Acta virol. **32** : 386—392, 1988

INHIBITORY EFFECT OF SULPHUR-CONTAINING PURINE NUCLEOSIDE ANALOGUES ON REPLICATION OF RNA VIRUSES: SELECTIVE ANTIVIRAL ACTIVITY AGAINST INFLUENZA VIRUSES

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Received August 18, 1987

Summary. — Four sulphur-containing purine nucleoside analogues: 6MP, 6-thioinosine, 6-methylthioinosine and 6-ethylthioinosine, were examined for antiviral activity against some RNA viruses. All compounds extensively inhibited the replication of influenza viruses but had no inhibitory effect on other RNA viruses: Sendai, RS, vesicular stomatitis and western equine encephalitis viruses.

Key words: anti-influenza thioinosine analogues; 6-thioinosine; 6-methylthioinosine; 6-ethylthioinosine; synergistic effect with ribavirin.

Introduction

RNA viruses comprise a number of important pathogens in man, e.g. the viruses causing Lassa fever and some haemorrhagic fevers, many viral encephalitides and recently, AIDS. Human beings have devised a strategy exclusively against those viral diseases by vaccination. However, some viruses, such as influenza viruses of which the antigenic phenotype frequently varies to escape neutralizing antibody, have not been completely eradicated by vaccination (Webster and Laver, 1975). On the other hand, antiviral chemotherapy, especially chemotherapy against RNA virus infection, has not been successfully developed for clinical use, unlike DNA virus chemotherapy such as acyclovir for herpesvirus infection (Schaeffer *et al.*, 1978).

Recently, Hasobe *et al.* reported a novel approach to developing antiviral agents (Hasobe and Saneyoshi, 1985a, Hasobe and Saneyoshi, 1985b, Hasobe *et al.*, 1985). In those studies, some purine nucleoside analogues which were studied extensively for antitumour activity in the past, were re-evaluated because of their highly specific antiviral activities. However, in the previous studies, a fish virus (infectious haematopoietic necrosis virus [IHNV] and fish cell line (chinook salmon embryo [CHSE-214]) system was described in

which all enzymatic reactions including macromolecular synthesis might take place at a low temperature, 15 °C. Therefore, we thought it would be intersting to find out if those agents are effective in the system of mammalian cells and viruses at 34—37 °C.

In the present study, among the agents effective against IHNV, 6-mercaptopurine (6MP) and three thioinosine analogues, 6-thioinosine (6TI), 6-methylthioinosine (6MeTI), and 6-ethylthioinosine (6EtTI), were tested. Their major biochemical effect is to inhibit the action of phosphoribosylpyrophosphate amidotransferase and the conversion of IMP to AMP and GMP, and caused an inbalance of the ribonucleotide pool. Through the entire study, the antiviral activity of these agents was compared with that of ribavirin as a positive control (Sidwell *et al.*, 1972) for the antiviral activity against the same animal RNA viruses in mammalian cells.

Materials and Methods

Virus and cells. Influenza viruses: A/Kumamoto/37/79 (H1N1), A/Fukuoka/1/80 (H3N2), and B/Yokohama/1/80 (B); paramyxoviruses: Sendai virus (Z strain) and respiratory syncytial (RS) virus (Long strain); rhabdovirus: vesicular stomatitis virus (VSV) (Indiana strain), toga-(alpha) virus: western equine encephalitis (WEE) virus (McMillan strain). Influenza viruses and Sendai virus were grown in embryonated eggs. RS virus was grown in HEp2 cells. VSV and WEE virus were propagated in BHK cells for plaque titration. Plaque titration of viruses: Influenza viruses were assayed on MDCK cells (Tobita *et al.*, 1975). For Sendai virus, LLC-MK2 cells were used (Sugita *et al.*, 1974). RS virus was assayed on HEp2 cells overlaid with 1 % methylcellulose (MC) containing Eagle's minimal essential medium (MEM) (Coates *et al.*, 1966). VSV and WEE viruses were titrated on BHK cells covered with MEM containing 1 % MC.

Assay of antiviral activity of the compounds. After virus adsorption to the cells, non-adsorbed viruses were discarded and overlay medium which included the test agent as described in the pertinent table or figure was added. Antiviral activity was expressed as the final concentration causing 50 % inhibition of plaque formation (IC_{50}).

Agents. The thioinosine analogues (6TI, 6MeTI and 6EtTI) were synthesized in our laboratory according to the method of Fox *et al.* (1958), Saneyoshi and Chihara (1967) and Johnson *et al.* (1968), respectively. Ribavirin and 6MP were kindly supplied by Yamasa Shoyu and Kojin Co. Ltd respectively. All experiments were repeated two or three times and mean values were calculated.

Results

As shown in Table 1, 6MP and the three thioinosine analogues inhibited the replication of influenza viruses. Plaque formation by the three subtypes of influenza virus was reduced below 50 % by less than 3 μg of the test compounds per ml. The efficacy of the five compounds tested to influenza viruses was in the order of 6EtTI, 6MeTI, 6MP, 6TI and ribavirin on every influenza subtypes. The most effective antiviral activity was attained by 0.71 μg/ml of 6EtTI to the replication of influenza A (H3N2) virus. On the other hand, the least effect was showed by TI of which a low concentration, 2.88 μg/ml reduced 50 % plaques formed by influenza B virus. The thioinosine analogues, however, did not show any inhibitory effects against other animal RNA viruses: Sendai, RS, VS, and WEE viruses.

The evaluation of a compound as an antiviral agent of course depends on its inhibitory activity against viral replication. But, it is also important

Table 1. Antiviral activity of 6MP and 6-thioinosine analogues against some RNA viruses

Compound[a]	IC_{50} (μg/ml) against							
	Influenza virus A (H1N1)	Influenza virus A (H3N2)	Influenza virus B (B)	Sendai virus	RS virus	VSV		WEE virus
						MDCK[b]	BHK[c]	
6MP	1.41 (709)[d]	1.49 (671)	2.40 (417)	> 100	1.00 >	ND[e]	> 100	> 100
6TI	2.11 (> 474)	2.02 (> 495)	2.88 (> 347)	> 100	3.20 >	ND	> 100	> 100
6MeTI	1.88 (> 532)	1.20 (> 833)	0.95 (> 1053)	> 100	1.00 >	ND	> 100	> 100
6EtTI	0.83 (> 1205)	0.71 (> 1049)	0.75 (> 1333)	> 100	3.20 >	> 100	> 100	> 100
Ribavirin	5.37 (186)	4.70 (213)	6.02 (166)	23.7	3.35	22.4	74.9	> 100

[a] Abbreviations: 6MP, 6-mercaptopurine; 6TI, 6-thioinosine; 6MeTI, 6-methyl thioinosine; 6-EtTI, 6-ethyl thioinosine.
[b] MDCK cells used for assay.
[c] BHK cells used for assay.
[d] Numbers in parenthesis mean antiviral index (AI) determined as follows: MCC/IC_{50} (see text)
[e] Not done.

that the compound has less cytotoxicity for host cells. In this study, four sulphur-containing purine nucleoside analogues were tested for possible cytotoxicity on confluent sheets of stationary cells rather than by inhibition of doubling of cells because the latter method is too sensitive and does not always reflect the cytotoxicity for stationary cells which are employed in the

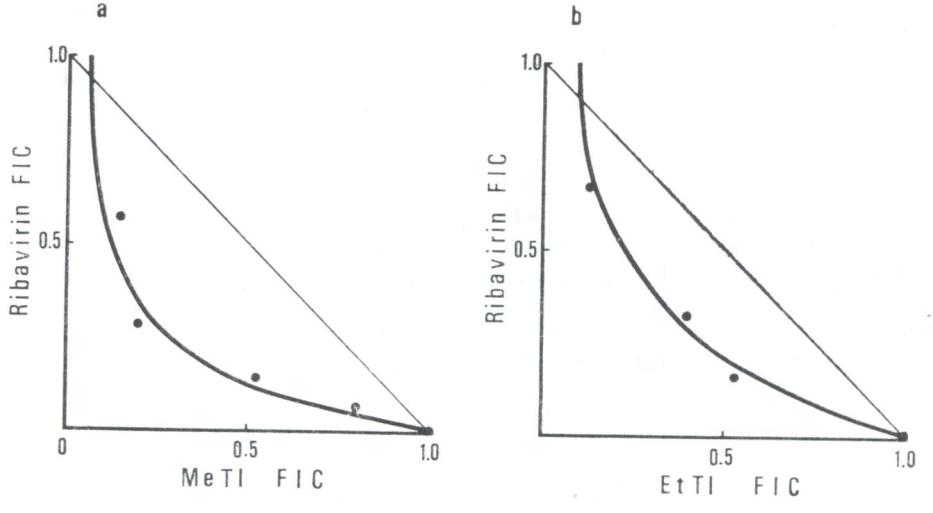

Fig. 1.
Antiviral isobolograms of MeTI (1a) and EtTI (1b) with ribavirin in MDCK cells infected with influenza B virus.
Each FIC value was recalculated from the IC_{50} of MeTI or EtTI and ribavirin in the combination test (see text).

plaque assay (data not shown). In fact, the cytotoxicity for confluent monolayers was scored as +1 to +4 and the concentration of compounds which showed +1 cytotoxicity was for convenience considered the minimal cytotoxic concentration (MCC) (Table 2). Consequently, it was found that sulphur-containing purine nucleosides possessed extensive low cytotoxicity for stationary cells except HEp2 cells, and in several combination of cells and compounds the MCC was not determined because of no cytotoxicity even at the highest concentration in this experimental system (Table 2).

Recently, Hasobe *et al.* showed that a test with a combination of an unknown agent and a compound with a known mechanism was useful for determining whether the test agent has the same antiviral mechanism as the reference agent (Hasobe *et al.*, 1986). In the present study, we tested the combination of 6MeTI or 6EtTI and ribavirin on influenza virus replication. The fractional inhibitory concentration (FIC) values were recalculated and plotted as an isobologram according to the method given in previous reports

Table 2. Cytotoxicity of sulphur-containing purine nucleosides

Compound[a]	MCC (μg/ml) in			
	MDCK	LLC−MK2	BHK	HEp2
6MP	1000	>320	32−10	3.2>
6TI	>1000	>320	32	3.2>
6MeTI	>1000	320	32	3.2
6EtTI	>1000	>100	320−100	32−3.2
Ribavirin	1000	320−100	>320	320

[a] see Table 1 for abbreviations.

(Hasobe *et al.*, 1986, Kirsi *et al.*, 1984). In such graph, when two agents act synergistically, the FIC value will be plotted in the left zone of the straight line. As shown in Fig. 1, the isobologram of the activity of 6EtTI against influenza B virus replication indicated a moderate synergistic effect with ribavirin (Fig. 1b). On the other hand, 6MeTI with ribavirin in MDCK cells exhibited striking synergistic effect (Fig. 1a).

Discussion

Ribavirin has been reported to be an excellent agent possessing a wide antiviral spectrum and low cytotoxicity in cultured cells (Sidwell *et al.*, 1972, Huffman *et al.*, 1973). However, in studies with experimental animals, the acute toxicity was evident (Huggins *et al.*, 1986). At present, it seems not always to be satisfactory for clinical use. In this paper we reported new antiviral agents for influenza viruses. Sulphur containing purine nucleoside analogues showed potent antiinfluenza virus activity. The IC_{50} values of each agent against the three subtypes of influenza virus did not show such variation. The narrowest range was 2.02 to 2.88 for 6TI and even the widest range, for MeTI, was 0.95 to 1.88. Therefore, it appears that some purine analogues substituted at the 6-thiol group, especially the analogues of 6TI, are extensively effective against three subtypes of human influenza virus. Of the thioinosine analogues, 6EtTI displayed the most potent antiviral activity, about six to eight times that of ribavirin.

However, on the contrary of our expectation, these agents did not possess antiviral activity against other RNA viruses. For RS virus, a member of a paramyxovirus subgroup, the IC_{50} was not determined because of the extensive cytotoxicity of the test agents for the monolayer of HEp2 cells (Table 2). The number of plaques of Sendai virus was also not reduced by up to 100 μg of any analogue per ml. The IC_{50} of ribavirin used as reference was 20−30 μg/ml, which was approximately four times the concentration of the agent needed to achieve a similar degree of inhibition of influenza viruses. This value seems to be reasonable in comparison with the results obtained by

others (Browne, 1981, Kirsi *et al.*, 1984). This tendency was also confirmed in the system of VSV and BHK or MDCK cells. The three thioinosine analogues and 6MP had no inhibitory effect on the growth of VSV but ribavirin was effective. However ribavirin, a wide-spectrum antiviral agent, had no effect on the growth of the togavirus, WEE virus (Table 1). This is not inconsistent with the results of Scholtissek's studies showing that ribavirin does not affect the growth of Semliki Forest virus (Sholtissek, 1976).

For antiviral efficacy, the antiviral index (AI), which is the MCC of a compound divided by the antiviral IC_{50}, is frequently used. In the present study the AI was determined for the influenza viruses against which the test compounds were effective. As shown in Table 1, the AI of ribavirin ranged from 160 to 210 for the three subtypes of influenza virus. Among the four compounds tested, only the AI of 6MP was determined and its values from 400 to 700 to for the three influenza virus subtypes were about three to four times those of ribavirin. For the three thioinosine analogues; 6TI, 6MeTI, 6EtTI, determination of the AI was impossible because of the inability to determine each MCC value. However, those AI values were apparently higher than that of ribavirin even at the greatest underestimation. The AI values of the test compounds for the other viruses were not determined owing to undecided IC_{50}. It is not known why 6-alkyl thioinosine analogues act on ifluenza virus but not on Sendai virus and other RNA viruses. As stated in this discussion, the antiviral mode of thioinosine analogues against mammalian RNA viruses is slightly different from that to fish viruses in the point that these agents are less or non effective against the rhabdovirus VSV. These compounds are known to depress the nucleotide pool of host cells. On the other hand, the antiviral mechanism of ribavirin has been defined as a bifunctional activity including the inhibition of cap formation and IMP dehydrogenase (Goswami *et al.*, 1979). The results in the combination experiments suggest that the two agents might have a different antiviral mechanism in MDCK cells from that of ribavirin. One possible explanation is that the antiviral effect on influenza viruses of these nucleoside analogues *in vitro* depends on their effect on the size of the ribonucleotide pool infected MDCK cells. The lack of inhibitory action of such analogues on Sendai, RS, VS and WEE viruses also suggests that the replication of the viruses might not depend on the ribonucleotide pool size in host cells. More detailed study from this point of view is a problem for the future. We are attempting to develop new derivatives of 6-alkyl thioinosine that will be effective against influenza and other RNA viruses.

Acknowledgement. We wish to express our gratitude to Dr. K. Nakajima for his critical discussion and reviewing the manuscript.

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