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DNA SEQUENCE SPECIFICITY OF ANTITUMOR AGENTS

Oncogenes as possible targets for cancer therapy

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Abstract

An examination of the DNA sequence specificity of guanine-N7 alkylation for nitrogen mustards and chlorethylnitrosoureas revealed that large variations in alkylation intensities existed among different guanines in the DNA sequence. The most striking finding was that most agents reacted preferentially at runs of G's, the degree of preference being much greater than would be expected from the number of G's alone. This correlated with the molecular electrostatic potential induced at the guanine-N7 position by the nearest neighbor base pairs. Uracil and quinacrine mustards, however, showed distinctly different reaction patterns from other mustards and a detailed examination has led to structural hypotheses to account for the differences. Certain regions of the genome including regions in some oncogenes and the Epstein-Barr virus have unusually high GC contents (>80% GC) which suggests that the antitumor effectiveness of alkylating agents may in part be due to selective reaction at certain regions in the genome. In fact certain mustards have been shown to exhibit enhanced reactivities with such regions in DNA fragments derived from the c-H-ras oncogene. The above findings point to the possibility of design of alkylating agents to optimise the selectivity of reaction with critical DNA regions. An alternative approach presently under investigation has emerged from an understanding of the characteristics of the sequence specific interaction of the natural oligopeptide antibiotics netropsin and distamycin in the minor groove of DNA. This has led to the synthesis of novel agents (lexitropsins) in which the binding specificity can be shifted from $(AT)_n$ in $(GC)_n$ in a predictable fashion. Thus the rational design of DNA sequence specific vectors linked to DNA reactive groups, such as alkylating or cleaving agents, could enable DNA damage to be delivered selectively to predetermined critical sites on the genome.

Key words: Chemotherapy; oncogenes, DNA sequence specificity.

This paper reviews 2 quite different approaches to the rational design of DNA sequence specific antitumor agents. The first examines the inherent DNA sequence selectivity of reaction of a class of alkylating agents, the nitrogen mustards, which are among the most useful drugs in cancer chemotherapy. A premise of this approach is the possibility that the antitumor effectiveness of alkylating agents may depend on preferential reaction at certain genomic locations. Once an understanding of the structural factors responsible for any sequence selective reaction is achieved it may be possible to design new alkylating agents with enhanced reactions with these specific targets, whilst reducing reaction with other sites that would yield deleterious side effects.

The other approach employs as model compounds the natural oligopeptide antibiotics netropsin and distamycin which are known to exhibit sequence specificity for $(AT)_4$ and $(AT)_5$ sites respectively. A detailed analysis of the structural requirements for the molecular recognition processes of these compounds has permitted chemical modification such that binding can be shifted from $(AT)_n$ to $(GC)_n$ in a predictable fashion. Such sequence specific compounds could be used directly as gene control agents, or their linking to DNA reactive groups, such as alkylating or cleaving agents, should permit the delivery of DNA damage selectively to predetermined critical sites on the genome, thus representing a novel class of rationally designed antitumor agents.

DNA sequence selective alkylation of guanine-N7 positions by nitrogen mustards

Mechlorethamine (nitrogen mustard, HN2, Fig. 1) was the first clinically effective anticancer agent (10), and derivatives, such as L-phenylalanine mustard (Melpha-

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lan), cyclophosphamide, and chloroambucil are still among the most useful clinical agents, despite their apparently non-specific chemical reaction mechanisms. Covalent binding may occur at many nucleophilic sites within nucleic acids and proteins, but DNA is probably the most important target with reaction predominantly at the N7 position of guanine. The requirement for antitumor activity of 2 alkylating groups within the mustard molecule suggests that the activity arises from the formation of crosslinks between macromolecular sites. DNA interstrand crosslinks and DNA-protein crosslinks have been observed in intact cells (8), and evidence for intrastrand crosslinks obtained indirectly (5).

A modification of the Maxam and Gilbert guanine-specific chemical cleavage technique for DNA sequencing has allowed for a direct examination of the guanine-N7 alkylation by nitrogen mustards at the individual base level (24). Briefly, plasmid DNA restriction fragments of known sequence are labelled at one end of one strand with ^{32}P using established procedures (26). The DNA is incubated with alkylating agent in 1 mmol EDTA, 25 mmol triethanolamine HCl, pH 7.2, for 60 min at 22°C. Reaction is stopped by the addition of a chilled solution containing sodium acetate, EDTA and tRNA, and the DNA precipitated with ethanol. Sites of guanine-N7 alkylation are quantitatively converted to strand breaks by treatment with 1 mol piperidine at 90°C for 15 min (25). The resulting single strand fragments are separated by polyacrylamide gel electrophoresis (26). Autoradiograms are scanned by microdensitometer, and the data directly corrected and analysed by computer (16).

All the nitrogen mustards examined exhibited large variations in alkylation intensities at different guanines within a DNA sequence (16, 24, Figs 2 and 3). The reaction intensities of several nitrogen mustards were closely correlated with that of the parent compound mechlorethamine in several different DNA segments. These included phenylalanine mustard, phosphoramide mustard, spiromustine, chloroambucil and mustamine. With all these compounds preferential reaction was observed at guanines that were located in the interior of guanine clusters. In contrast, isolated guanines were generally alkylated weakly. This was particulary striking when the guanine was followed by a cytosine on the 3′ side. Increased ionic strength produced the expected reduction in reaction rate but the order of ranking of guanine reactivities was preserved and the degree of selectivity was dependent on the charge on the mustard molecule. A similar preference for runs of contiguous guanines was also demonstrated for another class of clinically useful alkylating agents, the 2-chloroethylnitrosoureas (12).

Two compounds, uracil and quinacrine mustard (Fig. 1), showed significantly different alkylation patterns from the other mustards (Figs 2 and 3). In the case of uracil mustard the difference is due almost entirely to an enhanced reactivity of this compound for 5′-PyGC-3′ (Py

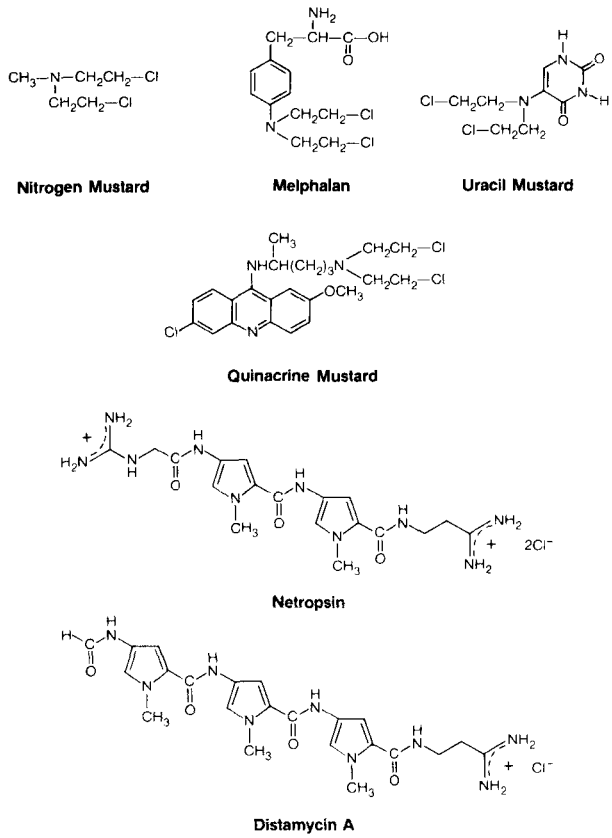

Fig. 1. Structures of the drugs described in the text.

=pyrimidine) sequences which are only weakly alkylated by other mustards. When such sites are excluded from consideration the remaining reaction intensities correlate well with other mustards. 6-Methyluracil mustard did not exhibit the specific reaction preferences of uracil mustard and behaved like other mustards.

The reaction intensities for quinacrine mustard showed the greatest discrimination between strong and weak sites of any mustard. The reaction in runs of guanines was particulary strong, whereas reaction at some sites was almost undetectable. A detailed examination revealed that the sequence preference was distinctive, unaffected by Mg or Na, and dependent on the 2 bases on the 3′-side of the reacting guanine in the following order of reactivity: GPu = TPu>GPy = TPy>AN = CN (Pu = purine, Py = pyrimidine, N = any base).

As a result of the above findings several questions had to be addressed. What might explain the enhanced reactivity of nitrogen mustards for runs of guanines? Can structural hypotheses be proposed to explain the unique properties of uracil and quinacrine mustards? And, can this knowledge be exploited in the design of new alkylating agents? In aqueous solution the nitrogen mustards decompose to form a highly reactive positively charged aziridinium ion. It would therefore be expected that reaction would occur at the most negative guanines. The effect

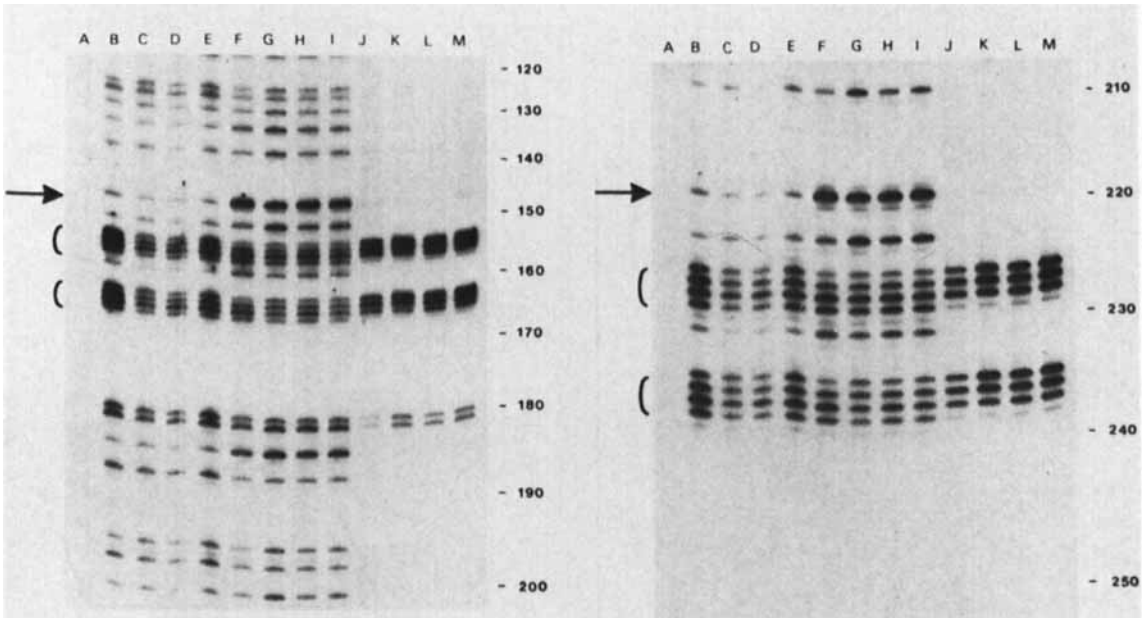

Fig. 2. Sites of guanine-N7 alkylation produced in a fragment of SV40 DNA following treatment with nitrogen mustard (mechlorethamine, lanes B–E), uracil mustard (lanes F–I) or quinacrine mustard (lanes J–M). Lane A is control, unalkylated DNA. The drug exposures were performed in either low ionic strength (25 mmol triethanolamine, lanes B, F, J) or containing 2 mmol Mg (lanes C, G, K), 100 mmol Na (lanes D, H, L) or 2 mmol Mg + 100 mmol Na (lanes E, I, M). The dose of drug was chosen to give approximately equivalent levels of damage in each case. The base number in SV40 DNA is indicated as are the runs of contiguous guanines (bracketed) and the most preferred sites of alkylation by uracil mustard (arrows).

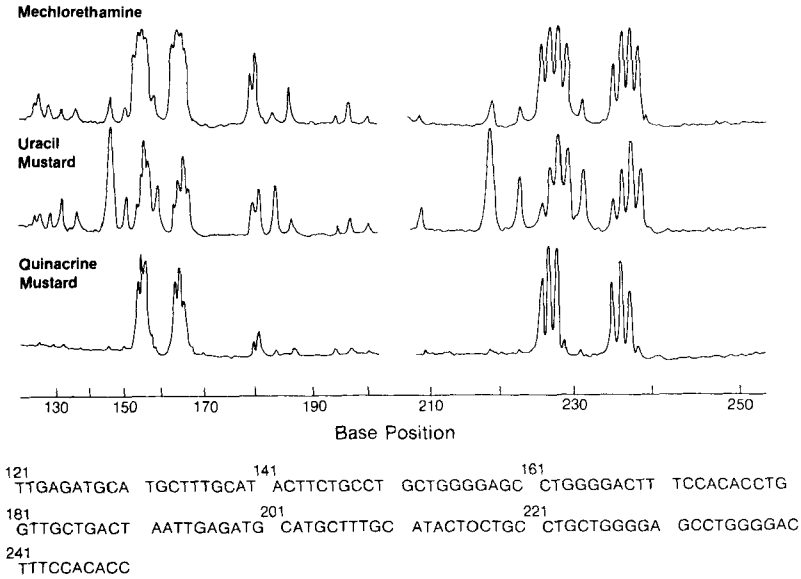

Fig. 3. Densitometric traces (from gel in Fig. 2) showing the different patterns of guanine-N7 alkylation by nitrogen mustard, uracil mustard and quinacrine mustard. The base sequence is also indicated.

of the nearest neighbor base pairs on the molecular electrostatic potential of a guanine-N7 position can be calculated according to Pullman & Pullman (27). The most negative sequence, GGG, was the most strongly alkylated in the case of most mustards, whereas the most positive sequences, which have a C on the 3′ side of the guanine, reacted only weakly. A correlation between the reaction intensity and molecular electrostatic potential was observed for all the mustards studied (correlation coefficient 0.75–0.90) with the exception of uracil and quinacrine mustards (Fig. 4).

Results with uracil and quinacrine mustards clearly

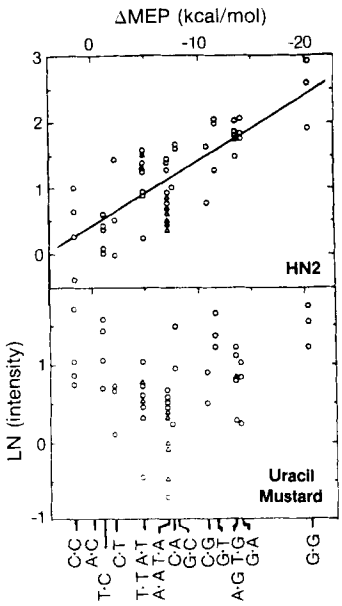

Fig. 4. Dependence of reaction intensity on molecular electrostatic potential (MEP) for nitrogen mustard and uracil mustard. The reaction intensities at various guanine-N7 positions in SV40 DNA are plotted against the effect of nearest neighbor base pairs on MEP as calculated by Pullman & Pullman (27). The corresponding base sequences, with the reacted guanine in the centre (indicated by .) are marked on the bottom of the graph. (Taken in part from (16)).

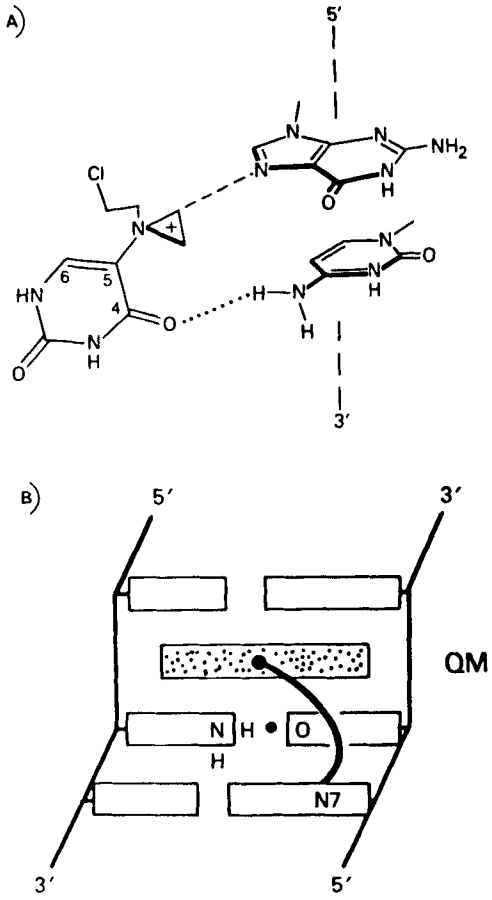

Fig. 5. A) Possible configuration of uracil mustard (aziridinium form) at the initiation of the reaction with guanine-N7, showing the proposed interaction of the uracil-04 atom with N-H of a 3′-cytosine. The attacking aziridinium carbon is modelled at a van der Waals distance from the guanine-N7 atom and in the plane of the guanine ring (from (16)).
B) Schematic view into the major groove showing a quinacrine mustard molecule intercalated between the first and second base pair 3′ to a guanine-N7 reaction site. The hydrocarbon side chain which tethers the mustard group to the quinacrine ring system is shown curving over an intervening thymine residue (from (16)).

demonstrate that the substituent attached to the nitrogen mustard group can impose a DNA sequence preference for reaction. In the case of uracil mustard computer modelling can help explain the observed preference for 5′-PyGC-3′ sequences. As the aziridinium ion of uracil mustard approaches the guanine-N7 the uracil-04 atom could interact with the N-H of the 3′-C, thereby countering the positive electrostatic influence of the latter on the guanine-N7 position (Fig. 5). The geometry of the proposed interaction would be improved if the guanine were displaced towards its sugar-phosphate backbone. This may occur when the guanine is situated between 2 pyrimidines as predicted by the Calladine-Dickerson rules (6). The interaction between the uracil-04 and the cytosine N-H would also be favored by a rotation about the uracil mustard C5–N5 bond, and a methyl group at the 6 position could block this rotation because of steric clash with the 2-chloroethyl group.

In the case of quinacrine mustard the reaction preference was found to rely on the 2 bases 3′ to the reactive guanine and not on the identity of the 5′ base. Models show that intercalation and guanine-N7 alkylation can only occur if the intercalation site is 3′ to the reacting guanine in right-handed B-DNA. It is also understandable why the base immediately 3′ must be a G or T. If it were an A or C there would be an amino group directly below the hydrocarbon side chain stretching from the intercalated quinacrine to the guanine-N7 reaction site (Fig. 5). This would be an unfavorable configuration compared to the case of G or T where there would be an oxygen atom in place of the amino group.

It is interesting to note that most mustards reacted weakly at 5′-GC-3′ sites, which are the sites of potential interstrand crosslinks. The exception to this was uracil mustard which showed preferential reaction at some of these sites suggesting that this agent may be superior at producing such crosslinks. In contrast, the selective reaction of most mustards for guanines in runs of guanines would favor the formation of intrastrand crosslinks.

It remains to be seen to what extent the reaction preferences are preserved in chromatin and intact cells. Nevertheless, DNA base sequence selectivity for alkylating

agents does exist and can be modified by altering the chemical structure thus presenting the possibility that new agents can be designed to react more and more selectively with critical regions of the genome.

Genomic targets for sequence specific agents

The question now arises as to which genomic locations would be targets for such agents, and which gene sequences would be suitable candidates for future more selective agents. Since the major reaction site on DNA for nitrogen mustards is the N7 position of guanine this fact alone would suggest that predominant reaction would occur in guanine-rich sequences. The additional finding that most mustards react preferentially in runs of guanines would further enhance the selective reaction with such sequences. The mammalian genome of warm-blooded vertebrates is a mosaic of very long (>>200 kilobases) DNA segments which are fairly homogenous in base composition and belong to a small number of major classes distinguished by differences in guanine-cytosine content (2). Included in this are regions of unexpectedly high GC content (>80%) which account for approximately 1.3% of the genome and include regions in the c-sis, c-abl, and c-Ha-ras oncogenes (29). The 5'-flank of the c-Ha-ras gene is particularly GC rich. It therefore seems possible that the antitumor effectiveness of alkylating agents may be due to selective reaction at such sequences.

When a hybrid pBR322-ras plasmid, ^{32}P end-labelled, is alkylated with low levels of mechlorethamine or phenylalanine mustard, cleaved with piperidine, and the products analysed on alkaline agarose gels 3 regions of strong alkylation are found in the GC-rich 5'-flanking region of the ras gene (13). Furthermore, an examination of the binding of [^{195m}Pt]cisplatin to restriction fragments of the hybrid plasmid indicated that fragments containing the 5'-flanking region of the c-Ha-ras gene bound 3–4 times more cisplatin per guanine than did reference fragments of pBR322. Cisplatin has previously been shown to attack preferentially runs of guanines (28). A similar pattern of enhanced binding was observed with [^{14}C]phenylalanine mustard. Thus the sequence preference of these agents entails hotspots of damage in the flanking region of the ras oncogene and suggests a possible mechanism for the antitumor actions of these drugs.

A search of the Genbank DNA data base for GC rich regions, as well as revealing a significant number of oncogenes (including human c-Ha-ras, c-mos, c-myc, etc.), also included a number of viral sequences, including the Epstein-Barr virus (EBV). In EBV, large regions of extraordinary GC-richness are located within the 3-kb repeats beginning about 3 kb from the replication origin and have features suggesting an important control function (1,14). The nuclei of tumor cells from the endemic African form of Burkitt's lymphoma contain multiple copies of the EBV genome and one or two doses of a nitrogen mustard (cyclophosphamide) can produce a dramatic regression of the tumor (4, 30). Such spectacular sensitivity to treatment with a single agent is not observed in the non-endemic form of the disease, which is rarely associated with EBV.

As the complex patterns of gene expression and control are unravelled specific sequences of biological interest emerge as potential sites of interference with sequence selective agents. To give one such example, a transcription factor, Sp1, has recently been described which binds specifically to a hexanucleotide sequence GGGCGG (GC-box) which is tandemly repeated 6 times in the 21 base pair repeat elements of the SV40 promoter (9). Preferential alkylation in these sites has been observed following nitrogen mustard exposure (unpublished observations). Such GC-box sequences are also found in the preferentially alkylated ras 5'-flank (7). SP1 has been shown to activate transcription and bind to the GC-box sequence in several viral and cellular promoters including herpes virus IE-3, IE 4/5, the human metallothionein promoter, the AIDS virus long terminal repeat (9), and in the promoter region of the human epidermal growth factor receptor proto-oncogene.

It can be argued that although sequence preferences do exist and may possibly be enhanced in the future, in general the alkylating agents described above are still not highly sequence specific since, in most cases, all guanines within DNA are alkylated to a greater or lesser extent. In addition, the sequence preferences for such agents are restricted to only a few bases. The more specific recognition of longer sequences requires a different approach, such as the development of DNA sequence specific oligopeptides.

DNA sequence specific oligopeptides

An alternative approach to developing DNA sequence specific antitumor agents has been to take as models the natural oligopeptide antibiotics netropsin and distamycin (Fig. 1) which bind within the minor groove of DNA and recognise $(AT)_4$ and $(AT)_5$ sequences respectively (11, 31). Detailed information of the DNA–netropsin interaction has been obtained from x-ray diffraction studies (17), and it is clear that the binding specificity is determined by a combination of hydrogen bonding, electrostatic interactions, and van der Waals contacts.

A close examination of the structural requirements for the molecular recognition of netropsin for its $(AT)_4$ sequence led to the suggestion that replacement of one or more pyrrole units by a heterocycle, capable of accepting a hydrogen bond from guanine(2)NH_2, should alter this preference to allow the recognition of GC in a predictable fashion. Such novel agents (termed lexitropsins or information–reading oligopeptides) containing one or more imidazole moieties instead of pyrrole units were synthesised (23). These compounds showed a decreased preference for AT sites (with a corresponding acceptance for GC

base pairs) in binding studies as determined by thermal melting and CD measurements (3). DNase footprinting experiments showed that these compounds recognised more sites than the parent compound netropsin, whilst still retaining a memory for AT sequences (23).

High field 1- and 2-dimensional ^{1}H-NMR techniques have provided valuable information on the structural, stereochemical and dynamic aspects of lexitropsin–DNA interactions. Initial studies showed that a prototype lexitropsin containing one imidazole moiety in place of the amino terminus pyrrole was located centrally in the decamer d[CGCAATTGCG]$_2$ (19). Thus although possessing the capability of recognising GC sites it still was biased for the original sequence of netropsin. A clue to overcoming this problem came from a consideration of the electrostatic component of the interaction.

It has been shown that in AT rich sequences the site of greatest negative potential occurs in the minor groove (18). Thus it would be expected that dicationic compounds would have a bias for such sites regardless of the sequence specificity imposed by hydrogen bonding. Accordingly a second generation of lexitropsins were synthesised in which the guanidiniumacetyl moiety of netropsin was replaced by the uncharged N-formyl end group characteristic of distamycin. These monocationic compounds were shown by footprinting to target cleanly for GC sites (15), such that the compound containing 2 imidazole moieties was specific for the sequence CCGT. Independent NMR analysis has shown binding of this compound to the sequence CCAT in the decamer d[CATGGCCATG]$_2$ (21), and to the sequence CCGT in the decamer d[(GATCCGTATG)-(CATACGGATC)] (20, Fig. 6).

It was deduced from the NMR studies that the reading of the 3′-terminal base pair was presumably dictated by the van der Waals interaction between the methylenes at the C-terminus of the lexitropsin and the DNA. These methylenes would enter into steric contact with the guanine-NH_2 of a GC base pair thereby forcing the recognition of an AT base pair. To test this a 'truncated' lexitropsin was designed and synthesised with only one methylene at the C-terminus. A detailed NMR analysis has revealed that on binding to the decadeoxyribonucleotide d[CGCAATTGCG]$_2$ whereas the parent compound binds to the central AATT, the 'truncated' molecule is displaced to read the sequence ATTG (22, Fig. 6).

Each refinement of the lexitropsin model gives information of specific contacts and, in turn suggests future avenues for development. Many of the lexitropsins so far developed show their own antitumor activity. It now seems possible to target for any sequence from $(AT)_4$ to $(GC)_4$ in a predictable way (Fig. 7). Targeting for longer sequences should be possible by adding further heterocyclic units. It is interesting to note, however, that the longest natural compounds (distamycin and anthelvencin) in this family of oligopeptides contain only 3 pyrrole units. Longer molecules may get 'out of phase' with the corre-

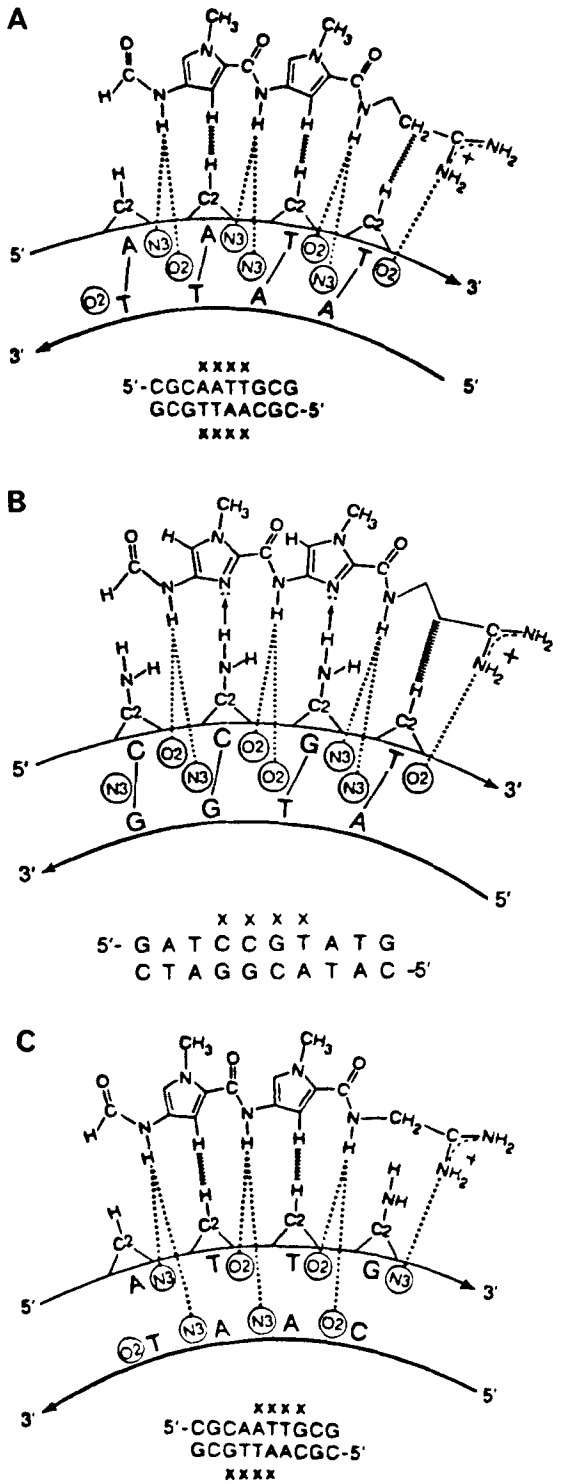

Fig. 6. Alignment of lexitropsins on oligonucleotide sequences as determined by high field ^{1}H-NMR. Panel A shows the binding of the parent lexitropsin (with N-formyl end group) to the AATT sequence. Panel B shows the recognition of GC sites after replacement of the pyrrole groups by imidazole. Panel C shows the effect of removal of one methylene group from the C-terminus of the lexitropsin thus allowing the recognition of a GC base pair at the fourth base in the sequence. In all cases dotted lines indicate hydrogen bonding and dashed lines represent intermolecular van der Waals interactions (from (20) and (22)).

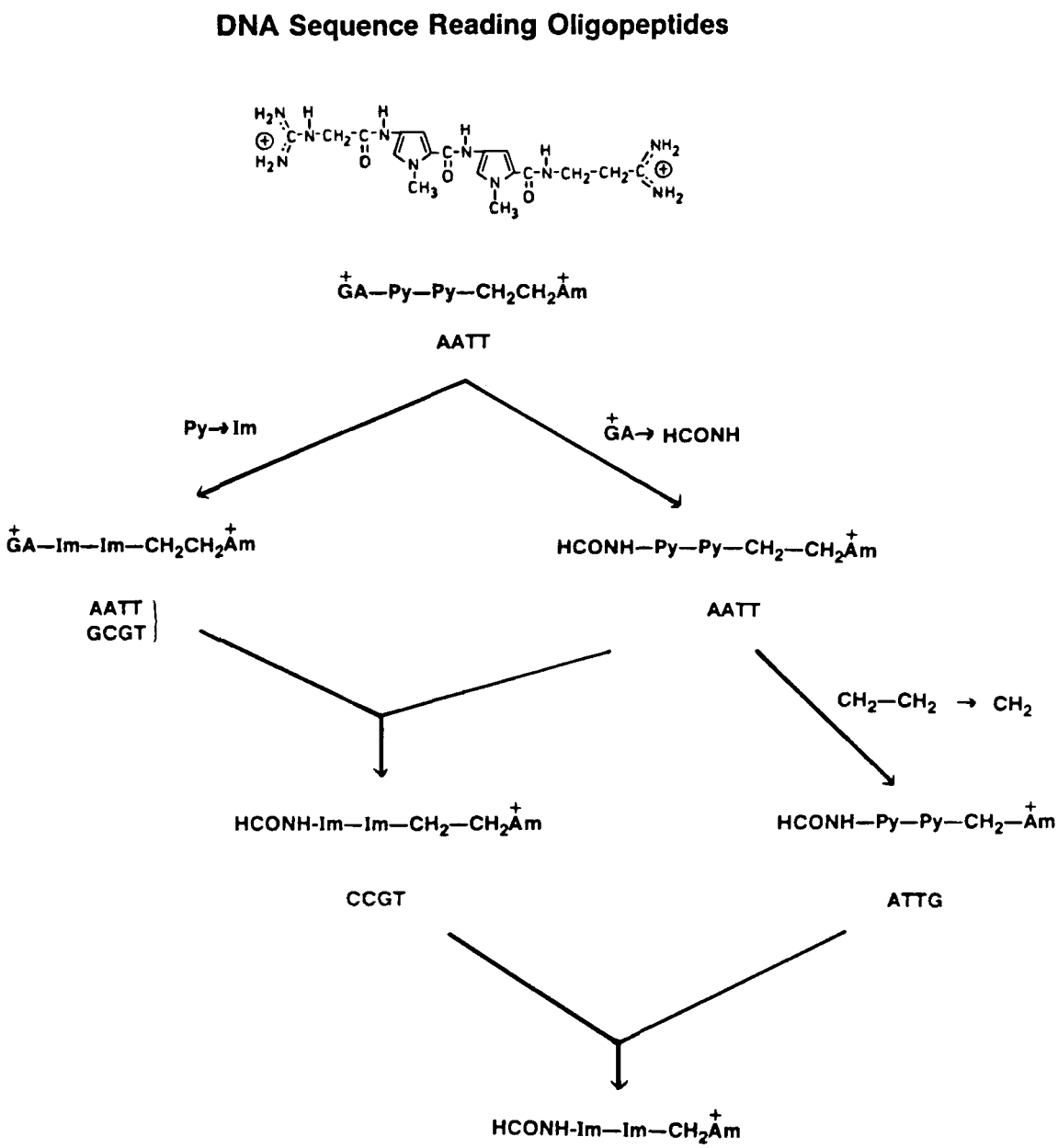

Fig. 7. The shift in binding specificity of DNA sequence reading oligopeptides from AATT to CCGG. Drug structures are abbreviated as follows: ĠA = guanidinium-acetyl, Py = pyrrole, HCONH = N-formyl, Im = imidazole, Ȧm = amidine. The sequence recognised, as determined by NMR, is indicated in each case.

sponding DNA sequence and this problem is presently being addressed using linkers of varying lengths.

Once specific targets have been identified oligopeptides can be designed to act as possible artificial repressors for the inactivation of genes such as oncogenes. Another logical approach would be to use them as vectors to deliver DNA damage to specific as yet undetermined critical sequences. The groups delivered could be e.g. DNA cleaving agents to essentially mimic restriction enzymes, or alkylating agents. One possibility presently under investigation is to link a photosensitizing agent to a sequence-specific vector to enable selective damage to be introduced into the DNA upon the appropriate light illumination. The expertise is now available to design a new generation of DNA interacting antitumor agents which will hopefully replace the 'shotgun' approach by a more rational means of delivering DNA damage to critical genomic sites.

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