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BIOCHEMICAL EFFECTS OF THE METHYL ESTER OF STREPTONIGRIN*

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Abstract—This paper reports the biochemical effects of the methyl ester of streptonigrin on human leukemic leukocytes and erythrocytes. A qualitative and quantitative comparison was made between this drug and its parent compound, streptonigrin. Both of these quinone antitumor antibiotics catalyzed the direct oxidation of NADH and NADPH via NAD–NADP diaphorase and caused depletion of cellular ATP. As the reduced form of both drugs was autoxidized to the active state, hydrogen peroxide was formed. The impaired leukocyte protein synthesis and enhanced DNA degradation caused by these drugs were thought to be secondary to ATP depletion and peroxide formation.

Although the biochemical effects of the two drugs were qualitatively similar, methyl ester streptonigrin (MeSN) was found to be considerably less effective than streptonigrin (SN). These findings *in vitro* correlate well with the observations that MeSN was less toxic than SN in tissue culture, animal, and man. A reduction in the biochemical effects of MeSN, as compared to SN, might explain the decreased toxicity noted with this drug. If the antitumor effects and the toxicity result from common biological mechanisms it would be predicted that MeSN would not provide a superior therapeutic ratio than its parent compound.

STREPTONIGRIN (SN), a quinoid antibiotic derived from *Streptomyces flocculus*, has been shown to have antitumor activity in human lymphoid malignancies.[1, 2] However, the usefulness of this drug in the treatment of hematologic malignancies was limited by severe gastrointestinal toxicity and prolonged thrombocytopenia. The methyl ester of streptonigrin (MeSN) was prepared at the John L. Smith Memorial Laboratory in an effort to find a derivative with a more favorable therapeutic ratio. MeSN differs from SN in the substitution of a methyl group for the hydrogen in the acid radical of the third ring (Fig. 1).[3] In animal tumor and tissue culture systems, MeSN was less toxic than SN while maintaining antitumor effectiveness.[4] Preliminary clinical studies indicated that MeSN had antitumor effects in patients with lymphomas and chronic lymphocytic leukemia, and more extensive studies in human malignancies were recommended.[5, 6]

The purpose of this study was to perform a qualitative and quantitative comparison of the mechanism of action of SN and MeSN and to relate these effects to previous studies in other systems.

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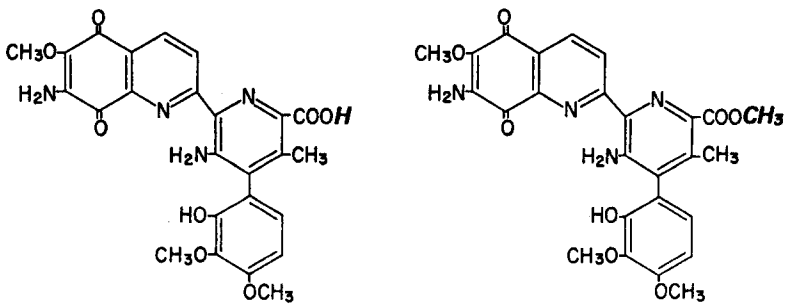

FIG. 1. Structure of streptonigrin and methyl streptonigrin.

EXPERIMENTAL SECTION

Intact cells. Leukemic leukocytes were obtained from patients having lymphocytic or granulocytic leukemia with leukocyte counts above 50,000 cells/μl[3]. The red blood cells were allowed to settle out of heparinized whole blood, and the leukocyte-containing plasma layer was aspirated after 30–45 min. Glucose (3·0 mg/ml) and sodium bicarbonate (1·0 mg/ml) were added to the leukocyte suspension. Red blood cells were isolated from normal blood by removing the plasma and buffy coat layers after centrifuging at 700 *g* for 10 min. The cells were washed three times in Krebs-Ringer phosphate solution and then suspended in an equal volume of this medium.

Energy metabolism. Respiration and anaerobic glycolysis of intact leukocytes and red cells were studied by conventional manometry. The drugs were tipped from the vessel side arm after the flasks had equilibrated. Respiration was expressed as cumulative oxygen uptake and anaerobic glycolysis by the amount of carbon dioxide released by the action of acid on bicarbonate. Cellular adenosine triphosphate (ATP) was extracted by homogenizing the cells in cold 5% trichloroacetic acid (TCA). The protein precipitate was re-extracted with cold 5% TCA and the combined acid-soluble fractions were extracted twice with ether and brought to pH 7·5 with 1 M potassium bicarbonate. The ATP content of the acid-soluble fraction was determined by the luciferase–firefly method.[7] Red cell glutathione was determined by the method of Beutler *et al.*[8]

Oxidation of NADH. NAD–NADP diaphorase was prepared from the soluble supernatant fraction of rat liver homogenates.[9] The rate of NADH oxidation in the presence of NAD–NADP diaphorase and catalytic amounts of SN or MeSN was recorded spectrophotometrically at 340 mμ.

Protein synthesis. Protein synthesis by intact cells was studied by incubating the leukemic leukocytes with L-arginine-U-^{14}C (4 mμmoles/ml) in a Dubnoff shaker at 37° and determining the incorporation of radioactivity into protein. The filter paper disk method of Bollum[10] was adapted to our system as a means of collecting acid-insoluble material. Aliquots (100 μl) of the reaction mixture were pipetted evenly onto 2·3-cm filter disks and rapidly dried under an infrared lamp; the protein was precipitated by plunging the disks into cold 5% TCA. The disks were washed with cold 5% TCA and then heated in 5% TCA at 90° for 7 min. This was followed by successive washes in ethanol, an ethanol:chloroform:ether mixture (2:2:1), and ether. Samples

were prepared for scintillation counting of radioactivity by placing the disks in the bottom of a vial and overlaying them with 10 ml of scintillation solution [1·9 g 1,4-bis-2-(5 phenyloxazolyl)-benzene (POPOP), 19·0 g 2,5-diphenyloxazole (PPO), 3·79 liters toluene].

Isolation of DNA. Leukocyte high-molecular weight DNA was isolated by homogenizing the white blood cells in 9 volumes of 2·0 M NaCl, 2·0% sodium lauryl sulfate, 4·0% sodium citrate, and 0·1% trimethylamine. After one volume of water-saturated phenol was added, the mixture was shaken vigorously and centrifuged at 700 *g* for 15 min. The aqueous phase was re-extracted with phenol and then washed twice with ether. Two volumes of 95% ethanol were added and the DNA was wound out, washed in 70% ethanol, then redissolved in 0·015 M NaCl, 0·0015 M sodium citrate (SSC/10). The quantity of DNA recovered was estimated in the spectrophotometer. For studies of melting and annealing curves of DNA, an additional purification step was employed. To 9 ml of DNA in SSC/10, 1 ml of 3 M potassium acetate and 0·001 M sodium versenate (pH 7·0) was added. While the solution was being stirred, 5·4 ml of isopropanol was added and the DNA wound out, passed through two rinses of 70% ethanol, and redissolved in SSC/10. The DNA isolated in this manner gave a 260 mμ/280 mμ O.D. ratio of 1·9. There was less than 5% contamination with protein and approximately 10% RNA as determined by the orcinol method.[11]

Reagents. Streptonigrin and methyl streptonigrin were generously provided by Dr. T. J. Medreck of Charles Pfizer & Co., Maywood, N.J. MeSN was insoluble in aqueous media and had to be dissolved in dimethylacetamide. For comparative data, SN was also dissolved in this solvent, and control experiments contained a similar amount of the solvent without drug. L-Arginine-U-^{14}C (250 mc-m.mole) was obtained from the New England Nuclear Corp., Boston, Mass.

RESULTS

Red cells consumed oxygen at linear rates in the presence of SN or MeSN, whereas no detectable amounts of oxygen were used in the absence of these drugs (Fig. 2). Cells incubated with SN consumed approximately five times as much oxygen over a 3-hr period as those incubated with an equal amount of MeSN. Oxygen uptake was markedly reduced when glucose was omitted from the medium. MeSN proved to be very insoluble and when suspended in an aqueous medium or in plasma failed to support oxygen uptake in both intact and lysed red cells.

Both SN and MeSN caused a non-stoichiometric disappearance of reduced glutathione (GSH) from red cells incubated without glucose for 3 hr (Table 1). SN proved to be approximately twice as effective as MeSN in this regard. Incubation with glucose minimized the decrease in red cell GSH.

Purified NAD–NADP diaphorase, with no acceptor other than oxygen, mediated the oxidation of NADH in the presence of catalytic amounts of SN and MeSN (Fig. 3). The rate of oxidation with SN was more than twice that with an equal amount of MeSN. When suspended in an aqueous medium, MeSN failed to show any activity in this subcellular system.

Both MeSN and SN had pronounced effects on the energy metabolism of the leukemic leukocytes (Fig. 4). Although there appeared to be little effect by either drug on leukocyte respiration, anaerobic glycolysis was inhibited, and there was a marked

decrease in cellular ATP levels. SN caused a greater and more rapid depletion of cellular ATP and had a more pronounced effect on anaerobic glycolysis.

Protein synthesis of intact leukocytes was inhibited by both drugs, and again SN was more active (Fig. 5). In the presence of SN the incorporation of amino acids into protein virtually ceased after 20-min incubation. With MeSN there was only a 40% inhibition of protein synthesis at 90 min.

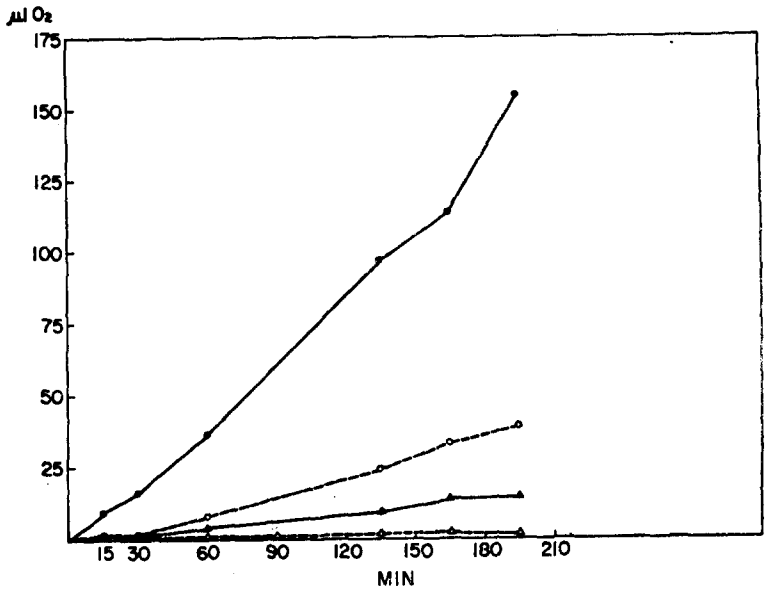

FIG. 2. Red cell oxygen uptake in the presence of streptonigrin (SN) and methyl streptonigrin (MeSN). The drugs were dissolved in dimethylacetamide except where noted. In the absence of drugs, red cells consumed no oxygen.
●—● SN 100 μg/ml; ○ - - - ○ MeSN 100 μg/ml; ▲—▲ MeSN; 100 μg/ml. No glucose; △ - - - △. MeSN 100 μg/ml in plasma or Krebs-Ringer.

TABLE 1. EFFECT OF METHYL STREPTONIGRIN AND STREPTONIGRIN ON RED CELL REDUCED GLUTATHIONE

Sample	RBC Reduced glutathione content (mM)	
	Incubated without glucose	Incubated with glucose
Control	1·210	1·588
Methyl streptonigrin (50 μg/ml, 0·1 mM)	0·891	1·520
Streptonigrin (50 μg/ml, 0·1 mM)	0·578	1·369

A 50% solution of RBC in Krebs-Ringer phosphate was incubated with MeSN or SN for 3 hr at 37°.

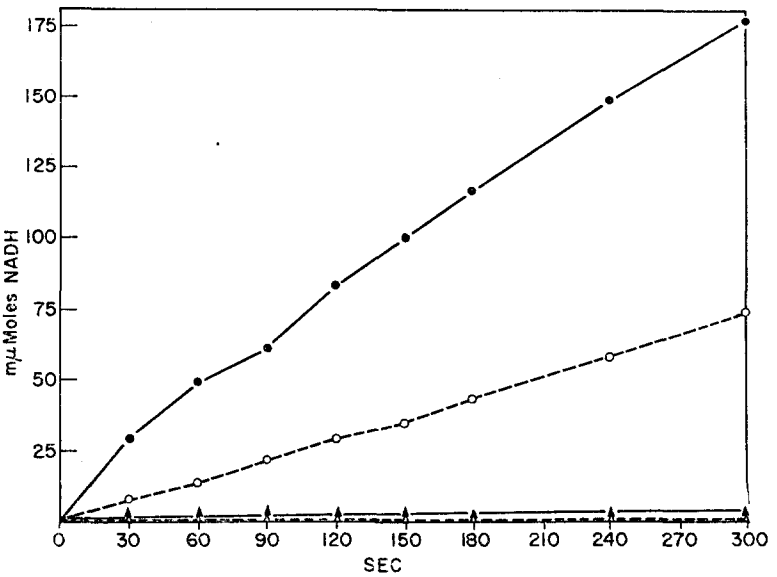

FIG. 3. Oxidation of NADH by rat liver NAD-NADP diaphorase in the presence of streptonigrin (SN) and methyl streptonigrin (MeSN). Each reaction contained 0·5 μmoles NADH, 250 μmoles Tris-HCl (pH 7·4) and 0·1 ml enzyme in a volume of 3·15 ml; SN and MeSN were added as indicated. ●—● SN 9·5 mμ mole; ○ - - - ○ MeSN 9·5 mμ mole; ▲—▲ MeSN in H_2O 9·5 mμ mole; △ - - - △ control.

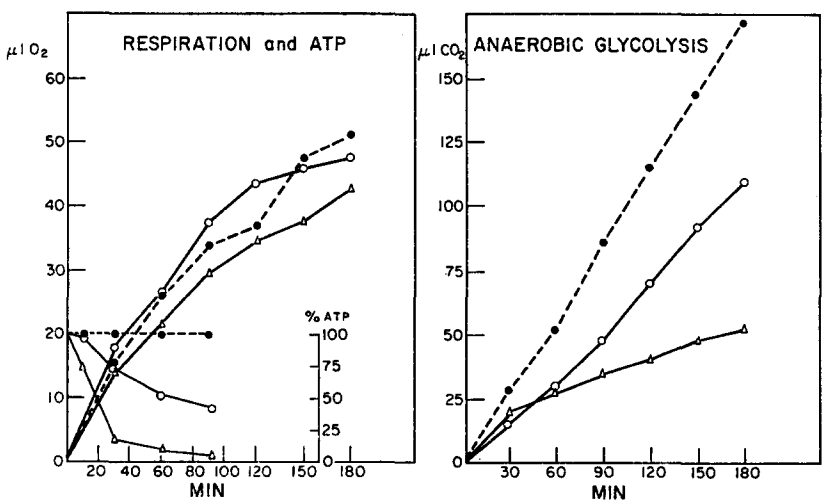

FIG. 4. Effect of streptonigrin (SN) and methyl streptonigrin on leukocyte respiration, anaerobic glycolysis, and ATP levels.
● - - - ● control; ○—○ MeSN 50 μg/ml; △—△ SN 50 μg/ml.

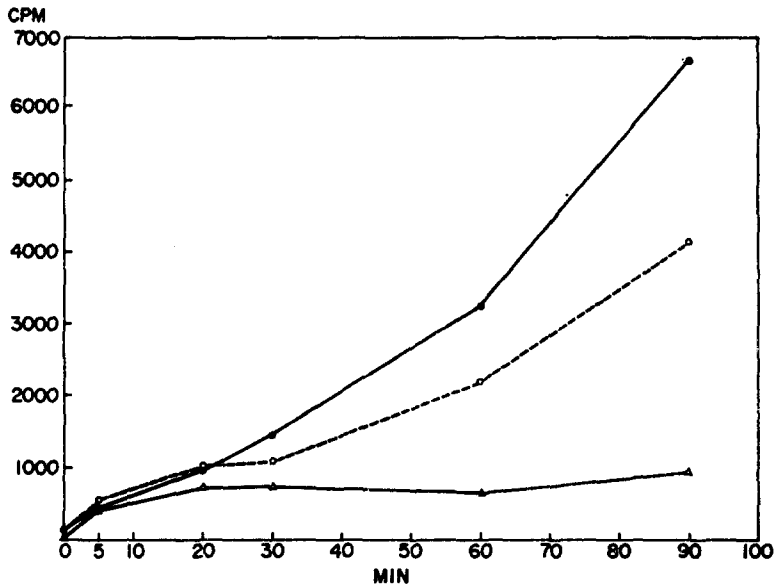

FIG. 5. Effect of streptonigrin and methyl streptonigrin on leukocyte protein synthesis. Leukocytes suspended in plasma with added glucose (3 mg/ml) and bicarbonate (1 mg/ ml) were incubated with L-arginine-U-^{14}C at 37°. Aliquots (0·1 ml) were obtained at various time intervals, and the amount of radioactivity incorporated into protein was determined.
●—● control; ○ - - - ○ MeSN 62·5 μg/ml; △—△ SN 62·5 μg/ml.

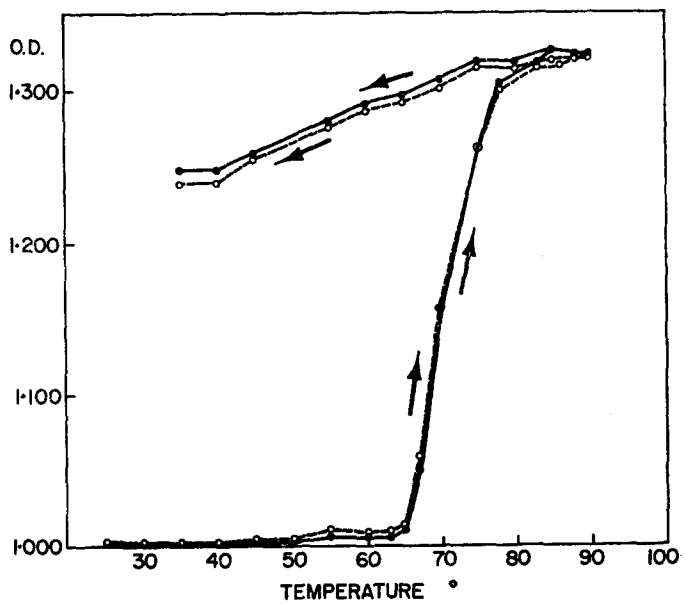

FIG. 6. The temperature melting curves of leukocyte DNA. DNA was extracted from cells incubated *in vitro* with and without methyl streptonigrin (MeSN), 50 μg/ml for 2·5 hr.
●—● control; ○ - - - ○ MeSN.

The quantity of high-molecular weight DNA isolated from leukemic cells incubated with SN or MeSN was consistently lower than that of control preparations (Table 2). SN caused a greater loss of DNA than did MeSN. The melting and annealing curves of the high-molecular weight DNA isolated from leukemic leukocytes incubated with MeSN did not differ from a control sample (Fig. 6).

TABLE 2. EFFECT OF STREPTONIGRIN AND METHYL STREPTONIGRIN ON LEUKOCYTE DNA

Sample	DNA Isolated (μg)
Zero time	390 (353–455)
Control	348
Methyl streptonigrin (50 μg/ml)	203 (189–217)
Streptonigrin (50 μg/ml)	162 (144–179)

White blood cells were incubated with SN or MeSN for 2·5 hr at 37°. The quantity of DNA recovered was determined spectrophotometrically.

DISCUSSION

Previous data from this laboratory suggested that SN had unique biochemical effects among active antitumor compounds.[12, 13] It was shown that SN catalyzed the oxidation of intra- and extra-mitochondrial NADH, which was associated with a progressive decline in cellular ATP levels. As a result of the autoxidation of reduced SN, oxygen consumption and hydrogen peroxide formation were observed. The inhibitory action on protein synthesis, and the promotion of RNA and DNA degradation by SN, were presumed to be secondary effects. It was concluded that the cytotoxic effects of SN were due to depletion of NADH and NADPH, interference with ATP production, and formation of peroxides.

Despite the considerably lower toxicity of MeSN as compared with SN, the present studies indicate that the two drugs have a similar mechanism of action. Both drugs supported a glucose-dependent red blood cell oxygen uptake, and both catalyzed the oxidation of NADH and NADPH via NAD–NADP diaphorase. The disappearance of red cell GSH after incubation with MeSN and SN is indicative of peroxide formation.[14] Glucose supported linear oxygen uptake and protected against the loss of GSH by the generation of NADH and NADPH. The two compounds caused similar impairment in the energy metabolism of the leukemic leukocytes, with marked lowering of ATP levels.

In intact leukocytes, both agents led to a loss of recoverable high-molecular weight DNA. MeSN, like SN, caused degradation of high-molecular weight DNA without evidence of cross-linking,[13] resembling the type of degradation seen with hydrogen peroxide,[15] but differing from mitomycin C, another quinoid antitumor antibiotic.[16] Protein synthesis in the intact cell was inhibited by SN and MeSN, presumably by the depletion of cellular ATP. The temporal and quantitative relationship between ATP depletion and the inhibition of protein synthesis is consistent with this hypothesis, as is the previous finding that SN had no effect on protein synthesis in a subcellular ribosomal system.[13]

Although the biochemical effects of the two drugs were similar, MeSN was considerably less active in all the systems studied. The differences were not due solely to cellular transport, since MeSN was also found to be less effective in cell-free systems. Although MeSN was insoluble in aqueous media, differences were noted when both drugs were dissolved in dimethylacetamide.

These biochemical findings *in vitro* are in accord with the studies in tissue cultures and animals which demonstrated that MeSN was less toxic than an equal amount of SN.[4] A reduction in the biochemical effects of MeSN, as compared to SN, might explain the decreased toxicity noted with this drug. If the antitumor effects and the toxicity result from common biological mechanisms, it would be predicted that MeSN would not provide a more advantageous therapeutic ratio than its parent compound.

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