Instituto Juan March
de Estudios e Investigaciones

21 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on
DNA-Drug Interactions

Organized by

K. R. Fox and J. Portugal

J. B. Chaires
W. A. Denny
R. E. Dickerson
K. R. Fox
F. Gago
T. Garestier
I. H. Goldberg
T. R. Krugh
J. W. Lown
L. A. Marky
S. Neidle
D. J. Patel
J. Portugal
A. Rich
L. P. G. Wakelin
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The lectures summarized in this publication were presented by their authors at a workshop held on the 15th through the 17th of November, 1993, at the Instituto Juan March.

Depósito legal: M-2.007/1994
I.S.B.N.: 84-7919-521-5
Impresión: Ediciones Peninsular. Tomelloso, 37. 28026 Madrid.

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DNA-DRUG INTERACTIONS

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T.R. Krugh - Structural Characterization of Drugs and Carcinogens Bound to DNA Oligomers.

F. Gago - Structure-Affinity Relationships for DNA Bifunctional Intercalators.

K.R. Fox - Kinetic Studies on the Interaction of Sequence Selective Ligands with Individual DNA Binding Sites.

Second Session
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INTRODUCTION

K. R. Fox and J. Portugal

It is generally considered that an improved understanding of the molecular basis of drug action will lead to the design of new compounds and advances in medical practice. Several important groups of antitumour agents are known to act by binding to DNA, interfering with its structure and function. A vital part of the search for new chemotherapeutic agents has been to investigate how these compounds recognise and bind to DNA. DNA-drug interactions are particularly amenable to investigation at the molecular level since the structure of the target (DNA) is known in considerable detail.

The aim of this workshop was to discuss progress in the field of DNA-drug interactions. The topics covered included:

- Factors governing the sequence-specific interaction of minor-groove binding drugs and intercalative agents with DNA.
- DNA damage by antitumour drugs and compounds which are covalently attached to the DNA.
- Design of new sequence-specific DNA binding molecules and modification of existing compounds.

The list of internationally renowned speakers covered both the interaction of ligands with DNA and the methods used to address these problems. These ranged from high resolution techniques such as molecular modelling, X-ray crystallography and NMR to chemical studies on the action of several DNA cleaving agents, solution studies using footprinting techniques, *in vitro* measurament of binding parameters, and cell biology studying the effects of drugs on growing cells.

In discussing the interaction of each drug with DNA several questions were addressed :

- How does it bind?.
- Does it have any sequence selectivity?.
- What effect does it have on DNA structure?.
- What are the kinetics of its interaction?.
- How does it cleave or modify DNA?.

In this workshop each of these questions are considered in depth for several DNA binding drugs, both natural and synthetic. We believe that the present volume containing the summaries of the different contributions will aid both the design of novel compounds as well as indicate new DNA target sites.

On behalf of all the participants we would like to thank the *Instituto Juan March* for generously supporting this workshop, and Andrés González in particular for looking after all the practical details.

Keith R. Fox (Southampton, UK) & José Portugal (Barcelona, Spain)

First Session

THE DEVELOPMENT OF BISINTERCALATING DNA THREADING AGENTS

LAURENCE P.G. WAKELIN AND WILLIAM A. DENNY

St. Lukes Institute of Cancer Research, Highfield Road, Rathgar, Dublin, Ireland and The Cancer Research Laboratory, Auckland University Medical School, Auckland, New Zealand.

DNA intercalating agents that bear two bulky sidechains on opposite sides of their chromophores have the capacity to bind in a manner where one sidechain comes to lie in each of the DNA grooves. Threading compounds of this type have two particularly interesting properties. Firstly, if the sidechains are chemically different and if their interactions with DNA are groove-specific then it may be possible to use such compounds as platforms for the delivery of reactive functional groups to DNA in a regio-selective manner. Secondly, the act of threading a bulky sidechain through the DNA helix necessarily slows both association and dissociation rates which has important consequences for the relationship between the DNA residence time of the ligand and its affinity for DNA. Specifically, threading agents offer the possibility of developing compounds that have moderate binding constants but very slow dissociation rates. These characteristics are precisely those required of anticancer agents whose mechanism of action relies on their ability to inhibit transcription by impeding the passage of RNA polymerase.

The naturally-occuring antitumour antibiotic nogalamycin is a DNA threading compound which we used as a paradigm for the design of simple intercalating threading agents. The essential structural determinants of nogalamycin include a bulky neutral hydrophobic sugar ring (a 'greasy ball') and a positively charged rigidly-held bicyclic sugar (a 'hook') disposed on opposite faces of an intercalating anthracycline chromophore. The 'geasy ball' lies in the DNA minor groove and the 'hook' makes specific hydrogen bonding interactions with GC basepairs in the major groove. Nogalamycin has an affinity for DNA of about 10^5-10^6 M^{-1}, has DNA residence times measured in many thousands of seconds and is a very potent inhibitor of RNA polymerase. We will describe our work on the development of nogalamycin-mimetics based upon 9-anilinoacridines substituted in the 4 position with a carboxamide sidechain bearing a positively charged hook. These compounds, which we term amsacrine-4-carboxamides, have affinites in the range of 10^5-10^7 M^{-1}, are very GC-selective and some have residence times of about 6 seconds. The latter should be compared with the 2 msec residence time of amsacrine, the parent compound that lacks the threading 'hook'. Whilst this is an impressive slowing of the dissociation rate it still falls far short of the performance of nogalamycin.

Our next step to increase the residence time of threading agents yet further was to try to harness the phenomenon of bifunctional intercalation to the cause. We reasoned that a threading compound comprised of two independently binding chromophores would have more difficulty dissociating than a simple monofunctional agent. Thus, we synthesised a series of compounds in which two 9-aminoacridine chromophores were joined together through their 9-amino positions with linkers of suitable lengths to permit the formation of one- and two-basepair sandwich bisintercalation complexes. All the compounds were substituted in the 4 position of each acridine ring with the positively charged carboxamide hook. We found that their affinities and residence times were strongly dependent upon the nature of the linker. A two-basepair bisintercalation threading complex dissociated with a residence time of about 70 sec compared to a value of 170 msec for the non-threading homologue lacking the hook. We are now synthesising rigidly-linked threading diacridines and varying the nature of the hook as well as its substitution pattern.

Structural Characterization of Drugs and Carcinogens Bound to DNA Oligomers.

Thomas R. Krugh

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One and two-dimensional NMR spectroscopy, energy minimization and molecular dynamics simulations have been used to characterize the structure and dynamics of anthramycin, benzo(a)pyrene, acetylaminofluorene, and aminofluorene bound to oligodeoxynucleotides in solution.

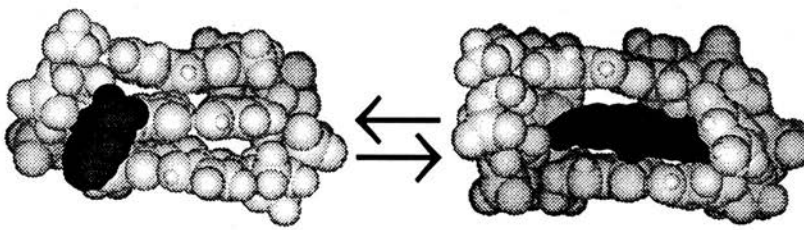
Anthramycin forms a covalent aminal linkage to the 2-amino group of guanine, forming a stable adduct which covers a 4 base pair region. There is little distortion of the duplex as anthramycin fits snugly into the minor groove, as shown from NOE interactions between anthramycin and DNA protons.

A covalent adduct of (+)-trans-*anti*-benzo[*a*]pyrene (BP) attached to a guanine amino group in the BP-modified oligomer d($C^1C^2T^3A^4T^5G^6T^7$[(+)-trans-*anti*-BP-G^8]$C^9A^{10}C^{11}$)• d($G^{12}T^{13}G^{14}C^{15}A^{16}C^{17}A^{18}T^{19}A^{20}G^{21}G^{22}$) duplex was synthesized. This BP-11-mer duplex exhibits NOESY crosspeaks between BP protons and the G^8, C^9, A^{16} and C^{17} nucleotide protons that clearly delineate the location and orientation of the BP moiety in the minor groove with its pyrene ring oriented in the 5' direction of the modified strand and situated near the sugar phosphate backbone of the unmodified strand. Chemical exchange crosspeaks indicate the presence of a second conformation of the BP-11-mer.

N-acetyl-2-aminofluorene adducts bound at the C_8 position of guanine have been synthesized and studied by two-dimensional NMR, energy minimization, and molecular dynamics. In the d($C^1C^2A^3C^4$[AAF-G^5]$C^6A^7C^8C^9$)• d($G^{10}G^{11}T^{12}G^{13}C^{14}G^{15}T^{16}G^{17}G^{18}$) modified duplex, referred to as the AAF-9-mer, eight of the nine complementary nucleotides form Watson-Crick base pairs. The AAF-G^5 and C^{14} bases show no evidence of hydrogen bond formation, consistent with the observation that the AAF-G^5 base adopts a *syn* conformation. Key distance restraints obtained from NOESY data recorded at 32 °C using a 100 ms mixing time were used in conformational searches by molecular mechanics energy minimization studies using the program DUPLEX. The final, unrestrained, minimum-energy conformation was then used as input for an unrestrained molecular dynamics simulation using AMBER 3.1 to further characterize the structure at the site of adduct formation. Chemical exchange crosspeaks are observed and thus the AAF-9-mer exists in more than a single conformation on the NMR time scale, although one conformation is predominant (≥70%). The structure of the predominant conformation of the AAF-9-mer shows stacking of the fluorene moiety on an adjacent base pair, while the distal ring of the fluorene moiety protrudes into the minor groove. The disprution of the duplex structure provides a clear rationalization for inhibitation of transcription, and for deletion mutations, should bypass occur.

A 2-aminofluorene (AF) modified deoxyoligonucleotide duplex, d($C^1A^2C^3C^4A^5$[AF-G^6]$G^7A^8A^9C^{10}$) • d($G^{11}T^{12}T^{13}C^{14}C^{15}T^{16}G^{17}G^{18}T^{19}$-$G^{20}$) is being studied as a model for the binding of the carcinogen, 2-aminofluorene, to the

c-H-*ras*1 proto-oncogene DNA sequence. Chemical exchange crosspeaks for DNA protons near the modification site and for fluorene protons in NOESY, ROESY and TOCSY spectra at 10.0 °C and 20.0 °C reveal the presence of two major conformations of the AF-modified duplex. The base protons, H1' sugar protons, and carcinogen protons for each conformation have been assigned by analysis of a 200 ms NOESY spectrum at 2.0 °C, pH 7.0 in D_2O. In one conformation of the AF-10-mer, AF^{ext}, the AF moiety is located in the major groove with the DNA maintaining a conformation similar to B-DNA; in the second conformation, AF^{int}, the fluorene is stacked within the duplex. Imino proton spectra reveal Watson-Crick base pairs for all ten complementary nucleotides in the AF^{ext} conformation and nine out of ten for the AF^{int} conformation. Distance restraints obtained from a 100 ms NOESY experiment at 2.0 °C were used in conformational searches by energy minimization. The two structures are shown below.



External-AF conformation
with AF-G•C intact

Inserted-AF conformation
with AF-G and C displaced

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Acknowledgments and Collaborators: This AAF-9-mer research was performed in collaboration with Suse Broyde and Rong Xu at NYU, Brian Hingerty at Oak Ridge, along with Suzanne F. O'Handley, Charles Bailey, David Sanford, and Cathy Lester at Rochester. The BP work was done by Matthew Fountain, and the AF work was performed by Linda Eckel. Anthramycin experiments were done in collaboration with David Graves (University of Mississippi) and Michael Stone (Vanderbilt). The National Institutes of Health and the Department of Energy (SB and BH) provided funding for these projects.

Structure-affinity relationships for DNA bifunctional intercalators.

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In the binding of bisintercalators to a DNA double helix a minimum number of events need be considered: i) diffusion of the ligand leading to a productive encounter with the polyanion; ii) unstacking of DNA bases coupled with, or followed by, stacking interactions with the drug's chromophores in a process involving considerable desolvation of both the drug molecule and the DNA groove to which it binds; iii) additional interactions with minor/major groove components resulting in further stabilization of the complex. Thus it can be seen that both enthalpic and entropic terms must contribute to the favourable binding free energy.

Traditional work in structure-activity relationships (SAR) for this group of compounds has dealt with the effects that introduction of new substituents or removal of existing ones have on the binding properties of a given drug. Among the best known examples are those provided by [*N*-MeCys^3,*N*-MeCys^7] TANDEM and TANDEM, triostin A analogues lacking either half or all of the N-methyl groups of cysteines and valines, respectively, whose binding specificity is dramatically reversed with respect to the parent compound[1]. Triostin A, in common with echinomycin, binds preferentially to CpG steps whereas the demethylated derivatives bind better to TpA[1,2]. Moreover, TANDEM shows a pronounced affinity for poly[d(AT)]•poly[d(AT)] to which it binds in a highly cooperative fashion[3].

Not until very recently, however, have molecular biology techniques allowed the modification of the other interacting species, *i.e.* DNA itself, in which turns out to be another very important aspect of SAR studies. Thus, it has been shown that removal of the exocyclic amino group of guanine (by replacement of this base with hypoxanthine) leads to the loss of echinomycin's specificity for CpG steps[4]. On the other hand, introduction of an extra amino group in the minor groove of AT regions (by replacing adenine with 2,6-diaminopurine) confers this same ligand enhanced binding affinity for any YpR combination other than the usual CpG step[5]. Furthermore, the affinity of echinomycin for these new binding sites in the modified DNA is at least one order of magnitude greater than that for normal DNA.

All of these findings reveal a complex interaction behaviour that is still far from being completely understood. Although x-ray and NMR techniques have provided a wealth of structural data on some of these complexes, computational chemistry tools can also be used in an attempt to deepen our understanding of the relative importance of the considerable number of forces involved in the binding process. In the light of our previous observation[6] that the conformational preference of A:T base pairs flanking CpG echinomycin binding sites could be strongly influenced by the electrostatic component of the stacking interactions between the heteroaromatic ring systems, we have investigated to what extent this hypothesis can be extended in order to account for the observed changes in binding specificity and affinity. This same study highlighted the unfavourable electrostatic interaction between the quinoxaline ring systems of echinomycin and the GC base pairs sandwiched between them. From this it was inferred that any favourable change in orientation or magnitude of the dipole moments of either the drug chromophores or the DNA base pairs could lead to

increased binding affinity. In order to explore the possible role of stacking interactions in determining the binding preferences of echinomycin, the charge distribution and the dipole moments for these non-standard base pairs were calculated. Molecular models were then generated for the complexes of echinomycin, TANDEM and [*N*-MeCys^3,*N*-MeCys^7] TANDEM with several DNA sequences, and the different contributions to their relative stability were evaluated.

We demonstrate that theoretical analyses of this kind can be of considerable value to gain further insight into the fine tuning of these drug-DNA interactions. In terms of DNA reading motifs it would seem that the classical definition of *optimal binding site* as a step comprising two adjacent base pairs falls somewhat short, and a longer sequence of at least *four base pairs* would be more appropriate for this class of compounds.

The present shortcomings and future developments of this type of techniques will also be discussed.

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Kinetic studies on the interaction of sequence selective ligands with individual DNA binding sites

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Various biophysical studies have shown that the dissociation of several sequence selective ligands from DNA is a slow process, with half lives of several minutes or more[1,2]. Most of these studies have used mixed sequence DNAs and therefore only provide average kinetic data. We have developed a variation of the footprinting technique for visualising the dissociation of sequence-selective ligands from individual binding sites within mixed sequence DNA[3]. In this technique a complex between the ligand and a radiolabelled DNA fragment is dissociated by addition of a vast excess of unlabelled DNA. The mixture is then footprinted at various times after addition of the cold DNA. Results for a number of antitumour antibiotics will be described, including actinomycin, echinomycin, TANDEM and mithramycin, measuring their dissociation from both natural and synthetic DNA fragments. We have also used this technique for measuring the association and dissociation rates for several intermolecular triplex helices.

Actinomycin (selective for the dinucleotide GpC), dissociates from mixed sequence DNAs with an average half-life of about 20 minutes at 20°C. The dissociation from each GC site is not identical and reflects the nature of the surrounding sequences. Experiments with synthetic DNA fragments reveal that actinomycin dissociates from GC sites surrounded by regions of $(AT)_n$ more slowly than those flanked by $A_n.T_n$. There is no difference between AGCT and TGCA. Examination of kinetic parameters of the regions of enhanced DNase I cleavage around each footprinting site confirm that these arise from ligand-induced alterations in local DNA structure, though these often decay more slowly than the footprint itself.

Echinomycin (a bifunctional intercalator selective for CpG), also shows a spectrum of dissociation rates from DNA. While dissociation from several sites on *tyr*T DNA is slow (>30 minutes) one site (ACGC) is much faster and is complete within 2 minutes. The related synthetic ligand TANDEM, which is selective for the dinucleotide TpA, especially when flanked by AT-rich regions dissociates much faster and is complete by 2 minutes. Dissociation from isolated TATA sites is faster than ATAT. In contrast, dissociation of TANDEM from blocks of $(AT)_n$ is much slower, and requires more than 30 minutes. The, and suggests a cooperative interaction of this ligand with $(AT)_n$ tracts.

In contrast the AT-selective minor groove binding ligand distamycin dissociates rapidly from DNA. At 4°C the footprints disappear before the first time point after adding the competitor DNA (<20s).

Similar studies with intermolecular triple helices reveal that triplex formation can take longer than 30 minutes. The rate of formation depends on the exact nature of the target sequence.

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Second Session

DNA MINOR GROOVE BINDING OF NONINTERCALATING AGENTS AND THEIR EFFECTS ON THE CATALYTIC ACTIVITY AND DNA BINDING OF GYRASE

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Many drugs and cytotoxic agents bind to DNA by nonintercalating means in the minor groove of double helical B-DNA and may act as inhibitors of enzymes in the DNA metabolism, e.g. they can specifically inhibit DNA and RNA polymerases (reviewed in 1). Most of these ligands show a high preference for AT base pair sequences. We have investigated the influence of minor groove binders on the catalytic activity of DNA gyrase, an essential enzyme of the DNA metabolism in prokaryotes. Using gyrase from *Streptomyces noursei* a different inhibitory effect on the enzyme activity of various ligands has been found depending on the chemical structure and DNA binding behaviour of the ligands. The molecular mechanism of the interference of three drugs with the gyrase binding on DNA was studied by applying a gel retardation assay using a 162 bp fragment from pBR322 containing a strong gyrase site. The results showed that binding of the enzyme to this DNA fragment is effectively inhibited by the GC-specific agent chromomycin A_3 but is poorly or not influenced by the AT-specific drugs distamycin A and netropsin. The specific inhibitory effect of chromomycin A_3 on DNA binding and cleavage reaction of gyrase may be related to a competitive and specific interaction of the ligand with GC-sequences located at and near the cleavage site. In contrast, the very

weak inhibitory potency of distamycin A and the ineffectiveness of netropsin are explained by the relatively low numbers of AT-affinity sites for these ligands present in the 162bp fragment. We further suggest that in presence of Mg^{2+} chromomycin A_3 binds as a dimer to the minor groove of the GGCC center and to other GC regions of the gyrase recognition site which strongly prevents the enzyme from DNA binding.

The molecular mechanism of the high inhibitory potency of chromomycin A_3 is discussed in terms of the structural model of the interaction of the gyrase tetramer with the DNA segment containing the cleavage site.

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Sequence-specific binding of Elsamicin to DNA

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Elsamicin A is a new fermentation product which appears to exhibit strong inhibitory activity against various tumors, and is presently undergoing Phase I clinical trials. Elsamicin A contains a novel aglycone chromophore, chartarin, in common with the antibiotic chartreusin, but differs in the presence of an amino-sugar sugar in its disaccharide moiety. Direct interaction with DNA is believed to be central to the mechanism by which elsamicin acts.

In order to analyze the sequence-selective binding of elsamicin A to DNA, we undertook a DNAse I footprinting study using various DNA restriction fragments [1]. The elsamicin binding sites can be mapped in regions which are rich in guanine+cytosine. We have also performed a complementary approach based on the use of different restriction enzymes containing, or not, the CpG step within or near their cleaving sequence.The sequence-specificity of the elsamicin A binding to DNA was investigated considering the inhibition of the rate of digestion of linearized pBR322 DNA by AatII, ClaI, EcoRI, HindIII and NruI restriction enzymes[2]. Elsamicin A inhibits the rate of digestion by NruI to a greater extent than it does for the other enzymes, thus evidencing the sequence-selective binding of elsamicin to GCG regions in DNA. Our results also show the important role of the neighboring sequences in the elsamicin A-DNA interactions and their effects on the cleavage by restriction enzymes.

Alternating GC-tracts have been shown to be related with the 5'-regions of various housekeeping genes, and may represent regulatory regions. Since these alternating CG sequences are among those required to observe the Z-form DNA, we consider whether the elsamicin binding to CGC sequences could be part of the mechanism used to inhibit transcription. The interaction of poly[d(G-C)] and poly[d(G-m^5C)] with elsamicin A has been studied under the B and Z conformations[3]. Both the rate and the extent of the B-to-Z transition are diminished by the antibiotic, as inferred by spectroscopic methods under ionic conditions that otherwise favor the leftt-handed conformation of the polynucleotides. Moreover, elsamicin converts the Z-form DNA back to the B-form. Circular dichroism data indicate that elsamicin binds to poly[d(G-C)] and poly[d(G-m^5C)] to form right handed-bound elsamicin region. The elsamicin A effect might be explained by a model in which the antibiotic binds preferentially to a B-form DNA, playing a role as an allosteric effector on the equilibrium between the B and Z conformations, thus favoring the right-handed one.

Furthermore, the ability of elsamicin A to cleave DNA in the presence of ferrous iron and reducing agents has been analyzed using experimental and theoretical approaches. Experimentally, the antibiotic causes DNA breakage in the presence of ferrous ions and a reducing agent. The DNA-

cleaving activity appears to be partially blocked by the action of superoxide dismutase and catalase. These results indicate that the elsamicin A aglycone moiety, chartarin, can be involved in the production of free radicals. We performed a broad theoretical study based in the quantum mechanical framework, which permits to determine the redox properties of elsamicin that lead to the generation of radical species. We suggest that the oxidation-reduction mechanism of the aglycone moiety of elsamicin (a lactone) leading to DNA breakage is different from the mechanism followed by other well characterized anti-cancer drugs, whose chromophore is a quinone [4].

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Structural Aspects of DNA Minor-Groove Recognition.

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The minor-groove of AT-rich B-form DNA is the site of binding of a range of synthetic ligands and natural products, some of which have established therapeutic utility. The minor-groove is also the locus of recognition for a number of proteins and protein domains. Structural studies by both NMR and X-ray crystallography have established the major features of recognition for a number of low molecular weight drugs and ligands and in particular crystallography has revealed the role played by changes in DNA sequence in the recognition process. A picture of the relative importance of the various factors contributing to binding stability and sequence preference, is now emerging, with information on the effects of factors such as groove width, hydrogen bonding and sequence changes.

Third Session

HOW DO DNA BINDING DRUGS WORK?

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Many drugs that bind to DNA are known to inhibit transcription and/or replication *in vivo*. The three-dimensional structure of a number of DNA-drug complexes have been solved. That allows us to think about the detailed manner in which the drug interacts with DNA. Sometimes these structures provide clues as to the manner in which the drug acts when polymerases are present. However, a number of interesting puzzles remain.

The structure of a number of DNA drug complexes will be surveyed in order to assist in drawing inferences about biological activity. This will include a number of intercalating drugs, such as Adriamycin and Daunomycin which are used in human chemotherapy. Other drugs that are not used clinically, but are very active biologically include Nogalomycin as well as Echinomycin.

The structure of a ditercalinium-DNA complex will be described, as well as the unique way in which this drug kills its host cell.

MAJOR GROOVE RECOGNITION OF DNA BY TRIPLE HELIX-FORMING OLIGONUCLEOTIDES AND OLIGONUCLEOTIDE INTERCALATOR CONJUGATES

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With the aim of controlling gene expression at the transcriptional level oligonucleotides or derivatives of oligonucleotides have been targeted to double-stranded deoxyribonucleic acid sequences. Canonical triple helices are formed when oligonucleotides containing thymines and cytosines bind to oligopurine.oligopyrimidine stretches of double-helical DNA. Cytosines provided that they are protonated on the N-3 position bind guanines whereas thymines recognize adenines. This recognition is specific and has a slow kinetics compared to that of a single stranded DNA recognition. Thus, the double-stranded target for triple-helix formation is restricted to a purine-pyrimidine sequence and an acidic pH is required for triple-helix formation.

In order to increase the stability of these short triple helices, especially at physiological pH, we have synthesized intercalator-oligonucleotide conjugates. Intercalation takes place at the triplex-duplex junction with "classical" intercalators such as acridine or psoralen derivatives. We have recently discovered intercalators (benzopyridoindoles) that bind more strongly to triple-helical than to double-helical nucleic acids. Oligoribonucleotides containing C and U as a third strand exhibit a stronger affinity for DNA duplexes than deoxynucleotide-containing analogs. 2'-O-methyl substitution in the riboses further enhances triple-helix stability. Triple helix formation can be also used in the antisense strategy. An oligopurine target sequence can be recognized by two oligopyrimidine sequences which form Watson-Crick and Hoogsteen hydrogen bonds. These two oligopyrimidines can be tethered to each other and form "oligonucleotide clamps" which can arrest nucleic acid-processing enzymes.

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SEQUENCE-RECOGNITION ELEMENTS AND CONFORMATIONAL CHANGES ASSOCIATED WITH BINDING OF SMALL MOLECULES TO DNA

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Most small molecules bind to DNA via the minor groove of the helix, and it has long been suspected that the exocyclic 2-amino group of guanine plays a key role in identifying their preferred binding sites. The purine 2-amino substituent not only obstructs access to the floor of the groove; it also disrupts the pattern of hydration, alters the electrostatic potential, and provides the only hydrogen bond donor available to interact with a ligand. We have sought to verify the notion that the 2-amino group of guanine is the critical element of recognition by removing it and/or shifting it. Removal amounts to converting guanosine nucleotides to inosines. Shifting the 2-amino group can be accomplished by relocating it on the A • T base pairs which then become 2,6-diaminopurine-thymine (DAP • T) pairs. Samples of *tyr* T DNA containing these modifications have been prepared using the polymerase chain reaction (PCR), and their interactions with selected peptide antibiotics have been measured. Actinomycin, which binds preferentially to GpC steps, and echinomycin, which bis-intercalates around CpG steps, were chosen as representative intercalators; netropsin was studied as a minor groove-binder selective for AT-rich clusters.

Inosine-containing *tyr* T DNA, which lacks the 2-amino group altogether, failed to bind echinomycin (judged by footprinting analysis) at any concentration up to 80 μM [1]. In order to investigate whether DNA containing inosine in only *one* strand would interact with the antibiotic, appropriately substituted samples of a synthetic 51-mer were prepared by PCR. Again, the binding of echinomycin to its cognate CpG sites was drastically impaired or even abolished when either one or both strands of the duplex lacked the guanosine nucleotides. By contrast, *tyr* T DNA entirely substituted with DAP residues in place of adenines provided an excellent substrate for binding actinomycin as well as echinomycin [2]. Examination of DNAse I footprinting patterns revealed that the effect of shifting the purine 2-amino group on to the A • T base pairs was to provoke a massive redistribution of binding sites for both antibiotics, such that the newly created sites containing DAP • T pairs were substantially preferred over the cognate GpC or CpG -containing sites (which still remained in the DNA samples examined). The new binding sites for actinomycin seemed to preserve the 5'Pu-Py3' arrangement of the canonical sites but with DAP-Py steps apparently preferred over GpC. Correspondingly, the new echinomycin binding sites preserved the 5'Py-Pu3' arrangement of the canonical sites but with Py-DAP steps strongly preferred over CpG. Indeed, the concentration-dependence of footprinting at particular sites suggested that for echinomycin there must be at least an order of magnitude increase in the affinity of the antibiotic for its new sites. The C_{50} for half-maximal protection of CpG sites by echinomycin in normal DNA is typically about 1-2 μM whereas the C_{50} for protection of TpDAP sites in the substituted DNA is more like 0.1 μM.

Substitution with inosine and/or DAP also had a marked effect on the sites of binding of netropsin. With inosine-substituted DNA netropsin could be seen to form footprints over practically the whole length of the DNA fragment, whereas with DAP-substituted DNA it produced no footprints at all. With DNA containing both inosine and DAP, i.e. DNA in which the purine 2-amino groups had been totally transferred from guanines to adenines, footprints were formed entirely at GC (now inosine • C) clusters and not at the canonical AT (now DAP • T) clusters. Taken as a whole, the results with inosine and DAP-substituted DNAs leave little room for doubt that the 2-amino group of guanine is the critical determinant for recognition of specific binding sites in DNA.

The nucleotide-substitution approach has also been employed to investigate whether Hoogsteen base pairs are formed around sites where echinomycin binds to DNA [3]. Because Hoogsteen pairs demand the formation of a hydrogen bond from N(3)-H of the pyrimidine base to N(7) of the purine, their formation can be prevented if 7-deaza-purine analogues are incorporated into the DNA in place of the normal nucleotides [4]. Again, *tyr* T DNA was prepared by PCR containing 7-deazadeoxyadenosine (c^7A) or 7-deazadeoxyguanosine (c^7G) residues in place of their natural counterparts. Quantitative footprinting with DNAase I was undertaken to determine accurate C_{50} values for half-maximal suppression of enzymic cleavage at all nucleotides affected by echinomycin binding [5]. No consistent differences between the values for natural, c7A-substituted and c7G-substituted DNAs were detected. These experiments also argue against any *anti-syn* conformational transition occurring in purine nucleotides associated with bis-intercalation of echinomycin, because any such transition should incur an energy penalty in c^7A or c^7G-substituted DNA yet none was observed.

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Sequence Selective DNA Minor Groove Binders as Potential Anticancer Agents

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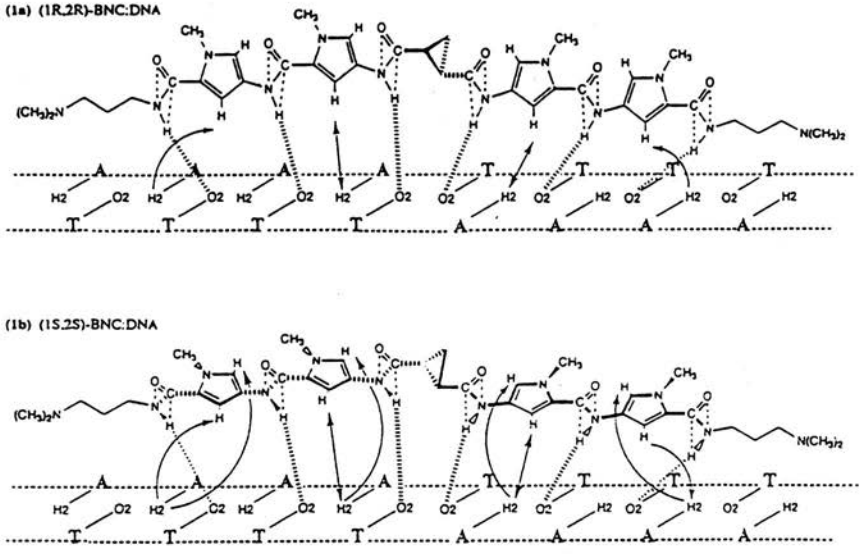
One of the most challenging problems in the use of drugs in the treatment of human disease is of specificity. Many antineoplastic and antiviral drugs interfere with the replication and transcription of DNA by binding to double-stranded DNA and here again a lack of specificity is in evidence. Consequently the action of such agents is non-selective and gives rise to side effects of varying severity. Therefore the question arises whether one should tailor the binding preference of DNA-binding agents to particular base sequences and thereby produce new drugs that might be more effective clinically and also serve as useful tools for the study of DNA binding in molecular biology. The principle of the use of oligonucleotides, or modifications thereof, while elegant is, in practice, plagued with difficulties of cellular uptake, intracellular instability, subcellular distribution, and diastereomer problems associated with backbone modification.

Recent advances in our understanding of molecular recognition between polypeptides and nucleic acids, together with the advent of new DNA sequencing and footprinting technology, permit the design of sequence selective agents. We have developed oligopeptides related to natural prototypes netropsin and distamycin but which are rationally designed to recognize specific DNA sequences. Such oligopeptide-based agents offer significant advantages over oligonucleotide probes including, ready cellular uptake, concentration in the cell nucleus and good intracellular stability.

The new "lexitropsins", or information-reading oligopeptides will serve as vectors for the delivery of DNA-interactive groups to sensitive sites. These groups include cleaving agents to produce miniature restriction enzymes and anticancer agents, as well as alkylators which also have provided good anticancer leads. At the same time the antiviral potential of the lexitropsins have been developed. We are aiming to develop lexitropsins for any predetermined 15 bp sequence (representing a unique target for the human genome) individual lexitropsins. A central problem in targeting longer sequences of DNA is related to the lack of phasing or spatial correspondence between repeat units in the ligand and the chiral DNA receptor. Chirality of minor groove binding ligands is anticipated to influence binding. This led us to explore a system of constrained dimeric arrangement of two netropsin units using optically pure C_{2v}-symmetric linkers:

(-)-(1R,2R)-bis(netropsin)-1,2-cyclopropanedicarboxamide (BNC) and
(+)-(1S,2S)-bis(netropsin)-1,2-cyclopropanedicarboxamide

Netropsin exhibits an entropy-driven preferential sequence specificity for minor groove binding to poly(dA)•poly(dT) *versus* poly(dA-dT); hence studies were performed on a duplex DNA $d(CGAAAATTTTCG)_2$ containing a 5'-AAAA segment. The following conclusions were drawn: Isohelical binding occurs for (1R,2R)-BNC with each netropsin subunit oriented against a 5'-TTTT site. Cyclopropane linker and N-methylpyrrole rings follow the right-handed twist of the



base pairs along minor groove of 5'-AAAATTTT. Complexes stabilized by single hydrogen bonds with thymidine-O_2 instead of three-center bifurcated bonds proposed for netropsin binding. Cyclopropane group is oriented away from DNA in the complexes of both enantiomers – this forces each netropsin to be aligned differently. Binding of (1R,2R)-BNC gives strand selectivity for 5'-TTTT. Less favorable binding of (1S,2S)-BNC results in alignment of the netropsin moieties against 5'-AAAA. Binding of (1S,2S)-BNC induces additional twisting between amide and N-methylpyrrole groups to accommodate hydrogen bonding to the walls of the groove.

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Fourth Session

SEQUENCE SELECTIVE BINDING OF ACTINOMCYIN D TO DUPLEX AND SINGLE-STRAND DNA

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The thermodynamic properties associated with the interaction of actinomycin D with DNA duplexes containing both classical -XGCY- and non classical -T(G)$_n$T- binding sites are examined. The influences of flanking base sequences on the binding parameters for the -XGCY-sequences are compared. These flanking base studies revealed that of the two classical actinomycin D intercalation sites examined (both containing -GC-steps), the -TGCA- sequence binds actinomycin D with a higher affinity than the -CGCA-sequence, yet binding to both of these sequences is quite high ($K_{int} = 10^6$ M^{-1}). The non classical -TGGGT-containing sequence was found to have comparable binding properties with the classical -XGCY- intercalation sites; however, the -TGGT- sequence exhibited a considerable drop in binding affinity, as well as marked changes in binding energetics. The binding of actinomycin D is demonstrated to be highly influenced by the sequence both at the intercalation site and by neighboring bases which flank the intercalation site.

In addition, actinomycin D was found to interact with single-strand DNA and this interaction was demonstrated to be highly sequence specific, with binding affinities ranging from 10^4 to 10^7 M^{-1} (strand). Our studies reveal single base changes within the non self-complementary 11-mers can result in drastic changes in binding affinities for these actinomycin D-single-strand DNA interactions.

Neocarzinostatin Chromophore as a Probe of Unusual DNA Structures

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The nonprotein chromophore of the enediyne antitumor antibiotic neocarzinostatin (NCS-Chrom) generates sequence-specific bistranded lesions in duplex DNA in the presence of thiol activating agents. NCS-Chrom intercalates between the DNA base pairs by way of its hydroxy-naphthoate moiety, so as to place the reactive enediyne portion in the DNA minor groove. Nucleophilic attack of the thiol at the C12 position of the chromophore initiates the process of cycloaromatization to form an indacene diradical intermediate as the DNA damaging species. The radical centers at C2 and C6 of the drug abstract hydrogens from the C5', C4' and/or C1' sites on the deoxyribose of the complementary DNA strands. At AG<u>C</u>•GC<u>T</u> sequences 1'-chemistry at the <u>C</u> residue generates an abasic site with a deoxyribonolactone moiety on one strand, and 5'-chemistry at the <u>T</u> residue generates a strand-break with a thymidine 5'-aldehyde on the opposite strand, the bistranded lesion being staggered two nucleotides in the 3'-direction. At AG<u>T</u>•AC<u>T</u> bistranded lesions are formed due to 4'-chemistry at the <u>T</u> residue of AG<u>T</u> and 5'-chemistry at the <u>T</u> residue of AC<u>T</u>. 4'-chemistry produces both strand breaks (3'-phosphoglycolate ends) and abasic sites (4'-hydroxylation product). Bistranded lesions involving abasic sites are believed to be mutagenic, whereas those consisting of double-stranded breaks may be lethal. Substantial deuterium isotope effects have been found, especially for the reactions involving 1'- or 4'-chemistry. Further, by following deuterium transfer from attack sites on the deoxyribose of DNA to the radical sites on NCS-Chrom, it has been shown that C2 on the drug abstracts hydrogen from C1' of <u>C</u> of AG<u>C</u>, and C6 abstracts hydrogen from C5' of <u>T</u> of GC<u>T</u> on the complementary strand, in support of a proposed model for drug-DNA interaction.

The precise chemistry of attack at AG<u>C</u>•GC<u>T</u>, however, is sensitive to the microstructure of the DNA at this site. For example, the placement of a stable G•T base-pair mismatch immediately 5' to the attack site alters the chemistry so that the 1'-chemistry at the <u>C</u> residue is essentially replaced by 4'-chemistry. This result can be understood in terms of the known crystal structure of the wobble G•T mismatch, where the 2-amino group of the G residue projects deeper into the minor groove so as to hinder access of the radical on the drug to the deep lying C1' position. On the other hand, placement of a relatively stable G•A mismatch on the 3'-side of the <u>T</u> of AG<u>T</u> is associated with the appearance of significant 1'-chemistry. These results emphasize the role of minor groove microstructure in determining the chemical mechanisms of DNA damage and underscore the potential usefulness of NCS-Chrom as a probe of DNA microheterogeneity.

NCS-Chrom activated by thiol also causes single-base bulge-specific cleavage, along with other intercalating/cleaving agents, such as bleo-mycin, methidiumpropyl-EDTA and ortho-phenanthroline. Selective cleavage occurs on the strand opposite the one containing the bulge, just 3' to the bulge, independent of the sequence. There is no cleavage at the bulge. These data are consistent with the known preference of inter-calating drugs for binding near or at a bulge; our data suggests that binding is at the bulge itself.

Recently we have found that NCS-Chrom, unexpectedly, site-specifically cleaves single-stranded or duplex DNA containing a two-base bulge only in the absence of thiol. DNAs, duplex or single-stranded, not containing the bulge structure are not attacked by the drug. Cleavage is at the nucleotide at the 3'-side of the bulge and is due to 5'-chemistry. Most interestingly, a new NCS-Chrom product is generated in this reaction due to the activation of the drug by an intramolecular process, under the influence of the DNA bulge conformation. It appears that the DNA, bent at the site of the bulge, participates in its own destruction. This reaction involves a novel mechanism of NCS-Chrom activation with the formation of an unusual drug reaction product. The mechanism of the reaction and the chemical structure of the drug product will be described. These studies extend the utility of NCS-Chrom as a probe of unusual DNA structures.

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Round Table Discussion on Design of Sequence-Selective DNA Binding Ligands

Moderator: J. William Lown

In order to define its scope and to guide the discussion the moderator proposed the following topics.

Antisense

1. Lack of consistency of results in different cell systems and in different laboratories.
2. Control sequences – the disturbing phenomenon of "antisense action" by random oligos often equals or exceeds that of the supposed specific probe.
3. Poor cellular uptake remains perhaps the most serious obstacle to further development.
4. The existence of 2^n diastereomers in an n-mer, backbone-modified to overcome intracellular nuclease degradation, remains a serious problem.
5. The inability of many backbone modified oligos to stimulate RNAse H in DNA:RNA hybrids requires attention.

Antigene

1. Poor cellular uptake and intranuclear access is a serious limitation for potential triplex-forming oligos.
2. Limited sequence recognition *via* triplex formation which limits potential targets.
3. Peptide nucleic acid (PNA) has promise but poor solubility and intracellular uptake limits its application.

Groove Binders

1. The newer lexitropsins exhibit good cell penetration and concentrate in the nucleus.
2. At present they show limited sequence recognition.
3. Phasing is important n the design of ligands aimed at targeting 14-16 base pairs.
4. Isohelicity and strand selectivity must be taken into account in designing such longer ligands.

Biology

1. What sequences should be targeted? *e.g.* protooncogenes, the MDR gene?
2. Solid tumors remain the most refractory targets. Are antisense or antigene strategies useful here?
3. Will antigene agents pose a risk as mutagens or carcinogens?
4. Can these newer agents offer advantages over existing chemotherapeutic drugs with respect to the resistance problem.

A lively discussion ensued that served the purpose of stimulating further debate on these issues during the workshop. The suggestion was made that such a round table discussion form a regular part of subsequent conferences in this field.

Fifth Session

Crystal structure of Anthramycin/C-C-A-A-C-G-T-T-G-G: Covalent Drug Binding Within the Minor Groove.

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Anthramycin, a pyrrolo[1,4]benzodiazepine drug related to tomaymycin and sibiromycin, is known to interact with the minor groove of DNA via a covalent bond to the N2 amine of guanine. Hurley and coworkers have shown that the preferred mode of binding is to guanine of a triplet sequence R-G-R, with R-G-Y and Y-G-R as secondary choices, and with least binding to Y-G-Y (R = purine; Y = pyrimidine). We have just completed the first x-ray crystal structure of a minor-groove-binding drug covalently linked to B-DNA, the 2:1 drug:DNA complex of anthramycin with C-C-A-A-C-G-T-T-G-G. The residual error or R factor is 22.5% for data out to a resolution of 2.3 Å.

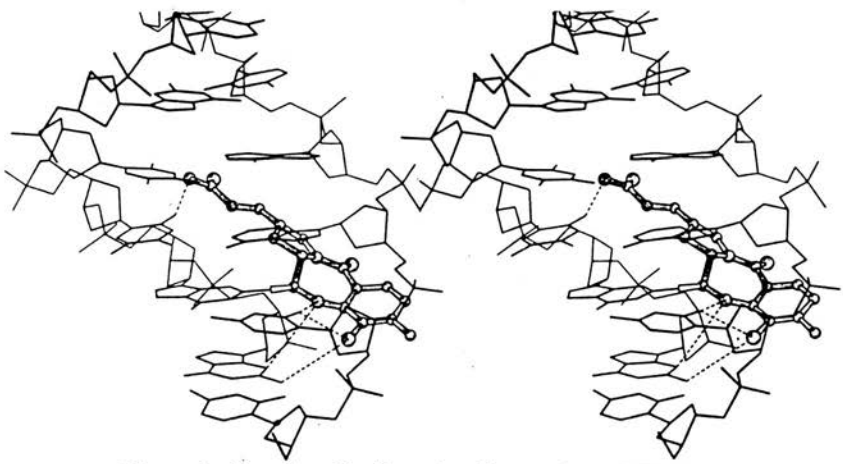

Figure 1. Covalent binding of anthramycin to B-DNA sequence T-G*-G. In this stereo view, the drug (ball-and-stick) sits in the minor groove behind the DNA (thin bonds). A covalent bond extends from the 7-membered ring to the N2 amine of guanine 9. One hydrogen bond (dotted lines) connects the anthramycin -CH=CH-CO-NH_2 "tail" to the C2 carbonyl oxygen of thymine 8; other H-bonds connect guanine 10 and cytosine 12 to the -OH at the "head" of the drug molecule and the NH of the 7-membered ring. Only the bottom half of the 10-base-pair DNA helix is shown; another anthramycin molecule binds to the upper half in the same manner.

One anthramycin molecule binds to the central G of sequence T-G-G near the 3' end of each chain of the B-DNA decamer helix, as shown in Figure 1. In addition to the covalent bond between 7-membered ring of the drug and guanine N2 amine, drug and DNA are connected by hydrogen bonds shown as dotted lines in Figure 1. DNA-drug complexes stack atop one another in the crystal in a way that promotes stacking contacts between anthramycin 6-membered rings.

These pyrrolo[1,4]benzodiazepines are important in that they represent some of the very few examples of minor-groove-binding drugs that exhibit a preference for GC base pairs rather than AT. The design of sequence-reading drugs requires that one know how to engineer GC-preferences as well as AT-preferences. T-G-G is an example of the intermediate binding site for anthramycin. We will investigate complexes with the optimal R-G-R site, such as C-A-G-A-C-I-T-C-T-G and C-G-G-A-C-I-T-C-C-G. These have the added advantage of isolating the drug-binding site from the ends of the helix. We also will examine complexes of tomaymycin, which cannot form the same hydrogen bonds as anthramycin because the -OH is positioned differently on the 6-membered ring. From these comparisons should come an explanation of the molecular basis for GC sequence specificity and strength of binding in this class of DNA-binding drugs.

DISSECTING THE FREE ENERGY OF ANTHRACYCLINE ANTIBIOTIC BINDING TO DNA

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The rational design of new anticancer antibiotics targeted toward DNA requires a thorough understanding of the DNA binding properties of existing compounds with proven clinical utility. A thorough understanding requires not only structural information about the drug – DNA complexes, but also a complete thermodynamic and kinetic characterization of the drug – DNA binding interaction. The clinically important anthracycline antibiotics (daunorubicin, doxorubicin) have become, arguably, the best characterized of the intercalating antibiotics, and as such are an important point of departure in efforts to design new DNA binding drugs. New thermodynamic results will be presented that allow us to attempt to dissect the DNA binding free energy of anthracycline antibiotics into its component parts, and to evaluate the contributions to the binding free energy of specific constituents on both the DNA and the drug. Binding results obtained using a series of synthetic deoxyoligonucleotides of varying sequence will be discussed. Binding studies using a judiciously selected series of anthracycline derivative will be described that begin to illuminate the contributions of specific chemical constituents on the antibiotic to DNA binding. Among the results of these latter studies, a quantitative evaluation of the electrostatic contribution to the binding free energy emerges. Further, the critical contribution of the daunosamine moiety to the DNA binding free energy becomes clear by comparison of the binding of anthracycline derivatives in which the daunosamine has been chemically or stereochemically altered. The anthracycline antibiotics daunorubicin and doxorubicin are unique in possessing both intercalating and groove binding moieties. We will report binding studies on the anthracycline aglycone moieties that will show quantitatively the importance of the dual binding mode of the parent antibiotics to their tight DNA binding. *Drs. Waldemar Priebe and David Graves, from the M. D. Anderson Cancer Center, Houston, Texas, and the University of Mississippi, Oxford, Mississippi, repectively , are important collaborators in this project.* *Supported by National Cancer institute Grant CA35635.*

SYNTHESIS OF A NOVEL OXYGENATED C8-LINKED PYRROLOBENZODIAZEPINE DIMER : A POTENT DNA-CROSS LINKING AGENT

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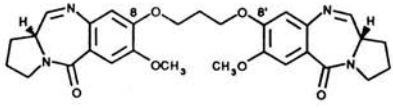
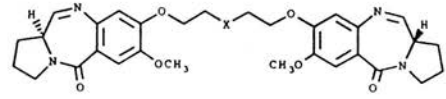
The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) such as anthramycin and DC-81 are a well known group of antitumour antibiotics that exert their biological activity through covalent interaction between the C-11 position of their central B-ring and N-2 of guanine in the minor groove of DNA[1]. They are known to be sequence selective with a preference for Pu-G-Pu triplets[1]. Recently, DSB-120 (**1**), the first example of a C8-linked PBD dimer was synthesized that consists of two DC-81 units joined through a propyl linker[2,3]. This compound has remarkable cytotoxicity and is one of the most efficient DNA cross-linking agents known to date. DSB-120 spans 6 base-pairs and has a preference for PuGATCPy or PyGATCPu sequences. In order to explore structure activity relationships, a number of analogues with homologous linkers have been prepared (i.e. **2**)[3]. The novel analogue (**3**) containing a central oxygen in a seven-atom linker has now been synthesized and evaluated for DNA-binding affinity and cytotoxicity.



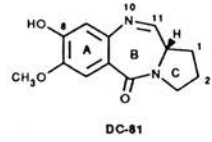
1: (DSB-120)



2: X = CH_2
3: X = O

The data in Table 1 show that **3** is a highly efficient DNA cross-linking agent (as measured by a plasmid cross-linking assay; $C_{50\%}$ = concentration of the drug required to effect 50% cross-linking of a sample of pBR322 DNA), approximately 3.5 times more potent than the equivalent dimer containing a pentyl linker (**2**). Interestingly, although the overall DNA binding affinity (as measured by thermal denaturation; ΔT_m = increase in DNA melting temperature for drug-treated calf thymus DNA compared to control) is identical for both compounds (i.e. ΔT_m = 8.1), **3** appears to be significantly less cytotoxic across the three cell lines examined.

			IC$_{50}$ (μmol)		
Compound	ΔT_m(°C)	$C_{50\%}$(μmol)	L1210	ADJ/PC6	K$_{562}$
1	15.1	0.055	0.01	0.0005	0.2
2	8.1	0.07	0.0045	0.0004	0.5
3	8.1	0.02	0.22	0.036	3.5
DC-81	0.7	---	0.38	0.33	NA



DC-81

Table 1: Thermal denaturation, cross-linking efficiency and *in vitro* cytotoxicity data.

A molecular modelling study of the interaction of **3** with specific sequences of DNA will be described and possible reasons for the differences between cross-linking efficiency and cytotoxicity in relation to the analogue **2** discussed.

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The CRC (Grant to JAH, TCJ, SN & DT), CRCT and UPEL are thanked for financial support.

MINOR DNA ADDUCTS OF PLATINUM COMPLEXES

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Numerous studies suggest that the antitumor action of platinum drugs is related to its ability to react with cellular DNA. The platinum drugs bind to DNA preferentially to guanine residues at the N(7) position, producing monofunctional adducts that can subsequently close to bifunctional lesions. Most of the structural information currently available pertains to the intrastrand cross-links of platinum between adjacent purines. 90 % of these adducts are formed in the reaction of DNA with *cis*-diamminedichloroplatinum(II) (cisplatin), which is one of the most effective anticancer drugs. Its minor DNA adducts are monofunctional lesions, interstrand cross-links and intrastrand cross-links between two nonadjacent purines.

Clinically ineffective *trans* isomer of cisplatin (transplatin) cannot form intrastrand cross-links between adjacent purines for sterical reasons. It has been, therefore, speculated that these DNA adducts of clinically effective cisplatin and its simple analogues are most likely responsible for antitumor activity of these drugs. Nevertheless, the DNA lesion (or lesions) of cisplatin and its simple analogues still remains (or remain) to be established conclusively.

It has been almost axiomatic that the monofunctional DNA adducts of platinum complexes do not affect DNA conformation. This view has been related to antitumor inefficiency of monofunctional platinum complexes.

The bifunctional platinum complexes can form DNA interstrand cross-links, but these adducts are not considered the lesions responsible for antitumor effect. This view is mainly derived from the observation that clinically ineffective transplatin forms more interstrand cross-links than does cisplatin. The latter speculation is, however, based on the assumption that both isomers form identical interstrand DNA lesions. However, DNA interstrand cross-links of transplatin have been studied less thoroughly so that the latter assumtion has no experimental support.

In the present work, we have extended physico-chemical and molecular biology methodologies to evaluate DNA distortions produced by the monofunctional adduts and interstrand cross-links of platinum complexes. It has been shown that monofunctional DNA adducts of platinum induce in DNA conformational distortions in a sequence dependent manner. The monofunctional binding at the guanine residues in the sequences Py-G-Py (Py is a pyrimidine residue) results in the breakage of hydrogen bonds between platinated deoxyguanosine and complementary deoxycytidine.

A systematic study of the interstrand lesion formed in DNA by clinically ineffective transplatin revealed that this lesion was formed between the sites in DNA, which were different from those involved in the interstrand cross-link of cisplatin. In addition, the DNA interstrand cross-links of cisplatin and transplatin induce in DNA different conformational alterations. Taken together, the role of the DNA monofunctional adducts and interstrand lesions in the mechanism of antitumor effect of platinum drugs deserves further systematic examinations.

ULTRASONIC VELOCITY MEASUREMENTS ARE A NEW METHOD FOR INVESTIGATIONS OF DNA-LIGAND INTERACTIONS.

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L.Marky, New York University, New York, U.S.A.

The ultrasonic velocity in a solution is a sensitive probe for the hydration of the solute. Any binding of a ligand to DNA is accompanied by an overlap of the hydration shells of the DNA and of the ligand and therefore can be detected by following the change of ultrasonic velocity. The modern technique of ultrasonic measurements makes it possible to carry out differential titrations in 0.2 ml of a DNA solution by adding ligands directly in an ultrasonic resonator cell, at DNA concentrations less than 1 mg/ml.

The ultrasonic titration curves for binding magnesium, netropsin (a minor groove ligand), ethidium and daunomycin (intercalators) to DNA duplexes of known sequences have been obtained. The stoichiometry of the DNA-ligand complexes, binding constants and structural characteristics of the complexes have been evaluated from these curves. It was shown that the structure of the magnesium-DNA complex depends on the nucleotide sequence. In this sense magnesium recognizes nucleotide sequences of a DNA double helix. The mechanism of the recognition is suggested. In case of netropsin, ethidium and daunomicyn the formation of secondary complexes have been detected and analyzed.

HYDRATION EFFECTS IN THE INTERACTION OF THE MINOR GROOVE LIGANDS NETROPSIN AND DISTAMYCIN TO POLYNUCLEOTIDES

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The non-intercalative ligands netropsin (Net) and distamycin (Dis) have been used to probe the relative hydration of the minor groove of eight synthetic polynucleotides of known sequence and composition. We used a combination of densimetric, calorimetric, and spectroscopic techniques to obtain complete thermodynamic profiles ($\Delta G°_b$, ΔH_b, ΔS_b and ΔV_b) for the association of Net and Dis to each polynucleotide. The interaction of each ligand to a given pair of isomer polynucleotides with identical composition and different sequences resulted in similar binding affinities. However, the duplexes with alternating purine/pyrimidine sequences generated much larger exothermic heats, less favorable entropies and larger volume contractions than did the corresponding homopurine/homopyrimidine duplexes. Furthermore, if we assumed similar structures for each pair of ligand-polynucleotide complexes, thereby creating thermodynamic cycles, the resulting differential thermodynamic profiles suggested that homopurine sequences in their unligated state are more hydrated than alternating purine/pyrimidine sequences. In addition, increasing the dG•dC content of the polynucleotides sharply reduced the affinity for the ligands, lowered the exothermic heats and raised the unfavorable binding entropies.

The overall results showed an enthalpy-entropy compensation with a $T_{comp.}$ of 320 K, characteristic of processes where water molecules play a vital role. This suggests that differential hydration primarily is responsible for the observed differences in the thermodynamic parameters. Another important finding was the apparent linear ΔS_b-ΔV_b correlation supporting the hypothesis that the observed volume changes reflect the coulombic-hydration contribution to the entropy. The slopes of the fitted lines, ΔS_b*vs* ΔV_b, for Dis and Net are about one half and one quarter, respectively, the slope reported for the protonation of small organic acids. These reflect a 14% volume compression of the water dipoles in the minor groove of the unligated duplexes. Thus, the amount of water release from the minor groove is believed to be a function of the DNA sequence. It is this effect that gives rise to the observed linear correlation because other entropic contributions appear nearly identical for these ligand-polynucleotide systems. This work was supported by Grants GM-42223 (L.A.M.) and GM-34938 (D.W.K.) from the National Institutes of Health, USA.

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Sixth Session

Structural Studies of Covalent Carcinogen-DNA Adducts and Antitumor Drug-DNA Complexes

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Memorial Sloan-Kettering Cancer Center
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My presentation will discuss recent structure determinations using combined NMR-molecular dynamics approaches on the following systems:

(1) Benzo[a]pyrene-N^2-guanine covalent adducts
(2) benzo[c]phenanthrene-N^6-adenine covalent adducts
(3) Sterigmatocystin Mycotoxin-N^7-guanine covalent adducts
(4) Duocarmycin-N^3-adenine covalent adducts at DNA duplex and triplex level
(5) SN-07 Anthracycline-N^2-guanine covalent adducts at DNA duplex and triplex level

The emphasis will be on covalently modified adducts positioned opposite their Watson-Crick complementary base, as well as positioned opposite other bases reflecting base substitutions mutations and opposite deletion sites reflecting frame shift mutations.

RELATIONSHIPS BETWEEN STRUCTURE, DNA ADDUCT FORMATION AND CYTOTOXICITY FOR TWO SERIES OF NITROGEN MUSTARD ALKYLATING AGENTS

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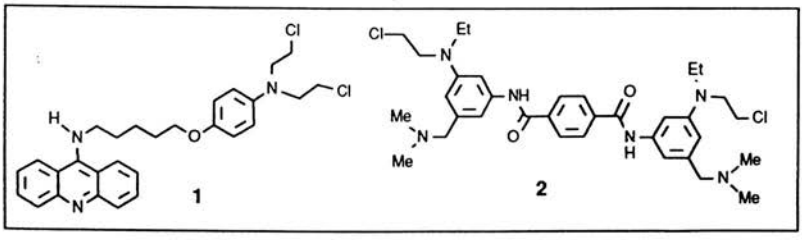
Compounds which can be selectively activated to cytotoxic species in the chronically-hypoxic regions found in solid tumour tissue are of interest as potential tumour-specific drugs. These compounds (hypoxia-selective cytotoxins; HSCs) comprise an hypoxia-selective trigger (usually reductively-activated) and a cytotoxic effector which is then released in the centre of the solid tumour. Nitrogen mustards, which have an ability to kill hypoxic (non-cycling) cells by a well-understood mechanism (cross-linking of DNA), are a promising class of effector molecules for HSCs. Their suitability as effectors depends on a number of factors, including the nature of the DNA adducts formed by them, and the susceptibility of these adducts to repair by cellular enzymes. We report here interrelationships between these properties for two series of DNA-targeted mustards, designed as potential high-potency effectors for HSCs.

For aniline mustards attached to a DNA-intercalating acridine moiety, the regiospecificity of alkylation depends on the length of the linker chain. While analogues tethered by a short chain alkylate as expected at the N7 position of guanine, longer-chain analogues (e.g. **1**) alkylate double stranded DNA almost exclusively at the N1 of adenine (the first aniline mustard reported to do so).

Aniline mustards attached to a DNA minor groove locating polybenzamide structure (e.g., **2**) also alkylate exclusively at AT sites, although the exact regiospecificity of alkylation appears to depend more subtly on structure. Preliminary evidence suggests alkylation at either A or T sites within AT-rich regions.



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POSTERS

NEW DNA INTERCALATING AGENTS DERIVED FROM PYRIDO[1,2-a]BENZIMIDAZOLIUM SALTS.

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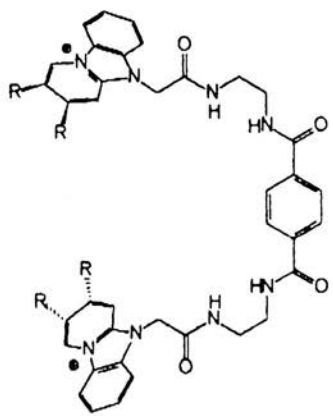
Following a research project on the synthesis of new DNA intercalating agents,[1] the preparation of different bisintercalating agents,[2] taking the complex ditercalinium-$[d(CGCG)]_2$ as a model,[3] is described.The methodology used to build the molecules, related to **1** and **2**, together with structural caractheristics and the results of different *in*



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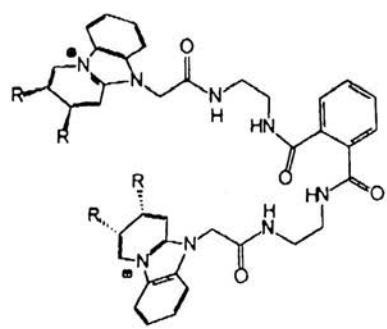

2

vitro evaluations will be presented.

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Probing DNA-Ligand Interactions using Circular Dichroism Spectroscopy

by Paula J. Bates & Alison Rodger*

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Circular dichroism spectroscopy has been used to investigate the binding of chiral transition metal tris-(1,10-phenanthroline) complexes to calf thymus DNA, poly(dA-dT) and poly(dG-dC). The results have been used to assess the merits of a number of proposed models for $[Ru(phen)_3]^{2+}$-DNA complexes.

The racemization of Λ- $[Co(phen)_3]^{3+}$ in the presence of $[Co(phen)_3]^{2+}$and DNA has also been studied. It would appear that the presence of DNA causes a dramatic increase in the rate of racemization. The kinetics of the reaction have been investigated using stopped-flow techniques.

Interaction with DNA of viologens based on phenanthridinium systems: new luminescent probes/photoreagents for polynucleotides.

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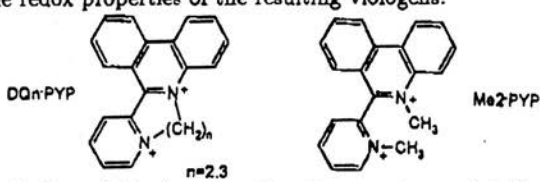
The *intercalative* binding of aromatic heterocyclic cationic molecules with DNA has been well documented in systems such as acridine dyes, ethidium bromide, anthracyclines, porphyrins, etc. *Viologens* are a group of cationic organic compounds derived from dialkylated 2,2'- or 4,4'-bipyridines; all of them are good electron acceptors towards a great variety of reductants, and have been widely used as pesticides due to their mutagenic power.
Most of viologens have been demonstrated to interact with DNA via groove binding; only recently, an intercalative binding mode has been proposed for two viologens derived from diazapyrene[1] and diazaperopyrene.[2] We have taken advantage of the high affinity of phenylphenanthridinium systems (e.g. ethidium) for DNA to design and synthesize a family of aza-analogues that might interact with polynucleotides via intercalative binding, with the aim of exploiting the redox properties of the resulting viologens.



In this way, quaternization of the heterocyclic nitrogen atoms of 6-(2-pyridyl)-phenanthridines (*pyp*) with alkyl chains has yielded several strongly luminescent viologens. Their interaction with DNA has been characterized by means of binding isotherms (via visible spectrophotometry), viscometric titrations, and CD. Molecular mechanics techniques have been employed in order to investigate structural and energetically the intercalative binding of these drugs into oligonucleotides. The redox potentials in water have been measured by cyclic voltammetry. The strong emission from the excited state (e.g. $\lambda = 556nm$ for DQ2-PYP) is quenched upon binding to DNA; experiments with mononucleotides suggest that the deactivation mechanism proceeds via a photoinduced electron transfer from the DNA bases. The obtained results open the possibility of employing these drugs as luminescent probes/artificial photonucleases for DNA.

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Acknowledgements. *The authors thank the Complutense University of Madrid and the Uriach-1838 private Foundation (Barcelona) for their generous support of this research through funding and doctoral grants to G.C. and M.C. G.-A. This work was partially supported also by DGICYT (Project PB92-0908).*

The Binding of 9-Hydroxyellipticine to DNA

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Derivatives of the naturally occurring plant alkaloid ellipticine show promise as anticancer agents[1]. Binding of ellipticines to DNA is thought to interfere with the action of the enzyme Topoisomerase II (an enzyme responsible for the interconversion of topological isomers of DNA) resulting in the formation of a 'cleavable complex' and subsequently breaks in the DNA strands. Intercalation of the ellipticine ring system between DNA base pairs is a necessary, though not by itself sufficient, component for determining antitumour activity.

CD, LD and UV absorption spectroscopies have been used to distinguish intercalation binding modes of 9-hydroxyellipticine from external stacking modes (as evinced by exciton signals in the CD spectra) previously suggested by Monnot et al for 7-hydroxyisoellipticines[2]. External stacking occurs more readily on mixed sequence CT-DNA than on the alternating pur-pyr sequences poly(dG-dC) and poly(dA-dT). The occurrence of external stacking at higher drug concentrations suggests a possible reason for the suppression of cytotoxicity at high drug doses.

Molecular dynamics simulations have been used to investigate the intercalative binding of 9-hydroxyellipticine to the dodecamer ATATATATATAT with 22 sodium ions and approx. 2400 explicit water molecules. Preliminary analysis of a 200ps dynamics trajectory shows the drug to be quite stable in the binding site with little disruption of base-pairing adjacent to the intercalation site.

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Visualizing the dissociation of small ligands from DNA

Michael C. Fletcher* & Keith R. Fox

We have developed a footprinting protocol for visualising the dissociation of sequence-selective ligands from individual binding sites in mixed sequence DNA (Fletcher & Fox, 1993). This technique has been applied to a number of antitumour antibiotics including actinomycin, echinomycin, mithramycin and nogalamycin, measuring their dissociation from both natural and synthetic DNA fragments.

Actinomycin, which is selective for the dinucleotide GpC, dissociates from mixed sequence DNAs with an average half-life of about 20 minutes at 20°C. Dissociation from each GC site is not the same and reflects the nature of the surrounding sequences. Experiments with synthetic DNA fragments reveal that actinomycin dissociates from GC sites surrounded by regions of $(AT)_n$ more slowly than those flanked by $A_n.T_n$. There is no difference between AGCT and TGCA. Examination of kinetic parameters of the regions of enhanced DNase I cleavage around each footprinting site confirm that these arise from ligand-induced alterations in local DNA structure.

Echinomycin, a bifunctional intercalator selective for CpG, also shows a spectrum of dissociation rates from DNA. While dissociation from several sites on *tyr*T DNA is slow (>30 minutes) one site (ACGC) is much faster and is complete within 2 minutes. Dissociation from a CG site flanked by blocks of $(AT)_n$ is slow (about 30 minutes) while it is apparently faster from a CG site surrounded by $T_n.A_n$ regions (<10 minutes). The related synthetic ligand TANDEM, which is selective for the dinucleotide TpA, especially when flanked by AT-rich regions dissociates much faster and is complete by 2 minutes. Dissociation from ATAA is faster than from ATAT. In contrast, dissociation of TANDEM from blocks of $(AT)_n$ is much slower requiring more than 30 minutes, and suggests a cooperative interaction of this ligand with $(AT)_n$ tracts.

Mithramycin, a GC-selective minor groove binding ligand, produces three footprints on *tyr*T DNA. Dissociation from isolated GG sites is much faster (about 30s) than from other GC-rich regions. Although the drug does not bind to A_nGCT_n it yields a footprint at a GC flanked by $(AT)_n$ from which it dissociates with a half-life of approximately 2 minutes.

The anthracycline derivative nogalamycin binds by spearing between the DNA base pairs, positioning substituents in both major and minor grooves. It has not absolute sequence binding preference but appears to recognise particular dynamic features of DNA structure. It binds best to regions of alternating purines and pyrimidines, especially to the sequence TGC. This ligand shows the slowest dissociation kinetics of the ligands so far investigated; several sites are still occupied more than 4 hours after commencing the dissociation process.

In contrast the AT-selective minor groove binding ligand distamycin dissociates rapidly from DNA. At 4°C the footprints disappear before the first time point after adding the competitor DNA (<20s).

DNA BINDING DOMAIN OF PHAGE Ø29 PROTEIN P6.

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B. subtillis phage ø29 has a linear double-stranded DNA with a terminal protein covalently bound at both 5´ ends. Replication is initiated at both genome ends by the viral DNA polymerase using the terminal protein as primer. The ø29 protein p6 is required *in vivo* for DNA replication and stimulates *in vitro* the initiation reaction by forming a multimeric nucleoprotein complex, in which DNA is wrapped around a protein core. The protein p6 binds in a cooperative way to DNA through the minor groove, and it has been proposed that DNA bendability is recognized as the signal for complex formation.

We have determined that the DNA binding domain of protein p6 is located at the N-terminal region, which contains the sequence: 1-AKMMQREITKTTVNVAKMV-19. Protein p6 mutants, obtained by site-directed mutagenesis, were tested for DNA binding, by DNase I footprinting. A change of R to A at position 6 gave rise to a mutant protein inactive in DNA binding. Changes in Q5, T9, and K 2, 10, and 17 produced mutant proteins with impaired DNA binding activities.

We have also tested for DNA binding, a peptide that contains the N-terminal 19 amino acids of wild-type protein p6, and the corresponding peptide with the substitution R to A at position 6 (R6A peptide).Although both peptides induce the same DNase I footprint pattern, the peptide R6A binds to DNA with 100-fold less affinity than wild-type peptide, in agreement with the results obtained with the mutant protein. The DNA minor groove binding drug distamycin competes with the wild-type peptide for DNA binding, suggesting that the peptide binds to the DNA through the minor groove, as it does protein p6. In order to study the peptide structure we have obtained the CD spectra of the peptide in the absence and presence of DNA. We observed that the α helical content of the peptide increased upon addition of DNA.

The role of stacking interactions in modulating the binding affinity of echinomycin for different oligonucleotides

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Departamento de Fisiología y Farmacología, Universidad de Alcalá de Henares (Madrid).

Echinomycin, a member of the quinoxaline family of antibiotics, binds to DNA by bisintercalation, generally sandwiching a CpG step. The binding selectivity of echinomycin for CpG sites is widely considered to be determined by a number of hydrogen bonds between the antibiotic and the guanine bases. However, the stacking interactions between the chromophores of the antibiotic and the adjacent base pairs must also play a role as the drug binds differently to $d(ACGT)_2$ relative to $d(TCGA)_2$, or to $d(ACGTACGT)_2$ relative to $d(TCGATCGA)_2$.

In the interaction between stacked heteroaromatic systems, the electrostatic term has been shown to determine their relative orientation, the van der Waals contribution being rather indiscriminate in comparison[1]. The reason for this lies on the polarity of the charge distributions, which can be accurately calculated by theoretical methods and represented in a simple fashion by the dipole moment vectors. We have previously shown[2] that: i) on Hoogsteen base pair formation, the dipole moment of an AT base pair alters its orientation and increases from a value of about 2 D found for the Watson-Crick conformation to more than 5 D; ii) the calculated dipole moment of the quinoxaline-2-carboxamide chromophores of echinomycin is also large, as supported by experimental measurement (4.15 ± 0.03 D). The conjunction of these two factors helped explain why echinomycin prefers binding to $d(ACGT)_2$ over $d(TCGA)_2$, and why Hoogsteen pairing schemes are only observed for the flanking AT pairs of the former complex.

We have now investigated the complexes of echinomycin with the octanucleotides $d(ACGTACGT)_2$ and $d(TCGATCGA)_2$ (named *A* and *T*, respectively) by molecular dynamics simulation techniques in aqueous solution, and a detailed analysis of the stacking interactions involving both the drug and the DNA base pairs has been performed. Our models suggest that, in addition to the drug-DNA stacking interactions, the stacking forces between the DNA base pairs are crucial to the stabilization of the complexes. Experimental work by NMR spectroscopy[3] has shown that echinomycin binds cooperatively and more strongly to the *A* sequence relative to the *T* sequence. Moreover, Hoogsteen AT pairs are transiently formed only in the *A* complex, in which the AT pairs were also observed to be destabilized relative to free DNA. We note that when the AT pairs adopt a Hoogsteen scheme in this sequence the arrangement of dipole vectors is very favourable. On the contrary, in the complex of echinomycin with *T* Hoogsteen AT base pairing would give rise to a different and unfavourable electrostatic interaction scheme and hence the Watson-Crick conformation is preferred for the AT base pairs of this complex, in consonance with the experimental observations.

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NEW DNA INTERCALATING AGENTS DERIVED FROM INDOLO[3,2-a]QUINOLIZINIUM SALTS.

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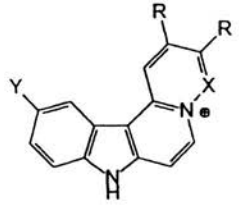
Following a research project on the synthesis of new DNA intercalating agents,[1] the preparation of different compounds, taking the complex ditercalinium-$[d(CGCG)]_2$ as a model,[2] is described.The methodology used to build the molecules, related to **1** and **2**, together with structural caractheristics and the results of different *in vitro* evaluations will be presented.



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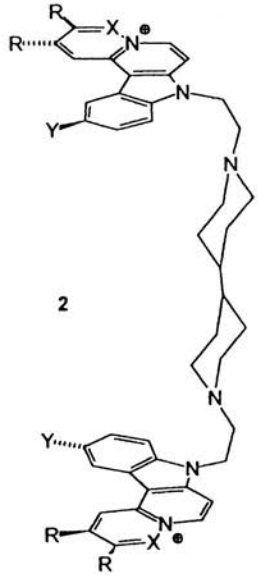

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The Sequence-specific HMG Box Peptide of TCF-1 Adopts a Predominantly α-Helical Conformation in Solution and Recognizes the Minor Groove of DNA.

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The High Mobility Group (HMG) 1 box is a protein motif that mediates DNA binding in a novel family of transcription-regulating proteins. Several members of this family, including the lymphoid specific proteins TCF-1 and LEF-1 and the mammalian sex-determining factor SRY, carry a single HMG box with affinity for the minor groove of the heptamer DNA binding motif -AACAAAG- or variations thereof. To initiate studies on the structural characteristics of the TCF-1 HMG box, we have expressed the 87-amino acid HMG box in milligram quantities in Escherichia coli and purified the soluble peptide to > 95% homogeneity. The peptide bound DNA with the same specificity as the complete protein and was capable of inducing DNA bending. Circular dichroism (CD) analysis revealed TCF-1 HMG box to adopt an approximately 60% α-helix/40% random coil conformation in solution. In the presence of an equimolar amount of double-stranded DNA containing the cognate motif, the CD spectrum changed significantly, implying the induction of a structuralmodification upon DNA/protein association.

MODELLING AND NMR STUDIES OF A SERIES OF POTENT DNA MINOR GROOVE CROSSLINKING AGENTS

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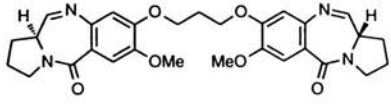
The sequence-specific covalent DNA-binding properties of a series of synthesised[1-3] C8-to-C8' linked pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) dimers (e.g. DSB-120), where the PBD units are linked by a 1,ω-alkanedioxy diether linkage, have been modelled extensively using combined molecular mechanics and dynamics techniques. PBDs form covalent bonds to guanine *via* the exocyclic amino-groups positioned in the minor groove of duplex DNA[4,5], and our modelling suggests that the separation of the two alkylating units in the PBD dimers would favour the formation of interstrand DNA crosslinks for separated guanines disposed on adjacent strands.



DSB-120

Calculations for covalent complexes of DSB-120 with defined d(CGY**G**X**X**C**Y**'CG) duplexes indicate: **X X** = AT >> AA(TT) ≈ GG(CC) > CG > TA > GC, and that **Y** = pyr > pur. In the most favoured crosslinked DNA-drug adduct, the molecule spans 6 DNA bases and actively recognises an embedded d(·GATC·) motif. Two directed (ligand)N–H···N3(Ade) hydrogen bonds are implicated in the recognition of spanned adenines within this crosslink site. Similar behaviour is computed for the homologous PBD dimer containing a $-O(CH_2)_5O-$ linkage, where the favoured binding site contains a d(·GAN**T**C·) motif (where **N** = A ~ T > C >> G).

The self-complementary 10-mer d(CIC**G**A**T**CICG) duplex was designed to test the interstrand crosslinking potential of DSB-120. High-field ^{1}H-NMR (500 MHz) examination of the 1:1 stoichiometric adduct shows that the duplex is crosslinked symmetrically *via* the minor groove N2-positions of the core guanines, with 11*S*, 11'*S* stereochemistry in the ligand and minimal DNA distortion. Refinement of the complex using molecular dynamics (*X-PLOR*) with dynamic annealing and using interproton NOE distance restraints from the 2D-NMR study gave a structure in which the crosslinked duplex retains an overall B-like DNA integrity.

The implications for DNA recognition by this series of PBD dimers will be discussed.

Acknowledgement: We thank the Cancer Research Campaign (U.K.) for financial support.

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Are DNA Triplexes A-form or B-form? Implications for Recognition.

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Recently, there has been discussion as to whether the (polypurine)·(polypyrimidine)·(polypyrimidine)-type triplexes have conformational features resembling A-DNA, as in the original fibre-diffraction-derived model, or more like B-DNA. Reasons for supporting the latter model include the determination by nmr of triplex sugar puckers, and the observation that a number of ligands which bind in the minor groove of B-DNA but not A-DNA, can bind to triplexes. Reported here are the results of two 160ps molecular dynamics simulations of the triplex $d(A)_{10}{\cdot}d(T)_{10}{\cdot}d(T)_{10}$, performed with explicit solvent under periodic boundary conditions, starting from both A- and B-type structures. Both simulations show similar behaviour, with partial unwinding of the helices (reduction in twist and increase in rise), and a preponderance of O1'-endo sugar puckers. The minor groove has a width of 6.3-7.3 Å and a depth of about 3.6 Å (as calculated using CURVES [1]). For comparison, the minor groove of canonical B-DNA has a width of 6.0 Å and depth of 5.5 Å, and for A-DNA the values are 10.9 and near zero, respectively.

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Post-translational regulation of P-glycoprotein in mammalian tumour cells expressing a distinctive drug resistance phenotype after exposure to fractionated X-irradiation.

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Human ovarian (SKOV-3) and Chinese hamster ovarian (CHO) tumour cells, following *in vitro* exposure to fractionated X-irradiation expressed a distinctive multiple drug resistance phenotype characterised by : (i) resistance to Vinca alkaloids and epipodophyllotoxins, whilst retaining sensitivity to anthracyclines, and (ii) overexpression of P-glycoprotein (Pgp) in the absence of any concommitant elevation in Pgp mRNA. To establish the mechanism associated with this Pgp overexpression in these X-ray-pretreated sublines (SKOV-3/DXR-10 and CHO/#DXR-10), the half-life of Pgp was examined in [^{35}S]-methionine-labelled cells after immunoprecipitation with two monoclonal antibodies to Pgp and compared to that of drug-selected 'classic' multidrug resistant (MDR) sublines. A reduced rate of Pgp turnover was identified in both human and hamster DXR-10 sublines, with a half-life of $\geq$ 40h, compared with 10 - 17 h in drug-selected MDR sublines. These results provide one of the first detailed examples of Pgp regulation by post-translational increased stability and demonstrate that the development of drug resistance following X-ray-pretreatment may arise by a mechanism distinct from that resulting after drug selection.

MOLECULAR DYNAMICS AND QUANTUM MECHANICS SIMULATIONS OF THE INTERACTIONS BETWEEN THE DNA AND CATIONS.

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Molecular Dynamic (MD) and ab initio methods have been used to study the interaction between B-DNA and cations. A fully hydrated dodecamer was used as a model of B-DNA, and 24 Na^+ counterions were used to mimic general cations interacting with the DNA.

The polarization is expected to have a key role in the interaction between an anion like the DNA and a cation like the Na^+. Accordingly, this effect cannot be longer ignored, but must be incorporated into the MD simulations. For this purpose, a preliminary MD simulation of the DNA + water + Na^+ system was performed at the NPT (P = 1 atm, T = 298 K) ensemble using the standard AMBER/AA Force-Field and periodic boundary conditions. These simulations allowed us to determine different patterns of interaction between the DNA and the Na^+. Selected configurations representative of the different patterns of interaction ("families") were used to recompute the ESP charges for the phosphate-Na^+ system using a mixed classical-ab initio 6-31 + G* method. The charges for the different "families" of configurations were weighted according to their probability factors, yielding to a set of configurationally-averaged charged. These new charges were used in additional MD simulations. These provide new "families" and new probabilities factors, which lead to the definition of a new set of charges. This iterative process was repeated until convergence.

The final ESP charges were used in two 150 ps MD simulations of the DNA + water + Na^+ system, which allowed us to determine the patterns of interaction between B-DNA and counterions, and the changes in the phosphate charge distribution due to the vicinity of water and counterions.

THE ANTI Z-DNA REACTIVITY OF Z-DNA FORMING SEQUENCES IS AFFECTED BY PLATINUM ANTITUMOR DRUGS.

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The effect of the binding of cis-diamminedichloroplatinum(II) (cis-DDP) and Pt-pentamidine (Pt-pent.) on the Z-DNA reactivity of potential Z-DNA forming sequences inserted in plasmids and the induction of the Z-DNA form in poly(dG-me^{5}dC).poly(dG-me^{5}dC) have been studied by ELISA and by CD spectroscopy, respectively. The results indicate that cis-DDP and Pt-pent. increases the Z-DNA reactivity of Z-DNA forming sequences in a plasmid containing a $(dG.dC)_{16}$ insert (pUCZ8) and in the pF18 plasmid containing a native Z-DNA forming sequence of Drosophila hydei. The molar ratio of platinum bound to nucleotides (r_b) to produce 50% of the induced Z-DNA reactivity was 0.10 for cis-DDP:pF18 and 0.15 for Pt-pent.:pF18 and 0.10 for both cis-DDP:pUCZ8 and Pt-pent.:pUCZ8. However, the efficacy of Pt-pent. to provoke in both plasmids the induced Z-DNA reactivity is about 2.5-fold higher than the capacity of cis-DDP. It was observed, moreover, that while Pt-pent. was capable of inducing Z-DNA reactivity in a GC-rich DNA sequence of the Hsp 70 protein of Trypanosoma cruzi inserted in pUC8 (p2M4EO3) and in sequences of pUC8, cis-DDP suppresses the Z-DNA reactivity existing in these plasmids. We show, in addition, that pentamidine also induces Z-DNA reactivity in all the plasmids indicated above. The CD spectra of poly (dG-me^{5}dC).poly(dG-me^{5}dC) showing that the drugs are able to induce the Z-DNA form suggest that the increase in Z-DNA reactivity observed in plasmids bound to the drugs, may be due to shifting towards a Z-DNA or Z-DNA like conformation of the Z-DNA forming sequences.

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Multiple mode of binding of the distamycin-analogue FCE-24517 with d(CGTATACG)$_2$. A 2D-NMR study supported by MM and MD calculations.

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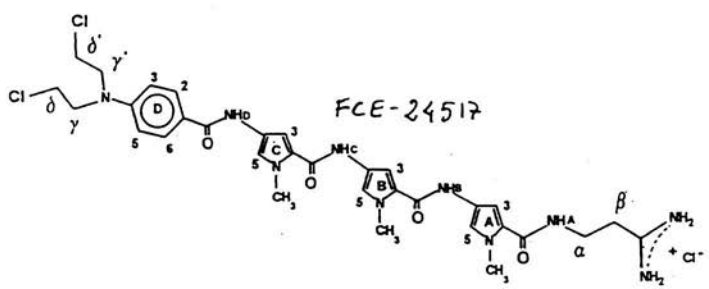
The interaction of a new distamycin analogue (FCE-24517, formed by a distamycin moiety linked to a nitrogen mustard) with the "AT"-rich oligodeoxynucleotide d(CGTATACG)$_2$ was studied by a combined usage of 2D-NMR techniques (ROESY, TOCSY and natural abundance ^{13}C, ^{1}H correlation). Proton and carbon resonances were assigned in terms of specific strand and residue.

The analysis at different temperatures of the NMR signals lead to the characterization of the complex from both a kinetic and a structural point of view. NOE contacts were found between the drug pyrrolic and propionamidinic protons and the H-2 and H-1' protons, located in the "TATA" segment.

In spite of the presence of an alkylating moiety, the drug was found to bind only reversibly to the minor groove of the octanucleotide, forming two 1:1 complexes, which are present in solution in equal amounts and in slow exchange. The presence of multiple equilibrium reactions was proved by means of NOESY and ROESY spectra, where all the chemical exchange cross-peaks were analysed. The mean time of residence in the double helix was thus measured.

The mobility of FCE-24517 within the minor groove was also studied by unrestrained molecular dynamics calculations.



DNA-Rifamycin SV interaction. Oxidative damage of DNA and the effect of antioxidants.

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Quinone containing-antitumor antibiotics are able to produce DNA damage by interacting with and/or free radical release during their oxidation mechanism. Activated free radical semiquinones can either react directly with biological targets such as DNA or RNA or generate the cytotoxic oxygen derived $0_2\cdot^-$ and ·0H. The formation of reactive oxygen species has been proposed as the basis for both the antitumor activity and for the cytotoxicity i.e. cardiotoxicity of anthracyclins[1]. Oxygen reactive species such as superoxide ($0_2\cdot^-$) hydrogen peroxide (H_20_2) and hydroxyl radical (0H.) are generated by different carcinogens, are mutagenic, transform cells, and are carcinogenic, and it is believed that they play a role in the increase of lipid peroxidation products whith age[2]. A common feature of these processes is the DNA damage induced during oxidative stress[2,3]. ·0H radicals have been shown to damage deoxyribose and to release thiobarbituric acid (TBA)-reactive materials both from DNA and from its sugar moiety[4]. Since the original observations in 1984 by Kasai and Nishimura[5], it has been clear that ·0H, which are considered the most reactive species of oxygen intermediates, mediate 8-0HdG formation. The identification of 8-0HdG is specially important since its presence in DNA is considered as a possible factor in carcinogenesis. Thus the presence of 8-0HdG in DNA template causes alpha-polymerase to miscode incorporation of nucleotides in the replicated strand[6] This effect is enhanced when the oxidized base is upstream of a pyrimidine (C), while little selectivity appears to be in the nucleotide inserted opposite 8-0HG[6]. These results are supported by recent data which clearly indicate the mutagenic activity of 8-0HdG[7].

DNA-drug interaction studies have been performed to investigate the damaging effect of Rifamycin SV and copper(II) ions on DNA, both "in vitro" and "in vivo",to define the possible reactive species involved in the degradation mechanism and to emphasize the role of free radical scavengers against the observed oxidative damage. Foot-printing studies, previously performed in colaboration with Dr. Portugal's group, show the binding of the drug to A and T rich regions of the HindIII and BamHII digested TyrTDNA fragment.

Incubation of calf thymus DNA in the presence of rifamycin SV induces a decrease in the absorbance of DNA at 260 nm. The effect was found to be proportional to the antibiotic concentration and enhanced by copper(II) ions. In the presence of rifamycin SV and copper(II), a significant increase in thiobarbituric acid-reactive (TBA-reactive) material is also observed. This effect is inhibited to different degrees by the following antioxidants: catalase 77%; thiourea 72%; glutathione (GSH) 62%; ethanol 52%; and DMSO 34%, suggesting that both hydrogen peroxide (H_20_2) and hydroxyl radicals (·OH) are involved in DNA damage. Rifamycin SV-copper(II) mixtures were also found to induce the production of peroxidation material from deoxyribose and, in this case, glutathione and ethanol were the most effective antioxidant substrates with inhibition rates of 91% and 88% respectively.

Electrophoretic studies show that calf thymus DNA becomes damaged after 20 min. incubation in the presence of both agents and that the damaged fragments run with migration rates similar to those obtained by the metal chelating agent 1,10-phenanthroline. The normal DNA electrophoretic pattern was found to be preserved by catalase, GSH at physiological concentrations, and by thiourea. No protection is observed in the presence of ethanol or DMSO[8].

Free radical production during rifamycin SV oxidation was assessed by E.P.R. studies revealing the formation of a semiquino species which was modified in the presence of copper(II) ions. These results are consistent with the damage induced by the antibiotic to isolated hepatocytes.[9]

The effect "in vitro" and "in vivo" of rifamycin SV on the formation of 8-hydroxy-2'-deoxyguanosine (8-0HdG) has also been investigated. Oxidative modification of 2'-deoxyguanosine has been measured as an indication of DNA damage using HPLC with electrochemical detection. Rifamycin SV in the presence of copper(II) ions induces the formation of 8-0HdG in calf thymus DNA. The effect is enhanced by increasing the antibiotic concentration and inhibited by catalase and hydoxyl radical (·0H) scavengers, such as thiourea and ethanol, depending on the rifamycin SV concentration. Reduced glutathione (GSH) inhibits DNA damage and this effect is proportional to the final concentration of the tripeptide in the incubation medium. A significant increase in the formation of 8-0HdG and of malondialdehyde (MDA) in rat liver DNA was observed only in GSH depleted animals after 5 days of rifamycin SV treatment[10]. These results support the involvement of H_2O_2 and ·0H in the mechanism of the oxidative modification of DNA achieved by rifamycin SV and emphasizes the antioxidant properties of GSH against free radical induced damage[11].

This work was supported by a Grand DGICYT PM89-0095 and PA92-0514 to G.T.S.

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THREE-DIMENSIONAL STRUCTURE OF THE A-TRACT DODECAMER d(CGCAAATTTGCG) COMPLEXED WITH THE DRUG HOECHST 33258

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Netropsin, distamycin and Hoechst 33258 are drugs with a common characteristic: their conformational planarity. This plays an important role in their interaction with the minor groove of DNA, in particular when AT base pairs are present. The DNA minor groove is narrower when the N2 atoms of the guanines are absent. This allows the drug to fit tightly in the groove.

We have determined the crystal structure of the complex d(CGCAAATTTGCG)-Hoechst 33258 at a resolution of 2.4 Å. The drug binds in the minor groove displaced from the center of the AT sequence covering from the A6.T19 base pair to almost the C10.G15 base pair. The piperazine ring is positioned over the T9.A16 base pair (Fig.1). The A4.T21 base pair is not covered by the drug.

Two three-centered hydrogen bonds are found between the drug and the DNA bases: N1(Hoechst)-O2(T19 and T7); N3(Hoechst)-N3(A18) and O2(T8). Other hydrogen bonds are formed between the drug and DNA deoxyribose atoms: O1(Hoechst)-O4'(T21) and the N6(Hoechst)-O3'(#C24).

In order to understand the conformational variability of the DNA and the influence of drug binding, the present structure is compared with other DNA dodecamers with different sequences or complexed with different drugs. The DNA molecule presents a high propeller twist in the AT base pairs as observed in previous dodecamer structures. A three-centered hydrogen bond occurs in the major groove between the N6 atom of A5 and the O4 atoms of T19 and T20.

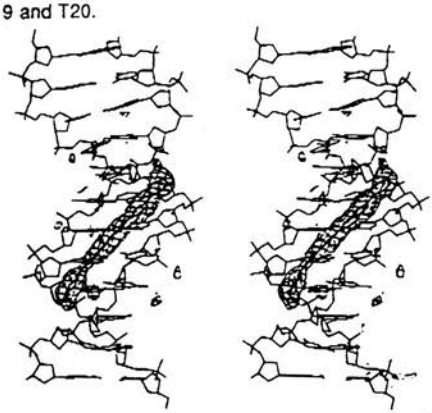

Fig.1 Stereo diagram of the final omit map showing the position of the drug in the DNA minor groove.

^{1}H NMR STRUCTURAL AND THERMODYNAMICAL ANALYSIS OF DRUG-NUCLEIC ACID INTERACTIONS: PROFLAVINE WITH DEOXYTETRANUCLEOTIDES OF DIFFERENT BASE SEQUENCE

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The interactions of proflavine (PF) and four self complementary deoxyribonucleoside triphosphates, 5'-d(ApGpCpT), 5'-d(ApCpGpT), 5'-d(GpCpGpC) and 5'-d(CpGpCpG), have been investigated by 1D and 2D 500 MHz ^{1}H NMR spectroscopy. Proflavine binds preferentially to the CG site of the tetramers in the duplex state and, as the four tetranucleotides contain 0, 1 and 2 CG sequences, the effect of binding with different flanking residual nucleosides can also be studied.

From a detailed analysis of the concentration dependences of proton chemical shifts of the interaction of PF with the tetranucleotides, the contributions to the multiple equilibria of the PF, the deoxytetranucleotides and their different complexes (1:1, 1:2, 2:1, 2:2) have been elucidated. The results show that PF binds preferentially to the CG site of the tetramers in the duplex state and to the reverse sequence (GC) in the monomer state.[1,2]

The structures of the intercalation complexes of PF with the tetranucleotides have been determined by a combination of 2D NOE spectra and the calculated induced limiting chemical shifts of the PF protons. It was found for the PF-tetranucleotide duplex complexes (1:2) that mutual orientations of the planes of CG base pairs and the dye chromophore do not depend on tetramer base sequence nor on the type of nucleoside flanking the ligand binding site. Nevertheless, the nature of the nucleotide sequence has marked influence on the magnitudes of the equilibrium association constants and, hence, on the probability of formation of such complexes in solution.

From a detailed analysis of the temperature dependences of the PF proton chemical shifts in the mixture with the tetranucleotides, the thermodynamic parameters (ΔH and ΔS) of different PF-tetranucleotide complexes (i.e., 1:1, 1:2, 2:1, 2:2) have also been determined.[3] The results show that hydrophobic interactions play a significant role in intercalative binding of PF to deoxytetranucleotide duplexes in aqueous solution. The results also show that the calculated melting temperatures (T_m) of the PF-tetranucleotide duplexes are 12-15K greater than the T_m for the tetranucleotides without the ligand and are also greater (5-7K) than T_m determined directly from the experimental temperature dependences of chemical shifts which are weighted averages for the multicomponent system.

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Reversal from Z- to B-form of poly[d(A-T)] induced by the antibiotics netropsin and distamycin.

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The antibiotics netropsin and distamycin represent the group of parent minor groove binders, which show a high preference for AT-sequences of B-DNA; whereas sequence-specific binding to A- and Z-DNA was not observed (1). However, interaction with the Z-form of poly[d(G-C)] of distamycin and some analogs may occur under certain conditions (2), but it is still unclear whether these ligands may bind AT-specifically to Z-DNA or they will convert it to B-DNA. To answer this question we have studied the binding of netropsin and distamycin to the Z-form of poly[d(A-T)], which exists in solution in presence of 95 mM $NiCl_2$ at 5 M NaCl (3). CD measurements showed that the transition from B- to Z-form of poly[d(A-T)] occurring in 5 M NaCl solution between 85 mM $NiCl_2$ and 95 mM $NiCl_2$ is inhibited by these ligands. Netropsin and distamycin form stable complexes with this DNA duplex polymer at 4 to 5 M NaCl (1). CD titration studies of netropsin and distamycin A with the B-form (at 5 M NaCl) and Z-form (at 5 M NaCl plus 95 mM $NiCl_2$) of poly[d(A-T)] indicated that these ligands induce a conversion from the Z- to B-form of the duplex polymer. Our data suggest that AT-specific interaction of netropsin and distamycin with DNA stabilizes DNA B-conformation but not the Z-conformation. Structural reasons of these differential binding behaviour of nonintercalators are discussed.

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Novel *bis*-platinum-complexes and their interaction with DNA.

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Inorganic metal complexes are frequently used for therapeutic purposes. Especially platinum complexes are administered in cancer diseases. The most important compounds in cancer chemotherapy are *cis*-diamminedichloroplatinum(II) (*cis*-DDP, cisplatin) and carboplatin. Cisplatin is used alone or in combination with other drugs mainly in the treatment of testicular and ovarian tumours with very good results. The biological target of cisplatin is the DNA. The reaction of cisplatin with DNA leads to several adducts that cause inhibition of DNA replication and cell death. The most abundant DNA modification, the d(GpG)-1,2-intrastrand crosslink, results in a profound distortion of the DNA *secondary* structure (bending and unwinding) which eventually leads to cytotoxicity. Although cisplatin is a potent antitumour agent, it also displays undesirable side effects, *e.g.*, extreme nephrotoxicity, nausea and vomiting. Emerging resistance to cisplatin is another major problem. Therefore, many other platinum compounds (cisplatin analogues) were synthesized as an alternative to cisplatin; some of them are at different stages of clinical trials. A recent interesting development are the dinuclear *bis*-platinum-complexes which display unique structural features in comparison with known cisplatin analogues, and therefore may react fundamentally differently and/or more effectively with DNA, *e.g.*, through enhanced formation of *inter*strand crosslinks. This different and stronger DNA binding could result in a better chemotherapeutic potential.

In our project novel *bis*-platinum-complexes in which two *cis*-$PtCl_2$ units are connected with a spacer will be synthesized. These complexes are thus potentially *tetra*functional. The spacer length can vary. The biochemistry and biology of these new compounds will be investigated, in particular:

- The molecular interaction with DNA (affinity, base specificity, influence of the neighbouring sequences, the kind of crosslinks, effects on the DNA secondary structure) will be analysed with chemical (DMS, OH radicals, ethylnitrosourea, $KMnO_4$, OsO_4, DEPC, BAA, CAA) and enzymatic (DNase I, exonucleases, single strand specific nucleases) means with the aim to determine whether these *bis*-platinum-complexes bind the DNA more strongly/differently than cisplatin.
- The effect of these compounds on DNA replication will be analysed quantitatively with the aim to ascertain whether they are more potent inhibitors than cisplatin.
- It will be investigated, whether these compounds possess antitumour activity and if they are active against cisplatin resistant cells.
- It should be answered if these compounds are suitable for further analyses as an alternative to cisplatin.

List of Invited Speakers

Workshop on

DNA-DRUG INTERACTIONS

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Organized by C. Belmonte and F. Cerveró. Lectures by C. Belmonte, G. J. Bennet, J. N. Campbell, F. Cerveró, A. W. Duggan, J. Gallar, H. O. Handwerker, M. Koltzenburg, R. H. LaMotte, R. A. Meyer, J. Ochoa, E. R. Perl, H. P. Rang, P. W. Reeh, H. G. Schaible, R. F. Schmidt, J. Szolcsányi, E. Torebjörk and W. D. Willis Jr.

2 **Workshop on DNA Structure and Protein Recognition.**
Organized by A. Klug and J. A. Subirana. Lectures by F. Azorín, D. M. Crothers, R. E. Dickerson, M. D. Frank-Kamenetskii, C. W. Hilbers, R. Kaptein, D. Moras, D. Rhodes, W. Saenger, M. Salas, P. B. Sigler, L. Kohlstaedt, J. A. Subirana, D. Suck, A. Travers and J. C. Wang.

3 **Lecture Course on Palaeobiology: Preparing for the Twenty-First Century.**
Organized by F. Álvarez and S. Conway Morris. Lectures by F. Álvarez, S. Conway Morris, B. Runnegar, A. Seilacher and R. A. Spicer.

4 **Workshop on The Past and the Future of Zea Mays.**
Organized by B. Burr, L. Herrera-Estrella and P. Puigdoménech. Lectures by P. Arruda, J. L. Bennetzen, S. P. Briggs, B. Burr, J. Doebley, H. K. Dooner, M. Fromm, G. Gavazzi, C. Gigot, S. Hake, L. Herrera-Estrella, D. A. Hoisington, J. Kermicle, M. Motto, T. Nelson, G. Neuhaus, P. Puigdomenech, H. Saedler, V. Szabo and A. Viotti.

5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

7 **Workshop on Transcription Initiation in Prokaryotes.**
Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
Organized by A. N. Barclay and J. Vives. Lectures by A. N. Barclay, H. G. Boman, I. D. Campbell, C. Chothia, F. Díaz de Espada, I. Faye, L. García Alonso, P. R. Kolatkar, B. Malissen, C. Milstein, R. Paolini, P. Parham, R. J. Poljak, J. V. Ravetch, J. Salzer, N. E. Simister, J. Trinick, J. Vives, B. Westermark and W. Zimmermann.

9 **Workshop on Control of Gene Expression in Yeast.**
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11 **Lecture Course on Conservation and Use of Genetic Resources.**
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15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
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16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
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19 Workshop on Viral Evasion of Host Defense Mechanisms.
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20 Workshop on Genomic Fingerprinting.
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The lectures summarized in this publication were presented by their authors at a workshop held on the 15th through the 17th of November, 1993, at the Instituto Juan March.

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