DNA Minor Groove Pharmacophores Describing Sequence Specific Properties

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The more that is known about human and other genome sequences and the correlation between gene expression and the course of a disease, the more evident it seems to be that DNA is chosen as a drug target instead of proteins which are built with the information encoded by DNA. According to this approach, small minor groove binding molecules have been designed to bind the DNA sequence specifically and thereby downregulate genes. Because of their lack of druglikeness, we plan to use them as templates for forthcoming virtual screening experiments to discover molecules with the same bioactivity and a different scaffold. In this proof of principle study, carried out with the software tool Catalyst, we present a model work for description of a ligand−DNA complex with the aid of pharmacophore modeling methods. The successful reproduction of sequence specificity of a polyamidic minor groove binding ligand is the precondition for later model application to virtual screening.

1. INTRODUCTION

Growing knowledge about the genome has prompted major research efforts aimed at the design of molecules that are able to manipulate gene expression. It is estimated that only 10−14% of the proteins encoded by the 30 000 human genes have appropriate binding sites for small molecules.[1] Therefore, a considerable part of the proteome still resists traditional drug discovery efforts. The interference with transcription control provides an instrument to generally modulate gene function[2] and has therefore a therapeutic impact[3−6] whenever the molecular basis of a given disease is well-known:[7] Instead of altering the function of a protein as traditional drugs do, they are able to prevent the protein's synthesis by interfering with the binding of transcription factors or activators in the promotor region of a gene. This strategy promises to be very effective—a smaller number of drug molecules is needed to target a single DNA sequence compared with the large amount of proteins which is synthesized with the information of this sequence.[2]

Two general approaches can be distinguished: either these molecules target messenger RNA (mRNA) or gene DNA.[10] On the one hand, mRNA is intercepted by antisense oligonucleotide-based drugs binding and degrading RNA, thus preventing target proteins from ever being made. Although developers are still confronted with problems,[11] industrial investments[12] as well as numerous publications[13−15] show the high potential of protein inhibition at stages prior to their synthesis. On the other hand, triple helix forming oligonucleotides, synthetic zinc fingers, and hairpin polyamides (Figure 1) are sequence selective representatives of molecules directly targeting gene DNA.[2] The scaffold of hairpin polyamides typically comprises a chain of heteroaromatic rings, mainly pyrroles, conjuncted by amide groups. In the middle of the chain its rigidity is interrupted by a flexible linker, which allows the molecule to assume the shape of a hairpin when interacting with the minor groove. A disadvantage of an ordinary polyamide in regard to sequence selective readout may be their relatively small reading frame of about 6 DNA bases. This has to be increased to address a single genomic site. On the other hand, a crucial feature for all substances active at the DNA is their ability to enter the cell nucleus. The higher the molecular weight of the compound, the smaller the chance to pass membranes. Because of their relatively small molecular weight the hairpin polyamides have the best chances to intrude the cell nucleus. In vivo studies show that polyamides indeed reach the DNA: inhibition of gene expression was found in cultured cells[16−18] as well as after feeding polyamides to *Drosophila*[19] or injection into a mouse model, where antifungal activity was found.[20] Successful nuclear uptake was also reported for several human cell lines.[21] These findings underline the potential of this class of minor groove binders.[22] Although the structure of the polyamidic minor groove binders has many advantages decisive for selective DNA readout, the scaffold also brings about severe disadvantages: Because of presumably poor metabolic stability due to the large number of amide bonds and the overall structure it is suspect to be very nondruglike.[74] It cannot be assumed to provide good oral bioavailability, which is a general aim in drug development.[1,23,24] Nevertheless, the great number of crystal structures of DNA in complex with

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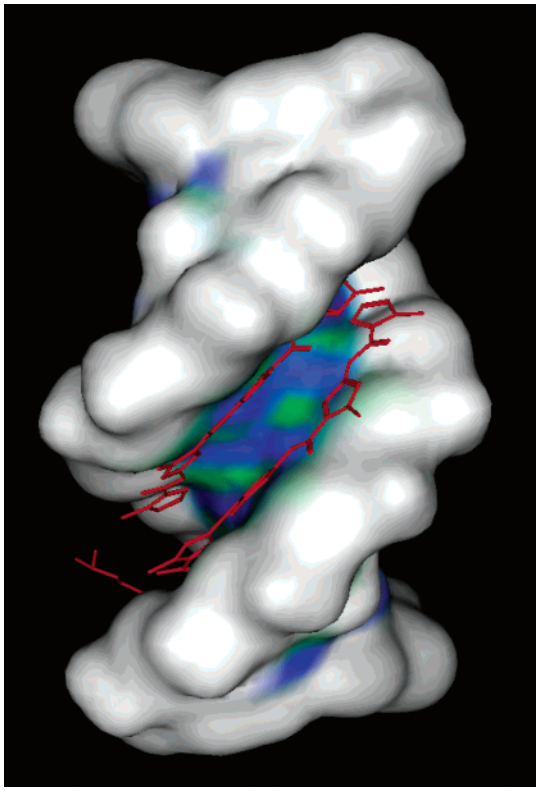

Figure 1. X-ray structure of a ligand in a complex with a DNA helix. The DNA is colored by pocket depth, blue spots symbolize hydrophilic regions, most of them cover hydrogen bond acceptor functions (PDB code: 1cvx by Kielkopf et al.[27]).

polyamides provides a good basis for rational drug design in a field that includes a number of major worldwide unmet clinical needs.[26]

An unfavorable but unavoidable structural element of a potential minor groove binder is the high number of hydrogen bond donors. They are required as counterparts of the high number of hydrogen bond acceptors in the minor groove. According to Lipinski's "rule of five" poor absorption and permeability become probable when the number of hydrogen bond donors exceeds five. However, Lipinski's analysis excluded structural classes containing large numbers of hydrogen bond donors a priori,[23] and examples of drugs containing large numbers of hydrogen bonds exist. Aminoglycosides are such examples for clinically relevant structures[25] that violate the "rule of five" in terms of donor functionality.

Distamycin, a natural product inhibiting DNA-dependent processes,[28] is a very simple but typical representative of the large group of polyamidic minor groove binders (Figure 2, polyamide **1**), thoroughly reviewed in the literature.[9,29−34] It became famous when it was found to bind to DNA as a dimer,[35,36] contacting each DNA strand separately. The scaffold served as a template for numerous more sophisticated compounds, because it bears some important advantages: [37] Each amide bond donates a hydrogen bond to the minor groove which in turn is covered with hydrogen bond acceptors. The distance between two donors on the ligand equals the distance between two bases.[38] The molecule is crescent-shaped and flexible enough to adopt a conformation which is complementary to the minor groove. The pyrrole rings can easily be modified or replaced by other heteroaromatic rings which are able to distinguish between DNA bases.[39,40] π-Stacking interactions[27] between the amides and the *N*-methylpolypyrrole rings enable the two parts of the dimer to stay close enough to fit into the groove. The combination of two stacked heteroaromatic rings is the repeating element which is responsible for reading the genetic code base by base. To ensure that the two parts of the dimer adopt only one defined arrangement, they are connected to form a hairpin.

To expand distamycin's DNA reading capabilities, rings with additional hydrogen-bonding functions and modified sterical properties were introduced to replace *N*-methylpyrrole: Imidazole with its acceptor function interacts with the exocyclic amine of guanine,[42−45] the only donor in the minor groove. Hydroxypyrrole has an additional donor to satisfy the second lone pair of thymine's carbonyl oxygen.[46,47] Moreover, the hydroxyl substituent is too bulky to stay next to guanine which requires the most space in the minor groove. With these two rings in combination with the original scaffold, a complete recognition of all base combinations is possible.[48] Which pair of building blocks is selective for a given base pair is summarized in the so-called pairing rules.[9]

In the years following the discovery of selective readout investigations concentrated on refining the minor groove binding molecules. Numerous aromatic[39,49−57] or aliphatic[58−60] building blocks and terminal groups[61,62] were tested. Moreover, covalent linkers between the dimer arms were varied,[41,63−66] including chiral[67] or doubled linkers[41,68] as well as linkers between whole hairpin ligands.[69,70] Structure variations were carried out to improve affinity, selectivity, shape, and flexibility, to extend the recognition site and to learn about sequence-recognition properties[71−73] not only leading to a better understanding but also to a large amount of data.

Poor druglikeness[74] and the amount of data available about DNA recognition by small molecules lead to the idea to describe the DNA−ligand complex with the aid of pharmacophore modeling methods and the plan to use the models for virtual screening. The very large number of molecules already synthesized and purchasable and the even much larger number of molecules that can be created by virtual combinatorial chemistry techniques[75] make it very likely to find new lead structures for DNA binding among them. Although virtual screening of compounds for their 3D-arrangement of interaction sites is a popular technique in drug discovery, and even though DNA has an exceptionally well-characterized structure, the vast majority of in silico studies have examined protein receptors. The small number of published studies which address the DNA minor groove as a target describes the application of docking methods.[26,76−82] No study has been published yet where the application of pharmacophore modeling to DNA complexes is reported. As they consist of relatively simple descriptors (each functional group is represented by a single point or vector in space), pharmacophore models are a good choice to be used to filter large databases containing 3D molecular structures.[83−85] There are numerous excellent reviews covering the current status and success stories about the application of pharmacophore models and virtual screening in drug design.[86−90] As pharmacophore modeling methods are not only optimized for speed but also for the description of interactions between small ligands and proteins, this proof of principle study will elucidate the feasibility to reproduce sequence preferences

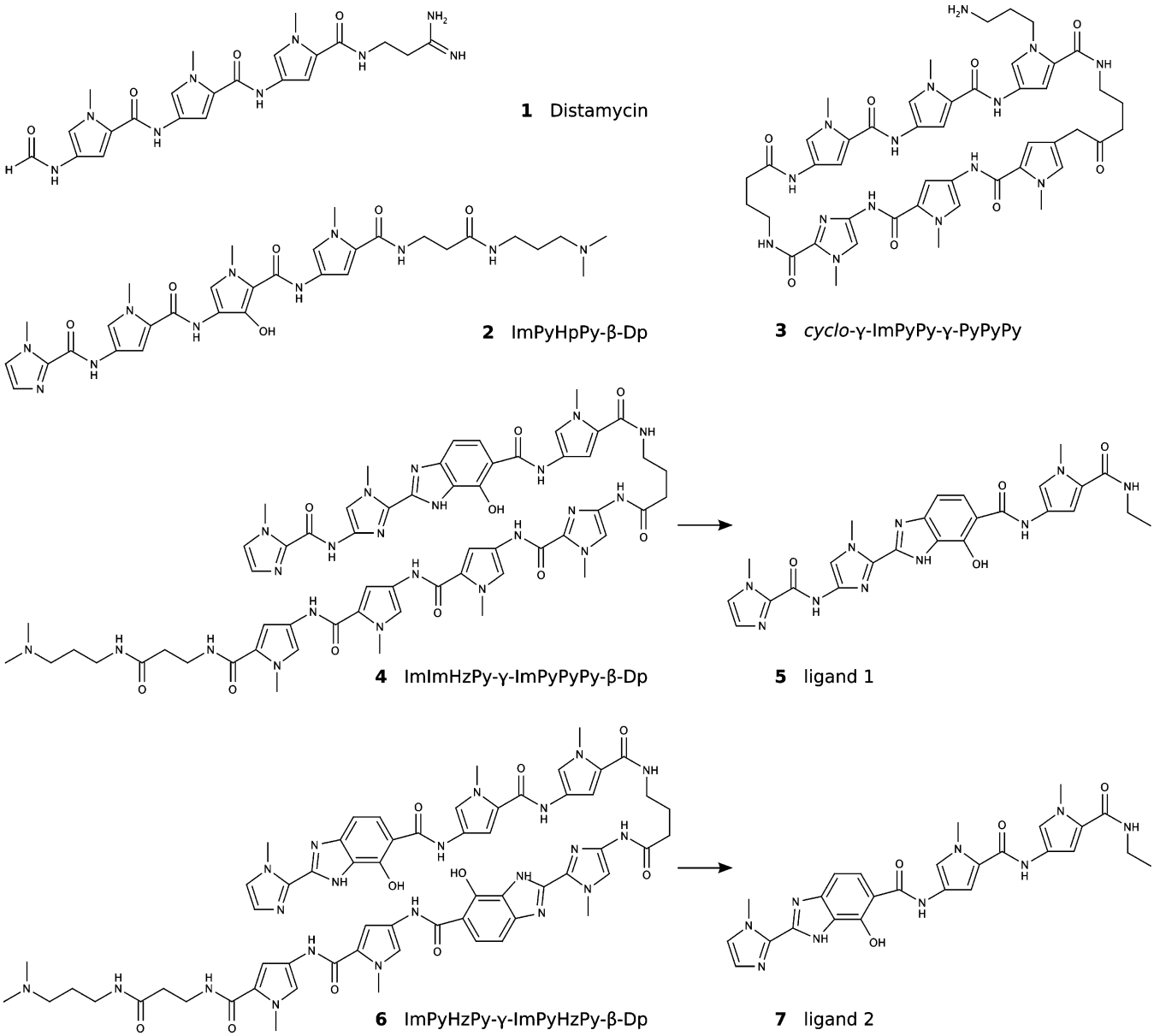

Figure 2. Chemical structures of polyamidic minor groove binders (Im = imidazole, Hp = hydroxypyrrole, Py = pyrrole, Hz = hydroxybenzimidazole, $\beta = \beta$-alanine, $\gamma = \gamma$-amino butyric acid, Dp = dimethylaminopropylamide). 1: Distamycin, 2: Sequence selective ligand with modified pyrrole units,[27] 3: Example of a cyclic polyamide,[41] 4: A typical hairpin ligand as used by Marques and co-workers,[55] 5: Because of software limitations only one-half of number 4 was used, in this study this structure is called 'ligand 1', 6: Another ligand from Marques and co-workers, 7: Ligand 2 is one-half of structure number 6.

of a given ligand. Only if a model fulfills this requirement, it will be useful for virtual screening in the future.

2. RESULTS AND DISCUSSION

To study the sequence specificity of a minor groove binding ligand we extracted information from DNA footprint experiments.[55] We have chosen footprint experiments as a basis for our study because crystal structures bring about some problems: On the one hand, most of them do not exceed 12 base pairs; on the other hand, crystal packing effects strongly modify DNA structures at both ends of the sequence. In footprint experiments the number of base pairs scanned by the ligand is much larger. A disadvantage of footprint experiments is the absence of detailed 3D DNA structure information. But although the DNA is flexible to a certain degree, the geometrical arrangement of hydrogen bond acceptors on the groove floor appears to be relatively insensitive to conformation alteration.[91] Moreover, docking to standard B-DNA structures was found to reproduce results from footprint experiments.[76] Therefore we decided that a canonical B-DNA structure would satisfy our requirements, and we do not have to rely on DNA structures determined by crystallography or NMR: 3D coordinates were automatically generated[92] with the sequence taken from the footprint experiment[55] providing the basis for model creation on the DNA.

The underlying experiment was taken from a study where a newly designed ligand (Figure 2, polyamide **4**, structure 1 in ref 55) with a new aromatic building block was tested against a DNA sequence consisting of 81 base pairs.[55] Hydroxybenzimidazole (Hz) was added to the minor groove binder construction kit to replace hydroxypyrrole (Hp) and its adjacent amide group. The latter was a benchmark to the field due to its ability to distinguish a T·A base pair from

Table 1. Ligand 1: Equilibrium Association Constants (K_a) Found in DNaseI Footprinting Experiments[55] in Comparison with the Fit Values Obtained by Pharmacophore DNA Screening

site no.	K_a [M^{-1}]	fit value
1	$\leq 1.0 \times 10^7$	
2	$\leq 1.0 \times 10^7$	
3	$5.7(\pm 0.4) \times 10^8$	4.13
4	$5.5(\pm 0.2) \times 10^9$	4.46

A·T, but it was also found to decrease ligand stability and affinity.[46−48,93−96] The footprint experiments were designed to investigate the hydroxybenzimidazole bicycle with regard to affinity and discrimination between the DNA bases. Within a strand of 81 base pairs four sequences were included which only differed at a central position where the hydroxybenzimidazole group was supposed to be situated. Two bases downstream and upstream from this site were chosen to suit the remainder of the ligand (see Figure 7 for the whole sequence—the sites designed for selectivity evaluation are highlighted). The ligand used for model generation was found to bind with high affinity ($K_a = 5.5(\pm 0.2) \times 10^9\ M^{-1}$) and sequence specificity to the sequence where thymine was situated next to hydroxybenzimidazole (site 4), preferring the T·A pairing over A·T by 10-fold and A,T over G,C by more than 50-fold. Affinity data—as far as published—are presented in Table 1. Site numbers correspond to those used in diagram 7.

Because specificity of DNA recognition arises from ligand interactions with the functional groups on the edges of Watson Crick base pairs,[52] hydrogen bonds play a key role for selective sequence readout in the minor groove. As a first test, the DNA was decorated with hydrogen-bonding pharmacophore features after conversion of the sequence information into 3D coordinates. Each base was described by an acceptor feature vector, except for guanine: it was provided with an additional donor feature (Figure 4). Coordinates of the base points of hydrogen-bonding features were directly taken from the 3D file, whereas the vector's projected points were generated by simple vector calculation. Their positioning was controlled to correspond to standard geometries as provided by the "Show Function Mapping" tool within Catalyst.

Taken together the features on the DNA form a long pharmacophore model, called "hypothesis" within the program Catalyst. It has the shape of a helix. In this state it cannot be compared with the ligand, because a pharmacophore model from the ligand's point of view would mainly consist of a set of hydrogen bonding donor features which cannot be directly compared with the DNA model. Therefore, each descriptor of the DNA model was reversed: The coordinates of the acceptor vector's base point became coordinates of the donor vector's projected point (Figure 4). Then the chain of descriptors was divided into two parts, each describing only one DNA strand. This was necessary to make the comparison between the ligand and the hypothesis work. We found that comparison between a whole hairpin ligand and a double strand hypothesis did not succeed, even if the hypothesis comprised only few bases. Presumably this limitation is due to improper conformation sampling. According to the division of the DNA model, the ligand was also divided into two halves. The remaining strand of features was cut into packages of five base pairs each, which was enough to be covered by a ligand (Figure 5).

For ligand preparation, half of structure **4** (Figure 2, ligand 1) was drawn within Catalyst. Optimal DNA screening results were achieved, when using a structure as a starting conformation for conformer generation, whose torsions have manually been adapted to resemble the molecules found in X-ray structures. Minimized or standard 3D conformations were by far less successful. A conformational model was generated with Catalyst: the BEST algorithm was used to achieve a thorough sampling of the conformational space.[97] Default settings for a maximum number of conformers (250) as well as for the energy limit (20 kcal/mol) were kept resulting in a set of 67 conformers. The fact that the structural details of the starting conformer strongly influences the results is a hint that this set does not meet the requirements of a thorough coverage of the conformational space. Increasing the maximum number of conformers or the energy limit in the conformer generation settings hardly helped. We found that an energy limit reduction from 20 kcal/mol to 10 kcal/mol does not influence the results, since the mapping algorithm hardly ever uses conformations other than the starting conformation.

After ligand and hypothesis preparation, a fit value was calculated by comparing the ligand with each hypothesis, resulting in a stepwise scan of the minor groove. The fit value is computed as follows

$$\text{Fit} = \sum_f \left[1 - \sum_c \left(\frac{D(c)}{T(c)} \right)^2 \right]$$

where D is the displacement of the feature on the ligand from the center of the location constraint, and T refers to the radius of the tolerance sphere.[98] A perfect mapping of features would result in a fit value equivalent to the number of features in the hypothesis. Each mapping process was conducted with Catalyst's command line tool 'citest': The BEST algorithm[83] was used. It not only takes prepared conformers into account but also allows subtle conformational adjustments. For our study this is especially helpful, because neither the automatically generated conformers nor the manually refined ones sufficiently reproduce the slight twist of the bioactive 3D configuration. To fully benefit from the conformational adjustments the energy threshold for the mapping was increased from 20 kcal/mol (default) to 50 kcal/mol.

With this first model, only consisting of hydrogen bond features, the collected fit values are somewhat higher for the four designed binding sites than for the rest of the 81 base pair strand but do not show any preference of the ligand for the experimentally confirmed binding site. This means that the description of the direction of the hydrogen bonds does not suffice to reproduce sequence selectivity. Therefore, some refinements had to be introduced to the model. Thymine was provided with an additional hydrogen bond feature to explicitly describe both carbonyl lone pairs pointing to the minor groove. Additionally, steric properties of the minor groove floor must be taken into account: An excluded volume sphere with the coordinates of guanine's exocyclic amine group and a tolerance sphere of 3 Å was added whenever guanine was described in the hypothesis, simulating the steric clash with the bulky hydroxyl group of hydroxybenzimidazole. To meet the restrictions caused by the simplification of the model to describe only one strand,

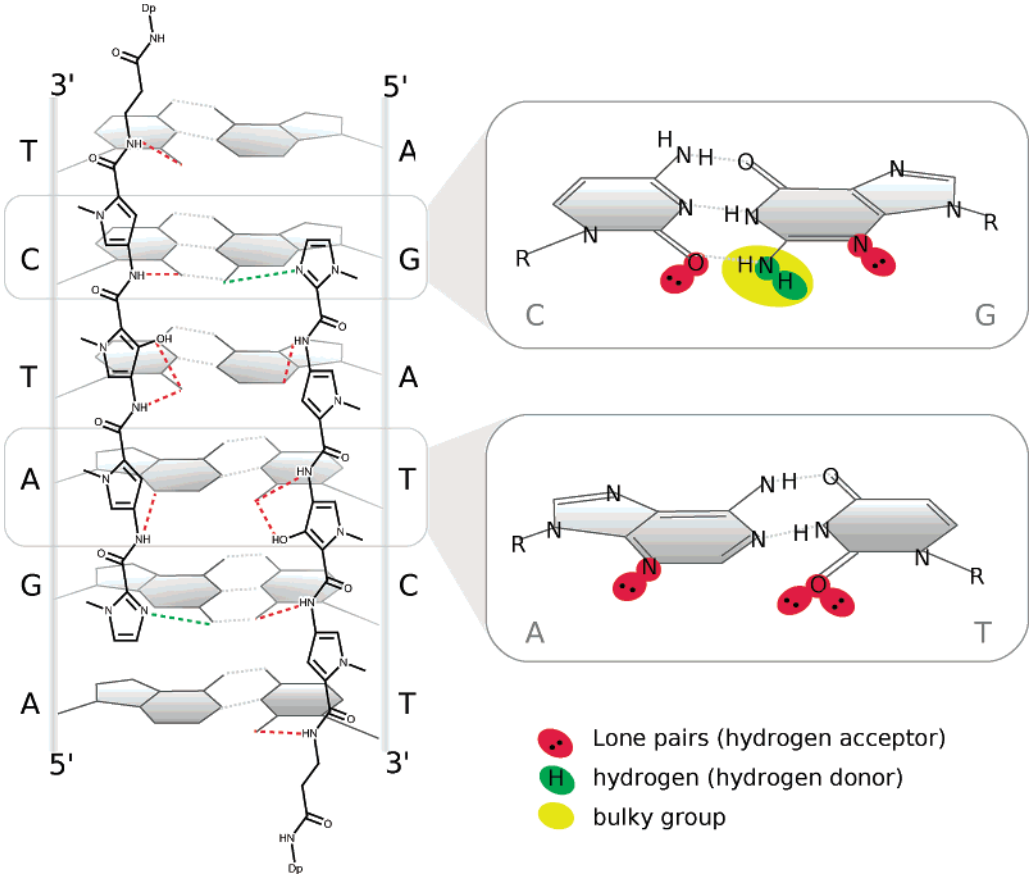

Figure 3. Left side: Schematic picture of a hairpin polyamide with modified heteroaromatic rings in complex with the DNA. All hydrogen bonds are shown as dashed lines. Right side: Functional groups and sterical characteristics in the DNA minor groove: carbonyl-O and heteroaromatic N provide lone pairs (dark red), guanine's exocyclic amine provides a hydrogen (green) for hydrogen bond formation, moreover it occupies more space than the other functional groups.

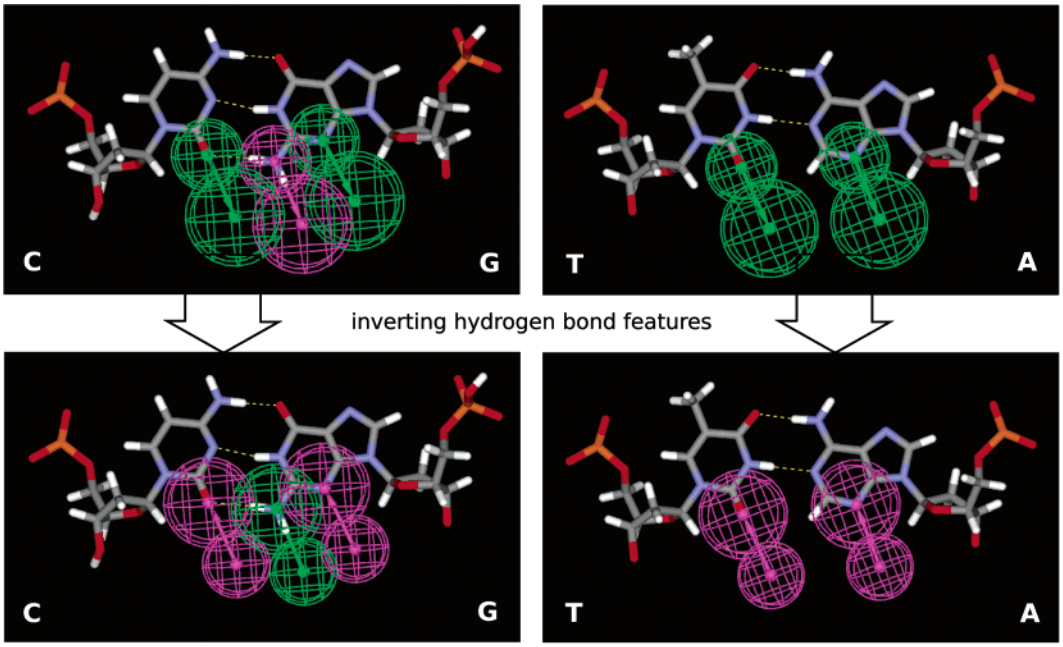

Figure 4. Each base pair is decorated with the hydrogen-bonding features that are decisive for selective ligand interactions at the minor groove floor. First row: features as automatically created by Catalyst, second row: features inverted to describe the interactions from the ligand's point of view.

further excluded volume spheres were added to each cytosine. They were built on the coordinates of the exocyclic amine of the opposite guanine to represent the steric influence of the opposite strand. Their optimal tolerance sphere radius was found to be 2.5 Å. The particular sizes of the radii were determined by the increased energy limit (50 kcal/mol instead of 20 kcal/mol) that was chosen to improve the conformational adjustments made by Catalyst's BEST compare/fit

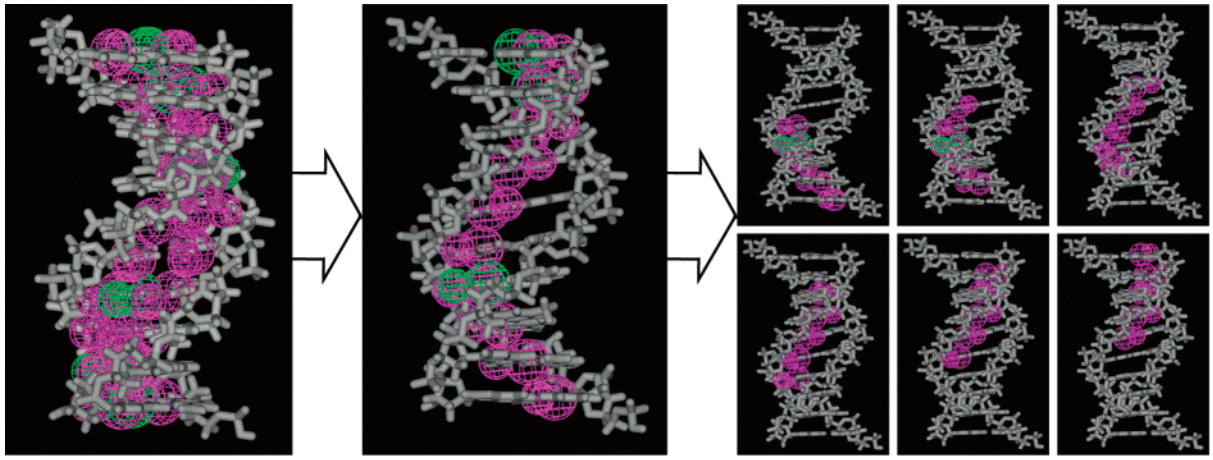

Figure 5. General steps toward DNA model generation: Creation of a hypothesis spanning the whole minor groove, division into two halves, cutting one-half into packages of five bases, where each packages starts at a different base step.

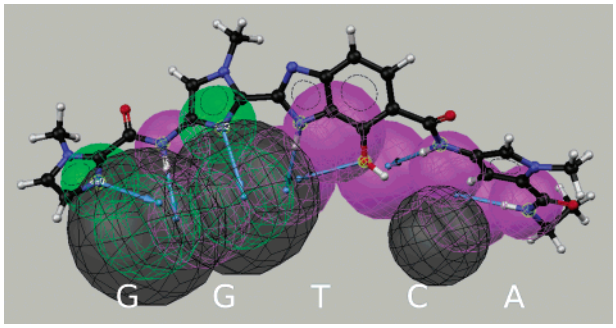

Figure 6. The ligand mapping to the hypothesis of the experimentally confirmed binding site.

algorithm. As each excluded volume is treated as a pseudoatom with VdW radius equal to its tolerance, the energy limit allows the ligand to slightly penetrate the excluded volumes. To not completely lose the effect of the volumes, they are quite large compared with the default radius of 1.5 Å. As a last step of model refinement the ligand was forced to map all features provided by the DNA, whereas the default settings would allow the algorithm to omit 2 features per hypothesis. Neither backbone information nor groove width constraints were incorporated in the model since their influence on ligand binding seems to be overruled by hydrogen bonding and steric repulsion at the minor groove floor.

Again, fit values were calculated for the ligand mapping to all hypotheses spanning 5 bases each. The diagram in Figure 7 shows the result: Each fit value was plotted right above the letter which denotes the first base that is part of the hypothesis. If the bar is completely missing, no mapping was possible in the compare/fit operation. A clear distinction between the designed binding sites (highlighted with gray background) and the rest of the DNA can be seen. The highest fit value is assigned to the experimentally confirmed preferred binding site (number 4, with dark gray background). Site 3, where thymine in the center of the binding sequence is replaced by adenine, does also have a high fit value. The dominance of these two bars corresponds to the experimental findings: The measured equilibrium association constant K_a for site 3 is only about ten times smaller than for site 4[55] (the exact numbers can be found in Table 1). The affinity to all other sites is considerably lower. The reason is that the discrimination between T·A versus A·T is in general less accurate than between T,A versus C,G base pairs.

A comparison between the calculated fit values and the experimental data is given in Table 1. It shows a correlation between the experimentally confirmed affinities and the fit values and therefore points out that reproduction of sequence specificity with the aid of pharmacophore modeling is feasible. On the other hand, these data should not be overinterpreted: Binding affinities are compared with numbers calculated from models that are optimized for selectivity prediction. They are lacking features that account for affinity only such as hydrophobic or features representing positive charges. They will be added for future screening studies but were not considered helpful for this study. The position of the two smaller bars means that the model suggests ligand−DNA interactions apart from the designed binding sites. In case of site 2 it means that the model correctly declares that there is no interaction with this site that was designed to demonstrate that the ligand is able to refuse sites containing G or C in their center. The appearance of the additional bars results from slight inaccuracies of the model: The OH group on hydroxybenzimidazole cannot be forced to exclusively interact with the third base of five as it seems to do in the experiments; its position also allows interaction with the fourth base. Moreover, the excluded volume sphere on C is penetrated by the ligand because of the high energy limit that is necessary for optimal performance of the compare/fit operation. However, the experimentators do not report exact fit values for sites beyond the designated binding sites, making the interpretation of these extra bars even more difficult.

As a validation of the method another ligand (Figure 2, polyamide **6**, structure 3 in ref 55) was investigated. Its arrangement of functional groups slightly differs from ligand 1. Especially the unused half of the ligand has more interesting rings. In footprint experiments this ligand was tested for its ability to discriminate multiple A·T from T·A base pairings: There were three predefined binding sequences where two DNA base pairs were varied within the parent-sequence context, 5′-GWWCT-3′ (W = A, T). For the whole sequence, see Figure 8, the sites designed for selectivity evaluation are highlighted. Discrimination of the varied base pairs should be accomplished

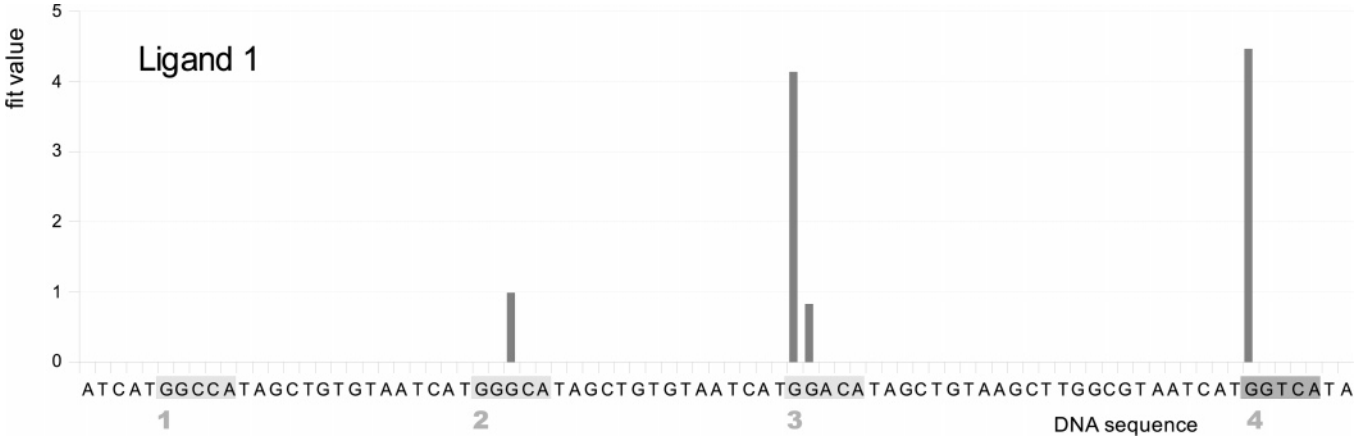

Figure 7. The diagram shows fit values for ligand 1 resulting from a DNA scan with the aid of the refined pharmacophore model. Every bar belongs to the hypothesis spanning five bases and starting with the base right under the bottom of the bar. The sequence GGTCA (dark gray) is the experimentally confirmed preferred binding site.

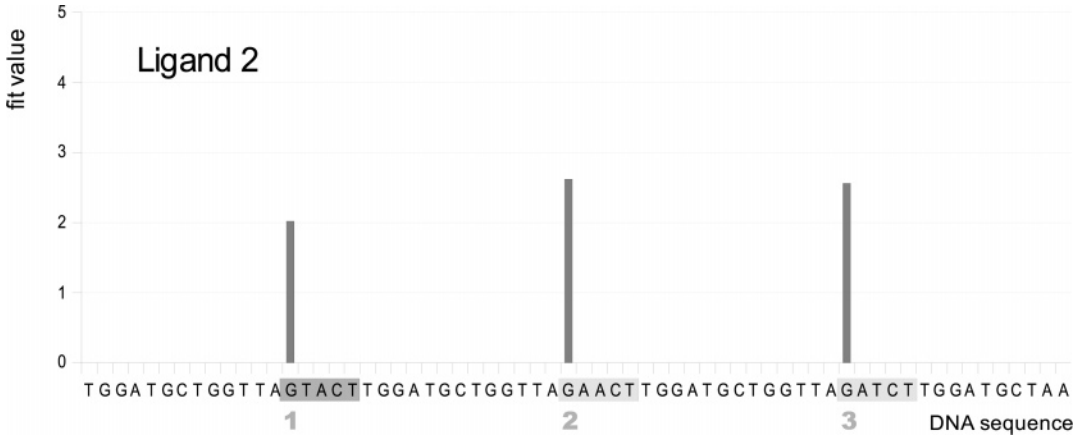

Figure 8. The diagram shows fit values for ligand 2 resulting from a DNA scan with the aid of our pharmacophore model. Every bar belongs to the hypothesis spanning five bases and starting with the base right under the bottom of the bar.

by the hydroxybenzimidazole of one-half of the ligand each. Therefore investigation with our method which only allowed usage of one-half of the ligand was especially interesting.

Ligand 2 was prepared as already described for ligand 1: Again, the success of the compare/fit operation strongly depended on the starting conformer used for the generation of multiple conformers. Therefore its rotatable bonds were also manipulated to resemble X-ray structures. DNA pharmacophore models of the new sequence were prepared as described for ligand 1. Fit values were calculated by mapping the ligand to each hypothesis and collected to give diagram 8. In general, the fit values are lower now, which is a hint that conformer adjustment to fit to the model did not work as well as for ligand 1. Raising the energy limit for the compare/fit operation beyond 50 kcal/mol does not help, because then there is also more energy to penetrate the excluded volumes. Remarkably, we again find a clear distinction between the predefined binding sites and the rest of the DNA. Only the heights of the bars do not completely correspond to the measured binding affinities. Site 1 is the designated binding site and should be preferred over the other sites about 10-fold. This experimental finding could not be reproduced by the model (for comparison of fit values with affinity data see Table 2). Most likely this weak point is due to the missing half of the ligand: Discrimination of A versus T at the first of the varied base pairs is accomplished by the regarded half of the ligand, and the second varied base pair was designed to test the influence of hydroxybenzimidazole within the other half of the ligand.

Table 2. Ligand 2: Equilibrium Association Constants (K_a) Found in DNaseI Footprinting Experiments[55] in Comparison with the Fit Values Obtained by Pharmacophore DNA Screening

site no.	K_a [M^{-1}]	fit value
1	$4.6(\pm 0.8) \times 10^8$	2.02
2	$3.2(\pm 0.4) \times 10^7$	2.62
3	$1.7(\pm 0.5) \times 10^7$	2.56

The time needed to scan the 81 base pair DNA is fairly short thanks to Catalyst's screening capabilities. Within the script which is used to scan the DNA step by step, each compare-fit operation requires about 1 s, when a restrictive model is used. Therefore the whole run is finished after slightly more than 1 min. In comparison, an analogous binding site prediction was done with docking methods:[76] Several ligands with polyamidic scaffold but slightly differing in composition of aromatic rings were moved along the DNA using simple simulation techniques. By applying a scoring function to the trajectory after energy minimization, the preferred binding site could be identified. This approach was very successful but far more time-consuming than our approach. Structure generation necessary for investigation of any 80 base-pair sequence was reported to require less than 1 h. Simulations with more than 80 base pairs did not

reach completion because of software memory limitations. In our case the resulting list of fit values is a composition of separate compare-fit operations and therefore not restricted in terms of DNA length.

3. CONCLUSION AND OUTLOOK

In this proof of principle study we could show that pharmacophore modeling methods are able to reproduce the experimentally found preferred binding sites of minor groove ligands. This is only the first but a decisive step toward model application to virtual screening. Especially the performance attracted our attention: Celerity is the crucial advantage of pharmacophore over docking methods. After some further small adjustments and refinements as the incorporation of additional information (hydrophobic and ionic properties of the ligand, the backbone as a limitation of groove width), we will start our virtual screening experiments chasing new active compounds which will occupy the DNA minor groove.

4. METHODS

The canonical 3D B-DNA structure was automatically generated within Sybyl,[92] version 7.1. The ligands were drawn within the software package Catalyst,[99] version 4.11 on a PC with a Pentium IV processor/3.0 GHz running Fedora Core 4 Linux. Catalyst generated multiple conformations for each ligand using the following settings: Best-quality conformation generation algorithm was used with default values for the maximum number of conformers (250) and the energy range above the minimum-energy conformer (20 kcal/mol). These settings prompted Catalyst to build 67 conformers for each ligand. Because of problems with displaying large DNA pieces in the graphical user interface of Catalyst, DNA based hypotheses were created with the aid of Perl scripts and Catalyst's command line tool 'hypoedit'. Mapping of the ligand to hypotheses and calculation of fit values were also done with the aid of a Catalyst command line tool ('citest') and scripts. If not mentioned otherwise in the text, default parameter settings of the programs were used. Structures (Figure 2) were designed with MarvinSketch.[100]

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