our studies in this area to completion. As a word of friendly advice, the Army Program is highly structured with specific tasks and expected times to complete each task. In some areas, such as routine chemistry or building a road, it may be possible to guarantee the passage of a milestone at a specific time. In our work, which is more creative, it would have been difficult to predict that we would have had to go through so many classes of ligand before we found the 11β compound, which shows promise. Moreover, we were quick to figure out that metabolism was inactivating our compound and we had strategies in mind to address the problem if it came up. But, again, this work took time, which put us off schedule. My suggestion is that such short grants where creative scientists are held to strict time limits is not in the best interest of the Program, which has the goal of bringing new approaches to the treatment of prostate cancer. The Program, by contrast, will attract more mundane "guarantee-able" approaches and not creative ones. If we are able to patch support together to continue this work, we shall be back and hopefully will have a more competitive work plan in place at some point in the future. But please give some thought to the words above as they are, in my view, in the best interest of both the Army and basic scientists such as myself.

Key Research Accomplishments:

- Chemical synthesis of steroid and nonsteroid compounds and assessment of their affinities for the androgen receptor.

- Synthesis and characterization of 11β-substituted-(Δ4,5;9,10 estra)-C6NC2-mustard. This bifunctional genotoxin has good affinity for the androgen receptor, while steroids substituted at the 7α position do not. The 11β compound demonstrated some specificity of its cytotoxic effects for AR+ prostate cancer cells.

- Synthesis of a radiolabeled derivative of 11β-substituted-(Δ4,5;9,10 estra)-C6NC2-mustard. This radioactive compound could be used to study the pharmacokinetics and metabolism of our bifunctional compound in cell culture and animal models.

Reportable Outcomes:

Presentations/Abstracts:

"Programmable therapeutics for prostate malignancies." Essigmann, J.M. and Croy, R.G., Presented at the Fifth Annual CaP-Cure Conference. September, 1998.

"Programmable drugs targeted at prostate malignancies." John M. Essigmann, Robert G. Croy, Hyun-Ju Park, John Marquis, Shawn Hillier, Beatriz Zayas, Gerald Wogan, & John Wishnok. Presented at Seventh Annual CaP-Cure Conference. October, 2000.

"Programmable drugs targeted at breast and prostate malignancies." Shawn Hillier, Beatriz Zayas, Hyun-Ju Park, John Marquis, Gerald Wogan, John Wishnok, Robert G. Croy & John M. Essigmann. Presented at BEH/ILP Conference, MIT, Cambridge, MA., November, 2000.

Employment Reveived:

Based on the training/experience supported by this award, Dr. Hyun-Ju Park was appointed Assistant Professor at the College of Pharmacy (51-255B), Sungkyunkwan University, 300 Chunchun-dong, Jangan-gu, Suwon, Kyunggi-do 440-746, Korea.

Conclusion:

The major portion of work during the grant period involved chemical synthesis of novel bifuctional compounds that form DNA adducts with high affinity for the androgen receptor (AR). Our strategy has been to attempt to incorporate molecules with known affinity for the AR into our desired bifunctional compounds containing an aniline mustard. Initial synthetic work focused in non-steroids such as derivatives of flutamide and phenylthiohydantoins. The ability of these molecules to bind to the AR proved to be very sensitive to structural alterations. So our attempts to use these relatively simple ligands was abandoned.

Incorporation of steroid ligands for the AR into our bifunctional molecular design also proved to be difficult. Dihydrotestosterone (DHT) – the natural ligand for the AR – was the initial focus of this portion of the synthetic work. Although some initial results with alkyl substitutions at the 17α and 7α positions were promising the affinities for the AR of the final bifunctional molecules containing DHT fell far short of our goals. We did have success with 11β-substituted estradien-3-one compounds. This lead compound consists of the 17α-OH-Δ4(5),9(10)-estra-3-one linked via the 11β carbon to the aniline mustard. This bifunctional compound binds to the AR and reacts with DNA to form covalent adducts. In cell culture, we found a modest increase in cytotoxicity toward prostate cancer cells that express the AR. Radiolabeled derivatives of the 11β-Δ4(5),9(10)-estra-C6NC2-mustard compound have been prepared. Pharmacokinetic studies in animal models will be underway shortly to assess its stability and bioavailability. We have also prepared a sufficient quantity of this compound to assess its toxic effects in the mouse.

Syntheses of structural variants of the lead compound are nearly completed. These compounds have a methyl or trifluoromethyl group at the 17α position. Our preliminary work with these compounds is promising in that they have improved affinity for the AR in competitive binding studies.

References: None Cited.

Appendices: No material enclosed.