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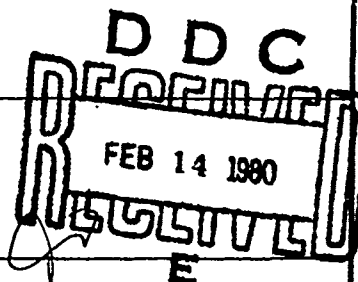
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Chemotherapy of Bacterial Plasmids

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Pharmacological approaches to plasmid chemotherapy are: 1) discovery of novel antibacterial drugs against which plasmids do not carry resistance genes, 2) molecular modification of existing drugs to render them non-susceptible to plasmid-encoded enzymes, 3) development of drugs which are selective inhibitors of plasmid DNA replication, 4) development of drugs which inhibit phenotypic expression of plasmid genes, and 5) development of drugs which are inhibitors of drug-inactivating enzymes that are plasmid gene products.

Bacterial plasmids are extrachromosomal, autonomous genetic entities which can be isolated as circular supercoiled duplex DNA. They are transmitted, especially among Gram-negative bacteria (Table 1), by conjugation without restrictions of bacterial taxonomy. R-plasmids possess one series of genes which determine the components of the bacterial conjugation system. In addition, they carry multiplicities of resistance genes whose products mediate bacterial resistance to a corresponding multiplicity of chemotherapeutic drugs, antibiotic and synthetic.

This essay presents a review of an emerging field. A practical chemotherapy of bacteria which harbor drug-resistance plasmids (R-plasmids) exists currently only in the trivial sense of administering antibiotics or synthetic drugs against which a given plasmid does not confer resistance. The subject of this article is the scientific basis of restoring chemotherapeutic drug sensitivity to bacteria which owe their drug resistance to plasmidic determinants.

Bacterial multiresistance to chemotherapeutic drugs, determined by R-plasmids, causes treatment failures of hospital infections, foremost in patients with a compromised immune system. The Deputy Assistant Secretary of Health of the United States reported in

1974 [1] that among 30 million acute hospital admissions per year, there occurred 300 000 infections with Gram-negative bacteria in which 100 000 fatalities were caused by bacteremia. Finland and Barnes [2] tabulated data on the incidence of Gram-negative bacteremia (per 1000 admissions) in a large city hospital: In 1935, before the introduction of bacterial chemotherapy, the incidence was 0.9 and the case fatality was 50%. From 1951, the incidence steadily increased to attain 8.7 in 1969 with a case fatality not different from that of the pre-chemotherapy era. By 1972, the fatality had risen to 57% [14].

R-plasmid-harboring bacteria also have caused large epidemics in populations at large. The first of these was a protracted epidemic of bacillary dysentery, caused by strains of *Shigella*, in Japan in the late 1950s. This led to the discovery that multiple drug resistance can be transferred among Gram-negative bacteria upon mixed cultivation. This history of the discovery of "R-factors" is summarized in Table 1 [3]. In 1969 and the following years, a devastating epidemic of shigellosis occurred in Central America [4] which involved more than 150 000 cases and, in Guatemala (a country of 5.6 million inhabitants) alone, caused 12 500 fatalities. "An enormous epidemic of typhoid fever has been raging in Mexico [5]" for several years which began in 1972 and abated in 1975. It was caused by a chloramphenicol-resistant strain of *Salmonella typhi*, also resistant to streptomycin, sulfonamides and tetracycline, and produced in excess of 10 000 reported cases [6] with an initial case fatality of 13.5% [5]. Coincidentally, an epidemic of chloramphenicol-resistant typhoid fever appeared in India in 1972; the causative strain was different from the Mexican strain [7]. More recent problems of plasmid-mediated drug resistance are the emergence of strains of *Haemophilus influenzae*, resistant to ampicillin [8] or chloramphenicol [9] and of *Neisseria gonorrhoeae*, resistant to penicillins [10].

R-plasmids have been found in bacteria which were

80 2 6 085

Table 1 Discovery of R-factors

Discovery	Point in time	Authors
Drug resistance is transferable by mixed cultivation	1959, November 1959, November	Ochiai et al. Akiba et al.
Transfer of drug resistance is not mediated by filtrable agents	1960, January 1960	Mitsuhashi et al. Akiba et al.
Drug resistance is transmitted regardless of the polarity of F agent	1960, January	Mitsuhashi et al.
Transferable drug resistance property is lost spontaneously during storage	1960, March	Mitsuhashi et al.
Transferable drug resistance property is eliminated by treatment with acriflavine	1960, June	Mitsuhashi et al. Fukasawa, Watanabe
Transmission of drug resistance is interrupted by blender treatment of mixed cultures	1960	Fukasawa, Watanabe
The term "R-factor" is introduced for the transferable resistance property	1960	Mitsuhashi
The range of transmission includes all species of the		
Enterobacteriaceae	1960	Harada et al.
<i>Vibrio comma</i>	1961	Baron, Falkow
<i>Pasteurella pestis</i>	1963	Ginoza, Matney
<i>Pseudomonas aeruginosa</i>	1971	Roe et al., Iyobe et al.
<i>Aeromonas</i> group	1972	Aoki et al.
<i>Bordetella bronchiseptica</i>	1973	Terakado et al.

isolated and lyophilized before antibiotic therapy became prevalent. One strain of *Escherichia coli*, exhibiting transferable resistance to streptomycin, tetracycline and bluensomycin, had been isolated prior to 1937 and lyophilized in 1946 [11]. Streptomycin was marketed from 1947, and tetracycline and bluensomycin had not been discovered in 1946. While R-plasmids, thus, have not arisen de novo in response to bacterial exposure to chemotherapeutic drugs, multiresistance as a global medical problem has been generated by the selective pressure of antibiotic usage, either in hospital practice or at large in societies with medically uncontrolled availability of antibiotics. One of the reservoirs of R-plasmids are farm animals, raised on antibiotics-supplemented feed [12]. Between 1960 and 1970, the production of antibiotics as feed-additives in the United States increased from 0.5 to 3.3 million kg per year [1].

There has been no lack of suggestions to restrict the medical use of antibiotics, topically, prophylactically, or without clearcut bacteriological rationale. Likewise, the agricultural use of antibiotics in animal husbandry has come under criticism. Unfortunately, there exists a perturbing trend of R-plasmid-harboring bacteria to persist, for example in the intestinal tract of individuals no longer receiving antibiotics [13]. R-plasmid-containing bacteria in antibiotic-fed farm animals likewise persist after the administration

of antibiotics is discontinued [14]. While particular ecological or genetic conditions may be responsible for observations of R-plasmid persistence in the human or animal bacterial flora [13, 15], this does not detract from the practical importance of the persistence phenomenon. The multiresistance problem, once created by extended and excessive drug pressure, may not disappear readily under limited efforts to lessen this pressure.

Pharmacological Approaches

The practical solution of the multiresistance problem should be the discovery and development of pharmacological approaches, i.e., of a chemotherapy of bacterial R-plasmids. The feasibility of anti-plasmid chemotherapy is being disputed (e.g., [16]). The current situation is comparable to that which has preceded most major advances in chemotherapy. Even 10 years after the introduction of salvarsan into practical medicine, the selective chemotherapeutic action of this drug as well as the basic theory of chemotherapy were questioned [1], and the advent of antibacterial and antiviral drugs [17] was preceded by the expressions of opinions that such drugs could not exist because of the prohibitively small size of their metabolic targets.

An examination of the prospects of R-plasmid chemotherapy must be based on a definition of its aims. A prophylactic approach in which an anti-plasmid drug would be administered, for example, to an entire hospital population would be unrealistic. The need is for *curative* compounds which can be administered to patients, suffering from infections with multiresistant bacteria, in order to resensitize such pathogens to conventional chemotherapeutic agents, either preceding antibacterial chemotherapy or, ideally, in combination with antibacterial drugs. The problem of the stabilization of R-plasmids in their host bacteria when these are grown in the presence of antibiotics for which the plasmids contain resistance determinants, has been cited [18, 19]. Such a selection of plasmids or of individual resistance genes [18, 19] will depend upon the mechanism of action of the anti-plasmid compound as well as that of the chemotherapeutic drug in combination. Nalidixic acid removed seven out of ten resistance genes from a plasmid, carried by *Klebsiella pneumoniae*, including that for nalidixic acid itself [20]. That plasmid resistance genes may "retreat to the safety of the chromosome [16]" by transposition under the pressure of combination chemotherapy has not been demonstrated.

I shall consider the following pharmacological approaches to plasmid chemotherapy:

- 1) Discovery and development of novel antibacterial drugs in the hope that the global plasmid population will not possess resistance genes against them.
- 2) Molecular modification of existing drugs in order to render them insensitive to attack by enzymes which are plasmid gene products.
- 3) Development of drugs which are selective inhibitors of plasmid DNA replication.
- 4) Development of drugs which selectively inhibit the phenotypic expression of plasmid genes, i.e., transcription and translation.
- 5) Development of inhibitors of those enzymes which are plasmid gene products and biochemically mediate drug resistance.

Novel Antibacterial Drugs

The phosphoglycolipid antibiotics, moenomycin (flavomycin) and macarbomycin inhibit the biosynthesis of the bacterial cell-wall peptidoglycan [21, 22] by interfering with the transfer of the lipid-bound precursor to the growing peptidoglycan [23]. Macarbomycin in limited studies [24] and flavomycin in extensive work [25] have been shown to inhibit *preferentially* bacteria which carry R-plasmids. Flavomycin is useful as a feed-additive in farm animals *in lieu* of ad

clinical antibiotics to their nutrition [23]. The reasons for the hypersensitivity of R⁺ bacteria to these antibiotics are not known. It is possible that the attachment of plasmids to a membrane fraction renders plasmid⁺ bacteria more sensitive to the actions of flavomycin or macarbomycin than bacteria which have unoccupied membranes.

Earlier, Kawakami and Landman had shown [26] that conversion of a strain of *Salmonella paratyphi* to stable L-forms by high concentrations of penicillin eliminated in a segregative manner resistance determinants from the R-plasmid R11 which had been transferred into these bacteria. The authors speculated that the conversion of the bacteria into spheroplasts eliminated not only the cell wall but also the mesosome fraction of the membrane which they proposed to be a potential attachment site of the plasmid.

A general use of flavomycin as feed-additive might reduce the size of the R-plasmid carrying bacterial pool in farm animals and certainly would stop the selective pressure of clinical antibiotics on this bacterial flora. It is not possible to predict whether or not such widespread use of flavomycin would ultimately lead to the emergence of plasmids with resistance genes for flavomycin.

Molecular Modification of Drugs

Certain enzymes that are plasmid gene products attack antibiotics by derivatization such as the acetylation of the two hydroxyl groups of chloramphenicol [27] or adenylation, acetylation or phosphorylation of the hydroxyl or amino groups of aminoglycoside antibiotics. While acetylation of chloramphenicol proceeds to inactivation of all the antibiotic present, the derivatization of the aminoglycosides is restricted in its extent, and the role of the derivatives in causing bacterial drug resistance is problematic [28].

An obvious pharmacological approach to overcoming resistance to those antibiotics which are derivatized by plasmid gene products would be molecular modification of the antibiotic molecules to delete those functional groups which are derivatized unless such deletion also causes loss of antibacterial activity.

One such experiment was performed inadvertently through the discovery of gentamicin. This aminoglycoside, first marketed in 1966, shows remarkable curative properties against bacterial infections of burn patients, septicemia, meningitis, and urinary-tract infections. Waitz and Weinstein [29] called attention to the absence of a hydroxyl group in the 3-position of the methyl-aminosugar moiety of gentamicin and suggested that this rendered the antibiotic non-susceptible to derivatization by plasmid gene products.

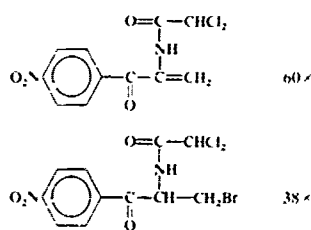


Fig. 1. Structure of chloramphenicol derivatives, lacking aliphatic hydroxyl groups [33]

However, one year after the publication [29], R-plasmid-mediated gentamicin resistance appeared. The biochemical basis of this resistance is N-acetylation, or O-adenylylation of other hydroxyl groups of the antibiotic [30].

The two hydroxyl groups of the propanediol moiety of chloramphenicol were considered indispensable for antibiotic activity [31, 32]. However, Kono and his associates [33] synthesized the two chloramphenicol derivatives (Fig. 1) which lack both hydroxyl groups. They are as active as chloramphenicol itself against sensitive bacteria and exceed the activity of the drug against resistant *Staphylococcus aureus* by factors of 60 and 38.

The overlapping substrate ranges [28] of the approximately 12 known aminoglycoside-derivatizing enzymes as well as synthetic-chemical problems would discourage efforts at molecular modifications, but the work of Kono et al. [33] suggests that congeners of chloramphenicol can be developed against which plasmid-determined resistance would be an unlikely prospect and which also might carry a lesser risk of inducing aplastic anemia, e.g., by replacing the aromatic nitro group with $-SO_3-CH_3$ [34].

Inhibitors of Plasmid DNA Replication

Growing cultures of plasmid-containing bacteria in broth media, containing aminoacridines at concentrations which were not growth-inhibitory, eliminated genetic [35] and phenotypic [19] indications of the functions of plasmids as well as biophysical evidence of the presence of plasmid DNAs [36, 37]. Hahn and his associates postulated, tested and showed that substances which bind to DNA by intercalation, possess the group property of eliminating plasmids from growing bacterial cultures [19, 38, 39]. In an unfortunate choice of expression, this activity has become known as "curing." Kinetic experiments have shown that plasmids were not literally eliminated from their host bacteria, but that "curing" compounds block selectively the replication of plasmid DNA. The non-segregation of plasmids into daughter cells produced

a kinetic course of plasmid loss from test cultures which resembled the growth curve of the plasmid⁺ progeny [40, 41]: the original plasmid⁺ population became progressively diluted with plasmid⁻ cells.

A few authors [42-44] have suggested that R-plasmid⁺ bacteria are more sensitive to growth inhibition by intercalants than the same organisms not carrying plasmids. An apparent elimination of plasmids from growing cultures would then, in actuality, be the result of a selective propagation of spontaneous plasmid⁻ segregants by differential growth inhibition. Such considerations cannot well explain numerous observations (rev. in [19]) of selective elimination of individual resistance genes from plasmids. For example, the kanamycin resistance gene is most easily eliminated by intercalants, and the frequency of this occurrence is a function of the relative stoichiometry of binding of these compounds to DNA (Fig. 2) [38]. The selective inhibition of R-plasmid DNA replication may be based upon the fact that circular supercoiled DNA has a higher affinity for intercalants than unconstrained DNA [45] and also is subject to stronger template inhibition by such compounds [46].

Among intercalative anti-plasmid compounds are the clinical drugs, daunomycin, quinacrine, quinine, chlorpromazine and chloroquine. However, there exist several arguments against their practical application as plasmidostatic drugs. Elimination of plasmids by these drugs in vitro does not attain 100% in overnight cultures, and such experiments require small inocula of the order of 10^3 bacteria per ml. The preferential suppression of R-plasmid⁺ bacteria by flavomycin was accomplished in continuous culture over a period of 5 days [25] but extensive efforts in the laboratory of this writer failed to maintain in a chemostat low bacterial titers of the order of 10^3 organisms per ml.

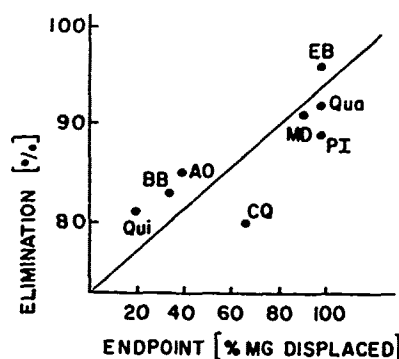


Fig. 2. Frequencies of elimination of the kanamycin determinant from the R-plasmid R1 in *Salmonella typhimurium* LL-2 by intercalants as a function of end points of displacement of methyl green from its complex with DNA. Eliminating concentrations 10^{-4} M; displacing concentrations 5×10^{-5} M. EB ethidium bromide; Qua quinacrine; MD miracid; PI propidium iodide; CQ chloroquine; AO acridine orange; BB berberine; Qui quinine [38]

Hence, a computer simulation of progressive R-plasmid loss in a continuous-growth system over prolonged incubation periods could not be tested. One hundred per cent plasmid elimination from strains of *Salmonella* was accomplished in vivo in mice but required treatment with ethidium bromide for one week [47].

Finally, there exist unexplained taxonomic differences among both plasmids and host bacteria as concerns susceptibility to plasmid elimination by intercalants [19, 44]. For these various reasons, it remains uncertain if an effective broad-spectrum plasmidostatic agent can be developed on the basis of the DNA intercalation principle.

A recently discovered bacterial enzyme system, DNA gyrase [48], catalyzes the ATP-dependent introduction of negative superhelical turns into double-stranded circular DNA. This activity is required for the replication of plasmids [49, 50]. One subunit of the enzyme is responsible for nicking and closing of circular DNA and is inhibited by nalidixic acid [51]. The second subunit introduces negative supercoiling at the expense of ATP hydrolysis and is inhibited by the antibiotic, novobiocin [52].

Empirical tests of the elimination of R-plasmids by nalidixic acid were based upon earlier knowledge that the drug operationally is an inhibitor of bacterial DNA biosynthesis (rev. in [53]). At a concentration of $6.25 \times 10^{-6} M$ which did not inhibit the growth of *Salmonella typhimurium*, nalidixic acid eliminated

all four antibiotic resistance determinants from the R-plasmid R1 with frequencies between 56 and 70% [19]. It is not known if this anti-plasmid effect of nalidixic acid was a result of an inhibition of plasmid DNA replication or of a selective inhibition of plasmid mRNA transcription [54]: it had been reported earlier [55] that nalidixic acid inhibited the synthesis of plasmid mRNA at concentrations which did not affect bacterial mRNA synthesis.

The anti-plasmid action of sodium dodecyl sulfate (SDS) and other anionic detergents of a chain length of > 10 carbon atoms (rev. in [19]) is probably related to the DNA nicking action of DNA gyrase [51]. SDS promotes double-stranded cleavage of circular DNA by the gyrase and an attachment of the denatured enzyme to the DNA by covalent bonds [52].

The antibiotic, novobiocin (Fig. 3) inhibits the ATP-dependent introduction of negative supercoils into circular DNA by blocking the binding of ATP to the DNA gyrase [52]. An anti-plasmid action of novobiocin has been investigated by McHugh and Swartz [56, 57]. The antibiotic eliminated 8 out of 14 plasmids

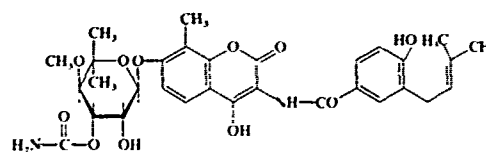
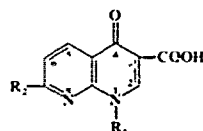


Fig. 3. Structure of novobiocin

Table 2. Effect of 1- and 7-position substitution of 1,8-naphthyridines on antibacterial activity



R ₁ (R ₂ = CH ₃)	Relative activity		R ₂ (R ₁ = C ₂ H ₅)	Relative activity	
	in vitro ^a	in vivo ^b		in vitro ^a	in vivo ^b
H	○	○	H	○	++
CH ₃	○	++	CH ₃ (NAL)	++++	++++
C ₂ H ₅ (NAL)	++++	++++	C ₂ H ₅	+++	+++
C ₃ H ₇	+++	+++	C ₃ H ₇	+++	○
i-C ₃ H ₇	+	+	i-C ₃ H ₇	+++	○
CH ₂ CH=CH ₂	+++	++	CH ₂ C ₆ H ₅	+	○
C ₆ H ₅	○	○	CH ₂ CN	+++	++
CH ₂ CF ₃	++++	++	COOH	○	○
CH ₂ COOH	○	○	CH ₂ OH (hydroxy-NAL)	++++	++++
CH ₂ CH ₂ OH	++++	++			
CH ₂ CH ₂ N(C ₂ H ₅)	○	○			
CH ₂ OCH ₃	+	++			

^a Minimal inhibitory concentration against either *Salmonella typhi* or *Escherichia coli* where ○ = ≥ 100 ; + = ≥ 50 ; ++ = ≥ 10 ; +++ = ≥ 5 ; and ++++ = $< 5 \mu\text{g/ml}$

^b Oral activity (ED₅₀) vs. lethal *Klebsiella pneumoniae* infection in mice where ○ = > 300 ; + = 300–200; ++ = 200–100; +++ = 100–50; ++++ = $< 50 \text{ mg/kg/day}$

in a series of bacterial strains, but the concentrations required were growth-inhibitory for the host bacteria. By agarose-gel electrophoresis it was shown that bacteria which no longer expressed drug resistance, no longer contained plasmid DNA [56]. Authors asked the question if novobiocin-insensitive plasmids possess specific DNA gyrase systems. The elimination of plasmids from 50% of clinical isolates of *E. coli* [57] appeared to be a plasmid-related sensitivity to novobiocin: it succeeded in novobiocin-resistant bacterial mutants.

Structure-activity data for nalidixic acid have been tabulated only for bacteria (Table 2) [53]. It would be logical, to develop such information for the anti-plasmid activities of nalidixic acid congeners in the expectation to detect compounds with selective effects on plasmid-associated DNA gyrases. The structural reasons of why novobiocin inhibits the DNA gyrase reaction in competition with ATP are not apparent [52]. The antibiotic is not known to affect any other ATP-requiring enzyme at low concentrations. The structure of novobiocin (Fig. 3) offers no obvious clue to the nature of the competition with ATP nor to molecular modification with a view to broadening the anti-R-plasmid spectrum of the antibiotic.

Drugs which Inhibit the Phenotypic Expression of Plasmid Genes

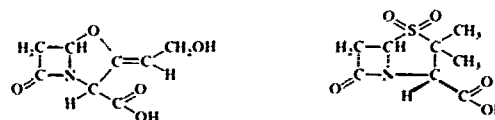
Drugs which inhibit the transcription of mRNA from DNA and its translation into proteins should inhibit the phenotypic expression of plasmid genes. But there are few biochemical indications that transcription or translation from plasmid genes differ from the corresponding bacterial processes. The possibility that nalidixic acid may function via inhibition of specific gyrase systems as a selective transcription inhibitor for plasmids [55] has been cited above. Rifampicin, an antibiotic which inhibits bacterial RNA polymerase, exhibits certain anti-plasmid actions (rev. in [19]). However, these might be attributed to an inhibition of the synthesis of a primer RNA which appears to be required to initiate plasmid DNA replication [19]. The antibiotic had no anti-plasmid effects in bacterial mutants, resistant to rifampicin [41].

Observations that the 4s and 5s RNA populations of a strain of *E. coli* which harbored the R-plasmid R1, contained components which hybridized with the plasmid DNA [58] have prompted a speculation that this plasmid may generate and utilize "a modified translation system". No mechanistic evidence of the operation of such a system is available which might conceivably provide a point of departure into the development of selective inhibitors of the biosynthesis of plasmid gene products.

In several strains of *Salmonella*, carrying an R-plasmid with resistance determinants for tetracycline, chloramphenicol, kanamycin and streptomycin, subculturing in the presence of tetracycline, a translation inhibitor, for 5 serial passages resulted in the complete loss of all resistance determinants, except that for tetracycline itself [59]. Since this phenomenon was restricted to *Salmonella* but was not observed with the same R-plasmid in *E. coli*, *Klebsiella*, *Proteus* or *Pseudomonas*, it was evidently not due to the inhibition of a plasmid-specific translation system.

Inhibitors of Drug-inactivating Enzymes that Are Plasmid Gene Products

Overcoming R-plasmid-mediated resistance through the use of compounds which inhibit plasmid gene products (enzymes) is currently that area of plasmid chemotherapy which appears closest to practical realization. This is the case for R-plasmids whose gene products are β -lactamases which destroy penicillins and cephalosporins through hydrolysis, i.e., not by derivatization but by degradation. Among such β -lactamase inhibitors are the empirically discovered biological product, clavulanic acid [60] from *Streptomyces clavuligerus*, and a semisynthetic penicillanic acid sulfone, designated as CP-45,899 [61] (Fig. 4). Additional β -lactamase inhibitors of natural origin and undisclosed structures have been cited in the literature [60, 62, 63]. Clavulanic acid, generally, has only



Clavulanic acid

CP-45,899

Fig. 4. Structures of clavulanic acid and of CP-45,899 [60, 61]

Table 3. β -Lactamase inhibitory activity of sodium clavulanate

Source of β -lactamase	Substrate*	I_{50} [μ g/ml]
<i>Pseudomonas aeruginosa</i> (Sabath type)	C	160
<i>Escherichia coli</i> JT410 chromosomally encoded	C	56
<i>Enterobacter cloacae</i> P99	C	10.0
<i>Klebsiella aerogenes</i> NCTC 418	P	0.03
<i>Escherichia coli</i> JT4, R-plasmid encoded	P	0.08
<i>Escherichia coli</i> JT39, R-plasmid encoded	P	0.08
<i>Proteus mirabilis</i> C889	P	0.03
<i>Pseudomonas aeruginosa</i> Dalglish (preparation contained some Sabath-type enzyme)	P	0.1
<i>Staphylococcus aureus</i> Russell	P	0.06
<i>Bacillus cereus</i> (Whatman Biochemicals Ltd.)	P	17.0

* C=Cephaloridine; P=Benzylpenicillin

weak antibiotic activity of its own. It inhibited a series of isolated bacterial β -lactamases to the extents shown in Table 3 [64]. At low concentrations, it sensitized those bacteria to inhibitions by ampicillin and cephaloridine whose β -lactamases were sensitive to clavulanic acid. Similar sensitizations were reported for β -lactamase-producing strains of *N. gonorrhoeae* [65, 66] and for certain strains of *S. aureus*, *H. influenzae*, *Bacteroides fragilis* and several Enterobacteriaceae while no sensitization was observed in *Pseudomonas aeruginosa* [66]. No factorially designed combination experiments to test for synergistic growth inhibitions by graded concentrations of clavulanic acid and of antibiotics with the isobologram method have been reported. At the time of this writing, no in vivo experiments with combinations of clavulanic acid and antibiotics had been published.

CP-45,899 inhibited β -lactamase enzymes, isolated from 7 different bacterial species. For enzymes which were chromosomal gene products, preincubation with the inhibitor was required. For example, the inhibition of a *Pseudomonas* enzyme increased with preincubation from 16 to 80%. The compound was 1000 times more active against plasmid gene products than against chromosomal gene products [67]. Sensitization studies to penicillins and cephalosporins by CP-45,899 in a variety of bacteria [68-70] produced in vitro results comparable to those with clavulanic acid, cited above. Factorially designed experiments with CP-45,899 and penicillin showed that the two drugs acted synergistically [71]. Systemic and local infections of mice with *S. aureus* and systemic infections with Gram-negative pathogens were successfully treated with combinations of CP-45,899 and ampicillin [72].

Derivatization of penicillanic acid by organic-chemical methods should be capable of yielding numerous congeners of CP-45,899 for testing as potential antiplasmid drugs in those bacteria whose resistance to β -lactam antibiotics is caused by plasmid-encoded β -lactamases. The information in [67-72] suggests that this line of drug development may be promising.

Conclusions

Increasing problems of bacterial multiresistance to chemotherapeutic drugs, caused by R-plasmids, are unlikely to be contained or to disappear through enforced limitations in antibiotic usage. They call for active pharmacological approaches to the chemotherapy of R-plasmids. The use of flavomycin as an animal feed-additive and the development of β -lactamase inhibitors for clinical use in combination chemotherapy are currently the most promising approaches.

- 1 Simmons, H.E., Stolley, P.D.: J. Am. Med. Ass. 227, 123 (1974)
- 2 Finland, M., Barnes, M.W.: Antibiot. Chemother. 21, 170 (1976)
- 3 Mitsuhashi, S., Iyobe, S., Inoue, M.: ibid. 20, 133 (1976)
- 4 Gangorosa, E.J., et al.: J. Infect. Diseases 126, 215 (1972)
- 5 Anonymous: Lancet 1975-I, 1230
- 6 Olarte, J., Galindo, M.: Antimicrob. Agents Chemother. 4, 597 (1973)
- 7 Anderson, I.S.: Top. Infect. Diseases 1, 59 (1975)
- 8 de Graaf, J., Elwell, L.P., Falkow, S.: J. Bacteriol. 126, 439 (1976)
- 9 Van Klingeren, J., van Embden, J., Dessens-Kroon, K.: Antimicrob. Agents Chemother. 11, 383 (1977)
- 10 Elwell, L.P., et al.: ibid. 11, 528 (1977)
- 11 Smith, D.H.: J. Bacteriol. 94, 2071 (1967)
- 12 Swann, M.M.: Report of the Joint Committee on the use of antibiotics in animal husbandry and veterinary medicine. London: Her Majesty's Stationary Office 1969
- 13 Richmond, M.H.: Top. Infect. Disease 2, 61 (1977); Jonsson, M.: Scand. J. Infect. Diseases 6, 339 (1974)
- 14 McGowan, J.F., Barnes, M.W., Finland, M.: J. Infect. Diseases 132, 316 (1973)
- 15 Levy, S.B., et al.: Top. Infect. Diseases 2, 43 (1977)
- 16 Davies, J.: ibid. 2, 343 (1977)
- 17 Dale, H.H.: Physiol. Rev. 3, 359 (1923); Bauer, D.J.: Mod. Trends Med. Virol. 1, 49 (1967)
- 18 Lebek, G.: Z. Hyg. Infektionskrankh. 149, 255 (1963)
- 19 Hahn, F.E.: Antibiot. Chemother. 20, 196 (1976)
- 20 Traub, W.H., Kleber, I.: Z. Bakt. Parasitenk. Abt. I, Orig. A. 229, 80 (1973)
- 21 Huber, G., Neseemann, G.: Biochem. Biophys. Res. Commun. 30, 7 (1968)
- 22 Suzuki, J., et al.: J. Antibiotics (Tokyo) 25, 94 (1972)
- 23 Huber, G., in: Antibiotics, Vol. V a. Berlin-Heidelberg-New York: Springer (in press)
- 24 Mitsuhashi, S., Iyobe, S., Hashimoto, H.: J. Antibiotics (Tokyo) 23, 319 (1970)
- 25 Watanabe, T., et al.: Johns Hopkins Med. J. Suppl. 2, 98 (1973)
- 26 Kawakami, M., Landman, O.E.: Biochem. Biophys. Res. Commun. 18, 716 (1965)
- 27 Shaw, W.V., Brodsky, R.F.: J. Bacteriol. 95, 28 (1968)
- 28 Davies, J., Kagan, S.A.: Top. Infect. Diseases 2, 207 (1977)
- 29 Waitz, J.A., Weinstein, M.: Med. J. Austral. Suppl. June 13, 19 (1970)
- 30 Benevise, R., Davies, J.: Ann. Rev. Biochem. 42, 471 (1973)
- 31 Hahn, F.E., et al.: Antibiot. Chemother. 6, 531 (1956)
- 32 Hahn, F.E., Gund, P.: Top. Infect. Diseases 1, 245 (1975)
- 33 Kono, M., et al.: J. Antibiotics (Tokyo) 22, 603 (1969)
- 34 Manyan, D.R., Arimura, G.K., Yunis, A.A.: Mol. Pharmacol. 11, 520 (1975)
- 35 Hirota, Y.: Proc. Nat. Acad. Sci. USA 46, 57 (1960)
- 36 Rownd, R., Nakaya, R., Nakamura, V.: J. Mol. Biol. 17, 376 (1966)
- 37 Courvalin, P.M., Carlier, C., Chabbert, Y.A.: Ann. Inst. Pasteur 123, 755 (1972)
- 38 Hahn, F.E., Ciak, J.: Antimicrob. Agents Chemother. 9, 77 (1976)
- 39 Allison, R.G., Hahn, F.E.: ibid. 11, 251 (1977)
- 40 Hohn, B., Korn, D.: J. Mol. Biol. 45, 385 (1969)
- 41 Riva, S., et al.: Antimicrob. Agents Chemother. 3, 456 (1973)
- 42 Yoshikawa, M., Sevag, M.G.: J. Bacteriol. 93, 245 (1967)
- 43 Yoshikawa, M.: Genet. Res. 17, 9 (1971)
- 44 Falkow, S.: Infectious Multiple Drug Resistance. London. Pion 1975
- 45 Bauer, M., Vinograd, J.: Progr. Mol. Subcell. Biol. 2, 181 (1971)
- 46 Richardson, J.P., Parker, S.R.: J. Mol. Biol. 78, 715 (1973)

- 47 Bouanchaud, D.H., Chabbert, Y.A. Ann NY Acad Sci 152: 305 (1971)
- 48 Gellert, M., et al. Proc Nat Acad Sci USA 73: 3872 (1976)
- 49 Sakakibara, Y., Tomizawa, J. ibid 71: 802 (1974)
- 50 Staudenbauer, K.L. Mol Gen Genet 145: 273 (1976)
- 51 Sugino, A., et al. Proc Nat Acad Sci USA 74: 4767 (1977)
- 52 Sugino, A., et al. ibid 75: 4838 (1978)
- 53 Goss, W.A., Cook, I.M., in Antibiotics Vol III, p 174 Berlin-Heidelberg-New York Springer 1975
- 54 Smith, C.L., Kubo, M., Imamoto, I. Nature 275: 420 (1978)
- 55 Puga, A., Tessman, I. Mol Biol 75: 99 (1973)
- 56 McHugh, G.L., Swartz, M.N. Antimicrob Agents Chemother 12: 423 (1977)
- 57 McHugh, G.L., Swartz, M.N. Abstr 18th Intersci Conf Antimicrob Agents Chemother, p 89 (1978)
- 58 Hogenauer, G., Hartmann, G., Ruf, C. Top Infect Diseases 2: 153 (1977)
- 59 Lebek, G. Z Hyg Infektionskrankh 150: 43 (1964)
- 60 Brown, A.G., et al. J Antibiotics (Tokyo) 29: 668 (1976)
- 61 Retsema, J.A., et al. Abstr 18th Intersci. Conf. Antimicrob. Agents Chemother, p 143 (1978)
- 62 Miyamura, S., Ochiai, H. Proc 5th Int. Congr. Chemother., p 218 (1973)
- 63 Umezawa, H., et al. J Antibiotics (Tokyo) 26: 51 (1973)
- 64 Reading, C., Cole, M. Antimicrob. Agents Chemother. 11: 852 (1977)
- 65 Miller, J.M., Baker, C.N., Thornsberry, C.: ibid. 14: 794 (1978)
- 66 Wise, R., Andrews, J.M., Bedford, K.A.: ibid. 13: 389 (1978)
- 67 Lu, K.P., Neu, H.C.: Abstr. 18th Intersci. Conf. Antimicrob. Agents Chemother., p. 142 (1978)
- 68 Retsema, J.A., et al.: ibid., p. 143
- 69 Aswapokee, N., Neu, H.C.: ibid., p. 144
- 70 Khan, W.N., et al.: ibid., p. 145
- 71 Elster, C., et al.: ibid., p. 146
- 72 English, A.R., et al.: ibid., p. 147

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