KINETIC ANALYSIS OF INHIBITION OF REVERSE TRANSCRIPTASE BY STREPTONIGRIN

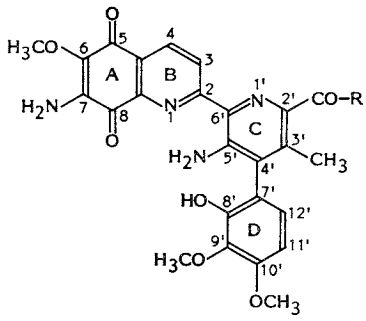
HIROMASA OKADA, YOSHIO INOUYE and SHOSHIRO NAKAMURA

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

(Received for publication September 24, 1986)

The antitumor antibiotic, streptonigrin (**1**), was first isolated from *Streptomyces flocculus*[1]. Later, the inhibition of avian myeloblastosis virus (AMV) reverse transcriptase by **1** was reported by CHIRIGOS *et al.*[2], while no inhibition was observed with methyl streptonigrin (**2**), suggesting the importance of the carboxyl group for this activity. To confirm this aspect, the biological properties of the newly synthesized streptonigrin-2′-carboxyl amide derivatives were studied comparatively with those of **1** and **2**. Unlike **2**, the amide-type derivatives unexceptionally inhibited AMV reverse transcriptase to the same extent with **1**, though their antimicrobial and cytocidal activities were much lower than those of **1**[3~5]. Moreover, the nature of these derivatives was shared by the quinoline quinone derivatives such as 6-methoxy- and 6-methoxy-7-methyl-5,8-quinoline quinones. On the basis of these observations, the quinoline quinone moiety of **1** is considered to be the minimum requisite for potent inhibitory activity of **1** against reverse transcriptase[6]. Concerning specificity, ID_{50} of **1** against reverse transcriptase was 3 μg/ml and those against DNA-directed DNA polymerase I of *Escherichia coli* origin and α of calf thymus origin were more than 160 μg/ml[5]. According to the findings of MIZUNO and GILBOE[7], and WHITE and WHITE[8], the inhibition of reverse transcriptase by **1** is speculated to result from the direct interaction with template-primer, even though the specificity of **1** for reverse transcriptase could not be explained by this mechanism. Contrary to this prediction, the inhibition of reverse transcriptase by **1** was only overcome when the concentration of reverse transcriptase was increased. The kinetic analysis of inhibition of reverse transcriptase by **1** is the subject of this paper.

Fig. 1. Structures of streptonigrin (**1**) and methyl streptonigrin (**2**).



1 R = OH

2 R = OCH_3

The activity of reverse transcriptase was assayed by the incorporation of [^{3}H]TTP into the

Table 1. Inhibition of reverse transcriptase by streptonigrin.

a) Effect of the concentration of enzyme.

Enzyme (units/ml)	% Inhibition
6.0	51
3.0	73
1.5	94
0.75	100

b) Effect of the concentration of TTP.

TTP (μM)	% Inhibition
100	71
25	70
6.3	70

c) Effect of the concentration of template-primer.

Poly(rA) (μg/ml)	Oligo(dT)$_{12\sim18}$ (unit/ml)	% Inhibition
80	0.32	72
20	0.08	62
5	0.02	57

The standard reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 30 mM NaCl, 100 μM TTP, 6 μCi/ml [^{3}H]TTP, 5 μg/ml poly(rA), 0.02 unit/ml oligo(dT)$_{12\sim18}$, 3.0 units/ml AMV reverse transcriptase and 3.0 μg/ml **1**. Incubation was carried out at 37°C for 1 hour and 50 μl aliquot of the reaction mixture was removed to determine the incorporation of [^{3}H]TTP.

Table 2. Effect of preincubation with reverse transcriptase or template-primer.

Treatment	% Inhibition
None	10
Preincubation with enzyme	62
Preincubation with template-primer	39

1 (0.1 μg) was incubated with reverse transcriptase (0.6 unit) or template-primer [0.5 μg of poly(rA), 0.002 unit of oligo(dT)$_{12\sim18}$] in a volume of 50 μl at 27°C for 12 minutes. The missing components were added to complete the reaction mixture (100 μl) and the whole was incubated at 37°C for an additional 30 minutes.

high molecular fraction as described in a previous paper[5].

The enzyme activity was measured in the presence of different concentrations of individual components of the reaction mixture, while the other components were kept at the same concentrations as those in the standard reaction mixture. The results summarized in Table 1 demonstrate that inhibition of reverse transcriptase by **1** is only reversed by increasing the enzyme concentration. Although no effect was observed by changing the concentration of TTP, the inhibition of reverse transcriptase by **1** was marginally enhanced by increasing the concentration of template-primer [poly(rA)-oligo(dT)$_{12\sim18}$]. These unexpected results motivated us to conduct a more detailed kinetic analysis.

In Table 2, **1** was incubated with either reverse transcriptase or template-primer prior to the initiation of enzyme reaction. In both cases, especially in the former one, the inhibition of reverse transcriptase by **1** was further enhanced over the value obtained without preincubation, implying that the interaction of **1** with not only enzyme but also template-primer facilitated the adverse effect of **1** on reverse transcriptase.

The initial rate of incorporation of [^{3}H]TTP was measured in the presence of 0, 5 or 10 μg/ml of **1**. The Lineweaver-Burk plot confirms that the depressed enzyme reaction is characteristic of non-competitive inhibition, *i.e.*, a decreased Vmax and an unaltered *Km* for [^{3}H]TTP incorporation (Fig. 2). Kinetic parameters as a function of the concentration of template-primer were also obtained in the absence or presence of **1**. The Lineweaver-Burk plot showed uncompetitive inhibition of **1** against [^{3}H]TTP incorporation by reverse transcriptase (data not

Fig. 2. Lineweaver-Burk plot of reverse transcriptase activity as a function of TTP concentration.

The initial rate of [^{3}H]TTP incorporation was measured in the absence (○) or presence of 5 (●) or 10 μg/ml (▲) of **1**.

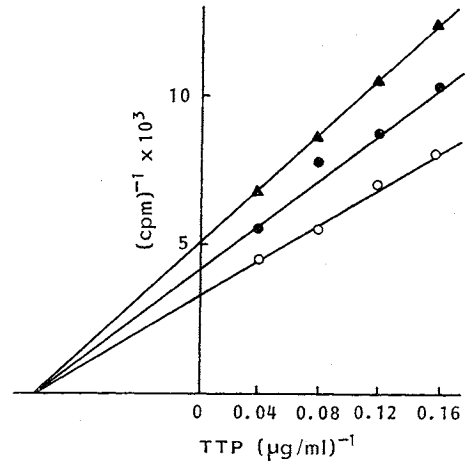
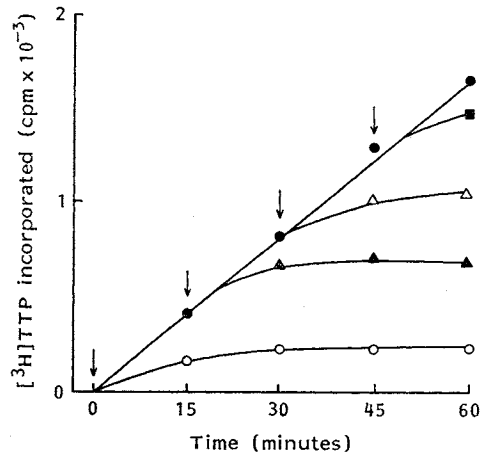


Fig. 3. Effect of delayed addition of streptonigrin.

1 was added at the times indicated by the arrows in the figure to a final concentration of 5 μg/ml.



shown), though the results were not typical and reproducible probably due to the interaction of **1** with template-primer as evident from the results shown in Table 2. In Fig. 3, **1** is added to the ongoing reaction system at different time intervals to see whether **1** inhibits reverse transcriptase at the initiation or elongation step. It is clear that **1** causes an instantaneous inhibition of reverse transcriptase whenever it is added to the reaction system.

MIZUNO and GILBOE[7], and WHITE and WHITE[8] placed much stress on the binding ability of **1** to DNA as the basis for its biological activities. However, according to the findings of LASZLO *et al.*[9~11] and ours[12], **1** primarily interferes with oxidative phosphorylation in mitochondria by playing a role of an electron acceptor in the oxidation of NADH by DT-diaphorase and the inhibition of DNA, RNA or protein synthesis is rather secondary to this effect. Recently, we observed that naphthoquinone derivatives with appropriate substitutions were active as an electron acceptor in the oxidation of NADH by *Clostridium kluyveri* diaphorase as well as an inhibitor of reverse transcriptase, whereas those inactive as an electron acceptor were also inactive as an inhibitor of reverse transcriptase[13]. By these observations and the experimental results in this paper, it may be concluded that **1** preferentially acts on the enzyme molecule in an enzyme-template-primer complex by a series of reactions including oxidation-reduction.

References

1) RAO, K. V. & W. P. CULLEN: Streptonigrin, an antitumor substance. I. Isolation and characterization. *In* Antibiotics Annual 1959~1960. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 950~953, Medical Encyclopedia Inc., New York, 1960
2) CHIRIGOS, M. A.; J. W. PEARSON, T. S. PAPAS, W. A. WOODS, H. B. WOOD, Jr. & G. SPAHN: Effect of streptonigrin NSC-45383 and analogs on oncornavirus replication and DNA polymerase activity. Cancer Chemother. Rep. 57: 305~309, 1973
3) MIYASAKA, T.; S. HIBINO, Y. INOUYE & S. NAKAMURA: Synthesis of novel streptonigrin-2-carboxyl amide derivatives by using 3,3'-(phenylphosphonyl)-bis(1,3-thiazolidine-2-thione). J. Chem. Soc. Perkin Trans. I 1986: 479~482, 1986
4) INOUYE, Y.; H. OKADA, S. K. ROY, T. MIYASAKA, S. HIBINO, N. TANAKA & S. NAKAMURA: Biological properties of streptonigrin derivatives. I. Antimicrobial and cytocidal activities. J. Antibiotics 38: 1429~1432, 1985
5) OKADA, H.; H. MUKAI, Y. INOUYE & S. NAKAMURA: Biological properties of streptonigrin derivatives. II. Inhibition of reverse transcriptase activity. J. Antibiotics 39: 306~308, 1986
6) INOUYE, Y.; Y. TAKE, K. OOGOSE, A. KUBO & S. NAKAMURA: The quinoline quinone as the minimum entity for reverse transcriptase inhibitory activity of streptonigrin. J. Antibiotics 40: 105~107, 1987
7) MIZUNO, N. S. & D. P. GILBOE: Binding of streptonigrin to DNA. Biochim. Biophys. Acta 224: 319~327, 1970
8) WHITE, H. L. & J. R. WHITE: Interaction of streptonigrin with DNA in vitro. Biochim. Biophys. Acta 123: 648~651, 1966
9) HOCHSTEIN, P.; J. LASZLO & D. MILLER: A unique, dicumarol-sensitive, non-phosphorylating oxidation of DPNH and TPNH catalyzed by streptonigrin. Biochem. Biophys. Res. Commun. 19: 289~296, 1965
10) KREMER, W. B. & J. LASZLO: Comparison of biochemical effects of isopropyridine azastreptonigrin (NSC-62709) with streptonigrin (NSC-45383). Cancer Chemother. Rep. 51: 19~24, 1967
11) MILLER, D. S.; J. LASZLO, K. S. MCCARTY, W. R. GUILD & P. HOCHSTEIN: Mechanism of action of streptonigrin in leukemic cells. Cancer Res. 27: 632~638, 1967
12) INOUYE, Y.; H. OKADA, J. UNO, T. ARAI & S. NAKAMURA: Effects of streptonigrin derivatives and sakyomicin A on the respiration of isolated rat liver mitochondria. J. Antibiotics 39: 550~556, 1986
13) TAKE, Y.; M. SAWADA, H. KUNAI, Y. INOUYE & S. NAKAMURA: Role of the naphthoquinone moiety in the biological activities of sakyomicin A. J. Antibiotics 39: 557~563, 1986