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Clofazimine Binding Studies with Deoxyribonucleic Acid [1, 2, 3]

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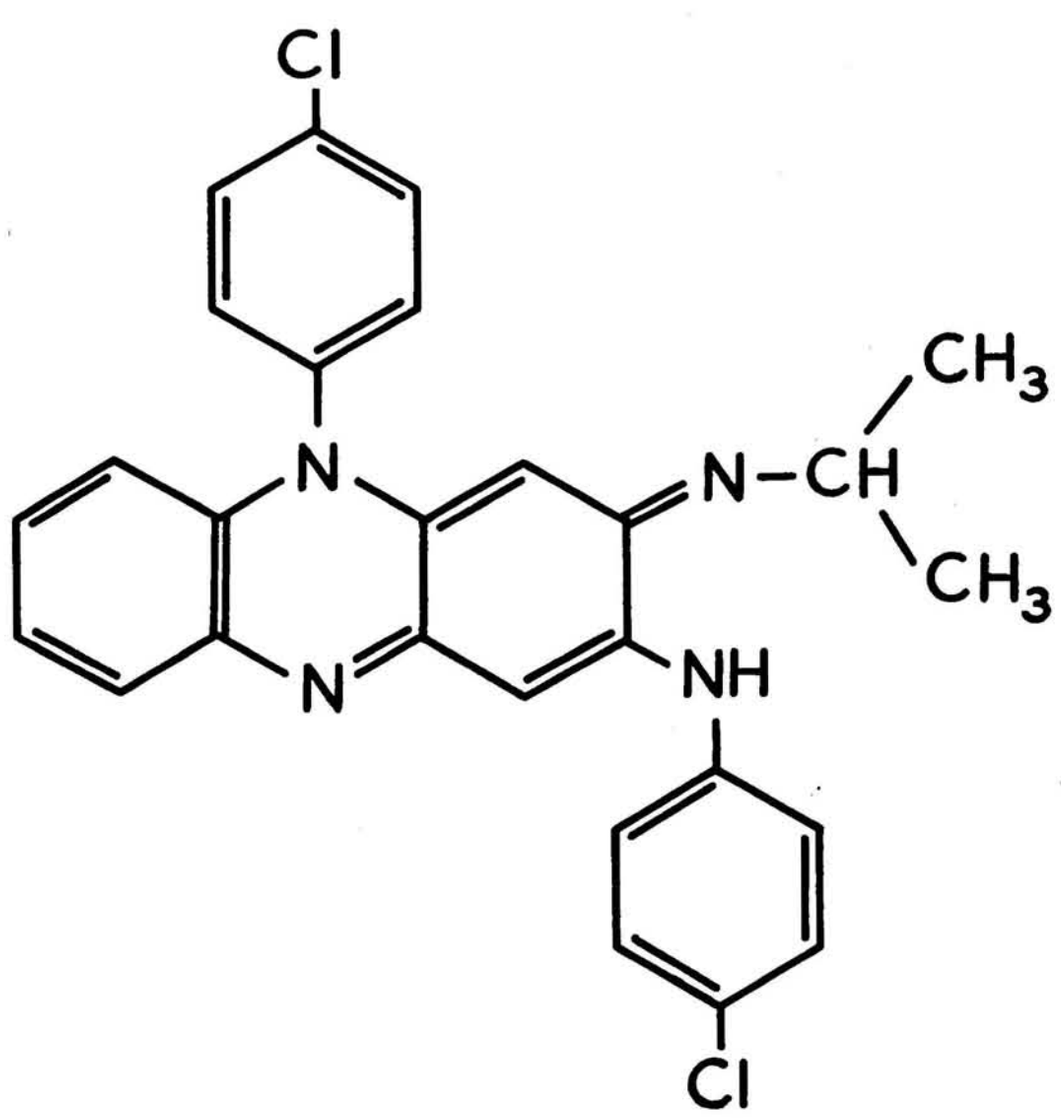
Clofazimine (B663) is a phenazine iminoquinone derivative (Fig. 1) used in the treatment of sulfone-resistant leprosy ([2, 21]). The antileprosy activity of the drug is not fully understood although Barry and co-workers suggested that the autoxidizable orange-red dye prevented the growth of mycobacteria through inhibition of aerobic respiration ([3]). The concept of an artificial electron acceptor inhibiting essential energy-yielding reactions of the respiratory chain was further used to explain clofazimine suppression of mitochondrial biogenesis during aerobic growth of *Saccharomyces cerevisiae* ([22]).

This report is concerned with the concept of an alternative mode of action for clofazimine that is unrelated to inhibition of respiratory chain function. The observation that clofazimine formed stable complexes with DNA ([19]) led to the development of a spectrophotometric assay that permitted a quantitative study of clofazimine-DNA binding. This work has characterized the primary binding site on the DNA strand. No evidence was found to indicate that clofazimine interacted through intercalative binding between the base-pairs of the DNA helix. The evidence was consistent with that of a minor groove binding drug.

MATERIALS AND METHODS

Spectrophotometric assay. DNA induced upfield shifts in the clofazimine absorption peak in the red region were recorded from 1 cm light-path cuvettes with a Cary Model 14 spectrophotometer using a 0-0.1 O.D. expanded scale and a high intensity light source. The assay mixture contained in 5 ml: 0.005 M ADA-tris buffer,[5] pH 7.0; dimethylformamide, 10% (v/v); and DNA, 250 μg/ml. Clofazimine was added at a final concentration of 6×10^{-6}M. In some experiments the solvent concentration was reduced to 7% (v/v). After mixing at room temperature spectral changes were measured at zero time. In the absence of DNA, clofazimine gave a sharp peak at 495 nm. Variations in clofazimine concentration followed Beer's law for one to two hours after which time departures occurred due to the slow precipitation of the drug. In the presence of DNA precipitation did not occur.

Methyl green displacement assay. The displacement of methyl green from preformed



CLOFAZIMINE (B.663)

FIG 1. Structure of clofazimine, 3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine. Formula, $C_{27}H_{22}Cl_2N_4$.

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[3] Presented in part at the U.S.-Japan Leprosy Meeting, Bethesda, Md., October 27-29, 1975 (Int. J. Lepr. **44** [1976] 133).

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[5] ADA = N-(2-Acetamido)-2-Iminodiacetic Acid.

bovine DNA-methyl green complexes by DNA interacting ligands was measured by the method of Kurnick and Radcliffe ([15]). The assay mixture contained in 6 ml: 0.005M ADA-tris buffer, pH 7.0; sufficient DNA-methyl green complex to give an O.D. of 0.500 at 640 nm (equivalent to 710 nanomoles of methyl green); the interacting ligand or drug was added at a final concentration of 5×10^{-6}M. The displacement assay was run to completion by incubating at room temperature for 24 hours. Dimethylformamide 7% (v/v) was added to prevent precipitation of insoluble compounds. Controls were included to correct for the slow spontaneous release of methyl green. The amount of methyl green released was calculated by measuring the decrease in O.D. at 640 nm in a Spectronic 70 spectrophotometer. A purified sample of methyl green (Chroma-Gesellschaft, Lot H-1A442) was used as standard. The methyl green displacement assays were run with an excess of complexed methyl green present. Calculation of the methyl green/ligand ratio approximated 12-14.

Biologicals. DNA's of varying base composition, synthetic polynucleotides, and complexed DNA-methyl green were obtained from Sigma Chemical Company, St. Louis, Missouri, or P-L Biochemicals Inc., Milwaukee, Wisconsin. DNA was extracted from *M. phlei* by the method of Mizuguchi and Tokunaga ([18]). The lyophilized nucleic acid polymers were slowly dissolved in 0.005M ADA-tris buffer, pH 7.0, with stirring and briefly dialysed against the same buffer to reduce excess salt content that interferes with the binding assay. Purified samples of clofazimine (B663), B1912 and B283 were provided by Dr. Vincent Barry, Dublin, Ireland.

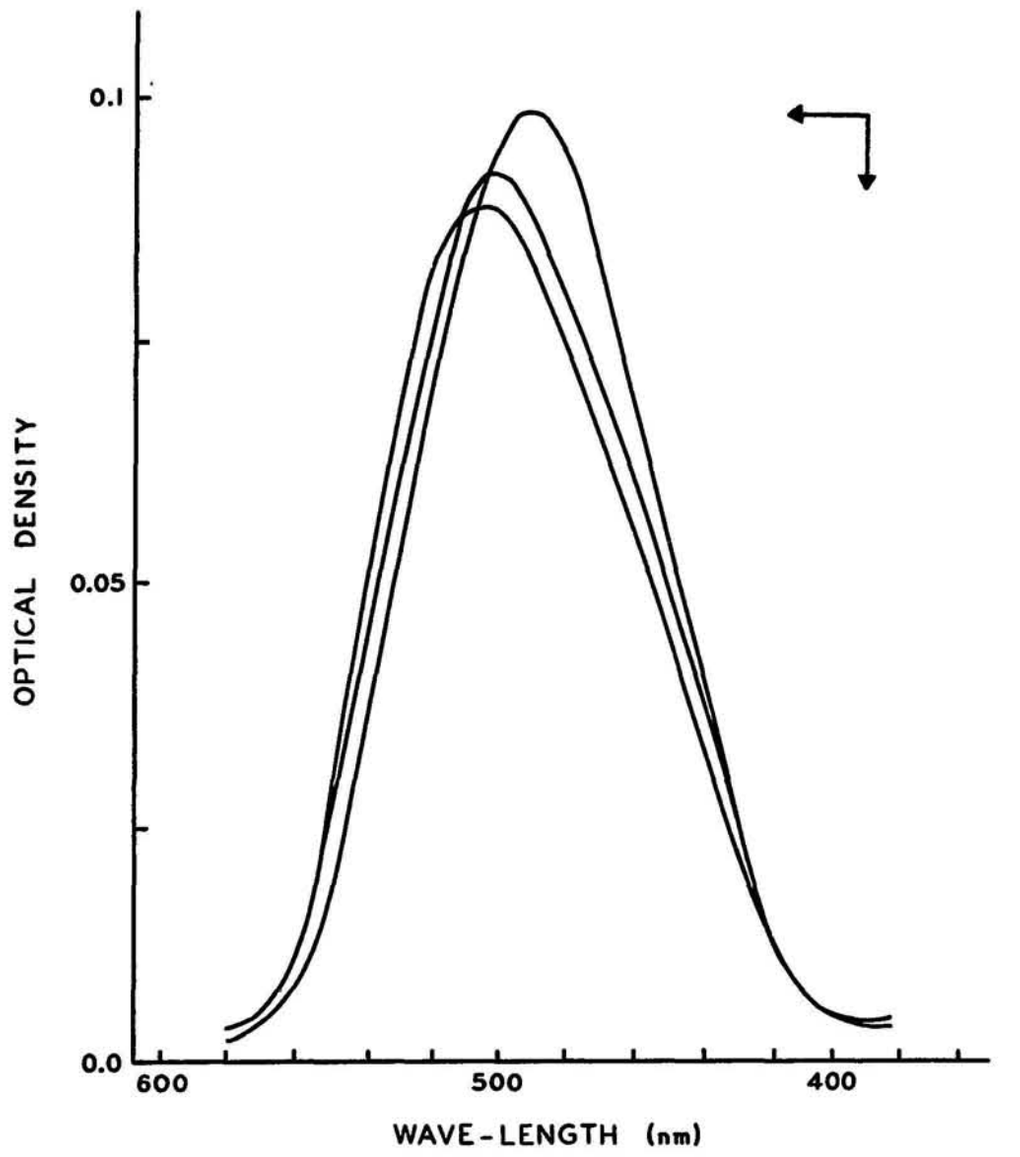

FIG. 2. Upfield red shifts and hypochromic displacement of the clofazimine 495 nm absorption peak due to binding to DNA from *M. lysodeikticus*. Top curve in the absence, center curve in the presence of 125 μg/ml, and bottom curve in the presence of 250 μg/ml of DNA.

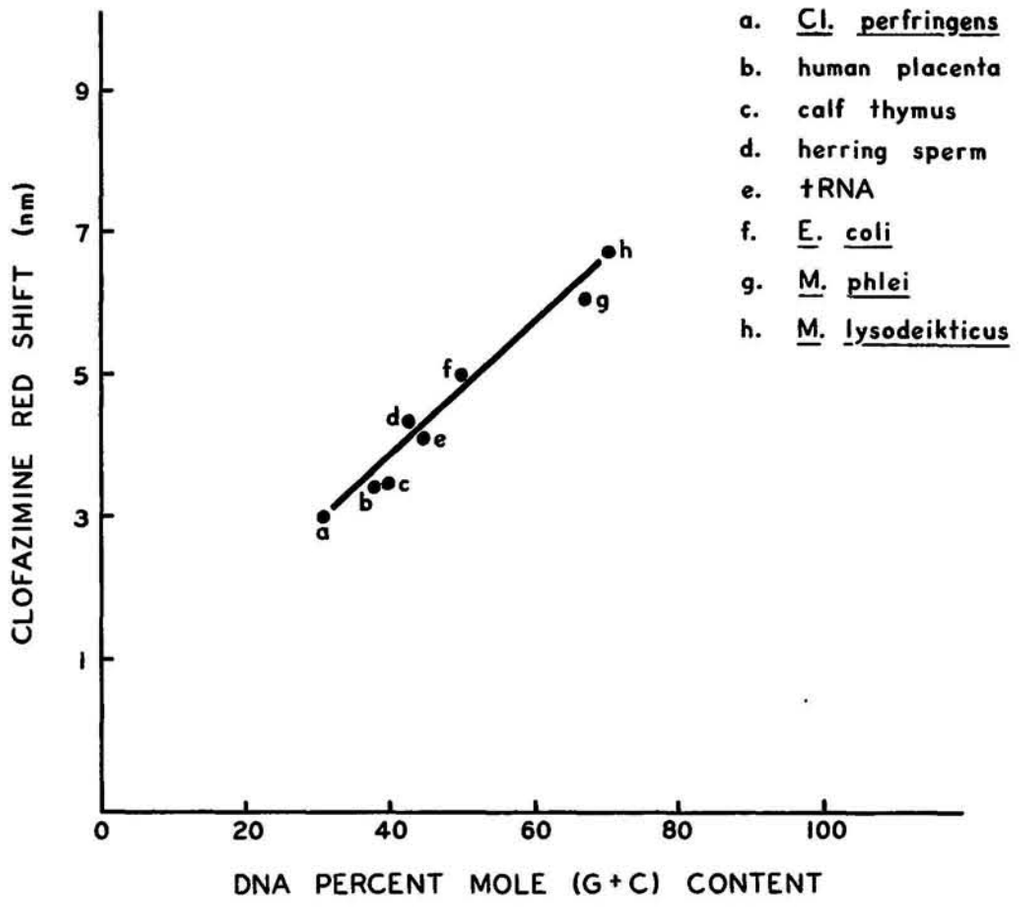

FIG. 3. Linear relationship between the amount of clofazimine upfield red shift and the G + C content of various DNA's.

RESULTS

Clofazimine interaction with DNA. The binding assay indicated that clofazimine will rapidly interact with various DNA strands, including human, with typical upfield red shifts and hypochromic displacement of the clofazimine 495 nm absorption peak (Fig. 2). The red spectral shift is attributed to the interaction of the heterocyclic phenazine ring system of the bound clofazimine with the nucleotide bases of the DNA strand ([4, 9]). An upfield red shift of 3 nm that occurred with human or bovine DNA was sufficient in magnitude to be observed as a visible color change. The complexed clofazimine does not readily dissociate from the DNA strand since it will sediment along with the strand during centrifugation.

Quantitation of the upfield red shift showed

that the change was proportional to the amount of added DNA. For DNA's with varying G + C content a linear relationship was found between the amount of red shift and the G + C content of the DNA strand (Fig. 3). It was noteworthy that mycobacterial DNA (G + C content 67-69%) gave a red shift that was approximately double that observed for human DNA (G + C content 38-39%). The data indicated that the guanine or possibly the cytosine bases played an important role in the binding process. When various DNA's were rapidly denatured by heating and cooling, so that their tertiary coiled helical structure was disrupted, essentially very little change occurred in the binding assay red shift data. This fact indicated that the coiled helix of DNA was not an essential requirement for drug binding and implied that base-pairing between strands was unessential.

Control experiments revealed that upfield red shifts did not occur when clofazimine was mixed with hyaluronic acid, chondroitin sulfate, glycogen or serum albumin. However, when clofazimine was mixed with yeast tRNA that had been stripped of amino acids a red shift of 4 nm occurred that was consistent with the G + C content of tRNA.

Synthetic polynucleotide strand binding. Double-stranded DNA was replaced in the binding assay by a number of synthetic polynucleotide strands. The absence of significant red shifts indicated that little base interaction occurred when clofazimine was mixed with single-stranded poly dA, poly dT, or poly dC. By comparison duplexes of poly dG, poly dC, as well as the single-stranded poly dG gave evidence of 5-7 nm red shifts. A careful quantitation of red shifts were made with the single polynucleotide strands poly A, poly U, poly C, and poly G. A significant red shift occurred when clofazimine interacted with poly G (Table 1). The poly G induced red shift was proportional to poly G concentration and resulted in progressive red shift hypochromic spectra with a common overlap or isosbestic point at 534 nm which supports the view that the spectral changes are due to a primary receptor site on the guanine base of the poly G strand. In the case of double-stranded DNA it was not possible to derive an overlap isosbestic point during spectral red shifts. The absence of a common overlap point implies that both primary base interaction and secondary electrostatic forces are associated in the binding process. The influence of secondary salt linkages on clofazimine binding to DNA appears to be cooperative with primary base interaction since high concentrations of NaCl will prevent binding of clofazimine to DNA.

TABLE 1. *Quantitated upfield red shifts due to clofazimine interaction with synthetic single-stranded polyribonucleotides.*

Synthetic polyribonucleotide	Upfield red shift (peak-to-peak, nm)
Poly A	2
Poly U	1-2
Poly C	2
Poly G	8-9

Methyl green displacement. The basic dye, methyl green, binds to the negatively charged phosphodiester bridge along regions of the narrow groove of the DNA strand ([14]). The dye can be displaced from its binding site by two different mechanisms. Dye displacement may occur due to stretching and local unwinding of the DNA double helix when flat planar molecules intercalate between the base pairs. A second type of displacement occurs when charged ligands are successfully able to compete with and displace methyl green from its binding site due to stronger binding forces. Although low concentrations of clofazimine complexes with bovine DNA, it was found that the binding mechanism did not result in any displacement of methyl green (Table 2). It was further found that clofazimine analogs B1912 and B283 did not displace significant amounts of methyl green. The structurally related basic phenazine dyes, phenosafranine, methylene violet, rhoduline violet and amethyst violet displaced small but progressive amounts of methyl green (Table 2). However, by comparison the known drug intercalators, ethidium bromide, hycanthone, lucanthone, quinacrine, and chloroquine all displaced significantly larger amounts of methyl green presumably due to stretching and local unwinding of the DNA strand (Table 2). The nonintercalative antibiotic, chromomycin A_3 ([24]), did not displace significant amounts of dye in the presence or absence of Mg ions. These data indicate that clofazimine, consistent with earlier data, did not interact with the DNA strand through a

TABLE 2. *Methyl green displaced from preformed DNA-methyl green complex by DNA interacting ligands.*

Ligand (5 × 10⁶M)	Methyl green released (nanomoles/24 hr.)
Clofazimine (B663)	0
B1912	0
B283	10
Phenosafranine	10
Methylene violet	26
Rhoduline violet	35
Amethyst violet	36
Phenazine	0
Ethidium bromide	152
Hycanthone	130
Lucanthone	194
Quinacrine	202
Chloroquine	133
Chromomycin A_3	21
Chromomycin $A_3 + MgCl_2$	47
$MgCl_2$	21

mechanism that was common to other intercalative drugs.

Mutagenesis. Two different types of bacterial growth assays have been used to determine mutagenic potential and possible DNA interaction effects of clofazimine. The histidine auxotrophic strains, TA98 and TA100, of *Salmonella typhimurium* were exposed to 0.2 to 7 μg/ml of clofazimine in the Ames type of assay ([1]). The back mutation rates were determined using an emulsifying agent, 3% (w/v) Cremaphor El, to maintain drug solubilization in the growth medium. The drug was tested both with and without the presence of phenobarbital-induced rat liver microsomes. Preliminary results have indicated that clofazimine did not exert any moderate to potent mutagenic effects as compared to drugs such as niridazole or hycanthone ([13]). Since it was found that clofazimine interacted with *E. coli* DNA (Fig. 3), a second type of bacterial growth assay was carried out to determine if this interaction involved the activity of the excision repair enzyme, DNA polymerase 1 or pol 1. Deficient *pol* A1 mutants of *E. coli* ([8]) did not show any increased drug sensitivity as compared to the parent strain. Thus it was concluded that excision repair processes were not involved with the clofazimine DNA interaction.

DISCUSSION

The clofazimine octanol/water partition coefficient or log p value is calculated from Hansch π values ([16]) to be +7.48. This indicates that the molecule is so lipophilic that its capacity to diffuse across cell membranes is limited. In terms of the "random walk" concept ([11]) the drug would tend to accumulate within the membrane matrix or precipitate on its surface. A log p value of +7.48 suggests that the molecule preferentially enters cells on a carrier molecule by pinocytosis or is phagocytosed as a colloidal particle. The most likely candidate for a carrier molecule is the lipoprotein components of the α- and β-globulin fractions which bind and transport the drug throughout the bloodstream ([5]). It has been suggested that the lipids from the lipoprotein carrier molecule give rise to the black osmiophilic rods or bands that are intimately associated with intracellular crystalline deposits of clofazimine ([17]).

It is evident from this work that clofazimine has the capacity to form stable complexes with anionic macromolecules such as DNA or tRNA, the degree of interaction being dependent upon the guanine base content. Furthermore, a re-investigation of the basicity of clofazimine has recently shown that the molecule has a pK_a of 8.35 ± 0.09 which is more basic than had previously been thought ([20]). Thus in the leprosy host-parasite relationship these facts raise the question as to whether the increased clofazimine interaction with high G + C mycobacterial DNA, possibly involving base sequence specificity, as compared to the reduced interaction with the lower G + C containing human strand may provide a sufficient basis for selective chemotherapeutic effects to emerge. Undoubtedly the very high log p value also has a contributory effect in reducing the concentrations of the drug that can cross the nuclear membrane to interact with human DNA particularly in nondividing cells. However in actively dividing human skin fibroblasts, clofazimine exerts an antimitotic effect ([7]) which may in fact result from interaction with macromolecules such as DNA or tRNA that are more "exposed" either in the nucleus or in the nucleolus during rapid cell division. In a sense it appears that clofazimine is an example of an antileprosy drug, which, in combination with a lipoprotein

carrier, has the capacity to participate in "lysosomotrophic chemotherapy" ([6]). Release of the drug from the carrier molecule due to phagolysosomal digestion would permit it to inhibit the growth of adjacent intracellular mycobacteria such as *M. leprae* by interacting with the parasites' DNA at the replication point ([23]) on the bacterial cell membrane.

The concept that clofazimine exerts its antimicrobial effect through DNA binding and inhibition of template function is not altogether irrelevant to the observation that clofazimine inhibits yeast mitochondrial biogenesis ([22]) since it is likely that this process is regulated by closed circular DNA oligomers detected in *S. cerevisiae* ([10, 12]) and thought to originate from this organelle.

It is proposed that clofazimine binds to DNA along the guanine sequence regions associated with the minor groove. The presence and orientation of the 2-amino group of guanine is likely to have an important influence on the binding. External binding may also involve secondary electrostatic sites on the charged phosphate groups of the phosphodiester backbone. It is evident from space-filling molecular models that the flat planarity of the drug's central phenazine ring (Fig. 1) is obstructed by both the chlorophenyl substituent attached to the ring N as well as the chloroanilino substituent being sterically out-of-plane. Such a structure would not be expected to intercalate between the base pairs of DNA.

The long-range challenge to understanding clofazimine binding mechanisms lies in the future therapeutic need to develop clofazimine structural analogs that are active against the inevitable emergence of clofazimine-resistant mutants of *M. leprae*. If such mutants were to emerge in patients that already harbored sulfone-resistant bacilli a double-resistant mutant would pose a serious problem for successful leprosy chemotherapy.

SUMMARY

1. The antileprosy drug, clofazimine, formed stable complexes with DNA and transfer RNA. A quantitative study was made of the spectral red shifts that occurred when clofazimine interacted with DNA. The red shift appeared specific for clofazimine binding to nucleic acid polymers.

2. The degree of clofazimine interaction with DNA was related to the G + C content of the DNA strand. As compared to the human strand, clofazimine interacted with the mycobacterial strand to give a larger red shift which was consistent with the increased G + C content of mycobacterial DNA.

3. It was found that clofazimine interacted with the synthetic single-standard polynucleotide, poly G, whereas little interaction occurred with poly A, poly C, or poly U. It was concluded that the guanine base region was a predominant site of clofazimine binding to DNA.

4. No evidence was found to indicate that clofazimine underwent intercalative binding between the base pairs of DNA.

5. It was proposed that clofazimine underwent binding along the minor groove region of DNA at appropriate base sequences which contain guanine. The resultant effect would inhibit template function of the DNA strand.

RESUMEN

1. La droga clofazimina, que se utiliza contra la lepra, formó complejos estables con ADN y ARN mensajero. Se hizo un estudio cuantitativo de los desplazamientos en el espectro rojo que se producían cuando la clofazimina interactuaba con el ADN. El desplazamiento rojo parecía ser específico para el enlace de la clofazimina con los polímeros de ácido nucleico.

2. El grado de interacción de la clofazimina con el ADN estaba relacionado con el contenido G + C de la hélice de ADN. Al compararlo con la hélice humana, la interacción de la clofazimina con la hélice de las micobacterias producía un desplazamiento rojo más intenso, que estaba de acuerdo con el mayor contenido de G + C del ADN micobacteriano.

3. Se encontró que la clofazimina interactuaba con el polinucleótido sintético de una sola hélice, Poli G, mientras se observaba escasa interacción con poli A, poli C o poli U. Se concluyó que la región de base de guanina era un sitio predominante de enlace de la clofazimina al ADN.

4. No se encontró evidencia que la clofazimina se enlarzara en forma intercalada entre los pares de bases de ADN.

5. Se propone que la clofazimina se enlaza a lo largo de la región del surco menor de ADN en las secuencias de bases apropiadas que contienen guanina. El efecto resultante inhibiría la función patrón de la hélice de ADN.

RÉSUMÉ

1. Un médicament antilépreux, la clofazimine, a formé des complexes stables avec l'ADN et l'ARN de transfert. Une étude quantitative a été menée pour étudier le décalage du spectre dans la gamme du rouge qui se produit lorsque la clofazimine réagit avec l'ADN. Une raie rouge semble spécifique pour la clofazimine liée aux polymères d'acide nucléique.

2. L'importance de l'interaction entre la clofazimine et l'ADN est liée au contenu G + C de la chaîne d'ADN. Comparée à la chaîne humaine d'ADN, la clofazimine a réagi avec la chaîne d'ADN mycobactérien en produisant un décalage vers le rouge plus important, qui correspondait à une quantité accrue de G + C dans l'ADN mycobactérien.

3. On a observé que la clofazimine réagissait avec le poly G, polynucléotide standard synthétique unique, tandis qu'elle ne réagissait que faiblement avec le poly A, le poly C, ou le poly U. On en a conclu que la région où se trouve la base guanine était un site privilégié pour la liaison de la clofazimine à l'ADN.

4. Aucune évidence n'a été relevée qui pourrait suggérer que la clofazimine est impliquée dans une lésion intercalaire avec les bases couplées de l'ADN.

5. On suggère que la clofazimine est soumise à une liaison au niveau de la région mineure (groove region) de l'ADN, au niveau approprié de séquences de bases contenant de la guanine. L'effet en serait dès lors une inhibition de la fonction de templat de la chaîne d'ADN.

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