*Antiviral Research* 14 (1990) 173–178
Elsevier



AVR 00438

Short Communication

# Inhibitory effect of a new antibiotic, guanine 7-N-oxide, on the replication of several RNA viruses

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(Received 9 October 1989; accepted 12 June 1990)

## Summary

Guanine 7-N-oxide (G-7-Ox) was examined for its antiviral activity against 9 viruses based on plaque reduction, neuraminidase activity reduction, a fluorescent antibody technique or ELISA. The following viruses were included in the tests: influenza, Sendai, simian virus 5 (SV5), respiratory syncytial, western equine encephalitis, Japanese encephalitis, vesicular stomatitis, rabies and polio. G-7-Ox showed broad anti-RNA viral activity against all viruses tested, except for poliovirus. Inhibition of persistent SV5 infection by G-7-Ox indicates that its antiviral activity is independent of cytotoxicity.

Guanine 7-N-oxide; Anti-RNA virus activity

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Guanine 7-N-oxide (G-7-Ox) was independently isolated from the culture broth of *Streptomyces* sp. in 1985 at two laboratories (Kern et al., 1985; Nishii et al., 1985). Nishii et al. showed that this agent had the unique property of possessing antitumor and antifungal activities, but not antibacterial activity. It was then examined for antiviral activity against fish viruses in cultured fish cells and was found to possess antiviral activity against RNA and DNA fish viruses (Hasobe and Saneyoshi, 1985).

For DNA viruses, some effective antiviral agents such as acyclovir, BVDU and BVaraU have been developed (Elion et al., 1977; Schaeffer et al., 1978; De Clercq et al., 1979; De Clercq and Walker, 1984; Machida and Sakata, 1984, Machida, 1986), but for RNA virus infections no new compounds have been made available

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for clinical use recently. We have now evaluated the activity of G-7-Ox against a number of mammalian RNA viruses. This report describes the broad-spectrum anti-RNA virus activity of G-7-Ox, which may be representative of a new category of antiviral substances.

In this study, the following 9 RNA viruses were used: influenza virus (IFV) A/PR8 (HON1), Sendai virus (Z), respiratory syncytial (RS) virus (Long strain), simian virus 5 (SV5) (strain 1243L), western equine encephalitis (WEE) virus (McMillan strain), Japanese encephalitis (JE) virus (Nakayama strain), vesicular stomatitis virus (VSV) (Indiana strain), rabies virus (Flury-HEP), and poliovirus type 1 (Sabin strain). IFV and Sendai virus were grown in fertilized hen's eggs and the chorioallantoic fluid was harvested for use. RS virus was grown in HEp2 cells. WEE, JE, rabies virus and VSV were propagated in BHK cells. For SV5 and poliovirus, primary monkey kidney (MK) cells were used.

Two kinds of cell cultures were used: BHK-21 cells were cultured in Eagle's minimal essential medium (MEM) with or without 6% calf serum. Primary MK cells were cultured in Earle's based medium containing lactalbumin hydrolysate with or without 2% calf serum (for growth and maintenance, respectively).

Antiviral activity was determined by 50% inhibition in each assay system (see Tables 1–3) and is expressed as the 50% inhibitory concentration ($IC_{50}$). Cytotoxicity was determined by two methods. The minimal cytotoxic concentration (MCC) for confluent monolayers of cells was determined as described in our previous report (Yamamoto et al., 1988). To investigate inhibition of growing cells, BHK or MK cells were seeded into 35-mm plastic dishes at a density of 2–3 $\times$ $10^5$ cells/well. The cells were incubated for 5 days, trypsinized and counted. The effective dose ($ED_{50}$) causing 50% cell growth inhibition is expressed as the concentration required to reduce the increment in cell number by 50%. The antiviral index (AI) is defined as the ratio of the MCC or $ED_{50}$ to the $IC_{50}$.

G-7-Ox showed broad antiviral activity against all viruses tested, except for poliovirus. Togaviruses (WEE and JE virus) and rhabdoviruses (VSV and rabies virus) grow well in BHK cells. For these viruses (except for rabies virus), the activity of G-7-Ox was assayed by plaque reduction. As shown in Table 1, WEE virus was slightly more sensitive to this agent ($IC_{50}$: 18 $\mu$g/ml) than JE virus or VSV, and G-7-Ox completely inhibited WEE virus plaque formation at 50 $\mu$g/ml. BHK cell proliferation was inhibited by 50% when 1.0 $\mu$g/ml of G-7-Ox was added, and this resulted in AI values lower than one, thus indicating a negative selectivity. On the other hand, confluent monolayers of BHK cells were not sensitive to the toxicity of G-7-Ox (MCC: 195 $\mu$g/ml). Thus, viral plaque formation was completely inhibited at G-7-Ox concentrations that were not toxic to the cells. Also, G-7-Ox effected an extensive reduction of plaque size at concentrations lower than the $IC_{50}$ (data not shown). With regard to rabies virus, the $IC_{50}$ was less than 3 $\mu$g/ml as determined by fluorescent antibody (FA) positive cell-counting.

IFV, Sendai virus and RS virus infect BHK cells abortively. Therefore, the activity of G-7-Ox against these viruses was determined using a special technique devised for this purpose. IFV and Sendai virus infection was monitored by their neuraminidase (NDase) activity (Table 1). This activity was reduced by 50% at 1 to 3 $\mu$g/ml of the agent. For RS virus, viral protein production was assayed by

TABLE 1
Antiviral activity of G-7-Ox against RNA viruses in BHK cells

| Virus | Assay | $IC_{50}$[a] ($\mu$g/ml) | AI[b] | |
|---|---|---|---|---|
| | | | $MCC/IC_{50}$ | $ED_{50}/IC_{50}$ |
| WEE | Plaque[c] | 18.2 ± 3.4 | 10.8 | 0.05 |
| JE | Plaque[c] | 30.0 ± 5.0 | 6.5 | 0.03 |
| VSV | Plaque[c] | 27.9 ± 7.1 | 7.0 | 0.04 |
| Rabies | FA[d] | > 3.0 | > 65.0 | > 0.33 |
| IFV[e] | NDase[f] | 2.2 ± 1.1 | 88.6 | 0.45 |
| Sendai[e] | NDase[f] | 1.1 ± 0.1 | 177.3 | 0.91 |
| RS | ELISA[g] | 4.1 ± 1.3 | 47.6 | 0.24 |

[a]Antiviral activity is expressed as the 50% inhibitory concentration ($IC_{50}$) for plaque reduction or inhibition of viral protein synthesis as compared to the control. Mean value for 3 experiments.
[b]AI was evaluated as described in the text. The MCC and $ED_{50}$ of G-7-Ox for BHK cells was 195 and 1.0 $\mu$g/ml, respectively.
[c]Plaques were formed in BHK cells incubated in Eagle's MEM including 1% methylcellulose and various concentrations of the agent. After 3 days, plaques were fixed with 10% folmarin and counted by methylene blue staining.
[d]The direct fluorescent antibody (FA) technique was used as described previously (Arai, 1985).
[e]BHK cells were infected at an MOI of 0.01. After 3 days of culture, the entire culture was solubilized with 0.75% Nonidet P-40 (Shell Chemical Co., U.S.A.) and the supernatant obtained by centrifugation at 3,000 rpm for 20 min was used as the enzyme source.
[f]Viral samples as the enzyme source were mixed with an equal volume of 1% fetuine (Gibco Laboratories, U.S.A.) in 0.2 M phosphate buffer or 0.1 M acetate buffer with 0.1% $CaCl_2$ for IFV or Sendai virus, respectively; and then incubated for 1.5–2 h at 37°C. The sialic acid released by the neuraminidase (NDase) was quantitated by the method of Aminoff (1961).
[g]Infected BHK cell cultures were freeze-thawed once and centrifuged at 3,000 rpm. The ELISA titer of supernatant was determined using an RS virus ELISA diagnostic kit (Dynabott Inc., U.S.A.).

ELISA. This virus also showed sensitivity to G-7-Ox ($IC_{50}$: 3–5 $\mu$g/ml).

Thus, the $IC_{50}$ values were determined separately for viruses that grow well in BHK cells and viruses that grow abortively. The $IC_{50}$ for the former group of viruses (WEE, JE and VSV) ranged from 10 to 30 $\mu$g/ml. For the latter viruses the $IC_{50}$ values were 1–5 $\mu$g/ml. Rabies virus belongs to the former group, showing good growth with a poor cytopathic effect in BHK cells, but its $IC_{50}$ value was like that of the latter group. The effect of G-7-Ox on this virus was not measured by counting plaques, but based on FA staining. Therefore, the difference in $IC_{50}$ noted between the two groups might have depended on the assay system used. This possibility was also supported by the finding that plaque size was greatly reduced at the latter group's $IC_{50}$ (data not shown).

In the case of the paramyxovirus SV5, hemagglutination (HA) titration and hemadsorption (HAd) tests of infected MK cells were performed. As shown in Table 2, viral HA was inhibited at an $IC_{50}$ of 0.5–1.0 $\mu$g/ml, a concentration that was lower than the MCC (10 $\mu$g/ml) for monolayers and also lower than the $ED_{50}$ (2.6 $\mu$g/ml) for doubling cells. The activity of G-7-Ox against SV5 was also confirmed by the HAd test; infection of MK cells was inhibited to one-fourth or less at 1 $\mu$g/ml of the agent. The finding that the AI (3.42) of SV5 in MK cells was greater than 1.0 suggests that G-7-Ox inhibited some stage(s) of viral growth

TABLE 2
Antiviral activity of G-7-Ox against SV5 in MK cells

| Concentration | HA[a]/0.5 ml (Mean[b]) | HAd score[c] | $IC_{50}$ ($\mu$g/ml) | AI[d] |
|---|---|---|---|---|
| 4 | < 4 | | | |
| 2 | < 4 | | | |
| 1 | 4–8 (6.7) | +~+ | | |
| 0.5 | 8–16 (11.1) | ++~+++ | 0.76 | 3.42 |
| 0 | 16 | ++++ | | |

[a]MK cells were infected at an MOI of about 1.0 in 96-well microplates. After 5 days culture, the HA titer of culture fluid was assayed using guinea pig red blood cells (GPRBC).
[b]Mean HA titer of 9 culture fluids.
[c]The HAd test was scored from +1 to +4, with +4 meaning that the entire surface of an infected cell was covered by GPRBC.
[d]Ratio of the $ED_{50}$ (2.6 $\mu$g/ml) for MK cells to the $IC_{50}$.

independently of its cytotoxicity. It is known that macromolecular synthesis by SV5 in MK cells is highly independent of the host cell metabolism and that viral-specific RNA synthesis is 1:100 or less of host RNA synthesis (Holmes and Choppin, 1966). Growth of SV5 was completely inhibited at 2 $\mu$g/ml of the agent (Table 2).

With respect to the antiviral mechanism of G-7-Ox, Hasobe et al. (1985, 1986) have suggested that this agent inhibits mRNA cap formation. If this assumption is correct, the growth of some picornaviruses like poliovirus would not be inhibited by G-7-Ox, because poliovirus does not possess a cap but a small protein, Vpg, at the 5′ terminal of its mRNA (Nomoto et al., 1977). The effect of G-7-Ox on the growth of poliovirus was tested in primary MK cell cultures. The results are summarized in Table 3. Primary (uninfected) MK cells showed cytotoxicity (+) at 10 $\mu$g/ml of the agent and the toxicity was increased to +2 at 20-40 $\mu$g/ml. On the other hand, poliovirus-infected cells showed a viral cytopathic score of +4 at a G-7-Ox concentration of 20 $\mu$g/ml and a score of +3 even at a concentration of 40 $\mu$g/ml. This suggests that poliovirus could still grow, even when MK cell metabolism was disturbed by G-7-Ox. At the end of this experiment, each culture was assessed for viral content. As shown in Table 3, virus infectivity was reduced by more than 2-fold only when the concentration of the agent rose to 80 $\mu$g/ml. On the other hand, IFV growth was completely inhibited in a parallel experiment, even at a concentration of 10 $\mu$g/ml.

Furthermore, we demonstrated that mRNA synthesis of WEE virus or VSV, as determined by [$^3$H]uridine incorporation, was not affected by G-7-Ox concentrations higher than the $IC_{50}$, but that the mRNA produced was not translated (data not shown). This finding is not inconsistent with the hypothesis that G-7-Ox might inhibit cap formation. However, these results only provide indirect evidence for the inhibition of mRNA cap formation and direct evidence is lacking. The agent may be converted to an active form (perhaps guanosine 7-N-oxide 5′-triphosphate) after uptake by the cells (Jackson et al., 1987), this possibility remains to be explored. In conclusion, although the precise antiviral mechanism of G-7-Ox is not known, it can be considered as a broad-spectrum antiviral agent active against a wide range

TABLE 3
Effect of G-7-Ox on poliovirus and IFV in MK cells

| Concentration ($\mu$g/ml) | Cytotoxicity | Poliovirus | | IFA |
|---|---|---|---|---|
| | | CPE[a] | Infectivity | HA[c] |
| None | | ++++ | $10^{6.75}$ | 64 |
| 10 | + | ++++ | $10^{6.50}$ | < 4 |
| 20 | ++ | ++++ | $10^{6.50}$ | < 4 |
| 40 | ++ | +++ | $10^{6.50}$ | < 4 |
| 80 | +++ | ++ | $10^{5.50}$ | < 4 |

[a]The cytopathic effect (CPE) of poliovirus was scored from + (about 25% cell destruction) to ++++ (complete cell destruction).
[b]Infected culture fluids were re-titrated based on the $TCID_{50}$ in 96-well microtiter plates. Mean values for 4 wells.
[c]Determined with chicken red blood cells.

of RNA viruses.

## Acknowledgement

We would like to thank Dr M. Nishii for supplying G-7-Ox and for his valuable advice. This work was supported in part by a grant-in-Aid for Fundamental Research from the Human Science Foundation.

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