Antiviral and immunoenhancing properties of 7-thia-8-oxoguanosine and related guanosine analogues

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DF SMEE, HB COTTAM, BS SHARMA, et al. Antiviral and immunoenhancing properties of 7-thia-8-oxoguanosine and related guanosine analogues. Can J Infect Dis 1992;3(Suppl B):41B-48B. 7-thia-8-oxoguanosine (TOGuo) is the first reported structure of a family of modified guanosine analogues exhibiting antiviral activity in rodent models. Its spectrum of action includes interferon-sensitive viruses such as alphaviruses, bunyaviruses, coronaviruses, flaviviruses, picornaviruses and, to a lesser extent, herpesviruses. Early treatment before or shortly after virus challenge is necessary to protect animals from mortality. The protective effect of TOGuo against Semliki Forest and Punta Toro viruses can be eliminated by co-treatment with antibody to alpha/beta-interferon, indicating that interferon induction is of prime importance for antiviral activity against these two viruses. Immunological studies indicate that the nucleoside induces alpha-interferon and activates B cells, natural killer cells, antibody-dependent cytotoxic cells and macrophages. TOGuo also has adjuvant activity, since its use in combination with a killed L1210 leukemia cell vaccine greatly reduced the mortality of mice inoculated with live L1210 leukemia cells compared with the vaccine used alone. Several related guanosine analogues show similar antiviral and immunoenhancing properties, including 7-methyl-8-oxoguanosine, 7-methyl-8-thioxoguanosine and 7-deazaguanosine. These studies indicate that certain modifications at the 7 and 8 positions of guanosine may confer antiviral and immunostimulatory properties to nucleoside analogues.

Key Words: *Analogues, Antiviral, Guanosine, Immune-enhancing*

Propriétés antivirales et immunostimulatrices de la 7-thia-oxoguanosine et autres analogues de la guanosine

RESUME: La 7-thia-8-oxoguanosine (TOGuo) est la première structure rapportée d'une famille d'analogues de la guanosine modifiée qui a un effet antiviral dans le modèle souris. Son spectre d'action inclut les virus sensibles à l'interféron tels les alphavirus, bunyavirus, coronavirus, flavivirus, picornavirus et, à un degré moindre, l'herpèsvirus. Il est nécessaire d'administrer le traitement rapidement avant ou après l'inoculation du virus pour prévenir la mortalité. L'effet protecteur de la TOGuo contre les virus de la forêt Semliki et de Punta Toro peut être éliminé par un traitement aux anticorps dirigés contre l'interféron alpha/bêta, ce qui indique que l'induction à l'interféron est d'importance primordiale pour l'activité antivirale dirigée contre ces deux virus. Des études immunologiques indiquent que le nucléoside déclenche la production d'interféron alpha et active les cellules B, les cellules tueuses naturelles, les cellules cytotoxiques anticorps-dépendantes et les macrophages. La TOGuo possède aussi un effet adjuvant. Utilisée en association avec un vaccin leucémique L1210 inactivé, elle réduit grandement la mortalité des souris chez qui on innocule des cellules L1210 vivantes par rapport au vaccin utilisé seul. Plusieurs analogues reliés à la guanosine semblent dotés de propriétés antivirales et immunostimulatrices similaires – la 7-méthyl-8-thioxoguanosine et la 7-deazaguanosine notamment. Ces études indiquent que certaines modifications effectuées au niveau des positions 7 et 8 de la guanosine pourraient conférer ces propriétés aux analogues des nucléosides.

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RECENT REPORTS IN THE LITERATURE SHOWED CERTAIN analogues of guanosine to have immunoenhancing properties in rodent models. In particular, 8-substituted (8-bromo and 8-mercapto) guanosines activate B cells (1), natural killer cells (2) and macrophages (2). A more potent compound, 7-methyl-8-oxoguanosine (MOGuo) was later synthesized and found to be immunologically active (3,4). At the time the present authors initiated their research, none of these compounds had been reported to possess antiviral properties in vivo. Because of the desired potency of MOGuo, they focused their efforts on synthesizing and testing guanosine analogues modified at the 7 and 8 positions. The most active compound in a new series, 7-thia-8-oxoguanosine (TOGuo), was evaluated for antiviral activity against Semliki Forest virus infections in mice. When found highly active (5), it was tested further against a number of DNA and RNA viruses, many of which were sensitive to the compound's effects in rodents (6-9). TOGuo has also been investigated for immunoenhancing properties in mice (5,10-13), and it exhibits properties similar to the nucleosides mentioned above. Other analogues of guanosine were later identified to be potent antiviral agents, including MOGuo (14), 7-methyl-8-thioxoguanosine (MTGuo) (14), and 7-deazaguanosine (7-dzGuo) (15). This report reviews the antiviral and immunological effects of these agents. Some new information is provided on the effects of TOGuo in animal systems, including a report of the compound's activity as a vaccine adjuvant in an L1210 leukemia model in mice.

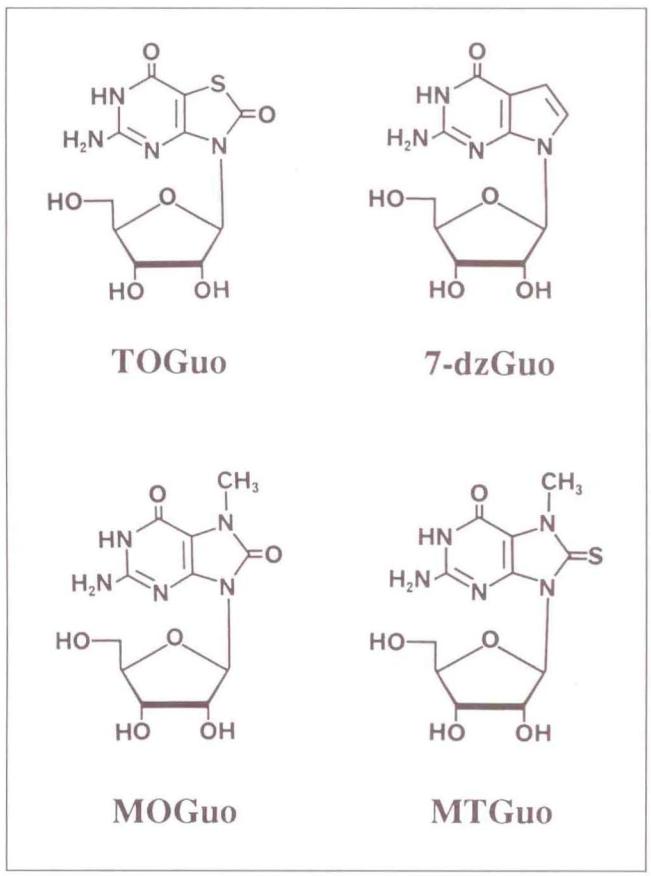

Figure 1) *Structures of 7-thia-8-oxoguanosine (TOGuo), 7-deazaguanosine (7-dzGuo), 7-methyl-8-oxoguanosine (MOGuo) and 7-methyl-8-thioxoguanosine (MTGuo)*

MATERIALS AND METHODS

Compounds: TOGuo, MOGuo, MTGuo and 7-dzGuo were synthesized at the ICN Nucleic Acid Research Institute by published procedures (5,14,16). The structures of these compounds are given in Figure 1. Each compound was dissolved in a solution of 2% sodium bicarbonate (pH 8.6 to 8.9) for animal studies or in cell culture medium for immunological assays. The placebo control for animal experiments was 2% sodium bicarbonate.

Virus infection models: All of the viruses used in animal studies were obtained from the American Type Culture Collection in Rockville, Maryland. The animals used were from Charles River Labs, Wilmington, Massachusetts, except for hairless mice which were from Jackson Labs, Bar Harbor, Maine. Most of the animal infection models have been discussed in recent publications (6-9) and are briefly described here. Eighteen to 20 g Swiss Webster (SW) female mice were injected intraperitoneally with 10 times 50% lethal doses of Banzi, encephalomyocarditis, herpes simplex type 1 or 2, San Angelo or Semliki Forest virus. The same procedure was used for Punta Toro virus using 10 to 12 g C57BL/6 mice. All of these viruses cause encephalitis. Murine cytomegalovirus was inoculated intraperitoneally into 10 to 12 g female SW mice, causing disease of all visceral organs (particularly the liver and spleen). Human coronavirus was injected intracerebrally into three- to four-day-old SW mice, resulting in encephalitis. Nonanesthetized three- to four-day-old Fischer rats were infected intranasally with rat coronavirus. Influenza B virus was administered intranasally to 10 to 12 g SW female mice. Rat coronavirus and influenza B cause pneumonia. Friend leukemia virus was inoculated intraperitoneally into 18 to 20 g SW female mice, resulting in splenomegaly by 21 days. Vesicular stomatitis virus was inoculated intranasally into 18 to 20 g SW female mice, causing encephalitis. Herpetic skin lesions were made on the backs of female hairless mice. Mice were first anesthetized, the skin near the shoulder and leg was abraded, then virus-containing medium was applied to the wounds.

Newly employed animal models reported here include infection of weanling (45 to 50 g) female Syrian hamsters with parainfluenza virus type 3, infection of three- to four-day-old Lewis rats with rat parvovirus (hemorrhagic encephalopathy of rats virus), infection of newborn (18 h or younger) SW mice with polyoma virus, and infection of 18 to 20 g SW female mice with lymphocytic choriomeningitis virus. The hamster model entailed intranasal inoculation of parainfluenza virus

(17). Four days later the lungs and nasal areas of each hamster were removed, homogenized in cell culture medium and titrated for virus in African green monkey kidney (Vero) cells. Rat parvovirus was inoculated intraperitoneally into infant rats (18). This resulted in death due to hemorrhagic encephalopathy at six to nine days. Lymphocytic choriomeningitis virus was inoculated intracerebrally into the brains of anesthetized mice (19) which caused death of the animals in six to 10 days. Newborn mice will develop tumours when inoculated with polyoma virus (20). In the present study, palpable tumours were evident in infected animals between two and four months after virus inoculation. Animals were either dead or were sacrificed at that time, and the numbers of tumours per animal counted.

The following statistical methods were used to evaluate antiviral activity: survivor increases (most models) and reduction in numbers of polyomavirus-induced tumours via Fisher exact test; decreases in Friend leukemia virus-induced splenomegaly via Student's *t* test; and reductions in parainfluenza virus titres via Mann Whitney U test. All analyses were two-tailed.

Animal treatment protocols: For most of the virus infections, treatments with guanosine analogues were conducted using divided daily intraperitoneal injections 24 and 18 h prior to virus inoculation (6-9). Exceptions to these regimens included the treatment of herpes virus infections at 48, 24 and 2 h before virus challenge (6,7), twice-weekly treatments of polyoma virus infections starting one month after virus inoculation and treatments of Punta Toro virus infections at 24 and 31 h after Punta Toro virus challenge (9). The treatment regimens were initially selected based upon published antiviral studies with pyrimidinones (21) which are also interferon-inducing agents that were evaluated against herpes simplex and Semliki Forest viruses.

Dosages of TOGuo and 7-dzGuo used in most experiments were 50, 100 and 200 mg/kg given in divided daily doses for one day only, except in the particular infections indicated above. A lower dosage of 25 mg TOGuo/kg was found to be active against Punta Toro, San Angelo and Semliki Forest viruses (6,7,9). MOGuo and MTGuo were evaluated at 25, 50 and 100 mg/kg; the 200 mg/kg dosage was not used as a compound-saving measure. The doses were selected based upon in vivo toxicity assays and efficacy experiments performed with TOGuo which was toxic (ie, caused weight loss or at least 10% mortality) at dosages above 300 mg/kg, and generally was inactive in antiviral experiments below 25 mg/kg. Treatment of mice with antibody to alpha/beta-interferon (obtained from Lee Biomolecular, Calfornia) was performed in conjunction with TOGuo treatments as described in Figure 2.

For most infections there were 10 to 12 animals per treatment or placebo group. The polyoma virus experiment had 20 mice per group, the human coronavirus had 33 mice per group and the rat

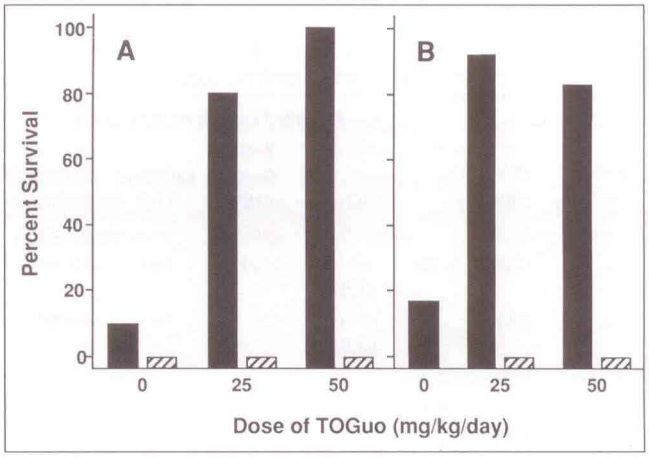

Figure 2) *Effects of antibody to alpha/beta interferon on the activity of 7-thia-8-oxoguanosine (TOGuo) against Punta Toro (**A**) and Semliki Forest (**B**) virus infections in mice. Solid bars indicate TOGuo or placebo (2% sodium bicarbonate); shaded bars indicate TOGuo or placebo plus 4000 units/day antibody. Half-daily doses of TOGuo or placebo were administered intraperitoneally 24 and 31 h after Punto Toro virus inoculation or 24 and 18 h prior to Semliki Forest virus infection; half-daily doses of antibody were given intraperitoneally 30 mins after each dose of TOGuo*

coronavirus experiments using TOGuo had 15 to 22 rats per group.

Interferon assay: Interferon was detected in mouse sera by titration on L929 cells followed one day later by infection with vesicular stomatitis virus (22). Plates (96-well) were evaluated microscopically for 50% inhibition of virus-induced cytopathology (CPE) three days later. The reciprocal of the lowest dilution inhibiting virus CPE by 50% was defined as the interferon titre in the sample. Discrimination between interferons alpha, beta and gamma was made by neutralization of interferon activity with antibodies to interferons (Lee Biomolecular).

Immunological assays: Freshly removed spleens were mechanically homogenized to create single cell suspensions. The red cells in the preparations were lysed for 1 min in 0.85% ammonium chloride. Subsequently, the remaining cells were washed twice in phosphate buffered saline then counted with a Coulter counter (Coulter Electronics, Florida). The number of cells used for immunological assays are given below. RPMI 1640 cell culture medium containing 10% fetal bovine serum, 10 mM HEPES buffer and 2 mM glutamine was used as assay medium.

Spleen cell blastogenesis assays were conducted using $1x10^5$ cells per well, three wells per sample (each spleen assayed separately) in 96-well plates incubated with 25 µg/mL of concanavalin A (Con A) – a T cell mitogen – for 48 h at 37°C. At 24 h, ^{3}H-thymidine (ICN Radiochemicals, California) at 0.5 µCi per well was added. At the end of the incubation period, the incorporated radioactivity collected on glass fibre filters was counted using a scintillation counter. Counts were

TABLE 1
Summary of antiviral activities of guanosine analogues against certain RNA viruses in animal models

Animal species	Infecting virus	Antiviral activity (reference) TOGuo	7-dz Guo (15)	MOGuo (14)	MTGuo (14)
Mouse	Banzi	++ (6,7)	+	+++	+++
Rat	Corona, rat	+++ (7,8)	nd	+++	+++
Mouse	EMC	+++ (6,8)	+	+++	+++
Mouse	Influenza B	— (6)	—	—*	—*
Mouse	San Angelo	+++ (6,7)	+++	+++	+++
Mouse	Semliki Forest	+++ (6,7)	+++	+++	+++
Mouse	VSV	+ (6)	+	+*	+*

*— 0-25% survival; + 25-50% survival; ++ 50-75% survival; +++ 75-100% survival at two or more doses. Death in the placebo group was generally less than 20%. 7-thia-8-oxoguanosine (TOGuo) and 7-deazaguanosine (7-dzGuo) treatments were 50, 100 and 200 mg/kg; 7-methyl-8-oxoguanosine (MOGuo) and 7-methyl-8-thioxoguanosine (MTGuo) were 25, 50 and 100 mg/kg. *Previously unreported information. EMC Encephalomyocarditis; nd Not determined; VSV Vesicular stomatitis virus*

expressed as mean thymidine incorporation ± standard deviation. Similar methods have been previously described (3,5). B cell blastogenesis assays were performed identical to T cell assays except that 20 µg of lipopolysaccharide (LPS) (*Escherichia coli* type 055:B5 [Sigma, Chemical Co, Missouri], a B cell mitogen) was used (3,5). Nucleosides (50, 100 and 200 µM) were in the culture medium during the entire time of incubation for both assays.

Natural killer cell activity was determined using spleen cells from mice treated 24 h earlier with 100 mg/kg of each compound or placebo. There were $1x10^6$ spleen effector cells per well (in 96-well plates) for effector-to-target cell ratios of 25:1, 50:1 and 100:1. The YAC-1 target cells were previously labeled with ^{51}chromium (100 µCi/mL for 1 h, then washed in phosphate buffered saline, incubated 30 mins, then washed again prior to use) before combining with the effector cells. The combined cells were incubated at 37°C for 4 h, centrifuged again for 5 mins at 500 *g*, then 100 mL of cell-free supernatant from each well was directly counted using a gamma counter. The percentage cytotoxicity for each sample was determined as described previously (10).

The macrophage activation assay was performed by pretreating mice four to five days by intraperitoneal injection of 3% thioglycollate medium (Difco Labs, Michigan), after which the peritoneal exudate cells were recovered (23). Nucleosides (50 µM) were incubated with 10^6 cells for 2 h, then chemiluminescence was determined in the presence of 250 µM luminol with and without 2 mg zymosan/mL (23). This assay has been reported previously (12).

Antibody-dependent cell cytotoxicity assays were performed using ^{51}chromium-labelled P815 tumour cells as targets and splenic lymphocytes as effector cells (11). The effector cells were obtained from mice treated 48 h earlier with 88 mg TOGuo/kg. Effector to target cell ratios were 12.5:1, 25:1 and 50:1. After 4 h of incubation, the percentage specific cytotoxicity was determined as previously described (11). Alloantisera to P815 cells was produced by a published method (24).

Vaccine adjuvant studies: Fresh murine L1210 leukemia cells harvested from DBA/2 mice were grown in RPMI-1640 medium supplemented with 25 mM HEPES buffer and 10% fetal bovine serum. L1210 leukemia vaccine was prepared by irradiating cells in a cesium source with a total of 6000 rads. The irradiated cells with and without TOGuo were inoculated intraperitoneally into DBA/2 mice. Vaccinations (10^4 irradiated cells per mouse) were administered at weekly intervals, and live L1210 leukemia cells (10^4 cells per mouse) were injected into mice one week after the last treatment. TOGuo was administered intraperitoneally at 150 mg/kg with or without vaccine. The vaccine dose (number of killed cells per mouse) was predetermined in titration experiments to not be effective by itself as a vaccine. The dosage of TOGuo was chosen because in other viral and immunological evaluations in mice the maximum effects were achieved between 100 and 200 mg/kg. There were 14 to 30 mice in each treatment group and 66 control (untreated) animals receiving only live leukemia cells. Deaths were recorded daily for 130 days. Statistical analysis of increases in the numbers of survivors was conducted using the two-tailed Fisher exact test.

RESULTS

Before conducting the animal studies, the four nucleosides were evaluated in cell culture for antiviral activity against herpes simplex virus type 2, mouse cytomegalovirus, parainfluenza virus type 3, Semliki Forest virus, adenovirus type 5, influenza A virus, visna virus and rhinovirus type IA (the authors' normal primary virus screen). None of the compounds showed antiviral activity at less than or equal to 320 µM (which is considered to be a high concentration). In addition, the compounds did not inhibit proliferation of leukemia cells at less than or equal to 320 µM, indicating a lack of antitumour activity. It was only when immunoenhancing activities were demonstrated that it was decided to evaluate these compounds in animals.

Antiviral activities were determined in several animal models of RNA virus infections (Table 1). All of the nucleosides were highly effective in preventing mortality in mice infected with rat corona, San Angelo and Semliki Forest viruses. MOGuo and MTGuo were slightly more protective to mice than TOGuo and 7-dzGuo in Banzi virus infections. 7-dzGuo was found to be less effective in preventing mortality from encephalomyo-

TABLE 2
Summary of antiviral activity of TOGuo against other RNA and DNA viruses in animal models

Animal species	Type of infection	Infecting virus	Activity (reference)
RNA viruses			
Mouse	Encephalitis	Corona, human	+ (7)
Mouse	Leukemia	Friend leukemia	— (7)†
Mouse	Meningitis	LCMV	—*
Hamster	Respiratory	Parainfluenza 3	—*†
Rat	Encephalitis	Parvo, rat	—*
Mouse	Tumours	Polyoma	—*†
Mouse	Hepatitis	Punta Toro	+++ (9)
DNA viruses			
Mouse	Visceral organ	MCMV	++ (7)
Mouse	Encephalitis	HSV-1	++ (7)
Mouse	Skin lesion	HSV-1	—*
Mouse	Encephalitis	HSV-2	++ (6)
Mouse	Skin lesion	HSV-2	— (6)

*— 0-25% survival; + 25-50% survival; ++ 50-75% survival; +++ 75-100% survival at two or more doses. Death in the placebo group was generally less than 20%. 7-Thia-8-oxoguanosine (TOGuo) treatments were 50, 100 and 200 mg/kg. *Previously unreported information. †Activity measured by reductions in splenomegaly, lung and nasal virus titres, and numbers of tumours for Friend leukemia, parainfluenza 3 and polyoma virus infections, respectively. HSV Herpes simplex virus; LCMV Lymphocytic choriomeningitis virus; MCMV Murine cytomegalovirus*

TABLE 3
Summary of immunological effects of guanosine analogues in mice

Immune parameter	Effect of nucleoside treatment on immune function (reference)			
	TOGuo	7-dzGuo (15)	MOGuo*	MTGuo*
B cell blastogenesis	++ (5)	—	++ (3,4)	+
T cell blastogenesis	—*	—	—	—
NK cell activation	+ (5,10)	+	+	+
Macrophage activation	+ (12,15)	+	+	+
Interferon-α induction	+++ (9,10)	+++	+++	+++
Interferon-β induction	— (10)	—	—	—
Interferon-γ induction	— (10)	—	—	—
ADCC enhancement	+ (11)	nd	nd	nd
Adjuvant activity	++*	nd	++ (3,4)	nd

*— No immunoenhancement; + two- to 10-fold enhancement; ++ 10- to 100-fold enhancement; +++ 100- to 1000-fold enhancement. *Previously unreported information. ADCC Antibody-dependent cellular cytotoxicity; nd Not determined; NK Natural killer; 7-dzGuo 7-deazaguanosine; MOGuo 7-methyl-8-oxoguanosine; MTGuo 7-methyl-8-thioxoguanosine; TOGuo 7-Thia-8-oxoguanosine*

TABLE 4
Effect of TOGuo in combination with an L1212 leukemia vaccine on survival of mice inoculated with live L1212 leukemia cells

Treatment given	Number of treatments (survivors/total (%))			
	0	1	2	3
None	0/66 (0)	—	—	—
TOGuo	—	0/14 (0)	0/30 (0)	0/22 (0)
Vaccine	—	0/14 (0)	4/30 (13)	7/22 (32)
Vaccine + TOGuo	—	0/14 (0)	27/30 (90)*	19/22 (86)*

*7-Thia-8-oxoguanosine (TOGuo) was administered intraperitoneally at 150 mg/kg with or without vaccine. Vaccine was 10^4 irradiated L1212 cells given intraperitoneally. Treatments were seven days apart; seven days after the last treatment, mice were challenged with 10^4 live L1212 leukemia cells. *Statistically different ($P<0.01$) from group receiving vaccine only*

carditis virus than were the other three compounds. Death due to influenza B virus could not be prevented by treatment with these agents, and only a moderate improvement in survival was achieved against vesicular stomatitis virus infections. All compounds had relatively the same degree of antiviral activity in each of the virus infections studied, except as noted above. These results suggest that the nucleosides may have common modes of action and similar in vivo potencies.

TOGuo was evaluated in a number of other animal infection models of DNA and RNA virus infections (Table 2). Against DNA viruses, the nucleoside partially protected mice from encephalitic infections caused by herpes simplex virus-1 and -2, and decreased mortality in murine cytomegalovirus infections. The severity of herpetic skin lesions was not reduced by TOGuo treatments, however. Against RNA viruses, TOGuo prevented mortality in the majority of mice infected with Punta Toro virus. The compound was much less protective against a human coronavirus infection, and showed no activity in Friend leukemia, lymphocytic choriomeningitis, parainfluenza, parvo and polyoma virus infections. To summarize the activities of TOGuo on virus infections (Tables 1,2), the compound was highly protective to mice infected with arthropod-borne viruses (Punta Toro, San Angelo, Semliki Forest and, to a lesser extent, Banzi), picornavirus (encephalomyocarditis) and respiratory (rat) coronavirus infections. The nucleoside was somewhat less effective in preventing mortality from herpes virus infections (herpes simplex virus-1 and -2, and murine cytomegalovirus) and was weakly active or inactive in 10 other virus infection models.

In immunological assays, the four compounds showed similar effects to enhance natural killer cell activity and to induce interferon. All compounds except 7-dzGuo activated B cells and none of the structures stimulated T cells. 7-dzGuo caused a greater activation of macrophages than the other nucleosides. TOGuo was also found to enhance antibody-dependent cell cytotoxicity functions (Table 3). The adjuvant activities of TOGuo and MOGuo were evaluated in different systems so cannot be directly compared. TOGuo enhanced

the effect of an L1210 leukemia vaccine in vivo (Table 4), whereas MOGuo enhanced humoral immunity in an in vitro antisheep red blood cell assay.

In many of the animal infection models presented here, the induction of interferon may be of prime importance to the compounds' antiviral activities. To validate this, mice were pretreated with TOGuo and antibodies to alpha/beta-interferon before Punta Toro and Semliki Forest virus challenges (Figure 2). Animals treated with the nucleoside only served as positive controls. In these studies, mice receiving TOGuo plus antibodies to alpha/beta-interferon died from the infections, whereas most animals receiving only TOGuo survived. Thus, the protective effect must have been due to the induced interferon. Other immune functions stimulated by TOGuo appeared to not be critical for antiviral activity against Semliki Forest virus (10). For example, treatment of mice with natural killer cell destroying antibody or with fumed silica (which kills macrophages) did not affect the ability of TOGuo to protect mice from lethal Semliki Forest virus infection. These same types of experiments have not yet been conducted with the other nucleosides.

Besides having possible clinical use as an antiviral agent, TOGuo may also be useful as a novel type of vaccine adjuvant. To demonstrate this potential, a vaccination study was performed in an L1210 leukemia model in mice (Table 4). L1210 leukemia was chosen because the cells are poorly immunogenic on their own. TOGuo by itself did not cause an increase in survival relative to untreated mice given live tumour cells, indicating that by itself its immunoenhancing activities do not constitute an antitumour effect. The vaccine when used alone only caused a slight increase in numbers of survivors. However, the vaccine (given two or three times) combined with TOGuo was markedly superior to the vaccine alone in preventing mortality. There was no statistically significant difference in survival between groups receiving TOGuo plus vaccine on two versus three occasions.

DISCUSSION

The virus-inhibitory effects of TOGuo, 7-dzGuo, MOGuo and MTGuo are most likely attributable to interferon induction in the host, with one or two exceptions – murine cytomegalovirus and encephalomyocarditis virus infections. Murine cytomegalovirus is not reported to be inhibited to a great extent by interferon (25), but has been shown to be quite sensitive to the action of natural killer cells (26). Because TOGuo is a good potentiator of natural killer cell activity in mice (5,10), this may be the reason for its antimurine cytomegalovirus activity. Against encephalomyocarditis virus, repeated experiments showed that 7-dzGuo was less effective than TOGuo. The two compounds differ in that TOGuo has B cell activating activity whereas 7-dzGuo does not. Whether B cell activation contributes to anti-encephalomyocarditis activity will require additional experimentation.

TOGuo (and the other nucleosides when evaluated) was not active against certain viruses known to be sensitive to interferon action which include human corona, vesicular stomatitis and influenza B viruses. In addition, herpesvirus infections of the skin were not treatable with TOGuo. Explanations for failure of the nucleoside to alter these virus infections may relate to the pharmacokinetics of the compound and its potential inability to distribute to the sites of virus replication to cause sufficient immune activation. In these studies, the human coronavirus was inoculated directly into the brain, and vesicular stomatitis virus was administered intranasally where it would rapidly reach the brain. Often the brain acts as a barrier to penetration of compounds, resulting in low levels of interferon in cerebrospinal fluid (27). As TOGuo had a positive effect on systemic herpes virus infections but not against herpetic skin diseases, these results suggest that the compound failed to reach the skin in a sufficient concentration to cause immune activation. The lack of effect of TOGuo against influenza B virus may be for an unrelated reason. Influenza viruses are inhibited by an interferon-induced cellular protein encoded by the Mx gene (28). The more common pathway of interferon activation (involving 2′,5′-adenylate synthetase) of cells to an antiviral state does not affect influenza virus replication. Mice that have the Mx gene will not die from influenza virus infection due to inherent resistance (29) and so do not make good models for studying antiviral agents. The present studies used mice lacking the Mx gene (death being the desired end point); the animals were, therefore, incapable of mounting an interferon response. This may account for the lack of antiviral activity of the interferon inducers against influenza B virus in SW mice.

In terms of immunoenhancing properties other than interferon induction, the activation of B cells appears to be a direct effect of 8-substituted guanosines on the cells, based upon detailed published experiments with 8-bromoguanosine (1), MOGuo (3,4) and TOGuo (13). The lack of a bulky substitution at the 8 position of 7-dzGuo may explain why it lacks B cell stimulatory activity. Interferon is known to be an activator of natural killer cells, so the effect of nucleoside treatment may be indirect. Studies published by the present authors indicate that part of natural killer cell activation cannot be neutralized by antibody to interferon (10). The activity of TOGuo on macrophages and on antibody-dependent cell cytotoxicity enhancement appears to be a direct effect.

The adjuvant effects of TOGuo were quite impressive in the L1210 leukemia virus model. In this infection, mice die within a few days after entry of leukemia cells into the brain. In order for mice to survive the infection, 100% of all tumour cells must be eradicated in the host.

The fact that TOGuo by itself did not alter mortality indicates that interferon and activated macrophages, antibody-dependent cytotoxic cells and natural killer cells (immune factors induced by TOGuo treatments) did not play a significant role in survival from the tumour challenge. TOGuo was also ineffective by itself in the Friend leukemia virus splenomegaly and polyomavirus-induced tumour models. In unreported studies, the nucleoside was quite effective in preventing or inhibiting the metastatic B16 melanoma tumour growth in mice. This effect could be abrogated by simultaneous treatment of the animals with natural killer cell destroying antibody, suggesting a role of these effector cells in tumour control. Because of the variety and diversity of tumour cell models, TOGuo definitely warrants further testing in other systems.

The mode of action of TOGuo to act as a vaccine adjuvant is not well understood, except that the compound has a direct B cell stimulatory effect involving the phosphoinositide signaling pathway and possible de novo synthesis of protein kinase C (13). In contrast, MOGuo was reported to stimulate specific antigen-dependent B cell immune responses independent of intracellular protein kinase C activation (4). Future investigations of the adjuvant effects of TOGuo or the related nucleosides should focus on tumour-specific neutralizing antibody production and the role of antigen presentation to B cells.

Besides the compounds evaluated here, many other related compounds have been reported to possess antiviral activity in rodents (30-32), presumably by the same modes of action. All of these are guanosine analogues substituted at the 7 and/or 8 positions of the modified purine ring. Certain sugar modifications are also acceptable to preserve antiviral activity, particularly changes at the 2′-position of the ribose moiety or 5′-substituted prodrugs (unpublished data). The use of these agents for the treatment of human infections or combined with vaccines will be the subject of future investigations.

ACKNOWLEDGEMENTS: This work was supported in part, by the US Army Medical Research and Development Command under Contract No. DAMD-17-91-C-1030. The views, opinions and/or findings contained in this report are those of the authors and should not be construed as an official department of the Army position, policy or decision unless so designated by other documentation. In conducting research using animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

REFERENCES

1. Wicker LS, Boltz RC Jr, Nichols EA, Miller BJ, Sigal NH, Peterson LB. Large activated B cells are the primary B-cell target of 8-bromoguanosine and 8-mercaptoguanosine. Cell Immunol 1987;106:318-29.
2. Koo GC, Jewell ME, Manyak CL, Sigal NH, Wicker LS. Activation of murine natural killer cells and macrophages by 8-bromoguanosine. J Immunol 1988;140:3249-52.
3. Goodman MG, Hennen WJ. Distinct effects of dual substitution on inductive and differentiative activities of C8-substituted guanine ribonucleosides. Cell Immunol 1986;102:395-402.
4. Goodman MG, Gupta S, Rosenthale ME, Capetola RJ, Bell SC, Weigle WO. Protein kinase C independent restoration of specific immune responsiveness in common variable immunodeficiency. Clin Immunol Immunopathol 1991;59:26-36.
5. Nagahara K, Anderson JD, Kini GD, et al. Thiazolo [4,5-*d*]pyrimidine nucleosides. The synthesis of certain 3-β-D-ribofuranosylthiazolo[4,5-*d*]pyrimidines as potential immunotherapeutic agents. J Med Chem 1990;33:407-15.
6. Smee DF, Alaghamandan HA, Cottam HB, Sharma BS, Jolley WB, Robins RK. Broad-spectrum in vivo antiviral activity of 7-thia-8-oxoguanosine, a novel immunopotentiating agent. Antimicrob Agents Chemother 1989;33:1487-92.
7. Smee DF, Alaghamandan HA, Cottam HB, Jolley WB, Robins RK. Antiviral activity of the novel immune modulator 7-thia-8-oxoguanosine. J Biol Response Mod 1990;9:24-32.
8. Smee DF, Alaghamandan HA, Bartlett ML, Robins RK. Intranasal treatment of picornavirus and coronavirus respiratory infections in rodents using 7-thia-8-oxoguanosine. Antiviral Chem Chemother 1990;1:47-52.
9. Smee DF, Huffman JH, Gessaman A, Huggins JW, Sidwell RW. Prophylactic and therapeutic activities of 7-thia-8-oxoguanosine against Punta Toro virus infections in mice. Antiviral Res 1991;15:229-39.
10. Smee DF, Alaghamandan HA, Jin A, Sharma BS, Jolley WB. Roles of interferon and natural killer cells in the antiviral activity of 7-thia-8-oxoguanosine against Semliki Forest virus infections in mice. Antiviral Res 1990;13:91-102.
11. Jin A, Mhaskar S, Jolley WB, Robins RK, Ojo-Amaize EA. A novel guanosine analog, 7-thia-8-oxoguanosine, enhances macrophage and lymphocyte antibody-dependent cell-mediated cytotoxicity. Cell Immunol 1990;126:414-9.
12. Ojo-Amaize EA, Rubalcava B, Avery TL, et al. Activation of the respiratory burst in murine phagocytes by certain guanine ribonucleosides modified at the 7 and 8 positions: Possible involvement of a pertussis toxin-sensitive G-protein. Immunol Lett 1989/1990;23:173-8.
13. Parandoosh Z, Ojo-Amaize E, Robins RK, Jolley WB, Rubalcava B. Stimulation of phosphoinositide signaling pathway in murine B lymphocytes by a novel guanosine analog, 7-thia-8-oxoguanosine. Biochem Biophys Res Commun 1989;163:1306-11.
14. Henry EM, Kini GD, Larson SB, Robins RK, Alaghamandan HA, Smee DF. Synthesis and broad-spectrum antiviral activity of 7,8-dihydro-7-methyl-8-thioxoguanosine. J Med Chem 1990;33:2127-30.
15. Smee DF, Alaghamandan HA, Gilbert J, et al. Immunoenhancing properties and antiviral activity of 7-deazaguanosine in mice. Antimicrob Agents Chemother 1991;35:152-7.
16. Ramasamy K, Imamura N, Robins RK, Revankar GR. A facile and improved synthesis of tubercidin and certain pyrrolo[2,3-*d*]pyrimidine nucleosides by the stereospecific sodium salt glycosylation procedure. J Heterocycl Chem 1988;25:1893-8.

17. Sidwell RW, Khare GP, Allen LB, et al. In vitro and in vivo effect of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) on types 1 and 3 parainfluenza virus infections. Chemotherapy 1975;21:205-20.
18. Nathanson N, Cole GA, Santos GW, Squire RA, Smith KO. Viral hemorrhagic encephalopathy of rats. I. Isolation, identification, and properties of the HER strains of rat virus. Am J Epidemiol 1970;91:328-38.
19. Buchmeier MJ, Welsh RM, Dutko FJ, Oldstone MBA. The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv Immunol 1980;30:275-331.
20. Andrewes C, Pereira HG, Wildy P. Papovaviridae. Viruses of Vertebrates, 4th edn. London: Bailliere Tindale, 1978:273-92.
21. Wierenga W. Antiviral and other bioactivities of pyrimidinones. Pharmacol Ther 1985;30:67-89.
22. Sidwell RW, Huffman JH. Use of disposable microtissue culture plates in antiviral and interferon induction studies. Appl Microbiol 1971;22:797-801.
23. Cheung K, Archibald AC, Robinson AF. The origin of chemiluminescence produced by neutrophils stimulated by opsonized zymosan. J Immunol 1983;130:2324-9.
24. Ojo E, Wigzell H. Natural killer cells may be the only cells in normal mouse lymphoid cell populations endowed with cytolytic ability for antibody-coated tumour target cells. Scand J Immunol 1978;7:297-306.
25. Chong KT, Gressner I, Mims CA. Interferon as a defence mechanism in mouse cytomegalovirus infection. J Gen Virol 1983;64:461-4.
26. Welsh RM. Regulation of virus infections by natural killer cells. Natl Immunol Cell Growth Regul 1986;5:169-99.
27. Stringfellow DA, Overall JC Jr, Glasgow LA. Interferon inducers in therapy of infection with encephalomyocarditis virus in mice. II. Effect of multiple doses of polyriboinosinic-polyribocytidylic acid on viral pathogenesis. J Infect Dis 1974;130:481-8.
28. Dreiding P, Staeheli P, Haller O. Interferon-induced protein Mx accumulates in nuclei of mouse cells expression resistance to influenza viruses. Virology 1985;140:192-6.
29. Pavlovic J, Jurcher T, Haller O, Staeheli P. Resistance to influenza virus and vesicular stomatitis virus conferred by expression of human MxA protein. J Virol 1990;64:3370-5.
30. Bontems RJ, Anderson JD, Smee DF, et al. Guanosine analogues. Synthesis of nucleosides of certain 3-substituted 6-aminopyrazolo[3,4-*d*]pyrimidin-4(5H)-ones as potential immunotherapeutic agents. J Med Chem 1990;33:2174-8.
31. Girgis NS, Michael MA, Smee DF, Alaghamandan HA, Robins RK, Cottam HB. Direct c-glycosylation of guanine analogues: The synthesis and antiviral activity of certain 7- and 9-deazaguanine c-nucleosides. J Med Chem 1990;33:2750-5.
32. Sanghvi YS, Larson SB, Smee DF, Revankar GR, Robins RK. In vivo antiviral activity of 5-amino-1-methyl-3-β-D-ribofuranosyl-pyrazolo[4,3-*d*]pyrimidin-7(6H)-one and related guanosine analogues prepared from formycin. Nucleosides Nucleotides 1991;10:1417-27.