Inhibitory Effects of Mitomycin-Related Compounds Lacking the C¹-C² Aziridine Ring

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Three chemical derivatives of mitomycin C cause loss of viability, inhibition of deoxyribonucleic acid synthesis, and inhibition of cell division in bacteria. Each of these drugs lacks the C₁-C₂ aziridine ring previously postulated to be essential for the bactericidal effect of mitomycin C.

There is ample evidence indicating that the principal cellular target of mitomycin C is the deoxyribonucleic acid (DNA) molecule (14). Among the several antibiotics which interfere with the genetic material of cells, mitomycin C is one of the few that seem to form covalent bonds with DNA (13, 17). The nature of these bonds is unknown. In 1963, Iyer and Szybalski (5) observed that mitomycin C can cross-link the complementary strands of DNA in vivo and, under appropriate conditions, in vitro as well (6). It was proposed by these authors (5) that the bactericidal effect of the mitomycins is the indirect consequence of the cross-links. In the details of this model, it was proposed that the reduced (hydroquinone) form of the drug molecule functions as a bifunctional alkylating agent: the aziridine group (at carbons 1 and 2; Fig. 1) and the carbamate group (at carbon 10) represent the two reactive centers of the molecule, capable of reacting with two nucleophilic sites in opposite strands of DNA (6).

As part of our studies on the chemical mechanism of the antibiotic action of the mitomycins, we examined the biological effects of three compounds structurally related to mitomycin: a hydrolysis product of mitomycin C (derivative Y) (12) and two synthetic analogues, Z (1) and X (10; see Fig. 1). Inhibition of bacterial growth by these compounds was reported earlier (1, 7, 10). Since each of these substances lacks the C₁-C₂ aziridine ring postulated to be essential for the cross-linking action of the parent drug mitomycin C, it was of interest to examine the biological effects of these compounds in detail and in direct comparison with mitomycin C.

MATERIALS AND METHODS

Derivative Y was prepared from mitomycin C by the method of Stevens et al. (12). Analogue Z [car-

bamic acid, methyl-, (1-ethyl-4,7-dihydro-5-methoxy-2,6-dimethyl-4,7-dioxo-3-indolyl)methyl ester] and analogue X [carbamic acid, methyl-, (5-(1-aziridinyl)-1-ethyl-4,7-dihydro-2,6-dimethyl-4,7-dioxo-3-indolyl)methyl ester] were kindly provided by Lederle Laboratories, Pearl River, N.Y. Mitomycin C was purchased from Kiowa Hakko Kogyo Ltd., Tokyo, Japan.

Three bacterial strains were used in the tests: a gram-positive coccus (*Diplococcus pneumoniae*), a gram-positive bacillus (*Bacillus subtilis* 168), and a gram-negative bacillus (*Escherichia coli* K-12). The mutant strain *E. coli* H135, showing sensitivity to a large number of chemically unrelated drugs (3), was kindly provided by H. L. Ennis of Roche Institute for Molecular Biology, Nutley, N.J. The bacteria were grown in commonly used synthetic media [medium C for *Diplococcus* (15); synthetic salts media for *B. subtilis* (11) and *E. coli* (2)], and the antibiotics were added to the cultures in the exponential phase of growth (growth monitored by nephelometry). Viable counts were determined by agar plating. Nucleic acid synthesis was measured by determining the amount of radioactive uracil (uracil-6-³H; 10 μ Ci and 5 μ g/ml of culture) incorporated into cold trichloroacetic acid-precipitable material, which was collected on membrane filters (Whatman, GF/A), dried, and counted in a scintillation spectrometer. For measuring incorporation into DNA, the samples were first treated with 1N NaOH at 37°C overnight and then precipitated with trichloroacetic acid. Incorporation into ribonucleic acid (RNA) was determined by subtracting the radioactivity in DNA from the total acid-precipitable counts.

RESULTS AND DISCUSSION

The results can be summarized as follows: (i) The analogues X and Z caused rapid and irreversible loss in the viable titer of pneumococci at concentrations below 10 μ g/ml (Fig. 2), whereas derivative Y did not. The actual concentrations used represent maximal concentrations which showed least interference with the

FIG. 1. Chemical structure of mitomycin C and compounds X, Y, and Z. The molecular weights are 334, 331, 320, and 320, respectively.

increase of cellular mass (measured by nephelometry) during the period within which 99.99% of the cells lost their viability. Phase contrast microscopy showed no changes in cellular morphology during this period. At 10 $\mu\text{g}/\text{ml}$ and after 1 hr of exposure (=1 generation time), derivative Y caused only about 50% loss of viable bacteria.

(ii) All mitomycin derivatives inhibited DNA synthesis (Fig. 3) and, somewhat less effectively, RNA synthesis (Fig. 4) in pneumococci at concentrations of less than 10 $\mu\text{g}/\text{ml}$. The concentrations shown in Fig. 3 represent the minimum needed to cause virtually complete inhibition of DNA synthesis. At this concentration and at lower concentrations as well, the drugs inhibited RNA synthesis less effectively than DNA synthesis. Nevertheless, a completely selective inhibition of DNA synthesis was never observed with the mitomycin derivatives. With the parent compound mitomycin C, such selectivity was observed only occasionally within a narrow range of drug concentrations (between 0.1 to 0.5 $\mu\text{g}/\text{ml}$) and for relatively short periods (maximum 0.5 generation time) after addition of the drug. An extremely sensitive dependence of the biological effects of mitomycin on the dose of the drug and the parameters of growth was noted earlier by other investigators (8).

(iii) Fluid cultures of *E. coli* K-12 were completely resistant to high concentrations (100 $\mu\text{g}/\text{ml}$) of each of the three mitomycin analogues, and the lowest concentration of mitomycin C which caused noticeable inhibition of growth was

TABLE 1. Inhibition of bacterial growth by mitomycin-related compounds^a

Drug	Concn ($\mu\text{g}/\text{disc}$)	Growth inhibition (width of growth inhibition zone, mm)		
		<i>B. subtilis</i> 168	<i>E. coli</i> K-12-H135	<i>E. coli</i> K-12
Mitomycin C	0.05	None	8.5	None
	0.1	1.5	12.5	None
	0.5	10.0	20.5	None
Analogue X	5.0	5.5		
	10.0	7.0		
	25.0		None	
	50.0		2.0	None
	100.0		6.5	None
Derivative Y	25.0	None	None	
	50.0		None	None
	100.0		2.0	None
Analogue Z	2.0	1.5		
	25.0		None	
	50.0		5.0	None
	100.0		8.5	None

^a Portions (0.1 ml) of late exponential cultures of the bacteria were spread on the surface of agar plates and then sterile glass fiber discs (Whatman GL/A, 2.45-cm diameter), wetted with solutions of the various drugs, were deposited on the agar surface. The width of the growth inhibition zone around the discs was determined after overnight incubation at 37 C.

0.5 $\mu\text{g}/\text{ml}$. In contrast, analogues X and Z at 20 and 10 $\mu\text{g}/\text{ml}$, respectively, inhibited the growth of a mutant derivative of this *E. coli* strain

FIG. 2. Effect of mitomycin derivatives on the viability of pneumococci. Portions of an exponentially growing pneumococcal culture were incubated at 37 C with mitomycin C and various mitomycin derivatives. At various times, further action of the drugs was terminated by 100- to 1,000-fold dilution into chilled growth medium (15), and the titer of surviving viable bacteria was determined by plating in agar. The concentrations of the drugs were as follows ($\mu\text{g/ml}$ of culture): compound Z, 2 (curve A); mitomycin C, 0.5 (curve B); compound X, 10 (curve C) and 5 (curve D); compound Y, 10 (curve E) and 5 (curve F).

(H135) which was isolated by Ennis and described as sensitive to a number of structurally and functionally unrelated antibiotics (3). Mitomycin C was also 10 times more effective against this strain (minimum growth-inhibitory concentration, 0.05 $\mu\text{g/ml}$). Similar results were obtained in agar inhibition disc assays. In *E. coli* K-12, mitomycin C showed no inhibition up to 0.5 μg per disc, and compounds X, Y, and Z up to 100 μg per disc. In the mutant strain H135, however, inhibition zones were obtained with all substances tested (Table 1). These findings suggest that the

FIG. 3. Inhibition of DNA synthesis by mitomycin derivatives. A pneumococcal culture was divided into several portions; each received 5 μg and 1 μCi of uracil-6-³H per ml and various mitomycin compounds. The tubes were incubated at 37 C and, at intervals, 100- μliter samples were removed to determine incorporation of the tracer into DNA and RNA (see Fig. 4) by the procedure described in Materials and Methods. A, Control culture; B, 5 $\mu\text{g/ml}$ of compound Y; C, 10 $\mu\text{g/ml}$ of compound Y; D, 1 $\mu\text{g/ml}$ of compound Z; E, 1 $\mu\text{g/ml}$ of compound X; F, 0.5 $\mu\text{g/ml}$ of mitomycin C.

FIG. 4. Inhibition of RNA synthesis by mitomycin derivatives. See legend of Fig. 3.

relative resistance of *E. coli* to mitomycin C and to various mitomycin derivatives (7) and analogues (16) is due to difficulties in cellular uptake of these drugs (3).

(iv) All three mitomycin derivatives caused inhibition of cell division in both *E. coli* H135

and *B. subtilis* 168 as evidenced by filamentous growth. Practically all bacilli taken from the outer rim of the inhibition zones (from agar inhibition disc plates) were long filaments many times the normal lengths of bacilli (Fig. 5). This finding indicates that, for each of the mitomycin

FIG. 5. Induction of filament formation by mitomycin compounds. Small samples were scraped off the outer rim of agar inhibition zones and examined by phase contrast microscopy. (1) Mitomycin C (0.5 μg per disc); (2) compound X (10 μg per disc); (3) mitomycin C (0.1 μg per disc); (4) compound Y (100 μg per disc). The bacteria were *Bacillus subtilis* 168 in 1 and 2 and *Escherichia coli* H135 in 3 and 4.

derivatives, there exists a dose range within which these compounds can selectively inhibit cell division for a period of several cell generation times. Induction of filamentous growth by sublethal dose of mitomycin C has been known for some time (4, 8, 9).

In summary, the two synthetic analogues, X and Z, mimic three characteristic inhibitory effects of the parent compound mitomycin C, namely: (i) rapid inactivation of the bacterium's reproductive capacity (loss of viability), (ii) inhibition of DNA synthesis, and (iii) selective inhibition of cell division. The third derivative, Y, inhibited nucleic acid synthesis and cell division and caused a relatively slow loss of viability at moderate concentrations.

These findings suggest that the presence of the aziridine ring, as in mitomycin C, is not essential for the characteristic inhibitory effects induced by this group of compounds. Thus one may reason that the observed cross-linking action of mitomycin C itself may either arise by a mechanism not involving the aziridine moiety, but rather some other portions of the molecule common to all compounds, or that the cross-links do involve the aziridine group but are of secondary or no significance with regard to the biological activities examined here. A similar suggestion has been made by the Lederle group based on their accumulated data of antibacterial activities of X, Z, and a whole series of closely related analogues (16). Finally, there is also a remote possibility that the synthetic compounds act by a mechanism unrelated to that of mitomycin C.

These conclusions must be taken into consideration in studies on the chemical mechanism of action of the mitomycins.

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