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Design of Novel Antiestrogens

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The physicochemical principle of "die and coin" complementarity proffered by Pauling and Delbruck and exemplified in Watson and Crick DNA was used to design new antineoplastic compounds. In search of an explanation for why certain molecules and not others are present in nature, biologically active small molecules were discovered to exhibit complementarity when inserted into cavities between base pairs in DNA. Ligands in the steroid/thyroid hormone/vitamin D family fit particularly well into the site 5′-dTdG-3′·5′-dCdA-3′. Degree of fit of various candidate compounds in the manner of a given hormone correlated with degree of hormonal activity. Hormone antagonists fit into the same site but in a different manner than the agonists. Computer graphics and energy calculations confirmed salient observations including the remarkable complementarity of estradiol and DNA. Using the above criteria, a new candidate antiestrogen, *para*-hydroxyphenyl-acetylamino-2,6-piperidinedione was successfully designed. Taken as a whole, these results coupled with recent independent findings raise the possibility that the mode of action of certain hormones and hormone antagonists may involve direct insertion into DNA mediated by classical protein receptors and other transcription factors.

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INTRODUCTION

Historically, the discovery of the specific mechanisms underlying biological processes has invariably resulted from a knowledge of the molecular structures involved. These have included studies with simple molecular models which have led to some of the most important scientific discoveries of the twentieth century. Based upon modeling considerations, Pauling *et al.* [1] proposed that protein structures could have α-helical and β-pleated sheet conformations. These predictions were later confirmed by X-ray crystallography and proved to be important in the function of certain proteins. Not long after Avery's laboratory provided evidence that genes were made up of nucleic acids [2], Watson and Crick [3] used model building to develop the double helical model of DNA. Well established theoretical models were also developed by Monod *et al.* [4] and Koshland *et al.* [5] for understanding allosteric transitions in proteins. More recently, Cram [6] received a Nobel Prize for host-guest chemistry which was based largely upon insights derived from simple Corey-Pauling-Koltun (CPK) space filling molecular models. Cram was able to make accurate predictions about structural recognition among various molecules and their biological actions that were subsequently confirmed in laboratory experiments. At the heart of these discoveries has been the principle of complementarity, i.e. the capacity of various molecules to fit together. Over 50 years ago, Pauling and Delbruck [7] provided a conceptual framework for complementarity as the "die and coin" fit of molecules. The quintessential examples of complementarity are obvious in the base pairs of DNA which led Watson and Crick [3] to predict a copying mechanism for genetic information.

Our laboratory has been advancing the hypothesis that complementarity can be extended to understanding why only certain small molecules and not others exist in nature as well as how certain small molecules regulate genes [8]. To be sure, it is well established that enzymes and receptor proteins, respectively are directly responsible for which molecules are synthesized and which have biological activity in nature. However, we

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have reasoned that since the genetic template ultimately also contains this information, direct complementary relationships might exist between the small molecules and the nucleic acids. This line of reasoning led the discovery based upon CPK models of complementary stereochemical relationships between nucleic acids and biologically active small molecules including steroid/thyroid hormones ([9], and references therein). For example, mammalian reproductive steroids were remarkable fits between base pairs in partially unwound double stranded DNA. These findings also led to the discovery that within any hormone class the degree of complementary fit into DNA correlated with degree of hormonal response [9, 10]. Although the hypothesis was put forth in 1977 that small molecular weight hormones in concert with chromosomal proteins may insert into DNA [8], we have cautioned against overinterpretation of results derived from hand held physical models particularly in the absence of experimental techniques to test the hypothesis. Recently, salient findings have been rigorously confirmed by both computer graphics and energy calculations [11–17]. These include the description of complementary complexes of steroids with DNA which fit Pauling and Delbruck's "die and coin" definition. Here, new findings are reported which provide a method to design estrogen agonists and antagonists. A discussion of the application of this approach to understanding gene regulation by steroid/thyroid hormone agonists and antagonists is also provided in light of recent experimental findings of ourselves and others.

EXPERIMENTAL

Molecular modeling was conducted on a Digital Microvax II computer with Sybyl 5.4 software (Tripos Associates, St Louis, MO) as described previously [11–15]. An Evans and Sutherland PS390 graphics computer equipped with a Stereographics viewer was interfaced by Ethernet to the Microvax. Structures of small molecules were obtained from: the Cambridge database [18]; modification of X-ray structures; construction with the Concord program; fragment libraries followed by energy minimization. Energy calculations were performed with the Sybyl force field using a 1.2 Å parameter for the van der Waals radii of hydrogen. The Gasteiger–Huckel method which includes σ and π bonding was used to calculate charges. The DNA was constructed as a double stranded dinucleotide from the Biopolymer module using the 3′-endo conformation for deoxyribose and Kollman charges. The DNA was unwound by twisting the 14 possible torsional angles on the sugar–phosphate backbone with the planarity and hydrogen bonding distances between heteroatoms on the base pairs maintained. During the unwinding process, adjustments were made to the DNA in order to best accommodate the size and shape of each hormonal ligand.

Insertion of the candidate ligands into DNA was accomplished using van der Waals dot and mesh surfaces coupled with the stereoviewer to guide the docking procedure and minimize poor steric contacts. The docking procedure was repeated several times to optimize the distances and directions of potential hydrogen bonds as well as to maximize van der Waals interactions. The conformations of functional groups on the ligands were also adjusted to maximize hydrogen bonding. The relative fit or "complementarity" of each ligand was calculated by measuring the optimal favorable energy change resulting from docking. A convenient method was to perform the docking procedure, define the ligand and DNA separately as aggregates and merge the molecules into a single complex. The change in van der Waals energy was used as a measure of steric complementarity; the change in electrostatic energy using donor hydrogens and acceptor heteroatoms was used to assess hydrogen bonding complementarity. The total fit of each ligand was evaluated by adding the change in kcal of the electrostatic and van der Waals energies. Thus, the greater the magnitude of the negative energy change resulting from complex formation, the more stable the complex and the better the fit.

Certain inherent limitations of the current methods have been described [11–14]. For example, the partially unwound site in DNA chosen in this study, i.e. 5′-dTdG-3′·5′-dCdA-3′ has been shown using various physical models to best accommodate steroid/thyroid ligands. To date, it has not been possible to examine all other possible sites or conformations of DNA and/or RNA. Rigorous examination of the sequence specificity of the fit of various ligands should become feasible when molecular dynamics techniques are available. Current experiments confirm previous suggestions that the position of water atoms surrounding the site will also be important in assessing degree of fit of ligands and greatly add to the specificity of such interactions [9]. It should also be emphasized that while it is straightforward to examine the relative fit into DNA of various agonists, it is difficult at the present time to compare in a quantitative manner the fit of antagonists. This is particularly evident for those antagonists having widely differing structures and thus very different ways of fitting into DNA. In this regard, attention must be also given to different DNA and ligand conformations as well as solvent effects.

RESULTS

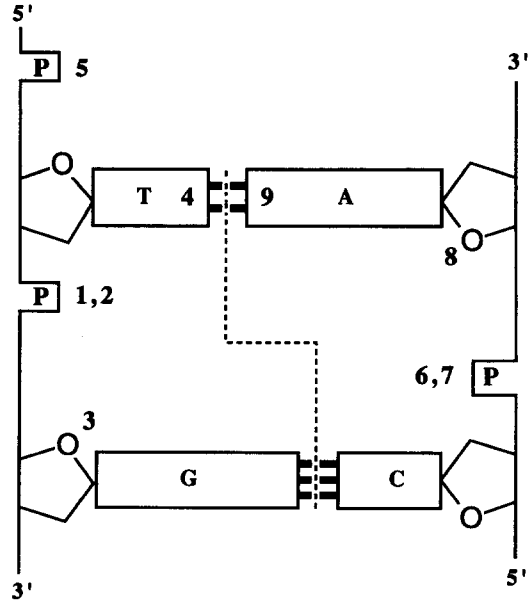
Complementarity of hormonal ligands inserted into DNA confirmed by computer modeling

A diagram of the site in DNA that various modeling approaches have shown to accommodate ligands of the steroid/thyroid family is provided in Fig. 1 along with the stereospecific linkages that each ligand formed to DNA heteroatoms. The results of computer modeling

applied to estrogens, androgens, progestins and thyroid hormones have confirmed earlier findings with space filling models (Fig. 2). Sample results of docking some of the hormones and related ligands into DNA using energy calculations are shown in Table 1. The fits of the natural hormones into DNA varied from approx. −36 kcal for the plant hormone gibberellic acid to approx. −60 kcal for estradiol and triiodothyronine (T_3). As noted in the past, each of the hormones was capable of linking both strands of double stranded DNA. Reported here for the first time is an exception, i.e. *trans*-retinoic acid which linked to only one strand (CpA).

Degree of fit into DNA of estrogens and synthetic analogs correlates with degree of uterotropic activity

In previous reports using physical models, when the relative fit into DNA of the natural hormone was



COMPOUND	1	2	3	4	5	6	7	8	9
Testosterone *	+						+		
Progesterone *		+					+		
Aldosterone		+			+		+		+
Cortisol		+	+	+	+		+		
Estradiol *	+					+			
Triiodothyronine (T_3) *	+					+†			
Retinoic Acid *						+			
1,25-(OH)$_2$ Vitamin D_3	+					+		+	

†Salt Bridge

Fig. 1. Diagram of site in DNA which accommodates steroid/thyroid/vitamin A and D ligands. Each ligand forms a unique pattern of complementary stereospecific donor/acceptor linkages to DNA at the positions numbered on the DNA and listed in the table. The dotted line on the diagram and the double line in the table divide linkages on opposite DNA strands. The numbering of atoms on the DNA designate: the phosphate oxygens on adjacent strands which can act as either proton acceptors (PO-; Nos 1 and 6) or proton donors (POH; Nos 2 and 7); proton acceptor at the O_4 of thymine (No. 4); proton acceptor at the 5′ phosphate of thymine (PO-; No. 5); proton acceptors at the O_4' of the deoxyribose sugars attached to guanine (No. 3) and adenine (No. 8); proton donor of the NH_2 of adenine (No. 9). The fits of ligands evaluated by computer modeling to date are designated with an *. Space filling models of T_3 [20] demonstrate that in addition to the salt bridge designated by †, T_3 can form a hydrogen bond between the 4′ hydroxyl group and either the phosphate group or O_4' of the opposite DNA strand depending upon the DNA conformation; computer models indicate that the preferred hydrogen bond is the former as shown. Water molecules appear to be required for certain linkages to the base pairs, e.g. the 11β-hydroxyl group of cortisol to the O_4 of thymine.

compared with that of various synthetic analogs, a correlation was discovered between relative degree of fit into DNA and relative hormone activity [9, 19]. When measured with energy calculations, degree of fit was confirmed to correlate with degree of hormonal activity. Interestingly, synthetic analogs with significantly greater hormonal activity than the natural hormone, e.g. 11β-acetoxyestradiol [19], isopropyldiiodothyronine [20] and 5α-dihydrotestosterone [11, 21] were in all cases reproducibly better fits into DNA than the respective natural hormones estradiol, T_3 and testosterone (Table 1).

We have reported with CPK models that alterations in the basic cyclopentanophenanthrene nucleus of the steroid or the positions of functional groups on the steroid skeleton result in poor fitting molecules [9, 22]. Using computer graphics and energy calculations, these observations have been confirmed [11]. In the case of estrogens, substitution of hydroxyl groups at only the 3 and 17β positions formed stereospecific hydrogen bonds to DNA; analogs with alternate ring patterns including ent-estradiol were relatively poor fits.

To further assess the putative correlation between fit into DNA and hormonal activity, the docking into DNA of a larger series of synthetic estrogens and related analogs selected from studies in our laboratories and the literature [19, 23–28] were evaluated. While the values for biological activity were obtained in different studies and under different conditions, a good correlation was nevertheless observed. As shown in Fig. 3(A), compounds with little estrogenic (uterotropic) activity did not fit well into DNA when compared to estradiol. Compounds which fit into DNA better than estradiol were more active than estradiol. There were no cases of poor fitting compounds which had good estrogenic activity nor were there any cases of good fitting compounds which possessed poor estrogenic activity. Available receptor binding data for those compounds evaluated in Fig. 3(A) were also compared with fit into DNA. No apparent correlation was observed [Fig. 3(B)]. No correlation was also observed between hormonal activity and receptor binding for these compounds [Fig. 3(C)].

To more closely examine these findings, further analysis was conducted on the 10 most hormonally active analogs for which receptor binding data were available. The conventional quantitative structure–activity relationship (QSAR) expression, i.e. $-\log$ of biological target properties, was employed (Fig. 3, bottom graphs; Sybyl Theory Manual [39]). A positive correlation was observed between fit into DNA and hormonal activity. Again no correlation was observed between fit into DNA and receptor binding. A negative correlation was observed between uterotropic activity and receptor binding for these compounds.

Docking of estrogen antagonists into DNA and drug design

As shown above for estrogens, molecules which fit into the same DNA site and in the manner of a given hormone (same hydrogen bonds) possessed agonist activity. Antagonists generally fit into the same site in DNA as the natural hormone but had differences in the type of fit. Specifically, the antagonists exhibited differences in the kind of hydrogen bonding and/or possessed structural features which extended out of the site into the major or minor grooves [9, 22]. These observations have now been confirmed by computer modeling (Fig. 4). For example, the antiestrogen 4-hydroxytamoxifen [40, 41] fit into the site which accommodated estrogen with a hydrogen bond between the phenolic hydroxyl and **C**p**A**; the amine side chain extended into the major groove and formed a hydrogen bond to O_4 of the base thymine. The total fit in DNA was -59.5 kcal. The antiestrogen LY 117018 [42] formed two hydrogen bonds between the phenolic hydroxyls and **C**p**A** and T**p**G; however, the amine side chain extended into the minor groove and formed a hydrogen bond to the O_2 of thymine. The total fit for LY 117018 was -88.6 kcal. The antiestrogen ICI 164384 [43] which is a 7α-substituted estrogen fit into DNA and formed two stereospecific linkages analogous to estradiol; the aliphatic side chain of ICI 164384 which contains an amide functional group extended into the minor groove and formed a hydrogen bond to the 5′ phosphate group attached to cytosine (**pC**p**A**). The overall energy of fit was -69.8 kcal.

Prospective studies were also conducted to design a compound which would fit the above criteria for an estrogen antagonist. Studies had shown that the dehydrated product of the urinary excretion product phenylacetylglutamine, i.e. 3-phenylacetylamino-2,6-piperidinedione, had weak activity as an inhibitor of estrogen stimulated MCF-7 (E3) cell growth *in vivo* and *in vitro* [44]. Given that this compound also exhibited little toxicity and had been shown with CPK models to fit quite well between DNA base pairs [45], it was a reasonable starting structure for modeling and potentially for drug design. Computer modeling confirmed that 3-phenylacetylamino-2,6-piperidinedione fit almost completely between base pairs in the site which accommodated estradiol. A single hydrogen bond was formed between the imino proton of the piperidinedione ring and a phosphate group (T**p**G), however, the overall fit of -28 kcal was unimpressive. To improve fit in the site, various substitutions were made to the basic nucleus including addition of various hydrogen bonding functional groups. The positioning of a hydroxyl group at the para position enabled a stereospecific hydrogen bond to be formed to C**p**A (Fig. 4). This resulted in a substantially improved overall energy fit of -51.9 kcal and thus a predicted improved biological activity for this analog. Sub-

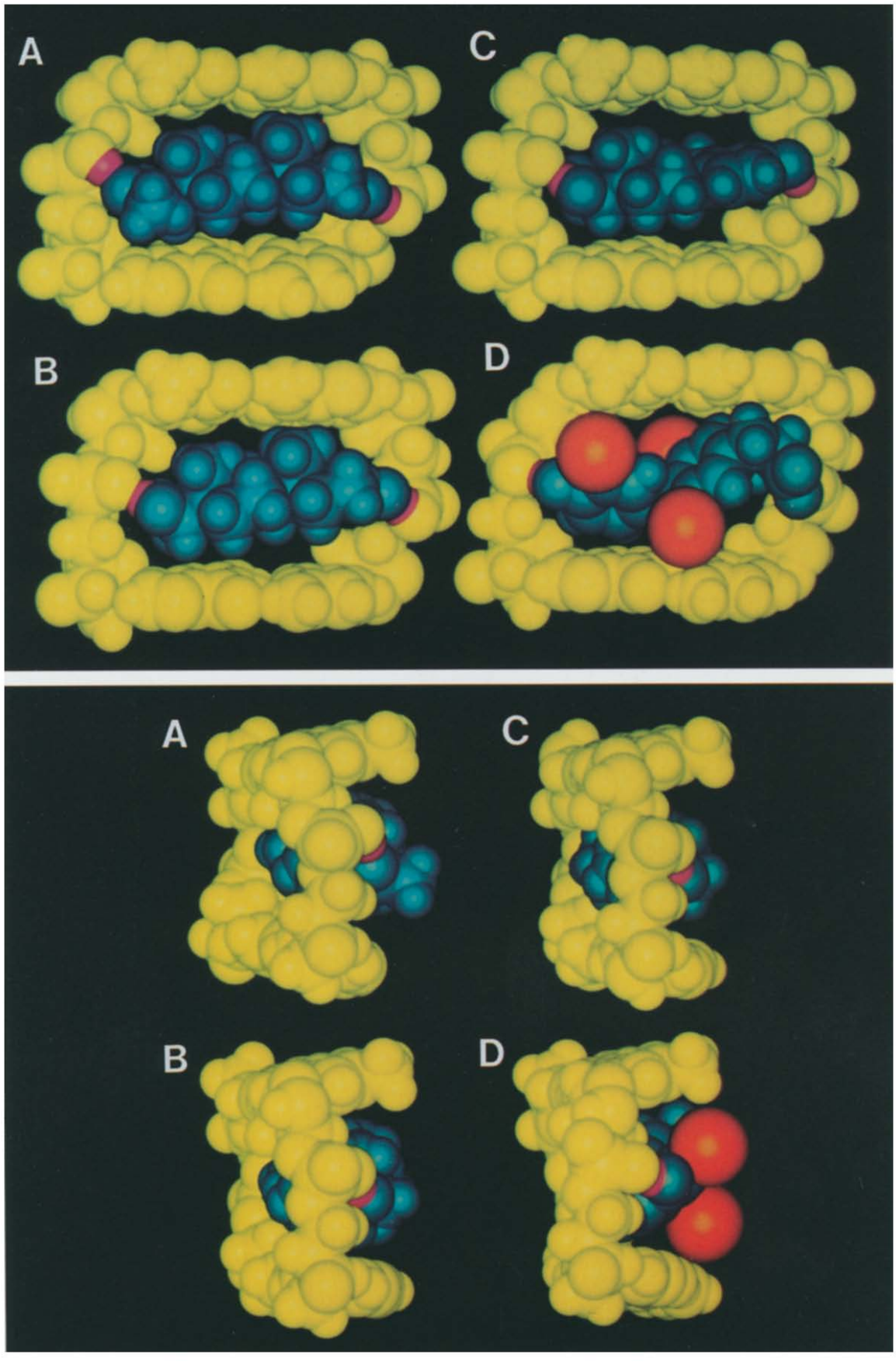


Fig. 2. Fit of hormonal ligands (blue) into DNA at 5′-dTdG-3′·5′-dCdA-3′ (yellow): (A) progesterone; (B) testosterone; (C) estradiol; (D) triiodothyronine (T_3). Protons involved in hydrogen bonding are colored magenta; the iodine atoms of T_3 are colored red. Top panel. View is from the major groove as in Fig. 1. Bottom panel. View is along the DNA backbone (dTdG).

Table 1. Small molecular weight hormones and related analogs confirmed to exhibit stereochemical complementarity when inserted between base pairs in partially unwound double stranded DNA using computer graphics and energy calculations

Hormone	Approximate energy of interaction	Site in DNA	Sterospecific hydrogen bonds	
Progesterone	−45.2 kcal	5′-dTdG-3′·5′-dCdA-3′	3-C=O ... HOP (A)	20-C=O ... HOP (G)
Tetrahydroprogesterone (3α,5α-THP)	−52.2 kcal	5′-dTdG-3′·5′-dCdA-3′	3-OH ... -OP (A)	20-C=O ... HOP (G)
Testosterone	−49.2 kcal	5′-dTdG-3′·5′-dCdA-3′	3-C=O ... HOP (A)	17β-OH ... -OP (G)
5α-Dihydrotestosterone	−50.0 kcal	5′-dTdG-3′·5′-dCdA-3′	3-C=O ... HOP (A)	17β-OH ... -OP (G)
Estradiol	−59.0 kcal	5′-dTdG-3′·5′-dCdA-3′	3-OH ... -OP (A)	17β-OH ... -OP (G)
11β-Acetoxyestradiol	−68.2 kcal	5′-dTdG-3′·5′-dCdA-3′	3-OH ... -OP (A)	17β-OH ... -OP (G)[a]
Thyroxine (T_4)	−32.2 kcal	5′-dTdG-3′·5′-dCdA-3′	NH_3 + ... -OP (A)	
Triiodothyronine (T_3)	−59.7 kcal	5′-dTdG-3′·5′-dCdA-3′	NH_3 + ... -OP (A)	4′-OH ... -OP (G)
Isopropyldiiodothyronine (3′-iPr-T_2)	−60.7 kcal	5′-dTdG-3′·5′-dCdA-3′	NH_3 + ... -OP (A)	4′-OH ... -OP (G)
Gibberellic acid (GA_3)	−35.5 kcal	5′-dTdA-3′·5′-dTdA-3′	3-OH ... -OP (A)	13-OH ... -OP (A)[a]

[a]Gibberellic acid [15] and 11β-acetoxyestradiol [19, 11, 53] form additional hydrogen bonds to the base pairs.

sequent synthesis and biological testing of *para*-hydroxy-3-phenylacetylamino-2,6-piperidinedione and related analogs demonstrated a good correlation between fit into DNA and antiestrogenic activity [44, 46]. Namely, in all biological experiments conducted, this analog had substantially greater activity than the unsubstituted parent compound. This included an IC_{50} in MCF-7 cells which was three orders of magnitude

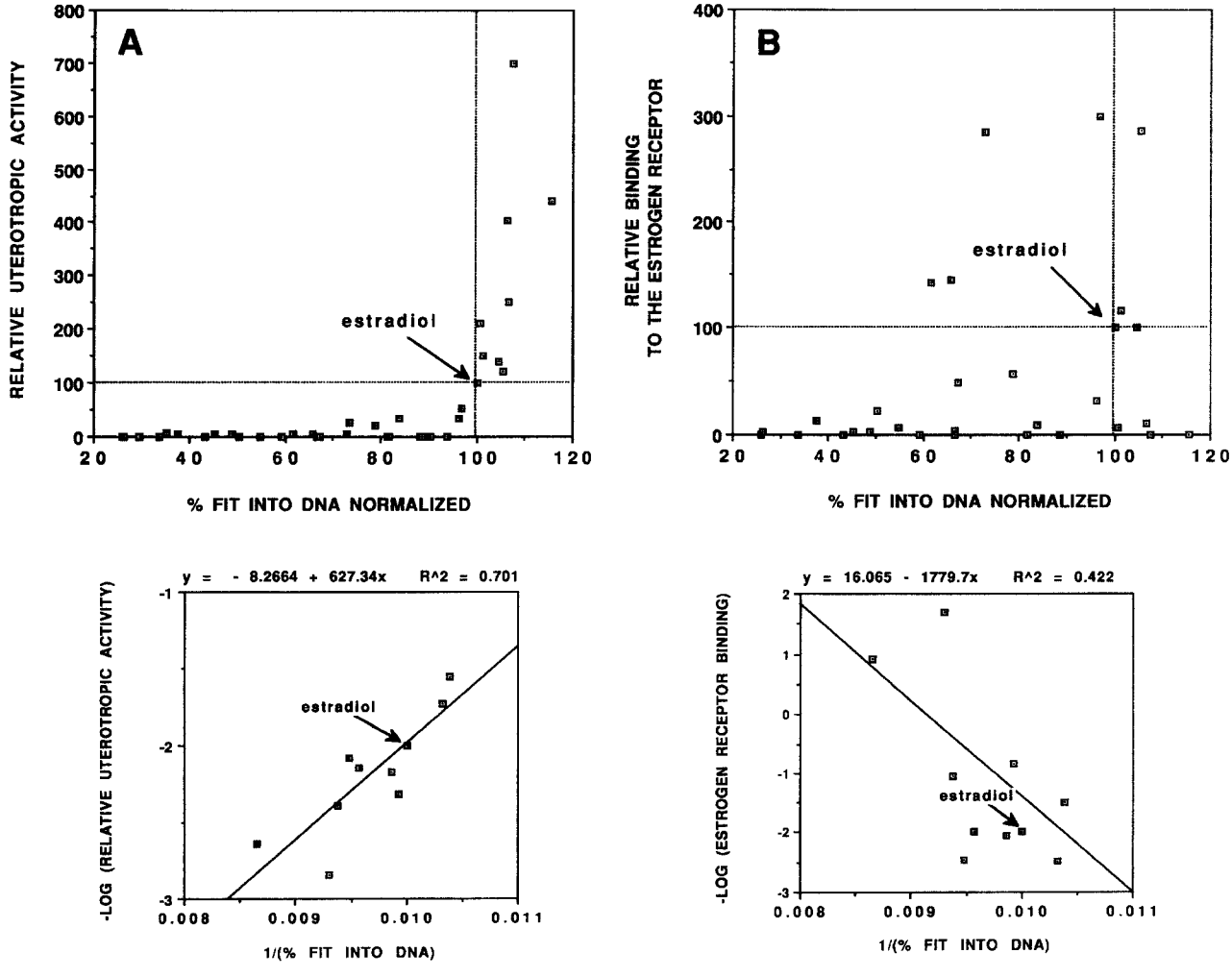
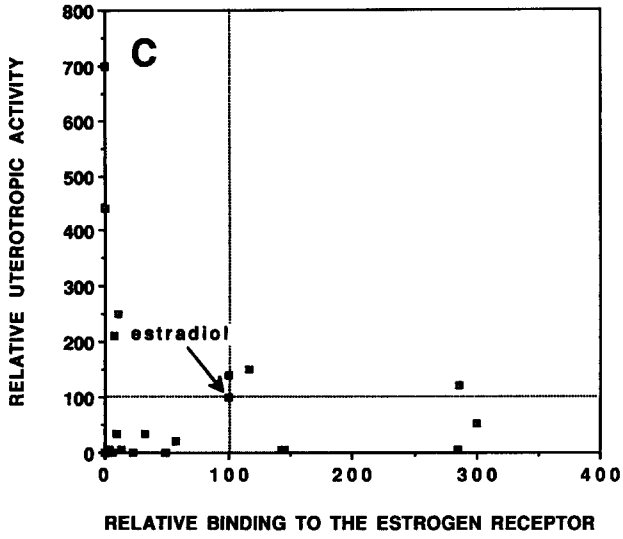


Fig. 3(A and B)—*legend opposite.*



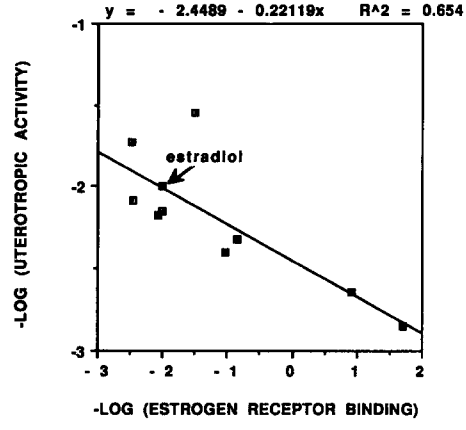


Fig. 3(C)

Fig. 3. Correlations of hormonal activity, receptor binding and fit into DNA measured with energy calculations. (A) Relative fit into DNA of ligands correlated with relative uterotropic activity; (B) relative fit into DNA of ligands correlated with relative binding to the estrogen receptor; (C) relative uterotropic activity of various compounds correlated with relative binding to the estrogen receptor. Below are analyses of a dataset containing the 10 most active compounds for which receptor binding data was available. All data points were normalized to the values of estradiol set at 100 and were derived from studies in our laboratories and the literature [19, 23–38, 53].

lower than 3-phenylacetylamino-2,6-piperidinedione and approached that of the well established antiestrogen tamoxifen.

DISCUSSION

The results presented here using computer graphics and energy calculations provide further confirmation that ligands in the steroid/thyroid/vitamin A and D receptor family fit between base pairs in DNA in a manner consistent with Pauling and Delbruck's [7] "die and coin" description of complementarity. Computer modeling has previously shown that the intermediates along the biosynthetic pathways of various hormones generally reflect increasing fit in DNA [14, 15]. The data provided in Table 1 are also consistent with this observation (progesterone < testosterone < 5α-dihydrotestosterone < estradiol; thyroxine < T_3). That degree of hormonal activity can be correlated with degree of fit into DNA [9–11] has also been confirmed using estrogens as an example. It follows that fit into DNA can be used to design new estrogen agonists. Ideally, super-agonists should fit completely within the site and exhibit better complementarity when measured with energy calculations. A good example is 11β-acetoxyestradiol which fits markedly better into DNA than estradiol by almost 10 kcal (Table 1) and is four times more potent as an estrogen than the natural hormone estradiol [19]. In fact, 11β-acetoxyestradiol is one of the most active estrogens known and to date is the best fitting synthetic compound into the estrogen site in DNA.

General criteria for designing hormone antagonists based upon observations made primarily with physical models have been described previously [9, 22]. Namely, the candidate ligand should fit into the same site but in a manner different from the natural hormone. Here, computer modeling demonstrates that the well known antiestrogens 4-hydroxytamoxifen, LY 117018 and ICI 164384 fulfill these criteria. Each of the antiestrogens fits into the site in DNA that accommodates estradiol and possesses at least one hydrogen bonding linkage to DNA which is different from estradiol. Moreover, unlike estradiol and estrogen agonists, each of these drugs has structural features which extend out of the DNA beyond the base pairs into either the major or minor grooves. Other hormone antagonists such as the antiprogestin RU486 have been shown to have similar features [13]. Because there is such a large number of candidate molecules which could fit the criteria, it should be possible to design antiestrogens with very widely differing structures. One example is *para*-hydroxy-3-phenylacetylamino-2,6-piperidinedione which was designed prospectively, synthesized and later found to have the predicted antagonist activity approaching that of tamoxifen in MCF-7 (E3) cells [44, 46, 47]. When compared with various piperidinedione analogs, *para*-hydroxy-3-phenylacetylamino-2,6-piperidinedione was the best fit into DNA and was by far the most active compound. It is interesting that this compound does not extend appreciably beyond the base pairs but has a different type of hydrogen bonding linkage (NH · · · -OP at TpG) than estradiol (OH · · · -OP at TpG). Perhaps of greater importance is the considerable conformational flexibility of the piperidinediones which is not present in the more rigid steroid skeleton. Such features need to be carefully considered in future drug design particularly if one mode of action of some estrogen agonists and antagonists actually involves insertion into DNA (*vide infra*).

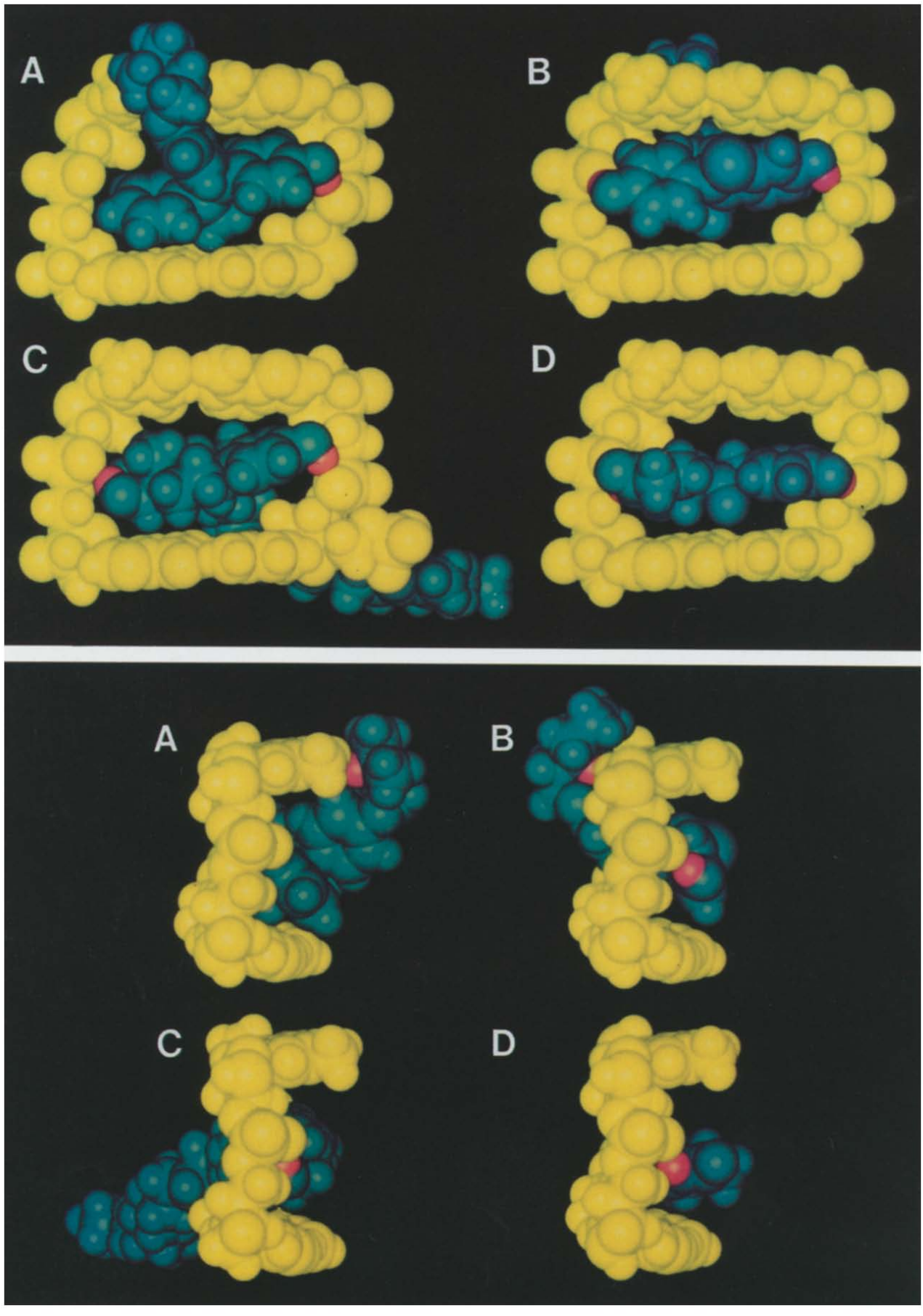


Fig. 4. Fit of antiestrogens (blue) into DNA at 5′-dTdG-3′·5′-dCdA-3′ (yellow): (A) 4-hydroxytamoxifen; (B) LY 117018; (C) ICI 164384; (D) *para*-hydroxyphenylacetylamino-2,6-piperidinedione. Protons involved in hydrogen bonding are colored magenta. The carbonyl oxygen of the amide group of ICI 164384 forms a hydrogen bond to a protonated 5′ phosphate group attached to cytosine. Top panel. View is from the major groove as in Fig. 1. Bottom panel. View is along the DNA backbone (dTdG).

Receptor mediated insertion of ligands into DNA

That there may be a stage in the mechanism of action of hormones in the steroid/thyroid/vitamin A and D family in which the ligands are inserted into DNA in concert with receptor proteins has been suggested in previous reports [8, 9, 11]. Sluyser [48] has also hypothesized that steroids may insert between base pairs. As previously reviewed [9], others including Huggins and Yang [49] and more recently Duax [50] have wondered whether steroids might contact DNA. However, various experimental studies have shown that steroids alone bind weakly to DNA. We determined that even the flat phytoestrogen coumestrol which would be expected to easily slide between base pairs bound poorly to synthetic oligonucleotides [51]. Thus, if steroids insert into DNA, other factors such as the receptor protein are most likely involved.

In our view, the possibility of ligand insertion into DNA must now be seriously considered in light of the correlation between hormonal activity and fit into DNA assessed by energy calculations coupled with the striking observation that super-agonists fit better than the natural hormone. This hypothesis does not conflict with the observation that the natural hormones cause specific conformational changes in the receptor protein which effect the binding of the receptor to hormone response elements (HREs) in DNA and is consistent with the conclusion of Beekman *et al.* [52] "that the ultimate role of the hormone lies in events after DNA binding, presumably in transcriptional activation". It does help explain why receptor binding does not correlate with hormonal activity. In fact, compounds which bind too strongly to the receptor relative to estradiol would be expected to have diminished activity. This is known to be the case for indenestrols which are metabolites of the synthetic estrogen diethylstilbestrol [38]. Compounds which bind weakly to the receptor could still have potent hormonal activity. Experimentally, this has been shown with 11β-methoxy substituted estrogens [26] and doisynolic acid analogs [32]. In our hands 11β-acetoxyestradiol, which to our knowledge is the most potent estrogen known, had $<1\%$ of the relative binding affinity of estradiol [53]. Similar findings using various estrogen analogs led Brooks *et al.* [36] to conclude that the extent and character of hormonal response is not directly related to receptor affinity.

The observation that steroid/thyroid hormones fit well between base pairs in the site 5′-dTdG-3′·5′-dCdA-3′ was originally based exclusively upon complementary matching of the ligand with various possible partially unwound sites in DNA using physical models. This observation led to the prediction that this site would be important in key regions of DNA including those which were regulated by steroid/thyroid hormones [9, 19, 20, 22]. The subsequent finding that many steroid/thyroid regulatory genes contain these sequences and they are invariant in the consensus sequences of the HREs which bind to steroid/thyroid receptors [54] supports this prediction. Moreover, 5′-dTdG-3′·5′-dCdA-3′ is present in the center of the half sites in the HREs.

While many of the ligands appear to fit well into 5′-dTdG-3′·5′-dCdA-3′, each hormone possesses unique stereospecific hydrogen bonds to DNA. These linkages are important in determining the type of hormonal activity elicited by a candidate ligand. In searching for common features among the linkages (Fig. 1), it became apparent that the ligands fall into two subclasses based upon their capacity to link to the CpA strand. Namely, testosterone, progesterone, aldosterone and cortisol bind to HOP (No. 7) whereas estradiol, T_3, retinoic acid and 1,25-dihydroxyvitamin D_3 link to -OP (No. 6). It is particularly striking that these ligands have been shown independently to fall into two subclasses with identical memberships based upon different classes of HREs [54]. Namely, the receptors for each member of the subclass recognize the same consensus sequences: GGTA**CA**nnn**TG**TYCY for androgens, progestins, mineralocorticoids and glucocorticoids; GGT**CA**nnn**TG**ACC for estrogens, thyroid hormones, vitamin D_3 and retinoic acid. These observations suggest that the HREs may be one site where the ligands are being inserted. The receptor protein possibly in concert with other transcription factors may bind to the HREs in such a way as mediate in a precise fashion the physicochemical properties of the site. This would include controlling the capacity of phosphate groups to act as proton donors or acceptors, the degree of unwinding and/or kinking [55] of the helix as well as the specific conformation of the DNA. Thus, the receptor would provide the exact environment necessary for insertion of the ligand. This possibility is supported by the computer models which demonstrate that different ligands can be accommodated within the same site but form complexes having different conformations of the DNA backbone (Fig. 2). The exact nature of hormonal response elicited (e.g. positive or negative regulation) would not be dependent primarily upon the structure of the ligand but on the HRE sequence and its interaction with the receptor [56]. The spacing and orientation of the various half sites (palindromes, pseudo palindromes, direct repeats etc.) which are known to vary among the HREs including the possible formation of cruciform structures would be potentially critical factors. The role of the ligand would be to assist in the regulation of the magnitude of the hormonal response via its insertion into the site. It follows that the receptor may not only provide the energy to create the site in DNA but also guide the ligand to the proper location in DNA and facilitate the insertion. Given that each of the steroids as well as T_3 and retinoic acid have wedge shaped structural features, these ligands may also aid in the uncoupling of hydrogen bonds between the base pairs

and separation of the DNA strands leading under the proper conditions to a facilitation of transcription. Recent *in vivo* experiments by Liehr *et al.* [57, 58] demonstrate that natural and synthetic estrogens can contact DNA and form covalent adducts with DNA. These findings are also consistent with the observations of DeSombre *et al.* that I^{123} labeled estrogens are radiotoxic to cells by damaging DNA in cells containing the estrogen receptor ([59] for further discussion see [11]).

Further evidence for the insertion hypothesis has been recently reported by Spanjaard and Chin [60] based upon experiments with truncated glucocorticoid receptors. Receptor constructs in which the hormone binding domain was removed unexpectedly stimulated transcriptional activity 2-fold in the presence of dexamethasone. Thus, the hormone was not enhancing transcription through binding to the receptor and must be acting in some other fashion, e.g. by inserting into the site created by interaction of the DNA with the DNA binding domain of the receptor. In the intact receptor, the observed tremendous enhancement of transcription mediated by the ligand would be due in part to the insertion of the ligand by the receptor. In summary, a convenient way to conceptualize the insertion hypothesis is to consider the DNA as a "secondary receptor" with the complementary ligand functioning as a transcription factor.

If receptor mediated insertion of hormones into DNA is part of the genomic mechanism of action, hormone antagonists could work at multiple levels. For example, an antagonist could bind strongly to the hormone binding site in the receptor thereby preventing insertion of the hormone. The antagonists could be inserted by the receptor into the site in DNA and form complexes similar to those reported here (Fig. 4). As is the case for agonists, it would also be possible for the antagonists to insert into DNA without assistance from the receptor. Such a mechanism might explain the activity of *para*-hydroxy-3-phenylacetylamino-2,6-piperidinedione [44] and new highly potent classes of antiestrogens [61, 62] which like certain agonists bind weakly to the estrogen receptor.

Very recently in the search for an answer to why nucleic acids contain pentose and not hexose sugars, Eschemoser and co-workers [63, 64] have synthesized DNA containing a six membered sugar (homo-DNA). Modeling studies of partially unwound homo-DNA which does not occur naturally reveal a complete loss of "die and coin" complementarity between the nucleic acid and the steroid/thyroid/vitamin A and D ligands [11]. This extraordinary finding provides further impetus for experimental testing of the insertion hypothesis.

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