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Antiestrogenic Piperidinediones Designed Prospectively using Computer Graphics and Energy Calculations of DNA–Ligand Complexes

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Drug design technology based upon DNA stereochemistry and now supplemented by computer modeling was used to design a novel compound to inhibit estrogen-induced tumor cell growth. A known compound 3-phenylacetylamino-2,6-piperidinedione (PP) was accommodated in partially unwound DNA in a manner consistent with criteria for antiestrogens. Examination of the PP–DNA complex revealed that substitution of a hydroxyl group at the para position (*p*-OH-PP) would provide a stereospecific hydrogen bond and a substantial increase in fit as assessed by energy calculations. The antiestrogen tamoxifen could also be accommodated within the site; analogous substitution of a hydroxyl at the 4 position resulted in a better fitting molecule. 4-Hydroxytamoxifen is a more potent antiestrogen than tamoxifen. Synthesis and subsequent evaluation of *p*-OH-PP as an inhibitor of estrogen stimulated MCF-7 (E3) human breast cancer cell growth demonstrated that *p*-OH-PP was more active than both PP and its hydrolysis product phenylacetylglutamine. As predicted, the order of fit into DNA correlated with the relative ability to inhibit estrogen-induced growth of tumor cells suggesting that the evolving drug design technology will be valuable in developing new drugs for breast cancer.

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INTRODUCTION

The development of new pharmaceuticals as well as other biologically active compounds has historically relied on serendipitous discoveries. Once a "lead compound" has been successfully identified, large numbers of structural analogs are generally synthesized and then evaluated in numerous *in vitro* and *in vivo* models. During this time consuming and expensive process, frequently no clear rationale is available to predict which analogs will be active. After an extensive database of compounds has been developed, attempts are then made to correlate structural properties and biological activities with the goal of designing the ideal drug. An obvious and very logical way to develop active compounds is to design them to fit into sites in appropriate macromolecules that ultimately govern their activity, e.g. specific sites in receptors or enzymes. However, given that with few exceptions relatively little is known about the stereochemistry of such sites, the task of designing active compounds remains formidable.

Molecular modeling has long been employed as a means to visualize three dimensional structural features of molecules as well as potential chemical interactions among molecules, e.g. ligands interacting with receptor sites. The more recent advent of computer modeling has provided a valuable complement to physical "hand held" models. Namely, elegant computer graphics methods permit the scientist to view and compare molecules in three dimensions with great accuracy. Moreover, computer energy calculations enable precise measurement of the relative interactions among molecules. A virtual arsenal of rapidly advancing hardware and software is now available including sophisticated quantitative structure–activity programs (for recent reviews see [1, 2]). Despite these remarkable advances, the design of a new drug can still be frustrating due to

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the difficulties described above, e.g. there is often little detailed knowledge of the structures of the macromolecules which give rise to a given activity. In the case of steroid hormone agonists and antagonists, for example, the stereochemical properties of ligand–receptor complexes are for the most part unknown.

Drug design using the stereochemistry of gene structure

Various modeling approaches in our laboratory over the past 15 years have been focused on exploring the hypothesis that gene structure, i.e. the stereochemistry of nucleic acids, contains structural information about naturally occurring small molecules ([3–5] and references therein). The rationale for this concept arose from the fact that genes encode the information for both enzymes and receptors which in turn govern the respective synthesis and biological activity of natural compounds. Thus, it occurred to us that the stereochemistry of small molecules might be reflected directly in the structure of the gene itself. In short, we have reported that many biologically active small molecular weight natural products, e.g. plant hormones and mammalian steroid hormones, have "lock and key" relationships with nucleic acids. Degree of fit of certain molecules into specific sites in DNA has been found to correlate with degree of biological activity [4, 6–8]. Hormone agonists generally fit into the same site and in the same manner as the parent hormone. Hormone antagonists generally fit certain portions of the site but possess distinct structural features which interact differently with the DNA [4–8]. Metabolic pathways were also reflected in DNA stereochemistry [4, 5, 8, 9]. When compared with the fit of a hormone, intermediates along the biosynthetic pathways of certain hormones possessed progressively increasing fit whereas inactive catabolites exhibited decreasing fit. Remarkably, the absolute stereochemistry of functional groups which result from various enzymatic steps matched the stereochemistry of potential hydrogen bond donor/acceptor linkages in the DNA structure. While the observations described above were made initially with physical models, e.g. Corey-Pauling-Koltun (CPK) space-filling models and Kendrew skeletal models, they have been confirmed recently using computer graphics and standard energy calculations [5, 10–14].

In this report, computer modeling was applied for the first time to examine the site in DNA which accommodates estrogens with the objective of prospectively designing novel antiestrogens. Using various physical models, estradiol had been previously shown to fit between base pairs in partially unwound DNA at the site 5′-dTdG-3′·5′-dCdA-3′ [4, 15]. Stereospecific hydrogen bonds were formed between each of the hydroxyl groups of estradiol and phosphate oxygens on adjacent DNA strands thereby forming a bridge between both sugar–phosphate backbones. The capacity of a given molecule to form hydrogen bonds analogous to estradiol as well as to be accommodated sterically within the same site in DNA correlated with estrogenic activity [4, 6, 7, 13]. Antagonists, e.g. tamoxifen and LY117018, fit in a different manner, i.e. they possessed different hydrogen bonding donor/acceptor linkages and/or had structural features which extended out of the site in DNA into the major or minor grooves [4]. In the search for novel structural frameworks to facilitate the design of potential antiestrogens, a novel piperidinedione compound, i.e. 3-phenylacetylamino-2,6-piperidinedione (PP), was examined (Fig. 1). PP was chosen because it had been previously shown to fit between base pairs in DNA [16], moreover, an increasing number of piperidinediones and closely related compounds have been shown to have antineoplastic effects including analogs which are being used in the treatment of breast cancer [17–22]. PP can be formed by simple dehydration of the natural product phenylacetylglutamine (PAG) [23] which is excreted abundantly in human urine. Earlier studies indicated that the PP had reproducible but weak antineoplastic activity [24–26] encouraging us to design more active analogs.

Here, we report the results of docking candidate ligands (Fig. 1) into DNA using computer graphics and energy calculations demonstrating that: (1) estradiol fits stereospecifically between base pairs in DNA confirming earlier published reports; (2) PP can also fit into the DNA site but in a different manner than estradiol; (3) the fit of PP can be substantially improved by substituting a hydroxyl group in the para position (*p*-OH-PP); (4) tamoxifen fits into the site with a portion of the molecule extending into the major groove; and (5) hydroxylation of tamoxifen (4-hydroxytamoxifen) analogous to that of PP results in increased fit in DNA. As predicted by the energy calculations, *p*-OH-PP was considerably more active than PAG or PP when tested for growth inhibition in MCF-7 (E3) human breast cancer cells. Similarly, the energy calculations are consistent with the fact that 4-hydroxytamoxifen is known to be more active than tamoxifen.

EXPERIMENTAL

Computer modeling

Computer modeling was conducted as described previously [5, 11] with Sybyl/Mendyl 5.41 (Tripos Associates, St Louis, MO) using a Digital Microvax II computer interfaced to an Evans and Sutherland PS390 graphics computer equipped with a Stereographics viewer. To evaluate the fit of candidate ligands and DNA, the two basic parameters generally used to examine interactions among molecules were employed: (1) steric fit was assessed by measuring the interaction of van der Waals surfaces; (2) electrostatic interactions (e.g. hydrogen bonds) were assessed by measuring charge interactions among potential donor/acceptor functional groups. Small molecule structures were obtained from: the Cambridge database [27]; modification of X-ray structures; construction with the Concord program or from fragment

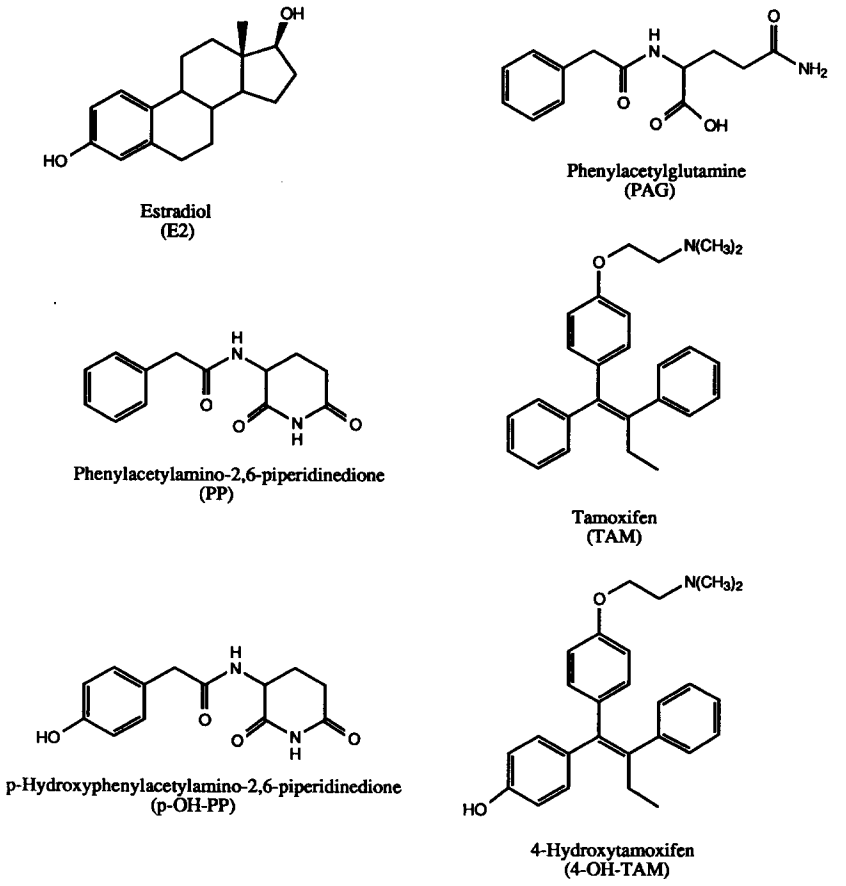

Fig. 1. Structures of compounds evaluated by computer modeling for fit into DNA.

libraries followed by energy minimization. Energies were calculated with the Sybyl/Mendyl force field with a 1.2 Å van der Waals parameter for hydrogen. Gasteiger-Huckel charges were calculated which include σ and π bonding. Partially unwound DNA depicting the site 5′-dTdG-3′·5′-dCdA-3′ was built with the 3′-*endo* deoxyribose conformation and Kollman charges.

The candidate ligands were inserted into the cavity between the base pairs in DNA using van der Waals surfaces and the stereoviewer to guide the docking procedure and minimize any obvious steric strain. Distances between appropriate heteroatoms were monitored interactively to optimize the direction and distances of potential hydrogen bonds. Donor/acceptor relationships were optimized by orienting functional groups on the ligands, e.g. by adjusting conformations to best fit the site. The force field was used to calculate the relative fit of each ligand by measuring the optimum favorable energy change resulting from docking the ligand. Steric fit was calculated from the change in van der Waals energy whereas hydrogen bonding fit was calculated from the change in electrostatic energy resulting from charge interactions of donor hydrogens and acceptor oxygens. The greater the magnitude of the negative energy change upon insertion of a given ligand into the DNA site, the more favorable the fit and the more stable the complex. The overall fit of the ligands was assessed by adding the change in kcal of the van der Waals and electrostatic energies and normalizing the value to that of estradiol which was assigned as 100% fit.

Biological evaluation in MCF-7 (E3) human breast cancer cells

A MCF-7 (E3) human breast cancer cell line purchased from the Michigan Cancer Foundation (Detroit, MI) was used to assess growth inhibitory effects of PAG, PP and *p*-OH-PP upon estradiol stimulated growth. The effects were also compared with those of the antiestrogen, tamoxifen (ICI America, Inc., Wilmington, DE). PP, PAG and *p*-OH-PP were prepared as described previously and added at various concentrations (10^{-8} to 10^{-2} M) [24–26]. Various concentrations of tamoxifen were dissolved in 95% EtOH and added at a 1:1000 fold dilution to achieve final concentrations of 10^{-10} to 10^{-6} M. The compounds were added at the time of plating cells along with estradiol; appropriate solvent controls were included. Cells were plated at a concentration of 50,000 cells/T25 flask in minimum essential media (MEM) supplemented with 0.1 mM HEPES, 0.2 mM glutamine, 0.1 μg/ml gentamicin, 10^{-6} M insulin, 10^{-11} M estradiol (Mann Lab, New York, NY) and 5% calf serum, in an atmosphere of 5% CO_2:95% air at 37°C. Cell culture reagents were obtained from GIBCO (Grand Island, NY). At 72 h, the cells were detached from the T25 flask using 0.05% trypsin and 0.02% EDTA at 37°C for 10 min and

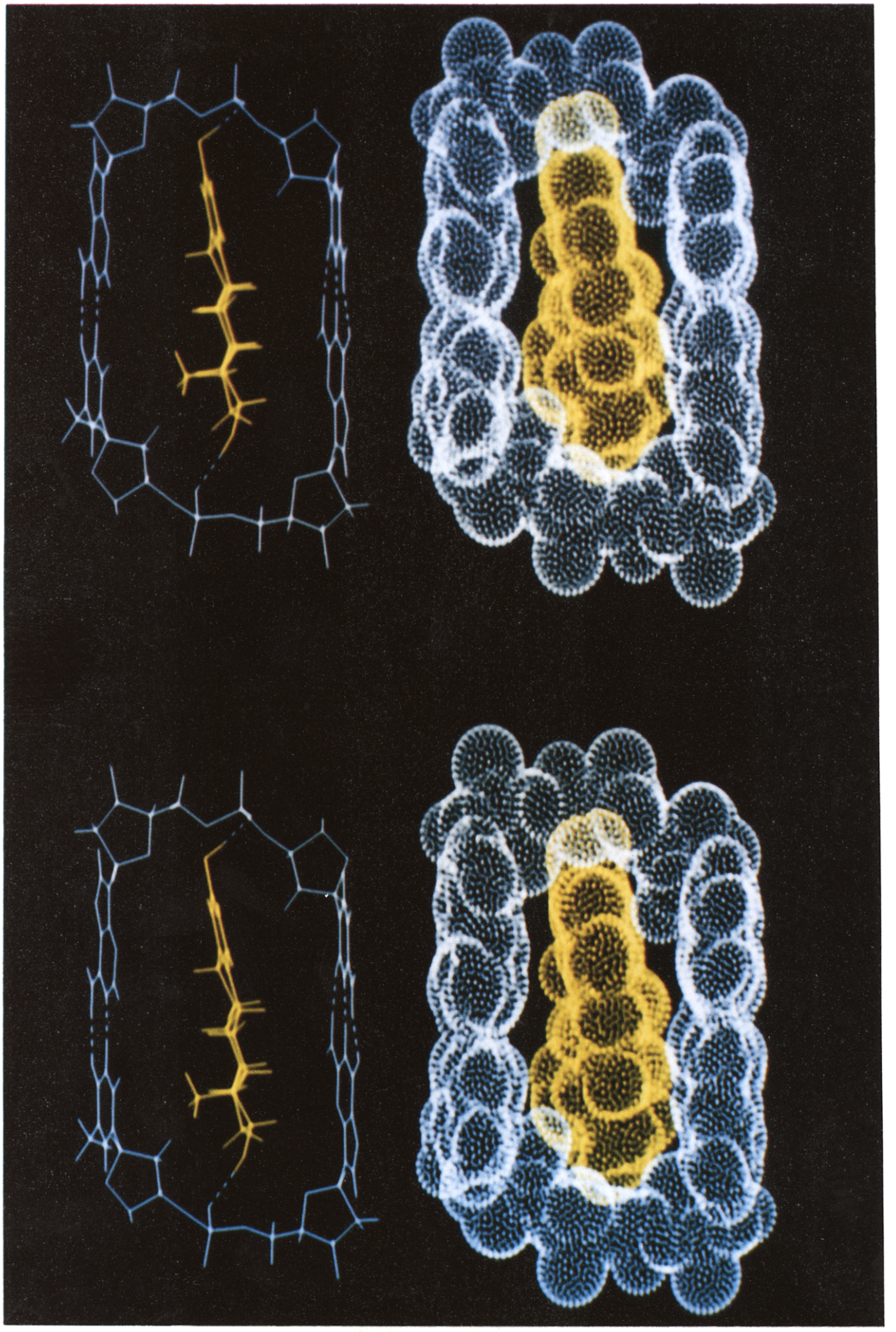

Fig. 2. Computer generated skeletal model (top) and the corresponding van der Waals dot surface model (bottom) of the fit of estradiol (yellow) inserted between base pairs in DNA at 5′-dTdG-3′-5′-dCdA-3′ (white). Hydrogen bonds are indicated by yellow dotted lines on the skeletal models. The view is in stereo (crossed) from the major groove with the TA base pair above and the GC base pair below. Computer generated space filling models of the estrogen/DNA complex have been reported recently [43].

allowed to grow over a 9 day period, changing media and test compounds every 48 h. An aliquot of cells was removed on day 9 and diluted into 10 ml of isoton (Coulter Diagnostics, Inc., Hialeah, FL); cell number was measured using a ZM model Coulter counter. Further details of the biological evaluation of these compounds is available and will be published elsewhere [24–26].

Caveats

While the use of the software and hardware described herein represent standard computer modeling techniques, there are certain inherent limitations. The results in kcal derived from energy calculations, e.g. docking energies of ligands fit into DNA, should not be interpreted as absolute values given that they were not empirically derived. However, the values are very useful in providing an accurate representation of the relative fit into DNA of a given series of compounds, for example, when examining the fit of estrogen agonists which are best accommodated completely between base pairs. While such information is also useful in examining the fit of antagonists, by virtue of the fact that antagonists can fit into DNA in a number of ways which include extension into the major or minor grooves, a wide variety of structural possibilities can be envisioned. Thus, in the general design of new active compounds, the structures of agonists are more limited than those of antagonists. For this reason, in the case of antagonists it is not yet possible to compare relative fit into DNA of different structural classes of antagonists which fit the DNA sites in a different manner (*vide infra*, piperidinediones vs tamoxifen). It should also be noted that both agonist and antagonist designs will be greatly improved when it becomes feasible to consider all possible conformations of a given ligand and DNA as well as effects of solvent, pH, ionic strength, other macromolecules (e.g. receptor proteins), etc. This information will be particularly useful in rigorously examining the sequence specificity of fit of molecules. Planned studies include consideration of water boundaries as well as molecular dynamics calculations which provide conformational flexibility to the molecules under examination.

RESULTS

Computer graphics demonstrated that estradiol could be inserted between base pairs in double stranded DNA at the site 5′-dTdG-3′·5′-dCdA-3′; two stereospecific hydrogen bonds were formed between each of the hydroxyl groups and negatively charged phosphate oxygens on adjacent strands [Figs 2 and 3(A)]. Energy calculations revealed that insertion of estradiol resulted in a van der Waals energy change of -16.321 kcal with an electrostatic change of -23.403 and -19.019 kcal due to the hydrogen bonds at the phenolic 3-hydroxyl to CpA (Hydrogen Bond 1) and at the 17β-hydroxyl to TpG (Hydrogen Bond 2) which were 2.663 and 2.659 Å, respectively. Thus, the total fit of estradiol in the site was -58.743 kcal which was normalized to a value of 100% in order to compare the fits of other ligands (Fig. 4). The normalized values for each component were 27.8% van der Waals, 39.8% Hydrogen Bond 1 and 32.4% Hydrogen Bond 2.

Attempts to insert PAG into DNA [Fig. 3(B)] resulted in a very weak electrostatic interaction between a terminal NH of the glutamine side chain and a phosphate oxygen at TpG (13.3%, Hydrogen Bond 2); the relative van der Waals fit was 11.1% with a total fit of 24.4%. Insertion of PP into DNA resulted in a 47.6% relative total fit. A hydrogen bond of 2.648 Å could be formed between the imino proton of PP and a phosphate oxygen at TpG (30.3%, Hydrogen Bond 2); the van der Waals fit was 17.3% [Fig. 3(C)]. *p*-OH-PP formed an insertion complex with DNA in which two hydrogen bonds of 2.710 and 2.653 Å were formed between the imino proton and a phosphate oxygen at TpG (28.4%, Hydrogen Bond 2) and the phenolic *p*-hydroxyl group and a phosphate oxygen at CpA (40.8%, Hydrogen Bond 1), respectively [Fig. 3(E)]. The van der Waals fit of *p*-OH-PP was 19.2% with a total overall fit of 88.4%.

Tamoxifen inserted between the base pairs with a total fit of 62.6%; the portion of the molecule containing the amine side chain extended out of the DNA into the major groove [Fig. 3(D)]. The energy calculations for tamoxifen were performed using charges for a quaternary amine thereby permitting a hydrogen bond between the amine and the O_4 of the base thymine. The electrostatic fit was 37.1% (Hydrogen Bond 3) with a 25.5% fit in van der Waals energy. 4-Hydroxytamoxifen also had an amine side chain which extended into the major groove and was a 101.2% total fit when inserted into DNA [Fig. 3(F)]. Two hydrogen bonds were formed in the 4-hydroxytamoxifen–DNA complex, i.e. the quaternary amine and the O_4 of thymine (37.1%, hydrogen Bond 3) and the phenolic 4-hydroxyl and the phosphate oxygen at CpA (37.3%, Hydrogen Bond 1); the van der Waals fit was 26.8%.

When examined for growth inhibition on estrogen stimulated MCF-7 (E3) cells (Fig. 5), PAG had little effect even at high dosage levels. PP inhibited growth with an IC_{50} of 3×10^{-3} M whereas *p*-OH-PP exhibited an IC_{50} of 7×10^{-6} M. The IC_{50} for tamoxifen was 1×10^{-7} M. Similar results were obtained when estradiol free calf serum was used and the very slow growth was stimulated to rapid growth by the addition of 10^{-11} M estradiol [28].

DISCUSSION

Computer modeling techniques described herein have confirmed earlier studies which employed physical models to demonstrate that estradiol is a remarkable fit between base pairs in DNA [4, 5, 15]. The estrogen molecule was accommodated completely between the base pairs and the locations of the hydroxyl groups at the 3 and 17β positions provided stereospecific hydrogen bonds to phosphate oxygens on adjacent strands.

Both the change in van der Waals and electrostatic energies upon insertion of estradiol into DNA were negative; the magnitude of the change indicated substantial favorable steric interactions and optimal hydrogen bonding linkages. Energy calculations demonstrating that the positioning of the functional groups on the cyclopentanophenanthrene skeleton as well as the chirality of the steroid nucleus manifest in natural estradiol are uniquely complementary to double stranded DNA will be published separately.

A main objective of this study was to identify and design new potential lead structures which would have antiestrogenic/antineoplastic properties. Based upon previous observations [4, 12], hormone agonists fit into the same DNA site and in the same manner as the parent hormone, i.e. they form stereospecific hydrogen bonds of the same type, length and relative strength. Antagonists on the other hand fit at least partially within the same site in DNA as agonists but have significant differences in either hydrogen bonding properties and/or possess structural features which can extend either into the major or minor grooves and form electrostatic interactions with the outside surfaces of the DNA. Using such criteria, the candidate ligand PAG would be a poor choice as an antiestrogen given that it was a poor steric fit relative to estradiol as measured by changes in van der Waals energy; moreover, in our hands, it was not possible to form reasonable hydrogen bonds concomitant with insertion of the molecule between base pairs. PAG was the poorest fitting molecule (24%) of those examined and thus would be expected to have little if any antiestrogenic activity. As predicted, PAG was essentially inactive when examined in estrogen stimulated MCF-7 (E3) human breast cancer cells (Fig. 5). The poor fit of PAG which is a urinary excretion product is also consistent with our general premise that inactive catabolites generally exhibit poor complementarity with DNA [5].

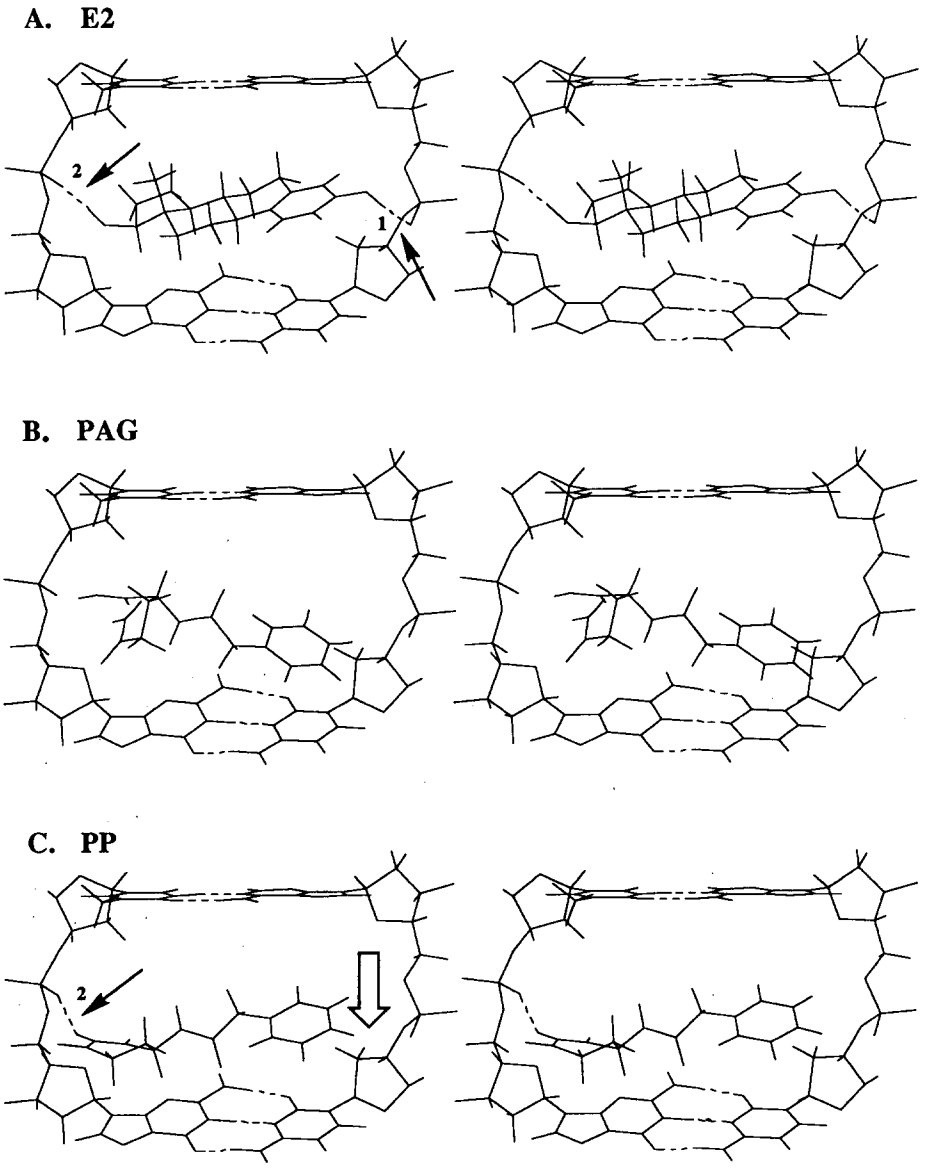

Fig. 3(A,B,C)—*legend opposite*

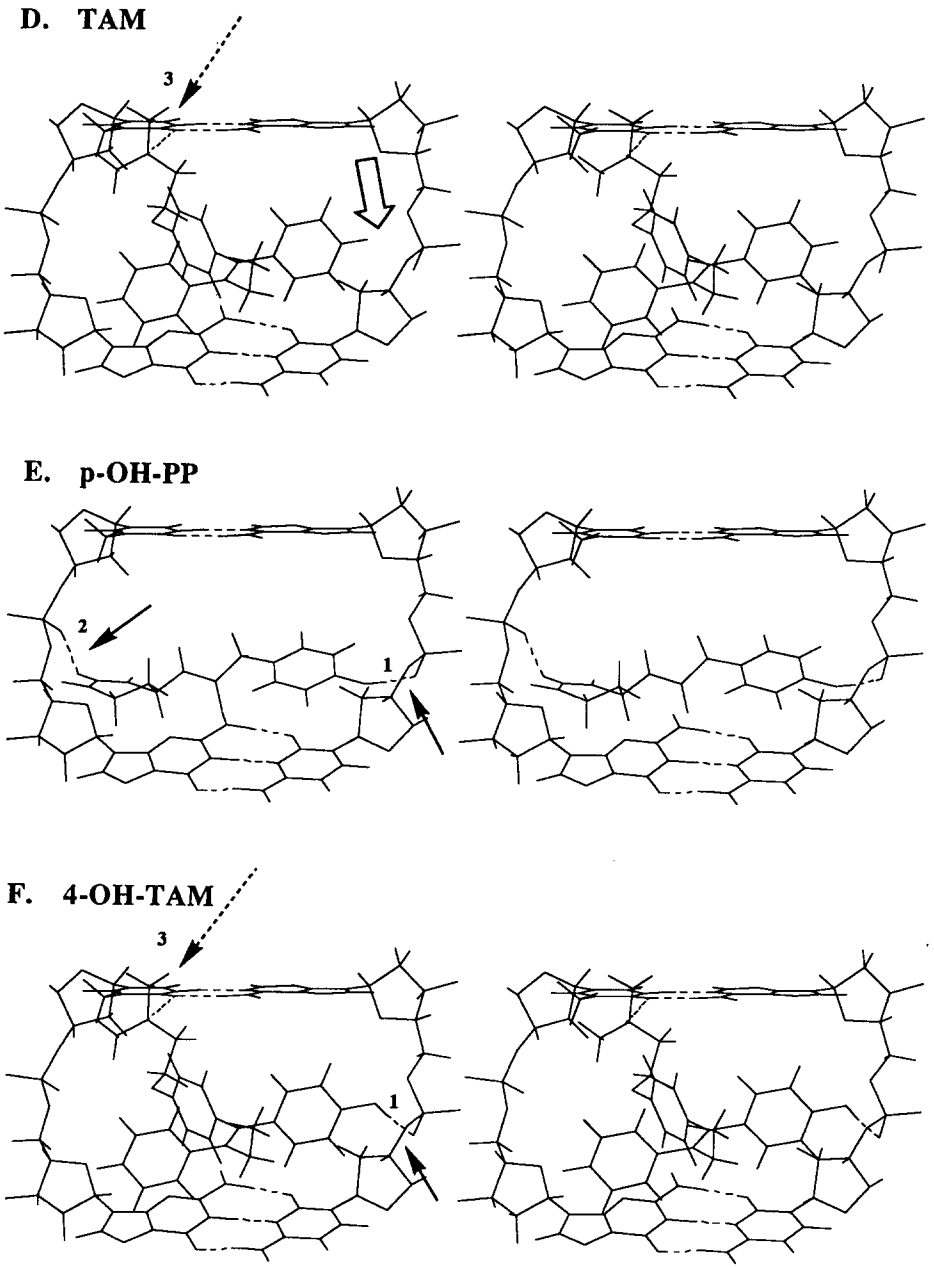

Fig. 3(D,E,F)

Fig. 3. Computer generated skeletal models (stereo) comparing the fit of candidate ligands (Fig. 1) into DNA from the same perspective as Fig. 2: (A) the estradiol–DNA complex with arrows 1 and 2 highlighting stereospecific hydrogen bonds between hydroxyl groups at 3 and 17β to CpA and TpG, respectively, thereby bridging adjacent DNA strands; (B) the PAG–DNA complex; (C) the PP–DNA complex with the hydrogen bond from the imine proton to TpG highlighted by arrow 2 and the open arrow indicating the proximity of atoms in the complex which could be modified to facilitate additional hydrogen bonding; (D) the tamoxifen (TAM)–DNA complex with the hydrogen bond 3 highlighted by a dotted arrow between the amine side chain extending into the major groove and the O_4 of thymine; analogous to C above, the open arrow indicates the close proximity of atoms in the complex which could be modified to enhance hydrogen bonding; (E) the *p*-OH-PP–DNA complex designed based upon the rationale in Fig. 3(C) resulting in two hydrogen bonds 1 and 2 bridging both sugar–phosphate backbones; (F) the 4-hydroxytamoxifen (4-OH-TAM)–DNA complex with two hydrogen bonds 1 (solid arrow) and 3 (dotted arrow) linking CpA and the outside surface of the base pairs at the O_4 of thymine, respectively. Note that the % hydrogen bond energy values presented in Fig. 4 were derived from the hydrogen bonds numbered on the complexes.

The piperidinedione PP which can be formed by dehydration of PAG was a much improved fit (48%) relative to PAG. PP formed a hydrogen bond between the imine and a phosphate oxygen at TpG whereas as stated above, no suitable hydrogen bonds were formed in the PAG/DNA complex. PP inserted into DNA further than PAG and thus had a better van der Waals fit. When compared with the estradiol–DNA complex, the hydrogen bond to PP was different from those in the estradiol–DNA complex in both magnitude and type.

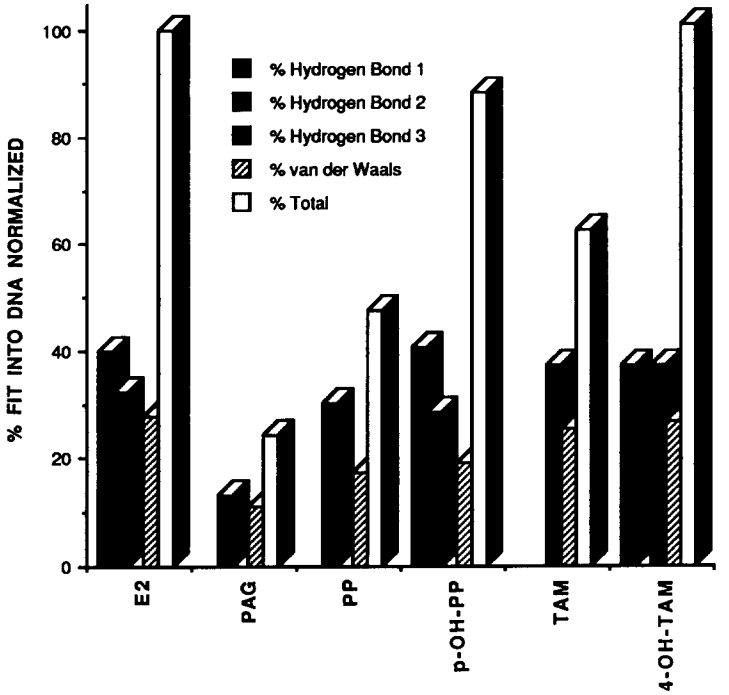

Fig. 4. Bar graph depicting the relative fit into DNA of candidate ligands (Fig. 1) using energy calculations. The individual bars represent the relative favorable change in electrostatic (hydrogen bonds 1–3) and van der Waals energy resulting from docking of a ligand along with the total of the values. The data for all ligands were normalized to the total fit of estradiol which was set at 100%. The respective hydrogen bond locations used in the electrostatic calculations are as depicted in Fig. 3 (see text).

The improved fit of PP in DNA relative to PAG indicated it should have greater antiestrogenic activity. As shown in Fig. 5, PP was found to have antiestrogenic activity. However, given the modest activity of PP in the MCF-7 assay (IC_{50} of 3×10^{-3} M) coupled with the observation that the overall fit of PP measured by energy calculations was still considerably lower than that of estradiol, the PP–DNA complex was examined

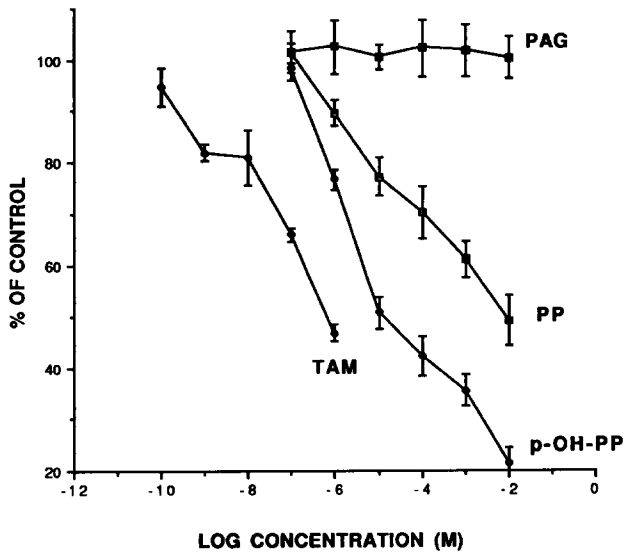

Fig. 5. Effect of varying amounts of PAG, PP, *p*-OH-PP and tamoxifen (TAM) on estrogen stimulated growth of MCF-7 (E3) human breast cancer cells. The data are a compilation of results from representative experiments [25, 29].

to determine whether it would be possible to design an analog of PP which would have significantly improved fit. One alternative was to form a hydrogen bond to the phosphate oxygen available at CpA. As shown in Fig. 3(C and E) this could be accomplished by inserting a functional group in the para position of PP. Indeed substitution of the hydroxyl group yielded a molecule *p*-OH-PP which could form a strong stereospecific hydrogen bond as measured by energy calculations. The fit of *p*-OH-PP into DNA (88%) was much greater than either PAG or PP leading us to predict that it should possess considerably higher antiestrogenic activity. These observations led to the synthesis of *p*-OH-PP and subsequent biological testing which revealed that it was more active by several orders of magnitude (IC_{50} of 7×10^{-6} M). In summary, the relative order of fit of these analogs as assessed by the energy calculations and thus the predicted activity correlates with order of biological activity found. Work in progress on a variety of analogs of PP using several biological assay systems further indicates that *p*-OH-PP by virtue of its relatively good fit in DNA is the most active analog [24–26, 28, 29].

There are a myriad of ways to design a potential antagonist based upon fit into DNA. For example, the classical antiestrogen tamoxifen which is used extensively in the treatment of breast cancer fits partially into the site that accommodates estradiol but contains an amine side chain which extends out of the DNA into the major groove and forms a hydrogen bond to the base pairs (63% total fit). As can be seen from the

hydrogen bonding heteroatoms linking both DNA strands) suggests that the stereochemical fit of the hormone may under certain conditions destabilize base pairing and facilitate separation of the DNA strands thereby modulating transcription.

In summary, the interaction of the hormone response elements with the receptor protein may govern the specificity of hormonal responses with the manner and degree of fit of the hormone into DNA governing the magnitude of the biological response. Binding of the hormone to the receptor protein would provide a means to guide the ligand to the proper site in DNA and possibly in concert with other transcription factors facilitate the insertion process. In this manner, the DNA could be considered a "secondary receptor" with the hormone functioning more like a transcription factor. Certain hormone antagonists, e.g. tamoxifen and the piperidinediones may fit into the site and interfere with action of the natural ligand. Given that this insertion hypothesis has been advanced previously [4] and more recently discussed in the context of new, published experimental findings [13], it will not be elaborated further here.

That the piperidinediones may also act by other mechanisms is suggested by the recent observation that PP and *p*-OH-PP decreases protein kinase activity [28]. These compounds also cause a dose dependent decline in estrogen receptor levels and inhibit estradiol induction of progesterone receptors. In these experiments, as predicted by modeling the activity of *p*-OH-PP was greater than PP and was similar to the classical anti-estrogen tamoxifen.

The basic rationale for the studies described here as well as for our past reports on the subject is that a rigorous study of gene stereochemistry will reveal critical information about the constraints nature has imposed on the structures of small molecules. On balance, the findings to date raise many more questions than they provide answers. Nevertheless, the evidence is mounting that gene structure will prove to be an invaluable source of information about biological structure, function, activity and metabolism.

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tamoxifen–DNA complex in Fig. 3(D), the phenyl ring proximal to the phosphate oxygen of CpA is positioned in the site such that the addition of a hydrogen bond donor functional group at the 4 position could form a stereospecific linkage. Substitution of this position with hydroxyl resulting in 4-hydroxytamoxifen enabled formation of a hydrogen bond. The fit of 4-hydroxytamoxifen (101%) was significantly greater than that of tamoxifen suggesting that it would be more active as an antiestrogen. Although not examined in our study, 4-hydroxytamoxifen has been previously reported to have considerably greater activity than tamoxifen [30] including when tested for growth inhibitory activity in MCF-7 cells [31].

As stated above, for many reasons including limitations of the computer modeling techniques, no interpretations about relative antihormonal activity should be made based upon energy calculations when disparate structures are evaluated, e.g. tamoxifen and the piperidinediones (Fig. 1). However, it is interesting that the structures of the most active compounds, i.e. estradiol, *p*-OH-PP and 4-hydroxytamoxifen have a common linkage between a phenolic hydroxyl group and the same phosphate oxygen (CpA). Also of interest is that in the case of PP and tamoxifen, criteria reported for the general metabolism of compounds to active structures based upon fit into DNA [4, 5, 9, 10], also predict that *p*-OH-PP and 4-hydroxytamoxifen would be active metabolites.

While the primary focus of this manuscript has been on designing new active piperidinediones using fit into DNA, the use of sites in DNA which biologically active estrogens fit into raises the provocative issue of how the findings relate to the classical understanding of the genomic mechanism of estrogen action which is known to be mediated by the estrogen receptor. The concept that small molecules including hormonal steroids in concert with specific binding proteins (receptor proteins acting as a major transcription factor) may regulate genes by stereochemical recognition of the ligand by DNA was initially advanced by our laboratories in 1977 [3]. Since the submission of this manuscript, new findings have been published on the biological effects of the piperidinediones [28, 32] including the observation that PP and *p*-OH-PP bind poorly to the estrogen receptor [28]. That weak binding to the estrogen receptor does not preclude antiestrogenic activity has also been reported recently in the case of two new classes of drug structures which are highly potent antiestrogens both *in vivo* and *in vitro* [33, 34]. Historically, many investigators have observed a poor correlation between the strength of binding of estrogen analogs and the magnitude of hormonal responses [35–38]. These observations led Brooks to conclude that binding affinity "is not directly related to the character or the extent of the response" [38]. Of particular interest are several estrogenic compounds which bind poorly to the estrogen receptor but are more potent than estradiol. For example, our laboratory has shown that one of the most active estrogen agonists, i.e. 11β-acetoxyestradiol which has four fold greater hormonal activity than estradiol [7], possesses $<1\%$ of the relative binding affinity of estradiol [39]. Taken as a whole, these results indicate that hormonal ligands have a critical gene regulatory function in addition to binding to the receptor. Given that we have observed a good correlation between degree of fit into DNA and degree of hormonal response [6, 13], it is reasonable to readdress the question of whether estrogens and antiestrogens might insert into DNA during their mode of action. For example, using computer graphics and energy calculations, 11β-acetoxyestradiol was confirmed to be a considerably better fit into DNA than estradiol [7, 13]. Because binding of steroid hormones to DNA without receptor has been shown to be weak ([4, 15] and references therein), estrogens as well as antiestrogens might insert into DNA in concert with the binding of estrogens receptors and other transcription factors to DNA. To our knowledge, there is no evidence to date which would preclude the insertion into DNA of estrogens, estrogen antagonists or other members of the steroid/thyroid family. Sluyser has made a similar suggestion on theoretical grounds [40].

Recent unexpected results of Spanjaard and Chin with glucocorticoids demonstrated that transcriptional activity induced by truncated constructs of the glucocorticoid receptor in which the hormone binding domain has been removed are stimulated two fold in the presence of dexamethasone [41]. Thus, it appears that the binding of the hormone to its receptor is not absolutely necessary for hormone action. Nevertheless, the hormone may act as an important transcriptional factor to enhance the rate of transcriptional activity. One possible mechanism for this action could be that the zinc finger DNA-binding domain which is common to all of the receptors in the steroid/thyroid superfamily [42] may create the cavity in DNA which can accommodate the hormone. Thus, the receptor protein upon binding to DNA in concert with other transcription factors may cause a specific conformational change in DNA. This would include unwinding giving rise to the site into which the ligand can be inserted. In the absence of either the receptor or the hormone, full transcriptional activity may not be elicited. It is conceivable that the insertion of the hormone may occur at the hormone response elements (HREs) which are known to interact with the zinc finger domain of the receptor. The composition, sequence and precise orientation of the HREs coupled with the composition and three dimensional properties of the receptor protein may govern the conformation and physicochemical characteristics of the site thereby providing a highly specific cavity that matches the exact stereochemistry of the hormone. Computer modeling has demonstrated as one might expect that the DNA conformations which accommodate progesterone, testosterone, estradiol and thyroid hormone are different lending support for this idea. The shapes (e.g. wedge shaped steroids and "propeller" shaped thyroid hormones [10]) and physicochemical features of the hormones (e.g. positions of

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