Antiviral Research, 19 (1992) 173–178

AVR 00567

New hypoxanthine nucleosides with RNA antiviral activity

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(Received 9 September 1991; accepted 27 February 1992)

Summary

A series of novel C-2 functionalized hypoxanthine and purine ribonucleosides have been synthesized and evaluated against exotic RNA viruses of the family or genus alpha, arena, flavi, and rhabdo. Both specific and broad-spectrum antiviral activities were discovered but only with hypoxanthine nucleosides.

Exotic RNA virus; Hypoxanthine nucleoside; Antiviral activity

Flavi, alpha, arena, and bunya viruses are RNA genomic viruses which exist worldwide and which are etiologic agents for a number of serious diseases (Hay and Bartkoski, 1982). The insect vectors of most of these viruses are mosquitoes and ticks. Of the 64 known viruses of the family Flaviviridae, about 28 have been associated with serious diseases in humans such as Japanese encephalitis, yellow fever, and dengue hemorrhagic fever (Burke et al., 1985; Halstead, 1988; Schlesinger and Schlesinger, 1986). The Bunyaviridae (>90 members) include the California encephalitis group (*Bunyavirus* genus), the *Hantavirus* genus, and the genus *Phlebovirus* which contains the Rift Valley fever virus and the sandfly fever group (Hay and Bartkoski, 1982; Rice et al., 1980; Schmaljohn et al., 1983). *Alphaviruses* (>20 members) are classified under the family, Togaviridae, and some of these viruses are etiologic agents

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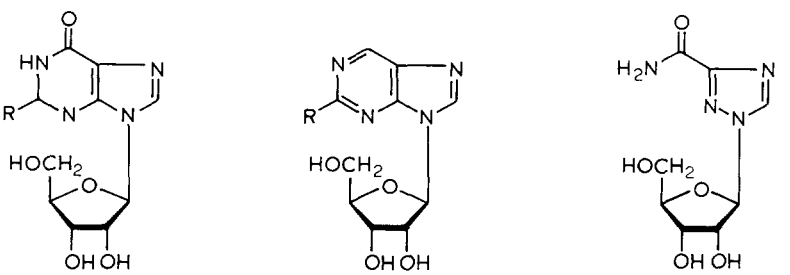
for encephalitic diseases such as Venezuelan equine encephalitis, Eastern equine encephalitis and Semliki forest and Chikungunya encephalitis (Schlesinger and Schlesinger, 1986; Soderland et al., 1985). The arena viruses (Hay and Bartkoski, 1982) include lymphocytic choriomeningitis (LCM) virus, Lassa fever virus, and the Tacaribe group of viruses (Machupo, Junin, Tacaribe, Tamiami, Parana, Latino, Pichinde and Atmapari). Only a few vaccines have been developed for these 'exotic' RNA viruses. However, those vaccines that do exist are often not readily available to the population at risk for infection. In addition, because of the many viruses and strains within a particular family or subgroup, vaccine development for a particular virus does not necessarily provide complete protection.

Although the use of nucleoside analogues as antiviral agents has made significant advances in recent years, broad-spectrum antiviral activity, particularly against RNA viruses, has not been generally observed. A few synthetic organic compounds have shown some antiviral activity against several of these exotic RNA viruses (Canonico, 1988), but a considerable amount of fundamental research work in this area remains to be done. In collaboration with the U.S. Army Medical Research Institute of Infectious Diseases, we have been involved in a program which has as its goal the discovery of new antiviral agents with broad-spectrum or specific antiviral activity against these exotic RNA viruses. We wish to report on the synthesis and in vitro RNA antiviral studies of a series of novel C-2 functionalized hypoxanthine ribonucleosides.

All of the target compounds (i.e. inosine and nebularine analogues, see Fig. 1) were synthesized by multistep procedures utilizing metal-mediated methodologies as key steps (Nair et al., 1987, 1988, 1989). The final products were purified by reversed-phase HPLC on Amberlite XAD-4 resin (C_2H_5OH/H_2O).



R = $-CH_2C(O)CH_3$, $-CH_2CH(OH)CH_3$, $-CH{=}CH_2$
$-CH(OH)CH_2OH$, $-CH_2CH_2OH$, $-CHO$, $-CH_2OH$
$-CH{=}CHCH_3$, $-I$, $-CH(CH_3)CH(OH)C_2H_5$

Fig. 1. Structures of the novel inosine and nebularine compounds used in this investigation and the structure of the known RNA antiviral compound, ribavirin.

In vitro antiviral assays were determined through inhibition of cytopathic effect (CPE) and through plaque reduction (PR) assays as follows:

CPE inhibition assay. Antiviral screening against Sandfly fever virus (Sicilian strain), Punta Toro virus (Adames strain), vesicular stomatitis virus (Indiana strain), Japanese encephalitis virus (Nakayama strain), Venezuelan equine encephalomyelitis virus (Trinidad donkey strain), and yellow fever virus (Asibi strain) was performed on Vero cells (except for VSV on L929 cells) in 96-well plates. Cells were pre-treated with DEAE-dextran (25 μg/ml) and 1% DMSO in 0.1 ml Hanks' balanced salt solution for 30 min at 37°C. This was removed and 0.1 ml containing 32 50% cell culture infectious doses of virus was added to each well and allowed to adsorb for 30 min at 37°C; 0.1 ml of test compound was added to appropriate wells. When virus control wells showed maximum CPE (VSV, VEE, and YF: 3 days, SFS: 5–6 days, PT: 5–7 days, JEV: 6–7 days post-infection), the wells were rinsed with PBS and the cells fixed with methanol. Fixed cells were stained with 10% Giemsa stain and examined microscopically for CPE. Antiviral activity was determined by calculating the drug concentration that reduced virus-induced cytopathic effect (CPE) by 50% (ED_{50}), calculated using a regression analysis program for semilog curve-fitting. The CD_{50} was determined by microscopic evaluation of treated uninfected cultures.

Plaque reduction assay. Antiviral screening against Pichinde virus (strain 4763) and Rift Valley fever virus (Zagazig strain) was performed by a plaque reduction assay on confluent Vero cells in 6 (PIC) or 24 (RVF)-well tissue culture plates. Each well was inoculated with 0.2 ml containing 50–100 pfu virus in MEM with 10% FBS, and virus was allowed to adsorb for 1 h. Drug containing agar (PIC) or agarose (RVF) was added immediately after the virus adsorption period. Plates were incubated for 3 days at 37°C after which cells were stained with 0.1% neutral red solution in PBS (PIC) or 4 days at 37°C after which the semi-solid agarose was decanted and cells were stained with crystal violet (RVF) to reveal viral plaques.

Antiviral data of the target inosine compounds (i.e. the C-2 modified hypoxanthine ribonucleosides) are summarized in Table 1. Although some antiviral activity was seen with many of the compounds synthesized, two of these compounds (**1** and **3**) have significant enough activity to deserve discussion. Both compounds have one degree of unsaturation in their modifying functionality. 2-Acetonylinosine (**1**) is active against the sandfly fever virus (SFS) with an ED_{50} of 0.32 μg/ml. Its CD_{50} in the Vero cell line used was of the order of 320 μg/ml. To the best of our knowledge, this is the most active compound known against the SFS virus. In terms of its ED_{50} value, it is almost two orders of magnitude more active than the well-known compound, ribavirin (Fig. 1), which has an ED_{50} of 28 μg/ml against this virus (Canonico, 1988). 2-Acetonylinosine (**1**) also appears to be very specific for the SFS virus as it is inactive against about 10 other exotic RNA viruses, some of which are

TABLE 1

Antiviral activity (in vitro) of novel inosine analogues[+] against exotic RNA viruses

Compound (inosine analogues: scheme 1)	Virus	Cell line	ED_{50} (μg/ml)	CD_{50} (μg/ml)
1 R = $-CH_2C(O)CH_3$	SFS	Vero	0.32	>320
	JEV	Vero	Inactive	
	PIC	Vero	Inactive	
	PT	Vero	Inactive	
	RVF	Vero	Inactive	
	VEE	Vero	Inactive	
	YF	Vero	Inactive	
2 R = $-CH_2CH(OH)CH_3$*	YF	Vero	237	320
3 R = $-CH=CH_2$	SFS	Vero	Inactive	
	JEV	Vero	3.2	10
	PIC	Vero	2.5	3.2
	PT	Vero	2.7	10
	RVF	Vero	24.1	24.1
	VEE	Vero	2.7	8.4
	YF	Vero	7.7	10
4 R = $-CH(OH)CH_2OH$*	RVF	Vero	182	250
5 R = $-CH_2CH_2OH$*	RVF	Vero	186	250
6 R = $-C(O)H$*	VSV	Vero	Inactive	
	VSV	L929	320	100
	PT	MK2	141	100
	PT	Vero	Inactive	
7 R = $-CH=CHCH_3$*	PT	Vero	217	100
8 R = $-I$*	PT	MK2	294	1000
	PT	Vero	Inactive	
9 R = $-CH(CH_3)CH(OH)C_2H_5$*	RVF	Vero	425	>250

[+]Other C-2 functionalized inosine and nebularine analogues synthesized showed little or no antiviral activity. *Not active against other RNA viruses listed below. Abbreviations for RNA viruses used in antiviral evaluation: JEV, Japanese encephalitis virus (*alphavirus*); PIC, pichinde (*arenavirus*); PT, punta toro (*phlebovirus*); RVF, Rift Valley fever (*phlebovirus*); VEE, Venezuelan equine encephalomyelitis (*alphavirus*); YF, yellow fever (*flavivirus*); VSV, vesicular stomatitis virus (*rhabdovirus*); SFS, sandfly fever, Sicilian, (*phlebovirus*).

listed in Table 1. The second compound, 2-vinylinosine (**3**), exhibits broad-spectrum activity against 6 RNA viruses with ED_{50} values mainly in the 2–3 μg/ml concentration range. This is unusual as there are very few compounds known that exhibit broad-spectrum RNA antiviral activity against the exotic RNA viruses. It should also be mentioned that the corresponding C-2 functionalized nebularine analogues (Fig. 1) also synthesized by us (Nair and Buenger, 1988b) were all inactive. It is likely that the nebularine analogues are not being activated by phosphorylation but further experimentation is needed to confirm this. The RNA antiviral activities of the hypoxanthine compounds

are presumably associated with inhibition of the RNA polymerases of the viruses by the triphosphates of these compounds. Inhibition of viral replication may also be possible at the monophosphate stage as the monophosphates of these hypoxanthine compounds are expected to be inhibitors of inosine monophosphate dehydrogenase (Malinoski and Stollar, 1981; Wong and Meyer Jr., 1984).

In summary, novel C-2 modified hypoxanthine and purine ribonucleosides have been synthesized and evaluated for antiviral activity against exotic RNA viruses. Both specific and broad-spectrum antiviral activities were discovered but only with the hypoxanthine nucleosides. The most significant activities were found with hypoxanthine nucleosides bearing unsaturation on a relatively small group at the 2-position. The design and synthesis of other RNA antiviral compounds using these leads are currently in progress.

Acknowledgements

We thank the U.S. Army Medical Research and Development Command for support of this research and for the antiviral evaluation data. Some of the antiviral data were determined at the Southern Research Institute, Birmingham, Alabama under contract to the USAMRDC. We thank Dr. William M. Shannon and co-workers for these screening results. V.N. thanks the University of Iowa for a Faculty Scholar Award.

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