THE SYNERGISIM OF NUCLEOSIDE ANTIBIOTICS COMBINED WITH GUANINE 7-*N*-OXIDE AGAINST A RHABDOVIRUS, INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV)†

MASAHIDE HASOBE and MINEO SANEYOSHI*

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

KIYOSHI ISONO

Riken, The Institute of Physical and Chemical Research, Wako-shi, Saitama 351-01, Japan

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Guanine 7-*N*-oxide was shown to have synergistic activity in combination with neplanocin A against a rhabdovirus, infectious hematopoietic necrosis virus (IHNV), as reported previously.

We examined further the antiviral activity of guanine 7-*N*-oxide in combination with other nucleoside antibiotics against IHNV. Synergism was seen between guanine 7-*N*-oxide and D-eritadenine or cordycepin. It is considered that compounds inhibiting RNA methylation show synergism with guanine 7-*N*-oxide.

Isolation and biological activity of guanine 7-*N*-oxide (Gua-7-oxide), a new antibiotic produced by *Streptomyces* sp., have recently been reported from several laboratories[2~5]. Moderate antiviral activity of this compound has also been demonstrated against DNA and RNA viruses derived from salmonids in our laboratory[6]. The mechanism of action of Gua-7-oxide against infectious hematopoietic necrosis virus (IHNV) is interesting. The incorporation of [^{3}H]uridine ([^{3}H]Urd) into viral RNA was not inhibited during viral RNA replication in IHNV infected cells as in the case of neplanocin A (Nep A). Gua-7-oxide combined with Nep A, an inhibitor of RNA methylation[7], showed synergistic anti-IHNV activity. If the inhibition site and the mode of inhibition of viral replication by both Gua-7-oxide and Nep A is the same, the combination of these agents should give an additive response. Therefore, the site of action of Gua-7-oxide may not be viral RNA methylation. Furthermore, because this compound did not inhibit the incorporation of [^{3}H]Urd into viral RNA, it was suggested that the mechanism of action of Gua-7-oxide could be the inhibition of viral RNA maturation possibly at the capping stage in infected cells.

Recently, we developed a convenient procedure for evaluation of anti-RNA viral agents in a microplate with 96 wells[8]. In the assay of drug combinations, this method is now also available for the evaluation of drug interaction. Thus we attempted to study the antiviral activity of drug combinations using this method.

In this paper, we report antiviral activities of several nucleoside antibiotics combined with Gua-7-oxide against IHNV.

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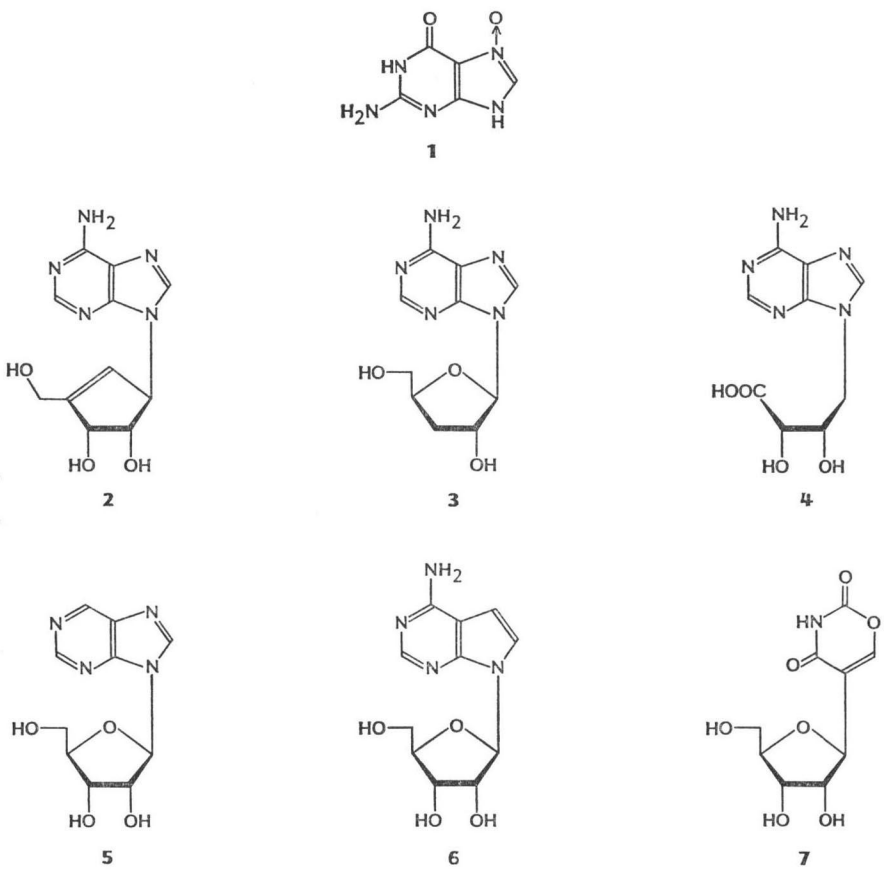
† This paper constitutes Part XXV of a series of Synthetic Nucleosides and Nucleotides Part XXIV[1].

This paper is dedicated to Professor NELSON J. LEONARD for the occasion of his 70th birthday and retirement from University of Illinois on this year.

Fig. 1. Chemical structures of the nucleoside antibiotics.

1; Guanine-7-oxide (Gua-7-oxide). **2**; Neplanocin A (Nep A). **3**; Cordycepin (Cordy). **4**; D-Eritadenine (D-Erit). **5**; Nebularine (Nebula). **6**; Tubercidin (Tuber). **7**; Minimycin (Mini). In this study, the name of each compound is abbreviated in parentheses.



Materials and Methods

Cells and Virus

Chinook salmon embryo (CHSE-214) cells[9] and infectious hematopoietic necrosis virus (IHNV)[10,11] were used in all experiments. The method for cultivation of CHSE-214 cells and the preparation of IHNV were reported previously[6,12].

Compounds

Gua-7-oxide was prepared from *Streptomyces* sp. by the method reported previously[2,4]. Cordycepin (Cordy) (Yamasa), D-eritadenine (D-Erit) (Tanabe Seiyaku Co., Ltd.), Nep A (Toyo Jozo Co., Ltd.), minimycin (Mini) (Kaken Kagaku) and tubercidin (Tuber) (Kaken Kagaku) were gifts from the indicated companies. Nebularine (Nebula) was synthesized by the method described previously[13]. The structures of these compounds are shown in Fig. 1.

Cytopathic Effect (CPE) Spot Reduction Method

The CPE spot reduction method using a microtiter plate with 96 wells was employed for the evaluation of anti-IHNV activity and the investigation of drug combinations. The details of this method were reported previously[6,8].

Table 1. Antiviral activity of Gua-7-oxide and nucleoside antibiotics used in combination.

Drug combination	Minimum FIC index[a]	FIC[b]	Amounts of drug required (μg/ml)		Decrease in IC_{50} (%)	Drug interaction
			IC_{50} combination	IC_{50} alone		
Gua-7-oxide		0.20	2.2	11.1	80	
	0.45					Synergistic
+Nep A		0.25	0.1	0.4	75	
Gua-7-oxide		0.40	6.2	15.5	60	
	0.74					Slightly
+Cordy		0.34	1.0	2.9	66	synergistic
Gua-7-oxide		0.12	1.7	14.4	88	
	0.42					Synergistic
+D-Erit		0.30	18.0	60.0	70	
Gua-7-oxide		0.50	7.0	14.0	50	
	1.04					Additive
+Nebula		0.54	0.7	1.3	46	
Gua-7-oxide		0.60	8.4	14.0	40	
	1.22					Slightly
+Mini		0.62	0.8	1.3	38	antagonistic
Gua-7-oxide		0.75	7.5	10.0	25	
	1.42					Antagonistic
+Tuber		0.67	0.4	0.6	33	

[a] Minimum FIC index was chosen from the isobologram.
[b] FIC, FIC of Gua-7-oxide and the combined drug present at the minimum FIC index point.

Viral RNA Synthesis

The procedure for detection of viral RNA synthesis on IHNV infected CHSE-214 cells was described in the previous paper[6].

Antiviral Activity in Drug Combination

The anti-IHNV activity in drug combination was examined by the CPE spot reduction method described above[6]. Compounds were assayed in duplicate at six or eight concentrations of one-third logarithmic dilution. The effect of this combination study was expressed graphically in statistically fitted isobolograms[14,15]. Each 50% inhibitory concentration (IC_{50}) obtained from CPE spot reduction assay of Drug-1 in combination was reexpressed as a fractional inhibitory concentration (FIC), that is, as a fraction of IC_{50} of Drug-1 used alone (Drug-1 FIC=Drug-1 IC_{50} in combination/Drug-1 IC_{50} alone). The resulting Drug-1 FIC was pared with the FIC of Drug-2 that was present in that combination (Drug-2 FIC=concentration of Drug-2 in combination/Drug-2 IC_{50} alone). The FIC for Drug-2, in the presence of each concentration, were determined by the same method.

Results

Antiviral Activity of Nucleoside Antibiotics

The antibiotics shown in Fig. 1 were tested for the anti-IHNV activity by CPE spot reduction method. The concentration of each compound required to suppress CPE spots to 50% was determined as 0.4 μg/ml of Nep A, 0.6 μg/ml of Tuber, 1.3 μg/ml of Mini and Nebula, 2.9 μg/ml of Cordy, 60 μg/ml of D-Erit and about 10 μg/ml of Gua-7-oxide (Fig. 2 and Table 1), respectively. The profiles of CPE spot reduction curves of compounds were cleraly dose-dependent (Fig. 2).

Effect on Viral RNA Synthesis

In order to measure viral RNA synthesis, actinomycin D (1.5 μg/ml) was added to inhibit cellular

Fig. 2. Antiviral activities of nucleoside antibiotics against IHNV by CPE spot reduction method.
○: Gua-7-oxide, ●: Nep A, △: Cordy, ▲: D-Erit, □: Nebula, ■: Mini, ▽: Tuber.
The compounds were diluted in one-third log dilution.

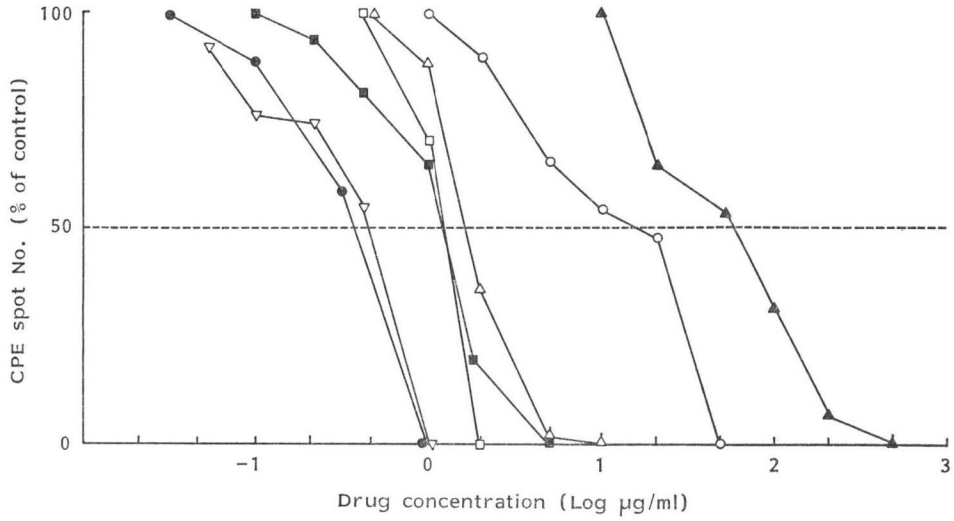
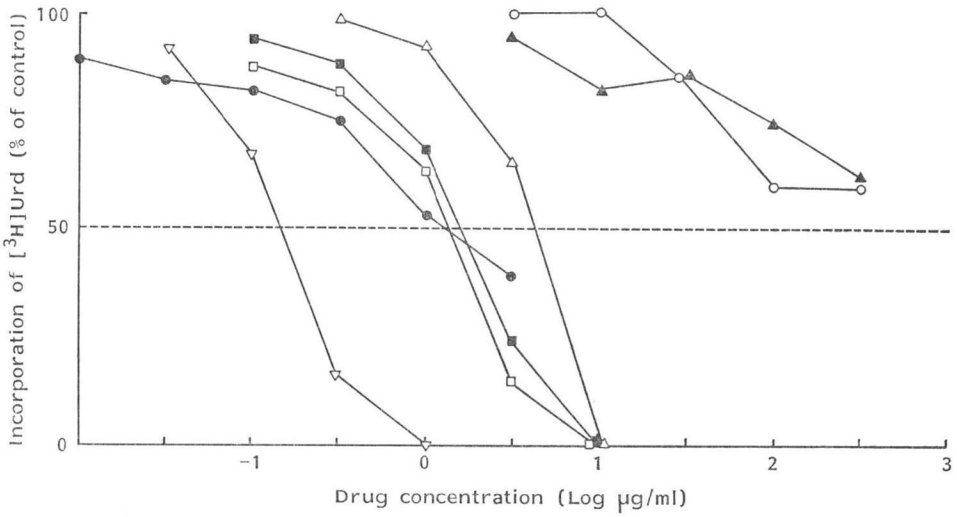


Fig. 3. The effect of nucleoside antibiotics on IHNV viral RNA synthesis.
○: Gua-7-oxide, ●: Nep A, △: Cordy, ▲: D-Erit, □: Nebula, ■: Mini, ▽: Tuber.
The 100% incorporation of [^{3}H]Urd is the value subtracted with the uninfected sample (3,000 cpm) treated with actinomycin D from the infected sample (30,000 cpm) treated with actinomycin D.



RNA synthesis. A peak of [^{3}H]Urd incorporation into IHNV RNA appeared between 12~15 hours post-infection (p.i.) (data not shown). Therefore, pulse labeling for 3 hours with radioactive precursor was performed at 12 hours p.i.

As shown in Fig. 3, Nebula and Mini showed inhibition profiles of viral RNA synthesis similar to that of antiviral activity using the CPE spot reduction assay. Although Cordy showed inhibition of viral RNA at a concentration of log 0.5 lower than that of anti-IHNV activity, the profile of inhibition was similar to that of the CPE spot reduction curve. D-Erit weakly inhibited viral RNA synthesis at a

Fig. 4. Isobolograms representing the anti-IHNV effects of antibiotics combined with Gua-7-oxide.

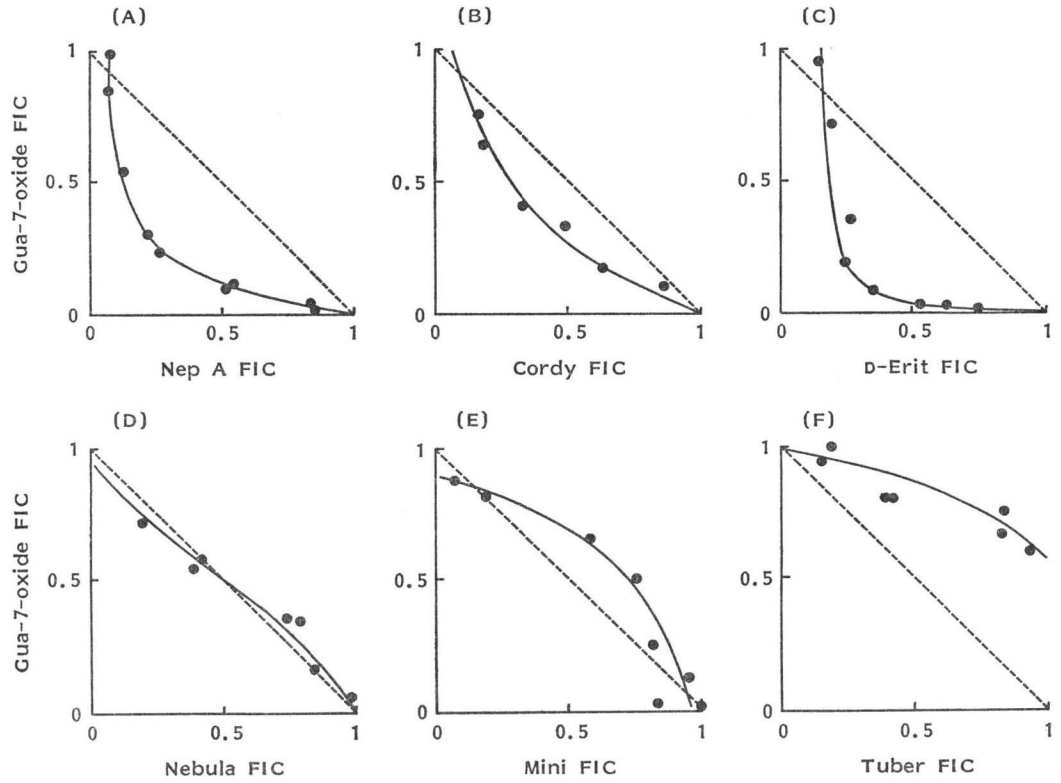
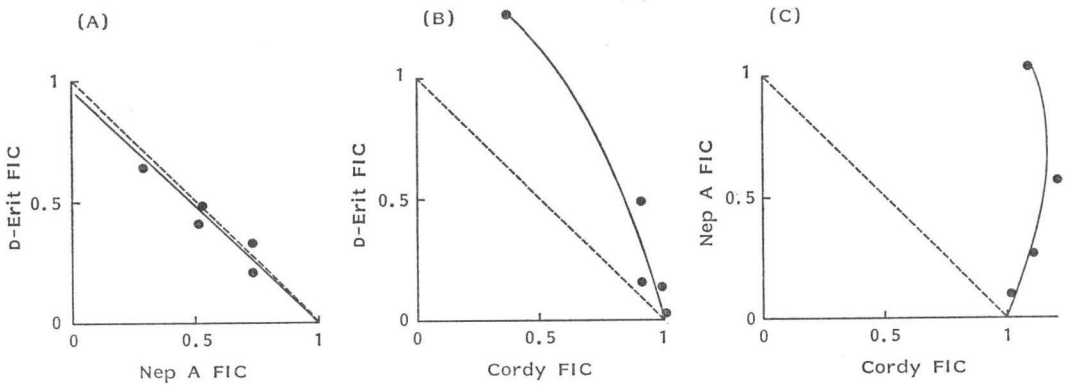


Fig. 5. Isobolograms representing the anti-IHNV effect of the combination of D-Erit and Nep A (A), D-Erit and Cordy (B) and Nep A and Cordy (C).



concentration of 320 μg/ml, which was equal to the 100% inhibitory concentration of CPE spots with IHNV infection. This profile of D-Erit was similar to that of Nep A and Gua-7-oxide as shown in Fig. 3.

Antiviral Activity in Drug Combination

The antiviral activities of Gua-7-oxide in combination with nucleoside antibiotics against IHNV are shown in Table 1. Effectiveness of drug combinations were expressed graphically in statistically

fitted isobolograms in Fig. 4. The isobolograms are used for quantitative analysis of the type of interaction among the compounds on inhibition of viral replication. FIC index values of less than 1.0 indicate a synergistic response. About a FIC value of 1.0 indicates an additive response, and FIC values greater than 1.0 are indicative of antagonistic response[14,15].

Minimum FIC indexes shown in Table 1 are less than 1.0 in the case of combination with Cordy, D-Erit and Nep A, 1.0 in combination with Nebula, and more than 1.0 in combination with Mini and Tuber. These data imply that Gua-7-oxide is synergistic with D-Erit and Nep A, slightly synergistic with Cordy, additive with Nebula, and antagonistic with Mini and Tuber.

Additionally, three compounds which showed synergistic response in combination with Gua-7-oxide were tested for interaction among them, *i.e.*, D-Erit and Nep A, D-Erit and Cordy, and Nep A and Cordy in combination. As shown in Fig. 5, the isobolograms indicated an additive response for D-Erit - Nep A, and antagonistic response for D-Erit - Cordy and Nep A - Cordy.

Discussion

In this paper, we examined the anti-IHNV activity of combinations of nucleoside antibiotics with Gua-7-oxide.

Synergistic response was observed for D-Erit, Cordy and Nep A when combined with Gua-7-oxide.

D-Erit is an inhibitor of *S*-adenosyl-L-homocystein hydrolase[16] like Nep A, and is considered to inhibit IHNV RNA methylation. Therefore the incorporation of [^{3}H]Urd into IHNV RNA on CHSE-214 cells treated with D-Erit showed no appreciable reduction (Fig. 3).

It is considered that Cordy is incorporated into RNA, resulting in polynucleotide chain termination[17], and inhibition of nucleic acid methylation *etc.*[18,19].

Besides the nucleoside antibiotics examined in this study, we have also observed that some synthetic nucleoside analogs show synergistic response in combination with Gua-7-oxide. Some of these synthetic compounds, *e.g.*, [*S*]-DHPA ([*S*])-9-(2,3-dihydropropyl)adenine), SIBA (5'-deoxy-5'-isobutylthioadenosine), D-eritadenine analog (D-eritadenineamide), are known to inhibit nucleic acid methylation.

It is concluded that the combination of Gua-7-oxide and an inhibitor of nucleic acid methylation results in a synergistic anti-IHNV response.

The antagonistic response with Tuber needs explanation. Tuber is known to be incorporated into oligonucleotide and behaves as an ATP analog[20]. If this is the case for IHNV-infected cells, IHNV messenger RNA (mRNA) synthesis might be inhibited at the stage of RNA maturation. Therefore, Gua-7-oxide could not function at the stage of mRNA maturation as described before[6], thus the anti-IHNV activity of this combination would lead to an antagonistic response.

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