ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1976, p. 77-80
Vol. 9, No. 1

Printed in U.S.A.

# Elimination of Resistance Determinants from R-Factor R1 by Intercalative Compounds

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Received for publication 10 June 1975

Eighteen deoxyribonucleic acid (DNA)-complexing compounds, among them 15 intercalative substances, and, additionally, nalidixic acid eliminated with different frequencies four antibiotic resistance determinants from the R-factor R1, carried by *Salmonella typhimurium*. Eliminating concentrations did not inhibit growth of the bacteria. The most active compound was "nitroacridine II" {1-diethylamino-3-[(6-nitro-9-acridinyl)amino]propanol}. When 14 compounds which had been tested at a standard concentration of $10^{-4}$ M were listed according to decreasing activities of elimination of the four resistance determinants, a nearly consistent activity sequence was revealed. Frequencies of elimination of the kanamycin resistance determinant correlated directly with the binding of compounds to DNA, i.e., with end points of their displacement of methyl green from the methyl green-DNA complex. We propose that the observed eliminations resulted from selective toxicity for plasmidic template DNA and inhibitions of R-factor replication.

During the past 15 years, a considerable literature has been published on the elimination of bacterial R-factors by a variety of chemical agents; this has recently been reviewed (13). We noted early (J. Ciak, S. Wormley, and F. E. Hahn, Bacteriol. Proc., p. 67, 1969) that among bacterial plasmid eliminating chemicals one category was comprised of substances that bind to deoxyribonucleic acid (DNA) by intercalation (15, 20, 30, 31). After it had been shown that R-factors chemically are DNA (11) and that bacteria from which all resistance determinants had been eliminated no longer contained R-factor DNA (8, 25), we assumed that intercalative eliminators of R-factors act by selective complexation with plasmid DNAs and, thereby, selectively inhibit their replication.

We postulated, therefore, that the capability of eliminating plasmids from bacteria is a group property of intercalating compounds and tested this hypothesis for the elimination of *lac* from F'*lac* and of R-factor R1 in strains of *Escherichia coli* (14). Tests of our hypothesis were subsequently carried out by others with the elimination of a nontransferable penicillin resistance plasmid in *Staphylococcus aureus* (3). The results of our work (14) were suggestive of the validity of our hypothesis, but we did not consider them conclusive because, among the five resistance determinants of R1, those for streptomycin and sulfonamide consistently segregated with the bacteria, i.e., were not eliminated.

For a conclusive test, we transferred the R-factor R1 from *E. coli* into *Salmonella typhimurium* LT-2, because salmonellae accommodate more frequent spontaneous (19) or chemically induced (5) eliminations of R-factors, and studied the eliminations of resistance determinants by a series of DNA-complexing compounds and by nalidixic acid. The results reported here support our hypothesis that inhibitors of DNA biosynthesis, foremost intercalative compounds as a group, eliminate resistance determinants at concentrations that permit growth and replication of R-factor-carrying bacteria.

## MATERIALS AND METHODS

**Bacterial strains and plasmid.** The R-factor in this study was R1, harbored by *E. coli* RS-2, a proline auxotroph (26). These bacteria had been used in previous studies in this laboratory (14). *S. typhimurium* LT-2 was obtained from the Department of Bacterial Immunology of our Institute.

**Media.** Complex media were antibiotic medium no. 3 (Penassay broth, Difco), tryptone broth (Difco), and meat extract agar. The minimal salts-glucose medium was prepared with Noble agar base (Difco) supplied with 50 μg of kanamycin per ml.

**Eliminating agents.** Nitroakridin 3582, nitroacridine II, and Hoechst 33258 (a substituted dibenzimidazole) were gifts from Farbwerke Hoechst, Frankfurt am Main, Germany, and tilorone was a gift from P. Chandra, University of Frankfurt, Germany. All other chemicals (Tables 1 and 2) were commercial preparations.

**R1 transfer.** The donor, *E. coli* RS-2, was grown

TABLE 1. *Percentages of elimination of resistance determinants from R1 in S. typhimurium*

| Compounds | Concn (M) | Kanamycin | Chloramphenicol | Streptomycin | Ampicillin |
|---|---|---|---|---|---|
| Nalidixic acid | $6.25 \times 10^{-6}$ | 70 | 56 | 60 | 64 |
| Nitroacridine II | $2.5 \times 10^{-5}$ | 99 | 96 | 96 | 97 |
| Daunomycin | $2.5 \times 10^{-5}$ | 86 | 80 | 77 | 81 |
| Proflavine | $2.5 \times 10^{-5}$ | 86 | 79 | 64 | 68 |
| Nitroakridin 3582 | $5 \times 10^{-5}$ | 82 | 69 | 70 | 69 |

TABLE 2. *Percentages of elimination of resistance determinants from R1 in S. typhimurium*

| Compounds at $10^{-4}$ M | Kanamycin | Chloramphenicol | Streptomycin | Ampicillin |
|---|---|---|---|---|
| Ethidium | 96 | 82 | 84 | 82 |
| Miracil D | 91 | 82 | 81 | 82 |
| Quinacrine | 92 | 79 | 81 | 71 |
| Propidium | 89 | 78 | 71 | 71 |
| Tilorone | 85 | 80 | 74 | 78 |
| *p*-Rosaniline | 87 | 71 | 73 | 66 |
| Acridine orange | 85 | 71 | 69 | 68 |
| Berberine | 83 | 68 | 70 | 62 |
| Quinine | 81 | 60 | 66 | 57 |
| Spermine | 81 | 56 | 71 | 58 |
| Chlorpromazine | 78 | 63 | 67 | 72 |
| Hoechst 33258 | 72 | 64 | 64 | 67 |
| Chloroquine | 80 | 58 | 52 | 53 |
| Methylene blue | 68 | 50 | 48 | 41 |

in Penassay broth for 18 h at 37 C. The recipient, *S. typhimurium* LT-2, was streaked on meat extract agar plates and harvested after 18 h of growth at 37 C. Bacteria were washed once and suspended in a 0.9% sodium chloride solution. The donor suspension was diluted 1:10, 1:100, and 1:1,000. Combinations of equal volumes of the three dilutions of the donor and the recipient suspensions were plated on minimal medium agar deficient in proline and containing 50 $\mu$g of kanamycin per ml. These plates were incubated at 37 C until colonies appeared. Bacteria from these colonies were inoculated onto meat extract agar, and colonies from this cultural passage were suspended in tryptone broth and tested for multiple antibiotic resistance on plates containing kanamycin, chloramphenicol, ampicillin, or streptomycin. Finally, the organism was serologically identified using *Salmonella* polyvalent antisera.

**Elimination experiments.** Stock cultures were maintained on meat extract agar containing either kanamycin or chloramphenicol. From these stock cultures, a MacConkey agar plate was streaked and incubated overnight. Bacteria from this plate were then inoculated into Penassay broth and incubated for 18 h. This liquid overnight culture was diluted by a factor of $10^4$ in Penassay broth and used as an inoculum for the elimination experiments, using our published method (14), which is a modification of the technique of Hirota (15). Frequencies of elimination of resistance determinants were determined as described previously (14).

## RESULTS

The R-factor R1 (26) used in this work carries determinants of resistance to kanamycin, chloramphenicol, ampicillin, streptomycin, and sulfonamide (14). Since our strain of *S. typhimurium* was unsusceptible to sulfadiazine prior to the transfer of R1, the present elimination studies were restricted to the four determinants of antibiotic resistance.

The standard concentration of DNA-complexing compounds in whose presence the test bacteria were grown was $10^{-4}$ M. However, a few substances were strongly growth inhibitory and, therefore, had to be studied at lower concentrations. Results with these compounds were listed in Table 1.

The most active compound was nitroacridine II which is a bis-demethoxy congener of Nitroakridin 3582. It eliminated, at $2.5 \times 10^{-5}$ M, all four resistance determinants with a frequency of 96%. Nalidixic acid, which inhibits DNA biosynthesis in gram-negative bacteria by an unknown mechanism, not involving drug binding to the DNA template (12), could only be used at $6.25 \times 10^{-6}$ M without inhibiting the growth of *S. typhimurium*; at this low concentration, nalidixic acid was moderately active in eliminating the four antibiotic resistance determinants of the R-factor.

Table 2 lists results obtained with compounds used at a standard concentration of $10^{-4}$ M. Hence, the data can be directly compared. All listed substances are known to form complexes with DNA. Hoechst 33258 does not bind to DNA by intercalation (4, 21). This type of binding is probably excluded for *p*-rosaniline and is, of course, not possible for spermine on structural grounds. The other listed substances are intercalators.

All compounds listed in Table 2 eliminated all four resistance determinants. The kanamycin determinant was invariably eliminated at higher frequency than the other three determinants. Preferential elimination of the kanamycin determinant from R-factor R11 in *Salmonella panama* has been previously reported for acriflavin (1) and ethidium bromide (5).

Compounds have been listed in Table 2 according to decreasing activities. This revealed

the existence of a hierarchical activity order with only a few inversions in the activity sequences, which may not all be statistically significant. We considered this to be evidence that a systematic effect had been studied and assumed that it reflected the abilities of the tested compounds to form complexes with DNA.

This assumption is borne out by Fig. 1. Here the percentages of frequency of the elimination of the kanamycin determinant are represented as a function of the end points of the displacement of methyl green from its complex with calf thymus DNA. A detailed study of this complex and of its dissociation by drugs has been completed in this laboratory by Krey and Hahn and is being published elsewhere. The correlation between the elimination of the kanamycin determinant and the methyl green-displacing ability of the eliminating compounds suggests that the measured eliminations are consequences of the formation of drug-DNA complexes.

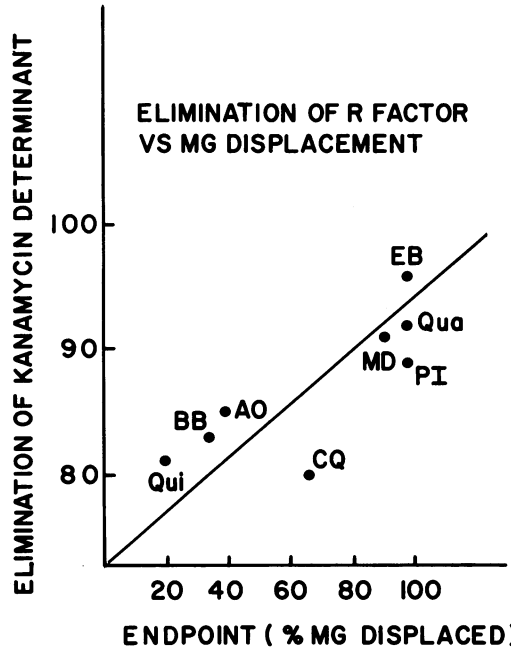


FIG. 1. *Frequencies of elimination of the kanamycin determinant from R1 in S. typhimurium LT-2 by intercalants as a function of end points of displacement of methyl green from its complex with DNA. Eliminating concentrations, $10^{-4}$ M; displacing concentrations, $5 \times 10^{-5}$ M. EB, Ethidium bromide; Qua, quinacrine; MD, miracil D; PI, propidium iodide; CQ, chloroquine; AO, acridine orange; BB, berberine; Qui, quinine.*

## DISCUSSION

The conclusion from our work is that DNA-complexing compounds, foremost those which bind to DNA by intercalation, have the group property of eliminating resistance determinants from bacterial R-factors. Eliminations of genetic markers of R-factors probably are direct results of inhibitions of plasmidic DNA replicons owing to the DNA-template toxicity of intercalators. Such template toxicities have, indeed, been demonstrated for daunomycin (10), proflavin (17), Nitroakridin 3582 (32), ethidium (29), miracil D (16), quinacrine (23), tilorone (6), quinine (23), and chloroquine (23).

Jacob et al. (18) noted the sensitivity of the replication of the F-plasmid to acridines to be greater than the sensitivity of the replication of *E. coli* to these compounds; our results are based upon an analogous sensitivity difference between plasmid and bacterial replication. The molecular basis of the selective toxicity of intercalants for plasmids may be the following conditions. Plasmid DNAs are circular supercoiled (7, 22), and DNAs of this conformation have a greater affinity for intercalative compounds (2) and are also subject to stronger template poisoning by intercalants than unconstrained DNAs (24). Progressive intercalation into supercoiled DNA produces first an unwinding of supercoils until the DNA becomes unconstrained circular, followed by upwinding into a supercoiled structure which owes its formation to the introduction of an excess of helical turns (2, 9). For substances tested in our work, such conformational transitions in supercoiled DNA have been demonstrated by Waring (28) with ethidium, proflavin, daunomycin, propidium, hycanthone (a miracil D congener), and chloroquine; in our laboratory we have made the same demonstrations for quinacrine, acridine orange, methylene blue, chlorpromazine, and quinine.

We propose, therefore, that the selective toxicity of intercalants for plasmid replication results from the greater affinity of such compounds for plasmidic supercoiled DNA and from the greater toxicity for supercoiled DNA templates, as compared with unconstrained DNA (24).

Considering the selective effect of nalidixic acid (Table 1) and anionic detergents (27) on plasmid replication, it is possible that these substances interfere with the attachment of plasmidic replicons to their replication sites on the bacterial membrane.

Finally, spermine, which stabilizes duplex DNA to strand separation, has been shown to inhibit the DNA-dependent DNA polymerase I reaction (22).

ACKNOWLEDGMENT

We wish to thank Louis S. Baron of our Institute for his helpful advice concerning the method of transferring R1 from *E. coli* to *S. typhimurium*.

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