J. Pharm. Pharmacol. 1986, 38: 353–356
Received October 21, 1985

Pharmacological activity of amantadine: effect of *N*-alkyl substitution

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Alkyl substitution on the nitrogen atom of the anti-parkinsonian drug amantadine resulted in changes in its pharmacological potency. Greater behavioural stimulation (i.e. increased motor activity) was observed following s.c. injection of several of the analogues, with optimal activity produced by *N*-n-propyl substitution. These molecular changes did not alter the activity of amantadine on inhibition of dopamine uptake or its weak affinity for dopamine receptors in-vitro. Several of the analogues were more effective than the parent compound in increasing the concentration of dopamine metabolites (suggesting an increase in dopamine utilization) following systemic injection, and these effects generally followed the same pattern as observed in the test of behavioural activity. These results provide further support for the concept that the activity of amantadine may be improved by molecular alterations, although the pharmacological basis for this activity remains obscure.

The mechanism of action of amantadine in Parkinson's disease is not well understood, although it is generally accepted that the drug acts via an increase in the pre-synaptic utilization of dopamine (DA). This may be accomplished by an increase in DA release (Von Voigtlander & Moore 1971), inhibition of DA uptake (Baldessarini et al 1972) or by some other, as yet unknown, mechanism.

Few structural modifications of amantadine have been developed which may serve to help clarify its mechanism of action or improve its clinical activity. The most widely studied analogue, memantine (3,5-dimethylamantadine; D-145), possesses greater pharmacological activity than amantadine (Costall & Naylor 1975; Henkel et al 1982). Other structural modifications of the amantadine molecule have also resulted in increased behavioural stimulation (Henkel et al 1982). This study was designed to investigate the effect of *N*-substitution on the pharmacological activity of amantadine. In studies of other dopaminergic agents, acting by direct receptor stimulation, *N*-substitution, especially *N*-n-propyl substitution, has increased pharmacological potency. This has been observed in a variety of chemical structures, including aporphines (Costall et al 1980), aminotetralins (Cannon 1983) and DA itself (Ginos et al 1979). While it is unlikely that amantadine also acts by direct DA receptor activation, the effects of *N*-substitution nevertheless warranted investigation and the results illustrate that this structural alteration does affect pharmacological activity.

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MATERIALS AND METHODS

Behavioural studies

Male CD-1 mice (Charles River Farms, Wilmington, MA) were used. Locomotor activity was measured in a 6-chamber Stoelting Activity Monitor after injection of the drugs or vehicle (distilled water). The mice were acclimatized to the chamber for 20 min, and then were injected subcutaneously and returned to the device for 120 min. Activity is expressed as 'counts' recorded by the monitor over the 2 h test period.

Biochemistry

The effect of the different drugs on the DA receptors was measured by their ability to displace [^{3}H]*N*-n-propylnorapomorphine (NPA) from membrane binding sites using the method of Costall et al (1980). In brief, rat striatum was homogenized in 0·32 M sucrose, centrifuged, washed and resuspended in 50 mM Tris buffer (pH = 7·6). This preparation was incubated at 37 °C for 20 min with [^{3}H]NPA (2 nM) and unlabelled drug. The incubation was terminated by filtration of the preparations under vacuum and washing with 15 ml of ice-cold buffer. Radioactivity bound to the tissue trapped by the filter was quantified by liquid scintillation spectrometry. Non-specific binding was determined in the presence of 1 μM (+)-butaclamol. The displacement of bound label by cold drug was plotted against log concentration and the IC50 (concentration which displaced 50% of specifically bound NPA) was determined by linear regression.

Inhibition of synaptosomal DA uptake was measured by a modification of the method of Koe (1976), as previously described (Gianutsos et al 1982). Rat striata were homogenized in 0·32 M sucrose and a standard P_2 pellet was obtained as described by Gray & Whittaker (1962). Aliquots of the synaptosomal suspension (approximately 10 mg protein ml^{-1}) were pre-incubated in modified Ringer buffer at 37 °C in the presence of drug. [^{3}H]DA (0·1 μM) was added and the incubation was continued for an additional 8 min. The uptake was terminated by rapid filtration under vacuum and the resulting pellet with trapped radioactivity was extensively washed, and was quantified by liquid scintillation spectrometry. Non-specific activity was obtained by incubation under otherwise identical conditions except that incubation was performed at 4 °C. IC50 values were obtained by linear regression as described above. All in-vitro experiments were performed in triplicate.

In-vivo activity was determined by measurements of the DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by HPLC with electrochemical detection, using a modification of the method of Ferrari et al (1982). Mice were injected with drug as described in the behavioural experiments and were killed 1 h later. Striata were removed and homogenized in 40% ethanol containing dihydroxycinnamic acid as an internal standard. After centrifugation, the supernatant was saturated with sodium chloride and was extracted twice with ethyl acetate. The organic layer was dried under nitrogen and the resulting residue was redissolved in the mobile phase consisting of 10% methanol in 0·1 M phosphate buffer (pH 5·1). An aliquot was injected into a Waters HPLC equipped with a Bioanalytical Systems LC4 electrochemical detector and the components were separated on a C18 column. Metabolites were quantified after integration of the area under the curve and comparison of internal standard to peak ratio. Standards of known concentration were assayed simultaneously.

Drugs

Amantadine HCl (Aldrich Chemical Co.) and amphetamine sulphate were used as standard compounds. Several mono *N*-substituted amantadines (*N*-ethyl, *N*-propyl, *N*-butyl and *N*-propyl memantine) and the disubstituted (*N,N*-di-n-propyl) amantadine, were synthesized by extensions of previous chemistry (Henkel et al 1982). All compounds were prepared as their hydrochloride salts and gave satisfactory microanalyses. Doses of drugs are expressed on a molar basis.

Statistics

Analysis of variance, followed by Tukey's Studentized Range Test was used to analyse the data statistically; $P < 0·05$ was used as the level of significance.

RESULTS

Effect of N-alkyl substitution on locomotor activity

The stimulatory effects of the various amantadine analogues on motor activity is summarized in Table 1. Data is expressed as % control; values from control mice (injected with distilled water) are expressed as 100%. Amphetamine, as expected, was a powerful stimulant of activity, while the unsubstituted amantadine was only weakly active. *N*-substitution altered the stimulant activity. *N*-butyl and especially *N*-propyl substitution markedly increased the stimulatory effect. These effects were generally dose-related. Curiously, the di-propyl substitution failed to produce a stimulatory effect; however, it is possible that it may be too hydrophobic (log $P = 5·2$) to exert activity. Memantine, the 3,5-dimethyl analogue of amantadine, also produced a powerful

Table 1. Effect of *N*-substituted amantadines on motor activity.[a]

Compound	Dose (mmol kg^{-1})	Motor activity (% control) (mean ± s.e.)
Amantadine	0·05	87 ± 44
	0·10	201 ± 68
	0·20	243 ± 58
Amphetamine	0·02	562 ± 141
	0·04	993 ± 232
N-Ethyl amantadine	0·05	255 ± 121
	0·10	92 ± 20
N-n-Propyl amantadine	0·05	119 ± 32
	0·10	771 ± 218
	0·20	999 ± 184
N-n-Butyl amantadine	0·05	299 ± 138
	0·10	352 ± 63
	0·20	489 ± 90
N,N-Di-n-propyl amantadine	0·05	107 ± 29
	0·10	116 ± 23
	0·40	74 ± 23
Memantine	0·025	266 ± 9
	0·05	377 ± 108
	0·10	916 ± 146
N-n-Propyl memantine	0·05	283 ± 40
	0·10	112 ± 22

[a] Mice were injected with drug and activity was monitored for 2 h as described in text. Values are expressed as percent of activity measured in saline-treated mice on the same day; N = 6–8.

stimulatory effect as expected. The *N*-propyl substitution failed to alter the stimulation induced by memantine.

Biochemical studies

In an effort to gain some insights into the mechanism for the behavioural effects produced by the amantadines, selected analogues were tested for their ability to inhibit DA uptake, bind to putative DA receptor sites and alter DA turnover. The results of these studies are described in Tables 2 and 3.

Table 2. Effect of *N*-substituted amantadines on striatal DA uptake and [^{3}H]NPA binding.

Compound	DA uptake IC50 (μM)[a]	NPA binding IC50 (μM)[b]
Amantadine	220	1000
N-Ethyl amantadine	*	5400
N-n-Propyl amantadine	*	1100
N-n-Butyl amantadine	*	*
N,N-Di-n-propyl amantadine	*	*
Memantine	210	520
N-n-Propyl memantine	64	700
Nomifensine	0·36	NT
Apomorphine	NT	0·0035

[a] Concentration of analogue which prevented the uptake of DA (0·1 μM) into striatal synaptosomes by 50% (see text).

[b] Concentration of analogue which displaced specific binding of NPA in striatal membranes by 50% (see text).

* Represents analogues for which no inhibition was observed at 0·1 mM concentration of drug. NT = not tested.

Table 3. Effect of *N*-substituted amantadines on striatal dopamine metabolites.

Treatment	Dose (mmol kg^{-1})	Metabolite concentration DOPAC	HVA
Saline		6·4 ± 0·4	5·3 ± 0·4
Amantadine	0·2	5·7 ± 0·6	4·4 ± 0·4
N-Ethyl amantadine	0·1	6·8 ± 0·3	7·8 ± 0·3
	0·2	5·8 ± 0·4	6·4 ± 0·8
N-n-Propyl amantadine	0 1	9·2 ± 1·2*	6·2 ± 0·3
	0·2	9·7 ± 0·8*	6·2 ± 0·7
N-n-Butyl amantadine	0·1	6·5 ± 0·5	8·7 ± 0·6*
	0·2	11·9 ± 0·4*	11·1 ± 0·4*
N,N-Di-n-propyl amantadine	0·1	5·2 ± 0·5	4·6 ± 1·2
	0·2	6·1 ± 0 2	7·1 ± 0·4
Memantine	0·05	10·2 ± 0·6*	11·9 ± 0·8*
	0·2	8·0 ± 0·4*	7·6 ± 1·0
N-n-Propyl memantine	0·05	10·8 ± 0·8*	6·5 ± 1·0

[a] Mice were injected with drug and were killed after 1 h. Striata were removed and assayed for DOPAC and HVA as described in text. Values represent means ± s.e.m. for 6–8 mice.

* Represents values significantly different ($P < 0·05$) from control.

The effect of the derivatives on striatal DA uptake is summarized in Table 2. Amantadine weakly inhibited DA uptake, but was 3 orders of magnitude less potent than nomifensine, a known DA uptake blocker (Hunt et al 1974). Some of the analogues were more potent than amantadine, but even the most potent had an IC50 more than 80 times that of nomifensine. Furthermore, there was no relationship between uptake inhibition potency and motor activity, since the most potent analogue for uptake inhibition (*N*-n-propyl memantine) was only weakly active in the motor behaviour test, while the most powerful behavioural stimulants were, at best, weakly active as uptake inhibitors.

The effects of the amantadines on the binding of NPA to putative DA receptors is also summarized in Table 2. Amantadine was essentially inactive being, at best, 6 orders of magnitude weaker than apomorphine, a standard DA receptor agonist. All of the tested analogues were more potent than amantadine, but even the most potent (memantine) had 1/10 000 the activity of apomorphine. Furthermore, there was no relationship between DA receptor affinity and the behavioural activity.

The effect of in-vivo administration of the amantadine analogues on the concentration of DA metabolites is summarized in Table 3. These studies were performed over a narrow range of behaviourally active doses. Amantadine itself was essentially inactive, producing a tendency to decrease levels of both metabolites (DOPAC and HVA) but this did not reach statistical significance. Several of the analogues were active, producing an increase in the concentration of DOPAC and HVA, although their effects did not always follow a dose-dependent relationship. The analogues which produced the largest effect (memantine, and the n-propyl and n-butyl analogues) were also the most active in the test of behavioural stimulation. All of the analogues which failed to increase significantly the levels of one or more metabolites also failed to produce more than a 3-fold increase in motor activity. Thus, there was some correlation between these two in-vivo measures. The only inconsistency was *N*-n-propyl memantine, which significantly increased DOPAC and HVA but did not produce a 3-fold increase in motor activity.

DISCUSSION

The results of this investigation clearly illustrate that *N*-alkyl substitution of amantadine results in an increase in behavioural activity as assessed by motor stimulation. It is interesting that the greatest effect was produced by n-propyl substitution. Similar effects (i.e. maximal activity produced by propyl substitution) have been observed in other studies with direct-acting DA agonist drugs (see Introduction), suggesting the possibility of a common

mechanism and/or binding site. However, the biochemical studies suggest that the amantadines have little affinity for typical DA receptor sites.

While it is clear that the pharmacological activity of amantadine may be improved by ring substitution (Henkel et al 1982) or by *N*-alkyl substitution, the neurochemical mechanism responsible for these effects remains elusive. Since amantadine finds clinical use in Parkinson's disease (Schwab et al 1969), a disease characterized by degeneration of DA-containing neurons (Hornykiewicz 1973), it was reasonable to investigate the role of DA in the behavioural effects of amantadine. Previously, DA uptake inhibition has been proposed as a possible mechanism of action of amantadine (Baldessarini et al 1972). There was, however, no correlation between uptake inhibition and the behavioural activity of the different analogues. Similarly, DA release could be considered as a likely mechanism. However, if the analogues were active in releasing DA from pre-synaptic storage sites, it should have been detected in the uptake assay (see Heikkila et al 1975). Therefore, pre-synaptic DAergic mechanisms did not appear to provide an explanation for the behavioural effects. Similarly, an action mediated by post-synaptic DA receptors appears to be equally unlikely as a mechanism since none of the analogues were able to displace labelled NPA except at very high concentrations. The possibility of a unique amantadine binding site (Allen 1983) remains to be tested.

Interestingly, in-vivo changes in the concentration of DA metabolites, an indicator of in-vivo DA release and utilization, did appear to correlate with the behavioural stimulation. Previous studies with amantadine have generally shown weak effects on dopamine utilization consistent with enhanced presynaptic activity (Fuller et al 1981; Cox 1975).

The reason for the discrepancy between the in-vivo and in-vitro results is presently unknown. It is possible that the analogues are metabolized to an unknown active species in-vivo. However, amantadine is considered to be generally metabolically stable (Vernier et al 1969) and the results obtained would appear to require that all of the analogues undergo metabolic activation since no significant activity was observed in any in-vitro test. A second possibility would be if amantadine indirectly influenced the activity of DAergic neurons through another neurotransmitter. The identity of this other potential transmitter or neuromodulator is also unknown. A detailed analysis of memantine, one of the most active of the amantadine analogues, failed to yield evidence of significant activity on adrenergic, GABAergic or opiate receptors (Osborne et al 1982).

In conclusion, alkyl substitution of amantadine imparts an increase in pharmacological activity, but the mechanism(s) responsible for this effect (and for the clinical activity of amantadine) remains obscure.

Acknowledgements

This research was supported by a grant from the United Parkinson Foundation. The technical assistance of Gale Morrow is gratefully acknowledged.

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