ELSEVIER

International Journal of Pharmaceutics 194 (2000) 15–20

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

3′-Deoxyribonucleosides and their derivatives as anti-amoebic agents

S. Ghosal *, B.N. KrishnaPrasad, M. Khanna, A.K. Dwivedi, S. Singh, A. Kumar, S.B. Katti

Central Drug Research Institute, Lucknow-226001, India

Received 10 August 1998; received in revised form 7 June 1999; accepted 27 July 1999

Abstract

In general nucleoside analogues were found to possess in vitro amoebicidal property against trophozoites of *Entamoeba histolytica*. The acid-labile nature of these compounds completely destroyed their ability to cure caecal amoebiasis of rats. However the therapeutic efficacy of one of these compounds yielded most promising results, with 10% cures when it was administered as enteric coated formulation.

Keywords: Anti-amoebic agents; Caecal amoebiasis of rats; *Entamoeba histolytica*; Nucleosides

1. Introduction

The consistently high incidence of amoebiasis renders drug development against this disease both a social obligation and a viable proposition to the pharmaceutical industry. Indeed a number of test agents belonging to a variety of chemical series have been evaluated for anti-amoebic activity against trophozoites of *Entamoeba histolytica*. Some of these compounds have been used clinically with varying degrees of success. The main reasons against their continued usage are the associated toxicity and occurrence of relapse despite adequate treatment (Ross, 1997). Currently imidazoles are being used in the treatment against amoebiasis. Among these the choice mainly rests on metronidazole (Powell et al., 1966). Efforts have been made to potentiate the efficacy of this compound (formulation with vioform/furazolidone) but with limited success (Martinez-Palomo and Cantellano, 1997). Thus search for a safe and effective anti-amoebic drug continues to remain an attractive proposition.

The biochemistry of *E. histolytica* offers a base for rational design of anti-amoebic agents. *E. histolytica* lacks the de novo synthetic pathways for purines and, therefore, depends on extracellular sources of purine bases and nucleosides as precursor for nucleic acid synthesis. The functioning of efficient purine salvage pathway is essential for the survival of this parasite (Boonlayangoor et al., 1978; Lo and Wang, 1985). Thus it should be possible to design selective inhibitors of the purine salvage enzymes of *E. histolytica* without affecting

* Corresponding author. Fax: +91-562-223405.

0378-5173/00/$ - see front matter
PII: S0378-5173(99)00263-X

the host. In fact growth inhibiting effect of nucleoside analogs have been reported (Booden et al., 1976; Eubank and Reeves, 1981; Das and Baer, 1991). Several pyrimidine and purine nucleosides have been found to inhibit the growth of amoebae under in vitro conditions with an IC_{50} value ranging from 0.14 to 0.82 μM. None of the nucleosides have been evaluated for in vivo antiamoebic activity. Perhaps, the deterrent reasons that can be anticipated are its poor biostability (acid-induced cleavage of the glycosyl bond) and host toxicity. However, these do not remain insurmountable problems. Recent data indicate that it is possible to reduce host toxicity with the help of nucleoside transport inhibitors specific for mammalian cells (Ogbunude and Baer, 1993). In view of the wide spectrum of the biological activity exhibited by 3′-deoxynucleosides it seemed logical to evaluate a few analogs of this series for antiamoebic activity and the results of these studies are presented in this communication.

2. Materials and methods

2.1. Synthesis of compounds

The synthesis of 3′-deoxynucleosides and their derivatives has been described earlier (Kumar et al., 1994) (Fig. 1). All the compounds reported in this paper have been thoroughly characterized (Kumar, 1996). During the glycosylation of guanine, gave not only N-9 substituted nucleoside but also the isomeric N-7 substituted compound. The assignment of position of the sugar moiety in the N-7 and N-9 (deoxyribofuranosyl) guanines was made primarily on the basis of comparison of their UV spectra with that of authentic N-7 and N-9 methylated guanines at acidic, alkaline and neutral pH reported in the literature (Jenkin et al., 1965). At acidic and alkaline pH these two isomeric forms show a distinct λ_{max} and they can be easily characterized.

2.2. Preparation of enteric coated formulation for animal feeding

A total of 0.5 g of compound 5a [N^2-acetyl-7-(2-*O*-acetyl-5-*O*-benzoyl-3-deoxy(*d*-ribofuranosyl)-guanine] and 2.5 g of cellulose acetate phthalate were dissolved separately in 25 ml of acetone. The solutions were mixed and left shaking at 35°C for 1 h to get a clear solution. The solvent was removed with the help of a mini spray drier. The encapsulated particle 20–60 mesh were selected and the resultant free flowing powder was suspended in 1.5% sodium carboxymethyl cellulose solution at the desired concentrations.

2.3. E. histolytica

A polyxenic culture of *E. histolytica* (2771 H_3) freshly isolated from the infected stool material of a symptomatic case of human amoebiasis was used in the study. The cultures were maintained in Robinsons' medium (Robinson, 1968).

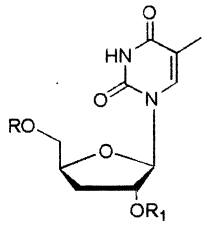
2.4. Amoebic inoculum

The amoebic inoculum was prepared according to the following criteria: ensuing the viability of trophozoites of *E. histolytica* prior to inoculation, reducing the excess bacterial flora accompanying the amoebae culture to enhance clarity for in vitro studies, to prevent sepsis in animal infections, and the rice starch added to the medium was such as to permit easy flow through a 26-gauge hypodermic needle. Thus the sediment from 48-h old cultures containing flourishing growth of *E. histolytica* were pooled and washed by low speed centrifugation in fresh overlay of the medium to remove excess bacteria. The sediment containing washed amoebae was inoculated into fresh medium to which a judicious amount of rice starch (particle size 400 mesh) and gentian violet was added. The amoebae were counted and divided into equal aliquots. From each tube four to five animals were inoculated to ensure similar inocula for infection.

2.5. In vitro screening of test compounds

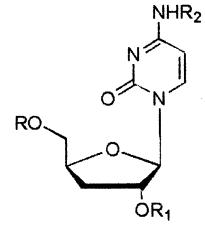
This was carried out according to the published method (Das, 1975). The stock solution of the test agent was prepared by dissolving it in a small

quantity of DMSO and water until a clear solution was obtained. The concentration of DMSO was below 5%, which is non-amoebicidal. Serial double dilutions of the stock solution were prepared in triple glass distilled water. Amoebic inoculum (0.1 ml) containing 2000–5000 trophozoites of *E. histolytica* was inoculated per well in each corner well of a three-cavity slide. The test agent (0.1 ml) in its known dilution was added per well and marked. The cavity was sealed with a cover slip. The slides were examined under a microscope 10×10 to record the initial observation, placed in moist chamber and incubated at 37°C. Microscopic observations were recorded at



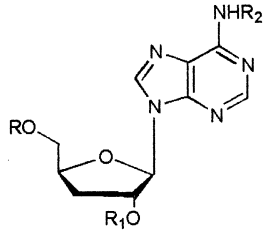
1

1a R=Bz; R_1=Ac
1b R=R_1=H



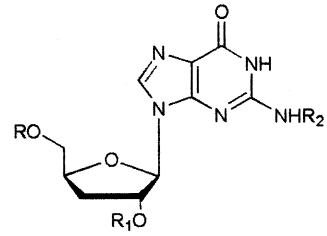
2

2a R=Bz; R_1=Ac; R_2=H
2b R=R_1=R_2=H
2c R=R_1=H; R_2=Bz



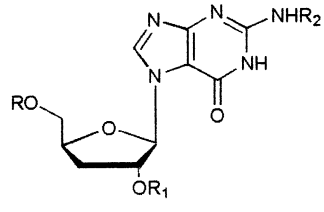
3

3a R=R_2=Bz; R_1=Ac
3b R=R_1=R_2=H
3c R=R_1=H; R_2=Bz



4

4a R=Bz; R_1=R_2=Ac
4b R=R_1=R_2=H
4c R=R_1=H; R_2=*iso*-butyl



5

4a R=Bz; R_1=R_2=Ac
4b R=R_1=R_2=H
4c R=R_1=H; R_2=*iso*-butyl

Fig. 1.

24- and 48-h intervals. Live amoebae remained refractive, motile and adhered to the glass surface. The dead amoebae were vacuolated, crumpled and detached from the glass surface. The standard drugs used were metronidazole and emetine hydrochloride, which were amoebicidal in vitro at 4 and 8 mg/ml, respectively.

2.6. Rats

Twenty-one-day-old weaned albino rats (Druckray strain) of either sex and weighing about 25 g, obtained from the Animal House of the Institute, were used in the study. They were housed in groups of five to six animals per cage. Food and water was provided ad libitum.

2.7. Experimental production of caecal amoebiasis in rats

Rats receiving balanced commercial pellet diet have generally been found to be refractory to experimental *E. histolytica* infection. Over the years the reasons responsible for aiding/inhibiting the development of amoebic infection in the rat caecum have been examined (KrishnaPrasad and Bansal, 1982, 1983; KrishnaPrasad et al., 1984; Leitch, 1988) and a method for the consistent production of caecal amoebiasis in rats has been developed. Accordingly the rats are fed on an autoclaved rice diet for 7 days prior to infection. The caecal contents of these rats attain a pH of 5.5–7.0 without the occurrence of free ammonia, which is toxic to these amoebae. Rats under ether anaesthesia were inoculated intracaecally at the time of laparotomy with 0.2–0.3 ml of amoebic inoculum containing 1×10^5 trophozoites of *E. histolytica* and the abdominal incision sutured. The rats developed caecal amoebiasis within 48 h with trophozoites of *E. histolytica* visible microscopically in the caecal contents and scrapings of the caecal wall. These animals are ready for evaluation of the therapeutic action of test agents.

2.8. Treatment schedule

Test agents were suspended in 1.5% sodium carboxymethylcellulose solution at the desired concentrations. The animals were orally administered with a test agent with the help of a feeding needle once daily for five consecutive days. A 3-day schedule was also included. The rats were sacrificed 48 h after the last dose with an overdose of ether anaesthesia and the caecum examined for the presence of *E. histolytica*. The reported method was used to evaluate the degree of infection.

Table 1
In vitro action of 3′-deoxynucleosides on trophozoites of *E. histolytica*

Test agent	In vitro activity (μg/ml)
1a	1000
1b	1000
2a	1000
2b	500
2c	500
3a	500
3b	500
3c	125
4a	NA
4b	250
4c	NA
5a	250
5b	500
5c	1000
2′-Deoxyadenosine	1000
2′-Deoxyguanosine	NA
2′-Deoxycytidine	NA
Thymidine	NA
Emetine hydrochloride	8
Metronidazole	4

3. Results and discussion

The in vitro action of the nucleoside analogues (1a–5c) along with standard anti-amoebic drugs against trophozoites of *E. histolytica* is presented in Table 1. The nucleosides 5a and 3c were found to be the most active compounds in the series. Tests carried out with naturally occurring 2-deoxynucleosides (dA, dG, dC&T) did not reveal any inherent amoebicidal property at concentrations of 1000 μg/ml. Compound 5a was first selected for evaluating its therapeutic efficacy against experimental caecal amoebiasis of rats.

Table 2
Therapeutic efficacy of 5a against experimental caecal amoebiasis of rats

Pure/formulation	Dose, mg/kg body wt. (days)	Rats cured/treated (%)	Average caecal score		
			Wall	Contents	Total
Pure	100 (5)	0/5 (0%)	2	2	4
Enteric coated	100 (5)	3/4 (75%)	0.5	0.5	1.0
Enteric coated	100 (5)	5/6 (83%)	0.16	0.16	0.32
Enteric coated	100 (5)	4/5 (80%)	0.0	0.0	0.0
Enteric coated	100 (5)	3/5 (60%)	0.0	0.0	0.0
Enteric coated	100 (5)	4/5 (80%)	0.0	0.0	0.0
Enteric coated	150 (5)	5/5 (100%)	0.0	0.0	0.0
Enteric coated	150 (5)	5/5 (100%)	0.0	0.0	0.0
Enteric coated	150 (5)	5/5 (100%)	0.0	0.0	0.0
Enteric coated	150 (3)	3/5 (60%)	0.0	0.0	0.0
Metronidazole	100 (5)	5/5 (100%)	0.0	0.0	0.0
Control	–	0/5 (0%)	3.6	3.6	7.2

Metronidazole was used as a drug standard. 5a was found to be ineffective at a dose of 100 mg/kg for five consecutive days (Table 2). These results suggested the possibility of an extensive depurination of this compound under strong acidic conditions present in the stomach thereby affecting its concentration in its active form at the site of action. This was confirmed by stirring the compound at room temperature with 0.1 N HCl pH 3.0. The reaction mixture was monitored after 30 min by TLC, which showed complete hydrolysis of the starting compounds thus confirming its instability in the gastric environment. Tests were conducted with enteric coated formulation of this compound to evaluate its therapeutic potential and the results are included in Table 2. The enteric coated formulation of 5a was found to indicate its true in vivo amoebicidal action. The therapeutic efficacy was recorded consistently in different batches of animals. Ten percent cures with total clearance of the amoebic infection, as determined through microscopic examination and culture methods, were consistently achieved with the enteric coated formulation of the compound fed at a dose of 150 mg/kg for 5 days. At the same dose administered for 3 days, 60% cures were recorded. The caecum of all the animals appeared normal with respect to their contents and the caecal walls. Where parasitic failures were observed their numbers could be recorded after prolonged microscopic examination.

The distinct difference in the amoebicidal property of the naturally occurring 2-deoxynucleosides (dA, dG, dC&T) and their analogues, especially the nucleosides 5a and 3c, further justify the purpose of this study. The in vivo tests were first started with 5a and confined to it. There are inherent problems in working with this class of compounds to understand their true potential both under in vitro conditions to demonstrate their amoebicidal property, and in vivo for their therapeutic potential. Given the labile nature of these compounds, the in vitro and in vivo results do not obviously reflect the true anti-amoebic end points, as the tests are conducted in the presence of serum or in animals. Yet significantly, on the molar basis 5a was found to be two and half times more effective therapeutically than the standard drug metronidazole used in this study. Development of techniques taking due consideration towards inexpensive production and stabilization of these molecules under biological conditions are essential towards logical conclusion of these studies.

Acknowledgements

The authors acknowledge RSIC for providing the spectral data of the compounds reported here. One of us (A.K.) thanks CSIR for financial assistance in the form of SRF. The technical support of A.P. Singh is also acknowledged.

References

Booden, T., Boonlayangoor, P., Albach, R.A., 1976. Incorporation of purines and pyrimidines in axenically grown *E. histolytica*. J. Parasitol. 62, 641–643.

Boonlayangoor, P., Albach, R.A., Booden, T., 1978. *E. histolytica*: uptake of purine bases and nucleosides during axenic growth. Exp. Parasitol. 45, 225–233.

Das, S.R., Baer, H.P., 1991. Inhibition of axenically grown *E. histolytica* by purine nucleoside analogs and actions of natural nucleosides. Trop. Med. Parasitol. 42, 161–163.

Das, S.R., 1975. A novel and rapid method for in vitro testing of antiamoebic agents against aerobic and anaerobic growing axenically or with bacteria. Curr. Sci. 44, 463–464.

Eubank, W.B., Reeves, R.E., 1981. Growth inhibition of axenic *E. histolytica* by tuberecidine and ara-A. Am. J. Trop. Med. Hyg. 30, 1900–1902.

Jenkin, S.R., Holy, F.W., Watson, E.J., 1965. 3′-Deoxynucleosides III: 3′-Deoxyguanosine. J. Org. Chem. 30, 2851–2852.

KrishnaPrasad, B.N., Bansal, I., 1982. Effect of caecal pH of rat on the development of experimental *Entamoeba histolytica* infections. Ind. J. Parasitol. 6, 97–98.

KrishnaPrasad, B.N., Bansal, I., 1983. Interrelationship between faecal pH and susceptibility to *Entamoeba histolytica* infections of rats. Trans. R. Soc. Trop. Med. Hyg. 72, 271–274.

KrishnaPrasad, B.N., Bansal, I., Nandi, R.C., Singh, S., Sarin, J.P.S., 1984. Correlation of naturally occurring concentrations of free ammonia in the caecum of rats with resistance to *Entamoeba histolytica*. Curr. Sci. 53, 371–374.

Kumar, A., 1996. Synthesis and physical studies on 2′, 5′-oligodeoxyribonucleotides. Ph.D. Thesis, University of Lucknow, Lucknow, India.

Kumar, A., Khan, S.I., Manglani, A., Khan, Z.K., Katti, S.B., 1994. Synthesis and antifungal activity of 3′-deoxyribonucleosides. Nucleosides Nucleotides 13, 1048–1058.

Leitch, G.J., 1988. Intestinal leuminal and mucosal microclimate H^+ and NH_4^+ concentrations or factors in the ecology of experimental amoebiasis. Am. J. Trop. Med. Hyg. 38, 480–496.

Lo, H.-S., Wang, C.C., 1985. Purine salvage in *E. histolytica*. J. Parasitol. 662–669.

Martinez-Palomo, A., Cantellano, M.E., 1997. Update on antiamoebic agent developments: clinical results and experience. In: Wolf, M.E. (Ed.), Burgers' Medicinal Chemistry and Drug Discovery, vol. 4. Wiley, New York, pp. 453–458.

Ogbunude, P., Baer, H.P., 1993. Nucleoside transport in parasites-current status and methodological aspects. Int. J. Biochem. 25, 471–477.

Powell, S.I., Wilmot, A.J., MacLeod, I., Elsdon-Dew, R., 1966. Metronidazole in amoebic dysentery and amoebic liver abscess. Lancet ii, 1329–1331.

Robinson, G.R., 1968. Laboratory cultivation of some human parasitic amoeba. J. Gen. Microbiol. 53, 9–29.

Ross, W., 1997. Antiamoebic agents. In: Wolf, M.E. (Ed.), Burgers' Medicinal Chemistry and Drug Discovery, vol. 4. Wiley, New York, pp. 429–452.