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COMPARATIVE EFFECTS OF STREPTONIGRIN DERIVATIVES ON TISSUE CULTURE CELLS

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Abstract—In tissue culture cells, streptonigrin methyl ester (MES) had 1/100th the activity of streptonigrin. MES inhibited the uptake of labeled precursors into RNA and DNA. Although its mode of action could not be distinguished from that of streptonigrin, this effect could not be accounted for by the liberation of free streptonigrin during metabolism.

With isopropylidine azastreptonigrin (IPAS), no evidence could be found of cytotoxicity or biochemical effects on nucleic acids in concentrations up to 10^{-3} M. The degree of binding of IPAS to cells was 70 times less than with MES. IPAS had no effect on DNA synthesis even when the cell membrane barrier had been disrupted; therefore lack of cell penetration could not be invoked as an explanation for its inactivity. Since bridging the aminoquinone function of streptonigrin resulted in loss of biological activity, it is suggested that the *o*-aminoquinone moiety may be important for the pharmacological action of streptonigrin.

STREPTONIGRIN, an antibiotic isolated from broth filtrate of *Streptomyces flocculus*,[1] was previously found to inhibit cell division and DNA and RNA synthesis in tissue culture cells.[2] Two derivatives of streptonigrin have been synthesized: streptonigrin methyl ester (NSC-54384, MES) and isopropylidine azastreptonigrin (NSC-62709, IPAS).* Both streptonigrin[3] and MES[4, 5] have been reported to have clinical efficacy, particularly against lymphomas. Preliminary studies with the streptonigrin derivatives in tissue culture cells showed that the structural modifications had reduced the cytotoxic properties of streptonigrin. The purpose of the current study was to investigate the biochemical effects of these derivatives and to compare them with the effects of the parent compound. The structures of streptonigrin and its derivatives are shown in Fig. 1.

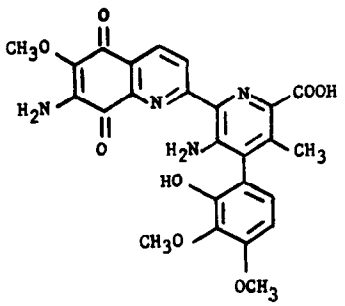
MATERIALS AND METHODS

Tissue culture cells. Mouse mammary tumor cells in ascites form (F66) adapted to suspension culture were used. The procedure for maintenance of cells, measurement of growth, and the examination for cell morphology were as previously described.[2] The cell concentration used in these experiments was 1×10^6 cells/ml unless specified otherwise.

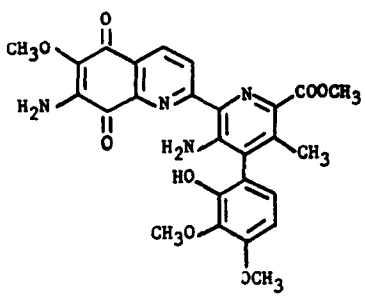
Materials. Streptonigrin methyl ester was used either as the commercially available solution in dimethylacetamide (2·5 mg/ml, lot 11-23-009), or as a solution in dimethylsulfoxide; IPAS preparation was available as a dry blend consisting of

* Generously supplied by Dr. T. J. Medrek, Chas. Pfizer & Co., Maywood, N.J., U.S.A.

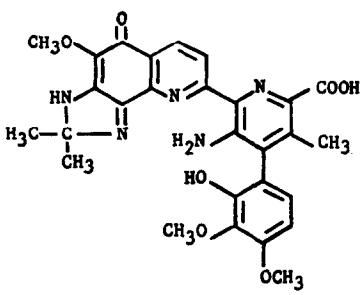
30 mg IPAS, 13 mg Na_2CO_3, 12 mg Na_2HPO_4, and 250 mg mannitol (lot 11-45-004). The control cultures for MES experiments contained corresponding amounts of the solvent used for the experiments, and the controls for IPAS contained equivalent amounts of the Na_2CO_3–Na_2HPO_4–mannitol mixture. The following radioactive compounds were used: 2-^{14}C-glycine (1·99 mc/m-mole) from Volk Radiochemical Co.; ^{3}H-thymidine (1·9 c/m-mole), ^{3}H-uridine (1·66 c/m-mole), ^{3}H-dCMP (2·5 c/m-mole), from Schwartz BioResearch, Inc.; ^{3}H-MES (1 μc/mg) and ^{3}H-IPAS (3 μc/mg) from Chas. Pfizer & Co.



Streptonigrin, $C_{25}H_{22}O_8N_4$



Streptonigrin Methyl Ester
$C_{26}H_{24}O_8N_4$



Isopropylidine Azastreptonigrin
$C_{28}H_{27}O_7N_5$

FIG. 1. Structure of streptonigrin, streptonigrin methyl ester, and isopropylidine and azastreptonigrin.

Incorporation of labeled precursors into nucleic acids and protein. Details of the individual experiments appear with the tables and figures depicting the results. The protein and RNA and DNA fractions were isolated, quantitated, and radioactivity determined as previously described.[2]

For experiments designed to estimate the binding of the drugs to cells, the cells were incubated for 1 hr with tritiated MES or IPAS with specific activity of 1 μc/mg. The cells were washed with fresh media, and then collected on a filter (type HA, Millipore, Bedford, Mass.). Radiactivity was assayed by placing the dried filters in vials containing 10 ml of scintillation mixture (6 g POP, 0·1 g POPOP, 1 l. toluene), and counting in a liquid scintillation spectrometer.

Cell sonicates were prepared by suspending 4×10^{11} cells in 10 ml medium and subjecting it to the Branson Sonifier in the cold (30 sec, max. intensity).

Thin-layer chromatography of extracts. Tissue culture cells were incubated with a medium containing MES (10^{-4}M). Aliquots of 10 ml were removed at 0, 0·5, 1, 2, and 4 hr after the addition of the drug, acidified to pH 4, and shaken with an equal volume of chloroform. The chloroform layer was removed and the extraction repeated twice more. The chloroform extracts were pooled and evaporated to 0·1 ml. Standards were prepared by treating 10 ml culture medium containing either streptonigrin (10^{-6}M) or MES (10^{-4}M) in a similar manner. Aliquots of the extracts were applied to thin-layer chromatography media, Gelman ITLC, type S.* The chromatograms were developed in benzene: acetone (9:1, v/v), dried, and sprayed with freshly prepared 1% ferric chloride–1% potassium ferricyanide.

RESULTS

Effects of MES. It was found that when dimethylacetamide was used as a solvent for MES, the solvent alone was inhibitory to cell growth at a concentration of 1·0 ml/100 ml medium or greater (Table 1). For subsequent experiments, MES was

TABLE 1. EFFECT OF DIMETHYLACETAMIDE ON CELL GROWTH

(ml DMA*) per 100 ml)	Cell growth (% control)
0·1	107
0·5	94
1·0	68
2·0	66

Cells were incubated at 37° for 24 hr.
* Dimethylacetamide.

TABLE 2. EFFECT OF MES ON CELL GROWTH

Conc. of MES (M)	Cell growth (% control)
10^{-4}	65
10^{-5}	72
10^{-6}	79
10^{-7}	103

Cells were incubated at 37° for 24 hr.

dissolved in dimethylsulfoxide which was found to be free from cytotoxicity at the levels used. The data in Table 2 show the effects of MES on cell growth. Cell multiplication was inhibited at concentration of 10^{-5} M and above.

When MES was added at a concentration of 10^{-4} M to the medium containing 2-^{14}C-glycine, it caused a reduction of incorporation of the label into RNA, DNA, and protein (Fig. 2). The inhibitory action of MES on the uptake of ^{3}H-uridine by RNA, and of ^{3}H-thymidine by DNA are shown in Figs. 3 and 4, respectively. The

* Gelman Instrument Co., Ann Arbor, Mich., U.S.A.

effect of varying the concentration of MES on the degree of inhibition of thymidine uptake by DNA and uridine uptake by RNA are shown in Fig. 5. The results show a dependency of per cent inhibition on the concentration of MES with no inhibition at 10^{-6}M and below. It also shows that the effect on DNA could not be dissociated

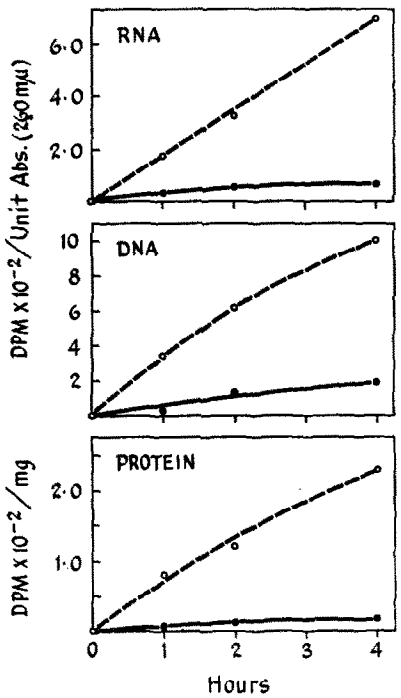


FIG. 2. Effect of MES on 2^{-14}C-glycine uptake; 10^7 cells were incubated in 10 ml medium containing 2^{-14}C-glycine (0·02 μc/ml); ○——○ control; ●——● MES, 10^{-4} M.

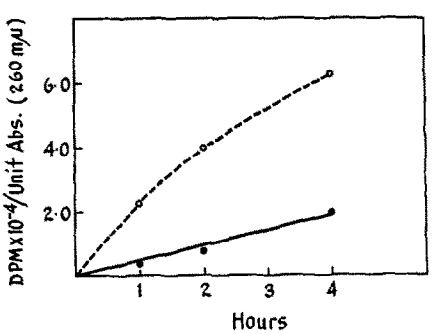


FIG. 3. Effect of MES on uptake of ^{3}H-uridine by RNA; 10^7 cells were incubated in 10 ml medium containing ^{3}H-uridine (0·2 μc/ml); ○——○ control; ●——● MES, 5×10^{-5} M.

from that on RNA. Since these effects are qualitatively similar to that previously observed with streptonigrin, the possibility was explored that MES could be hydrolyzed by the cells and the resultant streptonigrin could be responsible for its action. The thin-layer chromatogram of the tissue culture extracts are shown in Fig. 6. In

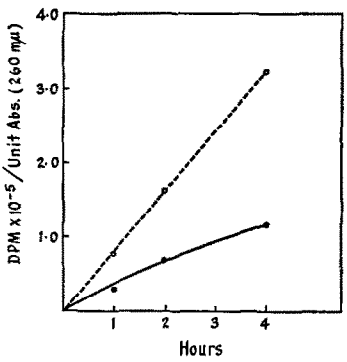


FIG. 4. Effect of MES on uptake of ^{3}H-thymidine by DNA; 10^7 cells were incubated in 10 ml medium containing ^{3}H-thymidine (1·0 μc/ml): ○———○ control; ●———● MES, 5×10^{-5} M.

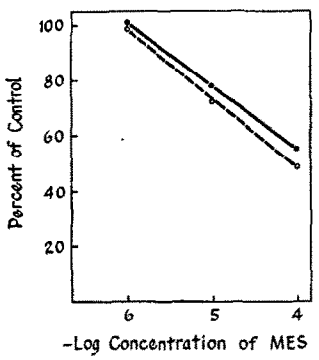


FIG. 5. Effect of varying concentration of MES on incorporation of ^{3}H-uridine into RNA (○———○) and ^{3}H-thymidine into DNA (●———●). Cells were incubated for 4 hr at 37° in medium containing 0·2 μc ^{3}H-uridine/ml or 1·0 μc ^{4}H/thymidine ml.

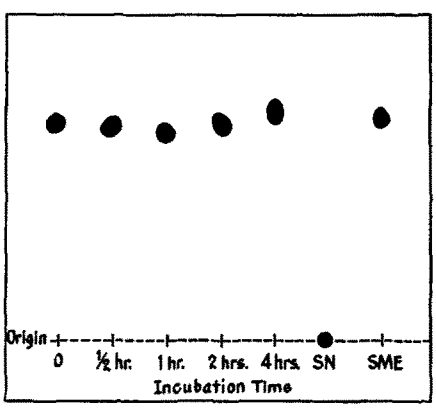


FIG. 6. Thin-layer chromatogram of extracts of tissue culture cells incubated with 10^{-4} M MES. SN = extract from tissue culture medium containing 10^{-6} M streptonigrin. MES = extract from tissue culture medium containing 10^{-4} M MES.

this system, streptonigrin remained at the origin while MES travelled near the front. The sensitivity of the method would have detected 1 per cent conversion of MES to streptonigrin, or concentration of streptonigrin which produced comparable toxic effects. Since no free streptonigrin was observed during the incubation period, the hydrolysis of MES, if any, was less than 1 per cent.

Effects of IPAS. In contrast to MES, little cytoxicity could be demonstrated with IPAS with concentrations as high as 10^{-3}M (Table 3). When IPAS was added to

TABLE 3. EFFECT OF IPAS ON CELL GROWTH

Conc. of IPAS (M)	Cell growth (% control)	Morphological appearance
10^{-3}	82	normal
10^{-4}	96	normal
10^{-5}	95	normal
10^{-6}	109	normal

Cells were incubated at 37° for 24 hr.

TABLE 4. BINDING OF ^{3}H-LABEL TO CELLS*

Labeled compound	Radioactivity (counts/min. per 10^6 cells)
^{3}H-MES	16,450
^{3}H-IPAS	225

*Cells were exposed to ^{3}H-MES (1 μc/mg) or ^{3}H-IPAS (1 μc/mg) at 10^{-5} M for 1 hr.

medium containing 2-^{14}C-glycine, there was no effect on the uptake of the label by RNA, DNA, and the protein fractions. Similarly, there was no difference in the uptake of ^{3}H-uridine by RNA and of ^{3}H-thymidine by DNA. The lack of demonstration of activity by IPAS raised the question of permeability of the cell membrane to this drug. The results in Table 4 show that after exposure of the cells to equal concentrations of MES and IPAS with identical specific activities, the binding of the label was 16,450 counts/min with MES and 225 with IPAS, or about 70-fold less binding with IPAS. In order to determine whether cell permeability played a role in the effectiveness of IPAS, the drug was added to a tissue system in which the cell membrane barrier had been disrupted by sonication. The synthesis of DNA was measured by incubating the cell sonicate with dATP, dGTP, TTP, and ^{3}H-dCMP. DNA was isolated, and the radioactivity assay data in Table 5 indicated that IPAS did not inhibit the uptake of the label by DNA. The results with MES are included to show that by contrast, a significant depression of incorporation of the label occurred with this compound.

DISCUSSION

Streptonigrin was shown to be a potent inhibitor of DNA synthesis in bacteria[6] and tissue culture cells.[2, 7] The exact mechanism responsible for this effect has not

been determined, but indication that a direct action on DNA is involved was suggested by the findings that streptonigrin induced phage production in lysogenic bacteria, initiated rapid breakdown of DNA in *Escherichia coli*,[8] produced chromosome breakage in human leukemic cells;[9] and the tritiated compound and/or its derivative was found to bind to DNA in tissue culture cells.[2] Streptonigrin also

TABLE 5. EFFECT ON CELL SONICATES*

Group	Radioactivity counts/min. (in DNA)
MES blank	120
MES	39
IPAS blank	84
IPAS	88

* Incubation mixture consisted of 2·5 ml cell sonicate, 250 mμmoles each of dGTP, dATP, and TTP, and 0·5 μc of ^{3}H-dCMP. Incubation was for 1 hr at 37°. The additions were as follows: MES blank, 0·025 ml dimethylsulfoxide; MES, 0·025 ml of 10^{-2} M solution of MES in dimethylsulfoxide; IPAS blank, 1·4 mg of Na_2CO_3–Na_2HPO_4– mannitol mixture; IPAS, 1·4 mg of Na_2CO_3–Na_2HPO_4–mannitol–IPAS mixture.

depressed RNA and protein synthesis in tissue culture cells. The latter appeared to be secondary to the effects of nucleic acids, while the former could not be dissociated from the effect on DNA, both nucleic acids being equally affected by the action of streptonigrin.

Other metabolic effects of streptonigrin have been reported with human leukemic cells in which an inhibition of respiration and anaerobic glycolysis took place,[10] and it was suggested that streptonigrin catalyzed NADH and NADPH oxidation.[11] Actinomycin D, which has been shown to inhibit RNA synthesis,[12] also inhibited respiration and anaerobic glycolysis in the leukemic cells,[13] suggesting that these antibiotics may also affect energy metabolism.

The action of MES could not be qualitatively distinguished from that of streptonigrin previously reported with tissue culture cells.[2] The highest concentration at which no effect could be seen was 10^{-6} M, while with streptonigrin this was 10^{-8} M; therefore MES has about 1/100 the activity of streptonigrin. Using splenomegaly assay system with Rauscher murine leukemia virus, McBride and co-workers[14] showed similarly that both streptonigrin and MES had antiviral activity, although higher doses of MES were necessary to obtain comparable results. The similarity of response to streptonigrin and MES could not be explained on the basis of liberation of streptonigrin from MES, since no detectable amounts of streptonigrin could be observed in tissue culture extracts. Likewise, when MES was administered to human cancer patients, there was no free streptonigrin detected in the urine.[15]

In contrast to MES, little effect could be demonstrated with IPAS with concentrations as high as 10^{-3}M. The low biological efficacy of IPAS could not be attributed to a lack of cell permeation since there was no effect on DNA synthesis when cell sonicates were used. Because bridging the aminoquinone function resulted in loss of activity, it is suggested that the *o*-aminoquinone moiety of streptonigrin is requisite for its pharmacological action. The absence of biological activity for IPAS is at

variance with the report of McBride *et al.*,[14] who found IPAS to have activity against Rauscher murine leukemia virus although it was less effective than MES. The reason for this difference in findings is unknown.

ADDENDUM

Since this manuscript was submitted, a paper by W. B. Kremer and J. Lazlo has appeared in this Journal (**15**, 111, 1966), which showed that streptonigrin and MES had similar biochemical effects on human leukemic leukocytes and erythrocytes, but MES was considerably less effective.

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