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In Vitro Antiviral Activity and Preliminary Clinical Trials of a New Adamantane Compound

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A compound, 1'-methyl spiro (adamantane-2,3'-pyrrolidine) maleate, chemically related to the antiviral drug amantadine, was tested for activity in vitro against a number of human respiratory viruses. By a variety of techniques, it was shown to be active against a wide range of human and animal influenza A viruses. The effect was, however, variable and ranged from high activity against two 1957 Asian strains to no observable activity against a 1971 strain. Like amantadine, the drug did not inhibit the growth of influenza B viruses. It was also inactive against a number of paramyxoviruses. Unlike amantadine, the drug did inhibit rhinoviruses, but to a lesser extent than myxoviruses. The coronavirus 229E was also sensitive to the action of the drug in vitro. Although an earlier trial in volunteers showed that, when given orally from 2 days before until 5 days after virus challenge, the drug was protective against infection with influenza A/Hong Kong/68 virus, a similar trial in volunteers challenged with rhinoviruses 2 and 9 revealed no useful activity against rhinoviruses in man.

Although many substances have been synthesized for potential activity against viruses, very few can be used in clinical medicine. One of these is 1-aminoadamantane hydrochloride (amantadine), which has been shown to be partly effective in influenza A infections of man (10, 19, 22, 25). As a consequence, a number of secondarily substituted adamantanes have now been produced for a study of structure-activity relationships (15). A compound, 1'-methyl spiro (adamantane-2,3-pyrrolidine) maleate (1:1) (Fig. 1), had greater activity in tissue culture, eggs, and mice than did amantadine, weight for weight, and also possessed a wider antiviral spectrum (F. E. André, unpublished data). Toxicological studies in animals and human pharmacological studies indicated that this drug was suitable for use in man when given orally. In a prophylactic trial it reduced the incidence of infections and severity of reactions of experimental Hong Kong influenza in volunteers (2). In this paper we shall describe the testing of the drug in vitro against a range of respiratory viruses and its use on a limited scale in additional human trials. The place of adamantane compounds in viral chemoprophylaxis and chemotherapy will be considered.

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MATERIALS AND METHODS

Influenza A viruses. All viruses were egg seeds. A/swine/Iowa/15/30 (Hsw1 N1) and A/Singapore/1/57 (H2 N2) were laboratory strains of unrecorded passage history. A/Okuda/57 (H2 N2), provided by Yoshiomi Okuno of Osaka, had had about 280 egg passages when received in this laboratory. A/Hong Kong/1/68 (H3 N2), two monkey kidney passages and one egg passage, was supplied by the World Influenza Centre, Mill Hill, London. A/Hong Kong/107/71 (H3 N2), early egg passage material, was obtained from the same source. Recombinant clones A/PR/8/34-Eng./939/69 (H3 N2), 64c and 64d, have been described by Beare and Hall (1) and by McCahon and Schild (16). Recombinant A/PR/8/34-Aichi/2/68 (H3 N2), X-31 (13), was supplied by the World Influenza Centre; A/swine/Wisconsin/1/66 (Hsw1 N1) and A/Turkey/Massachusetts/65 (Hav 6 N2), similarly obtained, had had four and six egg passages, respectively. Antigenic designations in parentheses are according to the World Health Organization (26).

Influenza B viruses. B/Lee/40, B/Taiwan/2/62 and B/Singapore/3/64 had had an unknown number of egg passages. B/Czechoslovakia/28/70 (seven egg passages) and B/Victoria/98926/70 (two monkey kidney passages and two egg passages) were obtained from the World Influenza Centre.

Paramyxoviruses. Parainfluenza type 1 (Sendai), an egg-adapted strain, had had an unknown passage history. Parainfluenza type 3 (HA1 strain) and parainfluenza type 3 (bovine strain) were adapted to growth in monkey kidney and bovine kidney tissue cultures, respectively.

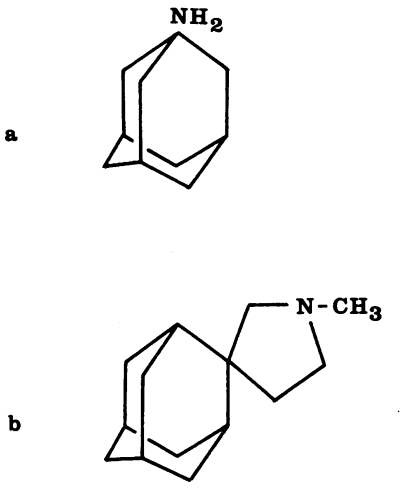

FIG. 1. *Structural formulas. a, 1-Aminoadamantane hydrochloride (amantadine); molecular weight, 188. b, 1'-Methyl spiro (adamantane-2,3'-pyrrolidine) maleate (1:1); molecular weight, 321.*

Rhinoviruses. Rhinoviruses (RV) types 1B, 2, 9, 31, and 43, used for experiments in vitro, were generally low-passage stocks derived from nasal washings of infected volunteers. Seeds were second, third, and fourth HeLa cell passages. RV9 used for plaque experiments was, however, fully adapted to growth in HeLa cells. RV2 and RV9 given to volunteers had been maintained by man-to-man passage only.

Coronavirus. The strain 229E was adapted to growth in L132 cells (4).

Antiviral compound. 1'-Methyl spiro (adamantane-2,3'-pyrrolidine) maleate (1:1) (spiroadamantane) was provided by Philips-Duphar B.V., Weesp, Holland, in powdered form and was diluted in tissue culture maintenance medium or in normal allantoic fluid to provide the concentrations needed. It was readily soluble in water, and it was possible to prepare concentrated stock solutions when necessary.

Assays of antiviral effects in eggs. Ten- to 11-day-old embryonated hen eggs were used. Injections of both virus and compound were by the allantoic route. Eggs were then incubated for 2 to 3 days at 33 C, and harvested allantoic fluids were tested for virus hemagglutinin with chicken red blood cells. Serial 10-fold dilutions were normally used for titrations in eggs, using 2 to 4 eggs per dilution. Titers were expressed as 50% egg-infecting doses.

Tissue cultures. Primary cultures of bovine kidney or monkey kidney were grown in Eagle basal medium with 10% calf serum by standard methods. Before use, cultures were washed free of serum and thereafter were maintained in Eagle medium alone. Rhinovirus-sensitive HeLa cells were grown and maintained by the methods of Stott and Tyrrell (21), and L132 cells were grown and maintained by the methods of Bradburne and Tyrrell (5). These two cell lines and the human diploid cell line MRC-5(11) were grown in Eagle basal medium with 10% calf serum. Maintenance media usually contained 1 or 2% of calf serum (5, 11, 21).

Titrations in tissue culture tubes. Three tubes were generally inoculated with each virus dilution. Influenza virus dilutions were 10-fold, and those for rhinoviruses and the coronavirus 229E were 3.2-fold (0.5 $\log_{10}$). Influenza virus end points were read by adsorption of guinea pig red cells (24) after a 4-day incubation; rhinovirus and coronavirus end points were read by cytopathic effect after a 5-day incubation.

Yield-reduction experiments. Groups of HeLa cell tubes, with or without spiroadamantane in the maintenance medium, were inoculated with about $10^{2.0}$ 50% tissue culture-infecting doses ($TCID_{50}$). No additional drug was used during the subsequent incubation period and there were no medium changes. On each of the 4 successive days, four tubes containing cells and maintenance medium were frozen, harvested, and stored to await subsequent titration in new HeLa cells. Similar experiments were performed in organ cultures of human embryo trachea (9). Cultures were treated for 18 h with drug or control fluid and were then inoculated with $10^{4.0}$ $TCID_{50}$ of virus. The virus was adsorbed for 5 h, residual inoculum was washed off, and fresh medium was added. Sampling, by harvesting of medium and replacement with drug-containing medium or with control medium, was performed daily for 4 consecutive days. Subsequently virus yields were assayed in HeLa cells.

Plaque assays. Plaque assays in primary bovine kidney for influenza viruses were performed by the method of Beare and Keast (3), and assays in rhinovirus-sensitive HeLa cells were performed by the method of Fiala and Kenny (7), modified by the substitution of 0.5% agarose for agar. Titers were expressed in plaque-forming units (PFU).

Trials in man. Human trials were invariably randomized and double blind (2, 20). Briefly, in the influenza virus studies, volunteers were treated with 140 mg of spiroadamantane per day. Capsules containing 70 mg of the drug were given orally twice daily from 2 days before virus inoculation until 5 days after it. A control group received identical capsules containing an inert placebo. Trial results were assessed on the basis of the incidence and severity of clinical reactions judged by daily examination, the presence of virus in nasal secretions on the 3rd and 4th days after virus inoculation, and development of fourfold rises in antibody titer in paired sera collected before and 18 days after virus inoculation. In the rhinovirus trials, the procedure was similar except that the daily dose of drug was 120 mg (60 mg orally twice daily), and virus excretion was examined additionally on the second day after virus inoculation. There was no preliminary antibody screen to select people who were serologically susceptible to the rhinoviruses used for challenge, and a proportion of the volunteers was therefore resistant to infection with any single rhinovirus serotype. In order to achieve a satisfactory proportion of infections in the control group, a double virus challenge was therefore performed comprising 25 TCD_{50} of RV2 and 50 TCD_{50} of RV9 (20).

RESULTS

Toxicity of the drug in tissue culture. In tissue culture experiments, the maximal drug concentrations used experimentally were also incorporated in maintenance media of uninoculated control cultures. These controls showed no evidence of toxicity on inspection by low-power light microscopy. At drug concentrations threefold higher than the maximum used experimentally, some granularity of the cell monolayer was generally observed.

Influenza viruses in tissue culture. It would have been desirable to assess the sensitivity of all viruses to the drug by means of plaque-inhibition tests. This was impossible with some influenza A viruses, however, since the group as a whole plaques irregularly and with low efficiency in tissue culture systems. Our purpose was to measure the activity of the drug against a wide range of viruses, and experiments could not therefore be confined to strains which were experimentally convenient. Hence plaque-inhibition tests were used when practicable and were supplemented by other techniques when necessary. However, influenza B viruses presented no problem since they plaqued easily and efficiently in bovine kidney cells (3), while a proportion of influenza A strains also plaqued in this system even though the number of PFU per milliliter was usually lower than egg infectivity titers (Beare and Keast, unpublished data).

The results of plaque-inhibition tests are shown in Table 1. A live vaccine strain (Asian subtype), A/Okuda/57, was highly sensitive to the action of the drug, and three recombinant influenza A viruses were rather less sensitive. A/Hong Kong/107/71, antigenically related to A/Hong Kong/1/68, appeared insensitive, as were four influenza B viruses chosen at random.

Because A/Hong Kong/1/68 plaqued poorly, its sensitivity to the drug was compared with that of A/Hong Kong/107/71 in tissue culture tubes (Table 2). It was fairly sensitive but less so than an Asian strain, A/Singapore/1/57. It may be that sensitivity to the drug is a feature of Asian viruses in general. The minimal concentration needed for viral inhibition under these conditions seemed to be 15 μg/ml. As before, there was no inhibition of growth of A/Hong Kong/107/71 or of influenza B viruses.

Experiments with influenza and parainfluenza viruses in embryonated eggs. The chicken embryo tolerated large amounts of drug, and experiments were performed with 50 to 500 μg per egg. Maximal inhibition of A/Hong Kong/1/68 occurred with 50 to 100 μg, and some inhibition of A/Hong Kong/107/71 was also seen but the inhibition was less than that of A/Hong Kong/1/68 (Table 3). Swine influenza viruses isolated in 1930 and 1966 were likewise inhibited, whereas a 1965 avian strain and parainfluenza type 1 (Sendai) were not (Table 4). It seemed, therefore, that most influenza A viruses were drug sensitive but that a significant number of A strains and all influenza B viruses were drug resistant.

TABLE 1. *Influenza virus plaque formation in bovine kidney monolayers: effect of introducing 32 μg/ml of spiroadamantane into the overlay*

Virus	Titer (log_{10} PFU/0.1 ml)	
	Control	With drug
A/Okuda/57	5.0	2.0
A/PR/8/34-Eng./939/69		
Clone 64c	6.5	4.4
Clone 64d	5.5	3.0
A/PR/8/34-Aichi/2/68		
Clone X-31	6.4	3.7
A/Hong Kong/107/71	7.3	7.2
B/Lee/40	6.9	6.9
B/Taiwan/2/62	>4.5	>4.5
B/Singapore/3/64	6.1	6.1
B/Czechoslovakia/28/70	6.3	6.6

TABLE 2. *Dose-response effect*[a]

Virus	Drug (μg/ml)			
	0	5	15	25
A/Singapore/1/57	6.5	6.5	4.1	3.5
A/Hong King/1/68	4.5	4.5	2.5	2.5
A/Hong Kong/107/71	5.5	5.5	5.5	5.5
B/Czechoslovakia/28/70	6.5			6.5
B/Victoria/98926/70	8.0			8.5

[a] Influenza virus titrations (log_{10} TCD_{50}/0.1 ml) in test tube cultures of bovine or monkey kidney were pretreated 2 to 18 h previously with concentrations of the drug indicated.

TABLE 3. *Dose-response effect*[a]

Dose of drug per egg (μg)	A/Hong Kong/1/68		A/Hong Kong/107/71
	0 h	−18 h	−18 h
0	8.0	6.7	7.5
50	>6.5	5.0	Not done
100	6.0	4.7	6.5
200	6.0	4.7	6.5
500	5.5	4.5	6.5

[a] Influenza A virus titrations (log_{10} 50% egg-infecting dose/0.1 ml) in embryonated eggs were inoculated with drug and virus at the same time (0 h) or with drug 18 h before virus (−18 h).

TABLE 4. *Dose-response effect*[a]

Virus	Drug (μg/egg)		
	0	100	500
A/Swine/Iowa/15/30	8.5	8.0	5.5
Swine/Wisconsin/1/66	6.5	4.5	4.0
Turkey/Massachusetts/65	7.5	Not done	7.5
Parainfluenza 1 (Sendai)	7.0	Not done	>6.5

[a] Influenza A and parainfluenza type 1 (Sendai) virus titrations (log_{10} 50% egg-infecting dose/0.1 ml) were determined in eggs pretreated with drug 18 h previously.

Paramyxoviruses in tissue culture. Plaques were produced in bovine kidney monolayers by human and bovine parainfluenza type 3 viruses, but there was no plaque inhibition by a drug concentration of 32 μg/ml in the overlay medium.

Rhinovirus inhibition in tissue cultures and organ cultures. Activity against rhinoviruses, although significant, was less than against influenza A viruses. A drug dose of 32 μg/ml in the overlay medium inhibited plaque formation by five rhinovirus types in HeLa cells (Table 5). The amount of inhibition seen could have been due to experimental error, and because the related substance, amantadine, was inactive against picornaviruses, the experiment was repeated in tube cultures but with similar results. No enhancement of the antiviral effect was produced by initial prolonged contact between drug and cells. Yield-titration experiments (Table 6) also confirmed antirhinovirus activity and it appeared that RV2 was somewhat more sensitive than the other strains, a finding which influenced the subsequent choice of viruses for trials in volunteers. Nevertheless, rhinovirus inhibition was not wholly clear cut, since it appeared to depend on the type of cells used in the assay system. In MRC-5 cells (unlike HeLa cells) there was no inhibition of RV2, RV9, or RV31, and in yield-reduction experiments in human tracheal organ cultures, there was no inhibition of RV2.

Trials of the drug against rhinovirus infections of man. Because of the drug's success in experimental Hong Kong influenza in man (2), a similar trial was performed with rhinoviruses types 2 and 9. The results of these trials were compared (Table 7). Although, as expected, the rate of infection with each individual rhinovirus was not high, a large part of the control group was infected with one or other of the two rhinoviruses. However, in contrast to the influenza trials, there was no evidence of a protective effect against rhinoviruses in man. No evidence of drug toxicity was observed in the volunteers.

TABLE 5. *Rhinovirus plaque formation in HeLa cell monolayers: effect of introducing 32 μg of drug per ml into the overlay*

Rhinovirus type	Titer (log_{10} PFU/0.1 ml)	
	Control	With drug
1B	5.2	4.7
2	5.8	4.8
9	5.6	4.7
31	4.2	3.3
43	5.2	4.2

TABLE 6. *Rhinovirus yields from HeLa cells*[a]

Rhinovirus type	Incubation period (days)	Yield (log_{10} $TCID_{50}$/0.1 ml)	
		Without drug	With drug
1B	1	1.7	1.2
	2	3.5	2.5
	3	5.5	4.2
	4	5.5	4.5
2	1	2.2	<0.7
	2	5.2	1.7
	3	7.5	3.7
	4	7.5	3.0
9	1	2.5	1.0
	2	4.5	2.5
	3	4.5	2.5
	4	5.5	3.5
31	1	2.0	1.2
	2	4.7	2.2
	3	5.5	3.5
	4	5.2	3.2
43	1	0.5	0.5
	2	3.5	3.0
	3	4.7	2.7
	4	4.7	3.2

[a] Maintenance medium contained 32 μg of drug per ml throughout the incubation period.

Coronavirus. Limited experiments were performed with the coronavirus strain 229E. Titrations in treated and untreated L132 cells suggested that the drug might possess significant activity against this virus group (Table 8). Activity was also detected when diploid human embryo lung cells were used as the host system.

DISCUSSION

Of antiviral compounds synthesized in the laboratory, amantadine has been the most successful of the drugs used for respiratory virus

TABLE 7. *Protection tests in man*[a]

Challenge virus	Treatment	Clinical reactions (grade)				Avg clinical score in infected volunteers[b]	No. of volunteers infected[b]	Virus excretion			Seroconversion		
		Severe	Moderate	Mild	Nil			A/HK/68[c]	RV2	RV9	A/HK/68	RV2	RV9
A/Hong Kong/68	Drug	0	2	2	24	6	14/28 (50%)	10/28 (36%)			11/28 (39%)		
	Placebo	2	4	4	19	11	22/29 (76%)	19/29 (66%)			18/29 (66%)		
RV2 + RV9	Drug	0	3	9	5	18	15/17 (88%)		8/17 (47%)	7/17 (41%)		4/17 (24%)	8/17 (47%)
	Placebo	1	2	5	9	14	16/17 (94%)		5/17 (29%)	9/17 (53%)		4/17 (24%)	9/17 (53%)

[a] Responses of volunteers treated with spiroadamantane or inert placebo to challenge with partly attenuated A/Hong Kong/68 virus or with RV2 and RV9 combined.

[b] People showing virus excretion, antibody rise, or both.

[c] $P < 0.05$ in respect of the difference between treated and untreated volunteers.

TABLE 8. *Dose-response effect*[a]

Drug concn (μg/ml)	Virus titer ($\log_{10}$ $TCID_{50}$/0.1 ml)	
	−0.5 h	−18 h
0	4.2	4.2
8	3.7	2.2
16	1.4	1.7
32	1.4	1.7

[a] Determinations of coronavirus 229E titrations in L132 cells to the maintenance medium of which drug was added 0.5 (−0.5 h) or 18 h (−18 h) before the virus.

infections. Hence its spectrum of activity and mode of action have been intensively studied (6, 8, 12, 14, 18, 23). It affects an early event in the replication of influenza A viruses and exercises a consistent, if sometimes marginal, effect in vivo. In a field where so little has been achieved, the study of chemically allied substances in an attempt to define structure-activity relationships seems to be worth a special effort. This has recently proved possible because of the synthesis of a number of new adamantanes (15), and the one which we have described in this paper, a spiroadamantane, is one of the most active and is appreciably more potent than 1-amino adamantane hydrochloride.

The results that have been obtained emphasize the problems involved in the design of broad-spectrum antiviral substances. Our drug inhibited the growth of some influenza A viruses but, like amantadine itself, was quite ineffective against influenza B viruses. Indeed, its activity against individual influenza A viruses was variable, and it produced quite different effects on viruses which were biologically similar and whose only distinguishing laboratory characteristics were their surface antigenic configurations. There is therefore no certainty that an adamantane compound which was effective in one influenza A outbreak would necessarily be so in a different outbreak. Our drug was also active in vitro against rhinoviruses, and presumably its mode of action on this group was quite different from that on the myxoviruses. To attempt to elucidate all its activities at the molecular level is not within the scope of this investigation.

There is, however, some encouragement to be derived from the study. In general, adamantane derivatives appear to produce a definite effect on members of the influenza A virus group, although this varies with different strains. Spiroadamantane also reduced the incidence and severity of experimental Hong Kong influenza in man, the virus of which was less sensitive to

its effects in vitro than was at least one other type A strain. Hence it is reasonable to suppose that the adamantane substances generally are a potential source of antiinfluenza drugs for man even if none of them do prove fully effective against all viruses. However, as regards the adamantane spiro compound under investigation, it appears that a very high degree of activity in vitro is necessary to produce a detectable effect in man. Attempts to compare the activity of a drug against widely differing viruses in vitro must necessarily be made in different host cells, and this may raise doubts as to the validity of the comparison. Although this drug clearly inhibited rhinovirus in vitro, its antirhinovirus activity appeared appreciably less than that against most strains of influenza A virus. Furthermore, the drug inhibited influenza virus in several different host systems in vitro, whereas rhinovirus inhibition occurred only in HeLa cells. However, it was also possible to compare the antiviral activity of this compound for influenza virus and rhinovirus in volunteers by using challenge trials carried out under strictly comparable conditions (except that in the rhinovirus trials the dose of drug was very slightly decreased). These experiments showed that, whereas the high activity against influenza virus in vitro was reflected in volunteers, the lesser activity against rhinoviruses in vitro was not accompanied by a useful antirhinovirus effect in man.

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