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ANTIVIRAL AND ANTIMETABOLIC ACTIVITIES OF FORMYCIN AND ITS N_1-, N_2-, 2′-*O*- AND 3′-*O*-METHYLATED DERIVATIVES

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Abstract—Formycin A, formycin B, and the N_1-, N_2-, 2′-*O*- and 3′-*O*-methyl derivatives of formycin A, were all examined for activity against vaccinia, herpes simplex and vesicular stomatitis viruses in primary rabbit kidney cells. The susceptibilities of calf intestinal adenosine deaminase to all the formycin A derivatives, relative to those of some adenosine analogues, were measured in order to take into account the possible effects of intracellular deamination on the antiviral and cytotoxic effects of the formycin derivatives. Formycin B was found to be inactive in all assay systems. Formycin A exhibited significant antiviral activity only against vesicular stomatitis virus, but it also proved relatively toxic to the host cells, appreciably inhibiting cellular DNA and RNA synthesis as measured by incorporation of labelled thymidine and uridine, respectively. Of the methylated analogues, N_1-methylformycin A (which was highly resistant to enzymatic deamination) and the 2′-*O*- and 3′-*O*-methyl derivatives of formycin A were totally inactive in all three viral assay systems. Only N_2-methylformycin exhibited relatively high activity against vaccinia virus, was not toxic to the cells, and did not affect cellular DNA and RNA synthesis.

The antibiotic formycin (also known as formycin A) and formycin B were originally isolated from culture filtrates of *Nocardia interforma* [1, 2]. Formycin A is a C-glycoside which is isomeric with, and a structural analogue of, adenosine (Fig. 1). It is readily deaminated by adenosine deaminase to formycin B, which is an isomer and analogue of inosine [2–4]. Interest in formycin A stems from the fact that it can replace adenosine in a variety of biochemical reactions [5, 6].

Formycin A, but not formycin B, was found to be an effective inhibitor of experimental tumours [7]. Analogous reports on the activities of the formycins against influenza A_1 [8] and a variety of other viruses [9] prompted us to undertake investigations on the *in vitro* activities of formycins A and B, and some new methylated analogues of formycin A, in several viral systems in primary rabbit kidney cells. The methylated analogues examined included N_1-methylformycin, N_2-methylformycin, 2′-*O*-methylformycin and 3′-*O*-methylformycin. The *O*′-methyl derivatives are of interest in relation to the previously reported deleterious effects of *O*′-methylation on the antiviral activity of ara-C* (1-β-D-arabinofuranosylcytosine) [10]. The *N*-methyl derivatives might be expected to exhibit different activities, because of possible tautomerism in the pyrazole ring of the aglycone, as illustrated in Fig. 1. Although attempts have been made to delineate such tautomerism by carbon-13 magnetic resonance spectroscopy [11, 12], the results do not show much more than that tautomerism probably exists. Studies have now been initiated in our laboratories to examine the tautomerism of

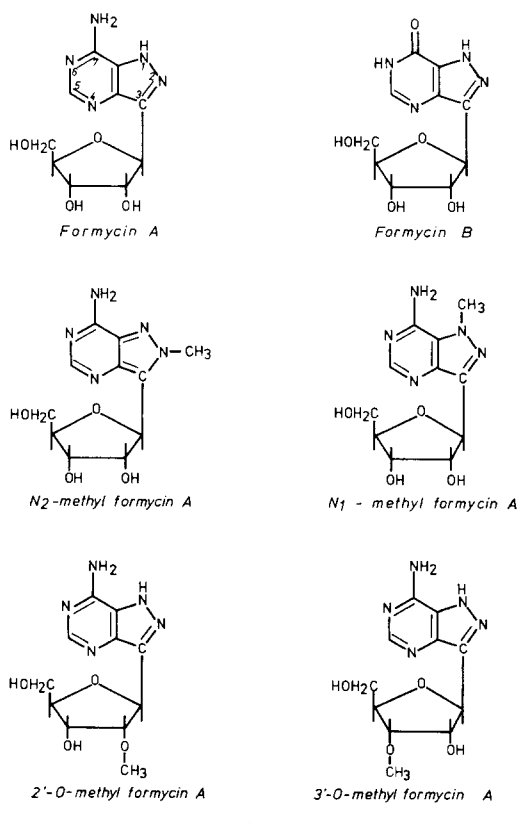

Fig. 1.

* Abbreviations used: ara-C, 1-β-D-arabinofuranosylcytosine, cytosine arabinoside, cytarabine, Cytosar®; IUdR, 5-iodo-2′-deoxyuridine; PRK, primary rabbit kidney; $CCID_{50}$, cell culture infecting dose 50 (dose infecting 50% of the cell cultures); MIC, minimal inhibitory concentration; MEM, Eagle's minimal essential medium.

formycins A and B by proton magnetic resonance spectroscopy and emission spectroscopy; preliminary findings suggest that the problem is complex and that tautomerism of the pyrimidine ring is also involved. Consequently, it was felt that the biological activity of fixed tautomers such as the N_1- and N_2-methyl derivatives might throw some light on the problem of tautomerism in formycin.

MATERIALS AND METHODS

Adenosine was obtained from Waldhoff (Mannheim, GFR) and deoxyadenosine from Serva (Heidelberg, GFR). Formycin A and formycin B were kindly donated by Dr. H. Isoyama (Meiji Seiki Kaisha Ltd., Tokyo) and Dr. R. K. Robins (ICN, Irvine, CA, U.S.A.), respectively. We are also indebted to the Cancer Chemotherapy Service Centre, N.C.I. (Bethesda, MD, U.S.A.) for a sample of N_2-methylformycin prepared by Townsend *et al.* [13].

2′-*O*- and 3′-*O*-Methyladenosine were prepared by diazomethane methylation of adenosine in a 1 mM methanolic solution of $SnCl_2$ [14] and the two isomers were fractionated on a Dowex (OH^-) column according to Dekker [15].

N_1-Methyl and N_2-methylformycins were prepared by diazomethane methylation of formycin A in methanolic medium. The two isomers (ratio 2:7) were separated on a preparative scale on a Dowex (OH^-) column, the procedure being based in part on the apparent different mobilities of nucleosides constrained in the *syn* conformation (8-bromoadenosine, 8-hydroxyisopropyladenosine, 6-methylcytidine) relative to those with a preference for the *anti* conformation (adenosine, cytidine). This procedure is much simpler than that based on treatment of the sodium salt of formycin with methyliodide [13]. Furthermore, in combination with the use of $SnCl_2$ as a catalyst for etherification of the sugar hydroxyls, the procedure has also been used to obtain the 2′-*O*-methyl and 3′-*O*-methylformycins, also reported by Robins *et al.* [14], as well as the four possible isomeric N_1 (N_2),2′(3′)-*O*-dimethylformycins, all of which were successfully fractionated by column chromatography, and identified by chromatographic properties, ultraviolet absorption spectra, and proton magnetic resonance spectroscopy (J. Giziewicz and D. Shugar, in preparation).

The rates of deamination of adenosine and formycin analogues were determined using Calbiochem calf intestinal adenosine deaminase (protein content 4 mg/ml; sp. act. 840 units/ml at 30°). The deamination rate was monitored in 10-mm spectrophotometer cuvettes by the change in absorption at λ_{max} upon conversion of the formycin A analogue to the corresponding formycin B analogue. For comparative purposes, the rates of deamination of adenosine and some of its analogues were measured under the same assay conditions. To 3 ml of substrate solution, 10^{-4} M in 0·01 M phosphate buffer pH 7·4, in a 10 mm spectrophotometer cuvette, were added 0·06 enzyme units. The rate of deamination at 34° was measured spectrally in a Unicam SP-8000 spectrophotometer by the time-dependent change in optical density at the absorption maximum. For those substrates which proved more resistant to deamination, the concentration of enzyme was increased 10-fold or more.

The antiviral activity tests were carried out in PRK (primary rabbit kidney) cell cultures grown to confluency in glass culture tubes. Eagle's minimal essential medium (MEM) was used as cell culture medium: it was supplemented with 10% calf serum for growth of the cells or 3% calf serum for maintenance of the cells. To explore the effects of the formycin derivatives on virus-induced cytopathogenicity, the cells were exposed to different concentrations of the compounds (40, 4, 0·4, 0·04, 0·004 μg/ml) in MEM (supplemented with 3% calf serum) either 24 hr before virus challenge, or immediately after virus inoculation, or both. Two reference materials were included in the antiviral tests: cytosine arabinoside (ara-C, Cytosar®), generously supplied by Upjohn (Puurs, Belgium) and 5-iododeoxyuridine (IUdR, IDU), provided by Ludeco (Brussels, Belgium). The cells were challenged with either of the following: vaccinia virus, herpes simplex virus or vesicular stomatitis virus. All viruses were added at 100 $CCID_{50}$ per tube and allowed to adsorb to the cells for 1 hr at 37°. Viral cytopathogenicity was recorded as soon as it reached 100% in the control cell cultures: at 2 days for vesicular stomatitis virus, at 3 days for vaccinia and herpes simplex virus. The antiviral activity is expressed as the minimal inhibitory concentration (μg/ml) of compound required to reduce virus-induced cytopathogenicity by 50%.

The effect of the formycin derivatives on host cell DNA and RNA synthesis was evaluated in PRK cell cultures grown to confluency in 55 mm Falcon plastic petri dishes (approximately 10^6 cells/petri dish). The cells were exposed to different concentrations of the compounds (200 or 40 μg/ml) in MEM (supplemented with 3% calf serum) for 24 hr, washed (3×) with MEM and then incubated for 30 min with either [^{3}H-*methyl*]thymidine (2 μCi/ml MEM/petri dish) or [^{3}H-5]uridine (1 μCi/ml MEM/petri dish). The sp. act. of [^{3}H-*methyl*]thymidine and [^{3}H-5]uridine amounted to 12 and 26 Ci/m-mole respectively. The plates were further processed for acid-insoluble radioactivity as described previously [10].

RESULTS

Enzymatic deamination of the formycin analogues. Since formycin A is known to be readily deaminated by adenosine deaminase [2–4], intracellular deamination of formycin A and its methyl derivatives might

Table 1. Rates of deamination of adenosine and formycin analogues by calf intestinal adenosine deaminase at 34°

Compounds	$t_{1/2}$ (min)*
Adenosine	2·5
2′-*O*-methyladenosine	3·5
3′-*O*-methyladenosine	42
2′-deoxyadenosine	4·0
Formycin A	9·5
N_1-methylformycin A	12,000
N_2-methylformycin A	86
2′-*O*-methylformycin A	13
3′-*O*-methylformycin A	44

* Time required for half-completion of the reaction.

Table 2. Effect of formycin analogues on virus-induced cytopathogenicity in PRK cell cultures

Compounds	Minimal inhibitory concentration* (μg/ml)		
	Vaccinia virus (PRK)	Herpes simplex virus (PRK)	Vesicular stomatitis virus (PRK)
Compounds added immediately after virus adsorption			
Formycin A	20	>40	2 (10–20)†
Formycin B	>40	>40	>40
N_2-Methylformycin A	1	>40	40 (40)
N_1-Methylformycin A	>40	>40	>40
2′-*O*-Methylformycin A	>40	>40	>40
3′-*O*-Methylformycin A	>40	>40	>40
Cytosine arabinoside	0·01	0·1	1
5-Iododeoxyuridine	0·1	0·2	>40
Compounds added 24 hr before virus challenge, not added thereafter			
Formycin A	40	>40	2 (10–20)
Formycin B	>40	>40	>40
N_2-Methylformycin A	10	>40	4 (4)
N_1-Methylformycin A	>40	>40	>40
2′-*O*-Methylformycin A	>40	>40	>40
3′-*O*-Methylformycin A	>40	>40	>40
Cytosine arabinoside	4	2	1
5-Iododeoxyuridine	>40	>40	>40
Compounds added 24 hr before virus challenge and added again immediately after virus adsorption			
Formycin A	10	>40	2 (10–20)
Formycin B	>40	>40	>40
N_2-Methylformycin A	0·2	4	4 (4)
N_1-Methylformycin A	>40	>40	>40
2′-*O*-Methylformycin A	>40	>40	>40
3′-*O*-Methylformycin A	>40	>40	>40
Cytosine arabinoside	0·02	0·1	0·4
5-Iododeoxyuridine	0·1	0·1	>40

* Required to reduce virus-induced cytopathogenicity by 50%.
† In parentheses: minimal toxic concentration (μg/ml), causing a morphological alteration of the cells.

be expected to affect their biological activity. The question of deamination is particularly relevant in view of the loss of antiviral activity of ara-C after it has been deaminated intracellularly by cytidine deaminase to ara-U (for review see Ref. 16). As shown in Table 1, both adenosine and formycin A became slightly more resistant to deamination when methylated at the 2′-*O*-position and considerably more resistant when methylated at the 3′-*O*-position. A marked increase in resistance to deamination was also observed after formycin A had been methylated at N_2, but the highest resistance to deamination was noted with N_1-methylformycin A (Table 1).

Antiviral activity. Vaccinia virus, herpes simplex virus and vesicular stomatitis virus were chosen as challenge viruses to assess the antiviral activity of the formycin derivatives and the reference compounds ara-C and IUdR (Table 2). As expected [17–19], ara-C suppressed the cytopathic effects of both DNA viruses and the rhabdo-virus at relatively low concentrations (MIC: 0·01, 0·1 and 1 μg/ml, respectively, when added after virus inoculation). IUdR was only effective against vaccinia and herpes simplex virus (MIC: 0·1 μg/ml), when added immediately after virus adsorption.

Formycin A exhibited some inhibitory effect on the cytopathogenicity of vaccinia virus (MIC: 10–40 μg/ml). This inhibitory effect was significantly increased upon substitution of a methyl group in the N_2 position: N_2-methyl formycin A was most effective when added immediately after the virus challenge; and, when it was administered before and after the virus, it approached the activity of IUdR (MIC: 0·1 μg/ml). Unlike N_2-methylformycin A, N_1-methylformycin A failed to inhibit vaccinia virus-induced cytopathogenicity, even at the highest concentration tested (40 μg/ml). Formycin B and the 2′-*O*- and 3′-*O*-methyl analogues of formycin A were also inactive at 40 μg/ml.

All formycin derivatives were inactive against herpes simplex virus-induced cytopathogenicity, except N_2-methyl formycin A which displayed some inhibitory effect (MIC: 4 μg/ml) when administered before and after the virus challenge.

Of all analogues, only the parent compound (formycin A) inhibited the cytopathic effect of vesicular stomatitis virus (MIC: 2 μg/ml). This concentration was 5- to 10-fold lower than the concentration at which formycin A itself caused a minimal but distinct morphological alteration of cells infected with vesicular stomatitis virus. N_2-methylformycin was also

Table 3. Effect of formycin analogues on cell morphology and DNA and RNA synthesis of PRK cell cultures

Compounds	Dose (μg/ml)	Toxicity*	[^{3}H-*methyl*]thymidine incorporated into DNA†		[^{3}H-5]uridine incorporated into RNA†	
			Gross counts	%	Gross counts	%
Control		−	3503	100	9299	100
Formycin A	200	+ +	168	4·8	447	4·8
	40	+	377	10·8	1577	17·0
Formycin B	200	±	4086	116·6	8752	94·1
	40	−	2625	74·9	7893	84·9
N_2-Methylformycin A	200	−	6756	129·9	9191	98·8
	40	−	4135	118·0	8591	92·4
N_1-Methylformycin A	200	−	3408	97·3	6688	71·9
	40	−	3206	91·5	5548	59·7
2′-*O*-Methylformycin A	200	−	7349	209·8	11146	119·9
	40	−	4652	132·8	8993	96·7
3′-*O*-Methylformycin A	200	−	9577	273·4	8408	90·4
	40	−	7967	227·4	7808	84·4

* Measured after the cells had been exposed to the compounds for 24 hr. Toxicity was recorded as amount of cytopathogenicity: −, none; ±, floating cells and debris but no disruption of monolayer; +, *ca* 25% disruption; + +, *ca* 50% disruption of monolayer. Average values for 4 petri dishes.

† Measured after the cells had been exposed to the compounds for 24 hr. Gross counts are expressed in counts/min per petri dish (average values for 2 petri dishes).

active against vesicular stomatitis virus, but at the concentration (4 μg/ml) required to block virus-induced cell damage by 50% it was itself responsible for a toxic alteration of the cells.

Antimetabolic activity. Cytotoxicity as well as incorporation of [^{3}H-*methyl*]thymidine and [^{3}H-5]uridine into host cell DNA or RNA were measured with cell cultures which had been exposed to 40 or 200 μg/ml of the formycin derivatives. None of these, except the parent compound formycin A, caused a substantial cytotoxicity and inhibition of DNA and RNA synthesis (Table 3). The 2′-*O*- and 3′-*O*-methyl derivatives exerted a stimulatory effect on thymidine incorporation. N_2-Methylformycin A neither stimulated nor inhibited thymidine or uridine incorporation. Formycin B also proved inert in this regard. Formycin A, however, reduced both thymidine and uridine incorporation by 95% at 200 μg/ml and by 80–90% at 40 μg/ml. We have previously shown that, in similar conditions, ara-C and IUdR inhibited PRK cell DNA synthesis by 80–90% at doses of 1 μg/ml and 200 μg/ml, respectively [19].

DISCUSSION

Although formycin A exhibited some activity against vaccinia and, even more so, against vesicular stomatitis virus, the doses at which such activity was observed were not much less than those resulting in a morphological alteration of the host cells and impairment of cellular DNA and RNA synthesis. Consequently, formycin A does not appear to be a suitable candidate as a specific antiviral agent, at least with the systems used in this investigation. Its cytotoxic effects suggest, furthermore, that its ready susceptibility to enzymatic deamination (Table 1) is of little relevance, since the deamination product, formycin B, does not exert discernible toxic effects on the host cells. The differential cytotoxic effects of formycin A and B are not limited to PRK cells. Formycin A has also been found to be toxic for HeLa [8] and chick embryo cells [9] at concentrations far below those required for the cytotoxicity of formycin B [8].

Takeuchi *et al.* [8] reported inhibition of influenza A_1 virus multiplication by both formycins A and B, and Ishida *et al.* [9] found formycin A to be active against vaccinia, polio, influenza and vesicular stomatitis virus in chick embryo and HeLa cells, when tested by three different methods (drug-through-agar diffusion, drug-in-agar dilution and ordinary drug dilution assay in culture tubes). However, the minimal doses of formycin A required to inhibit polio and vaccinia virus in HeLa and chick embryo cells [9], did not markedly differ from those required to cause a morphological alteration of the cells. These results point to a lack of specificity in the antiviral activity of formycin A, at least as far as polio and vaccinia virus are concerned. Some specificity was claimed for the activity of formycin A against influenza and vesicular stomatitis virus, since virus-inhibiting effects were obtained at concentrations 10- to 15-fold lower than those eliciting cytotoxicity [9]. A similar toxicity to activity ratio was observed with formycin A in PRK cells infected with vesicular stomatitis virus (Table 2). It is questionable, however, whether this 'therapeutic' margin is sufficiently large to justify further antiviral trials with formycin A.

Of particular interest are the results obtained with N_1-methylformycin and N_2-methylformycin. The former, notwithstanding its virtually complete resistance to deamination (Table 1), did not exhibit antiviral activity, nor did it affect cellular DNA or RNA synthesis. Although it is conceivable that the resistance of N_1-methylformycin to deamination is due to steric hindrance by the N_1-methyl substituent, which is adjacent to the amino group, it is pertinent to note that the susceptibility of cytidine to cytidine deaminase is not appreciably reduced when the H-5

of the pyrimidine ring is replaced by a methyl, or even an ethyl, group (E. Krajewska, personal communication).

On the other hand, the N_2-methyl analogue inhibited the cytopathic effect of vaccinia virus at a concentration (0·2 μg/ml, when added before and after virus challenge) far below that (>200 μg/ml) impairing normal cell morphology and cellular DNA and RNA synthesis (Tables 2 and 3). The antiviral index or ratio of the minimal toxic dose (causing a morphological alteration of the cells or an appreciable inhibition of cellular DNA or RNA synthesis) to the minimal effective dose (required to suppress virus-induced cytopathogenicity by 50%) calculated from these data would seem higher than 1000, and, accordingly, N_2-methylformycin may be considered as a specific antiviral agent, at least against vaccinia virus in PRK cells.

Consistent with the differential effects of N_1- and N_2-methylformycin on vaccinia virus-induced cytopathogenicity in PRK cells is a preliminary report that N_2-methylformycin exhibits a quantitatively higher level of activity than N_1-methylformycin against leukemia L-1210 [20]. Since N_2-methylformycin is sensitive to deamination, albeit 10 times less than formycin itself (Table 1), it seemed advisable to examine the antiviral activity of N_2-methylformycin B in our assay systems. In preliminary tests, N_2-methylformycin B failed to inhibit vaccinia virus-induced cytopathogenicity, even at 40 μg/ml. Thus, the relatively high susceptibility of N_2-methylformycin A to deamination (as compared to N_1-methylformycin A does not appear to seriously affect its inhibitory effect on vaccinia virus. Similarly irrelevant is the deaminase susceptibility of formycin A, both in terms of cytotoxicity and antiviral activity (see above). However, PRK cells may contain deaminases which differ from those present in calf intestine. Therefore, additional experiments have been designed to establish the rates of deamination of adenosine and formycin and their derivatives in PRK cells.

The lack of antiviral activity of the 2′-*O*-methyl and 3′-*O*-methyl derivatives of formycin A (Table 2) is perhaps not entirely unexpected in view of the previous demonstration of the abolition of activity of ara-C upon methylation of the 2′, 3′ or 5′ hydroxyls [10]. Because of these results, no attempts were made to assay the four isomeric $N_1(N_2)$,2′(3′)-*O*-dimethylformycins.

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