Amantadine: an antiviral and antiparkinsonian agent

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ABSTRACT: Amantadine is an antiviral agent that specifically inhibits influenza A virus replication at a micromolar concentration. This drug is also very effective in the treatment of human Parkinson's disease. Other important clinical applications of this agent have been studied recently, ranging from viral infections, e.g. herpes, herpes zoster neuralgia to granulomatosis and from neuroleptic extrapyramidal movement disease to depression and cocain dependence. Biological and pharmacological activities of amantadine presented in this paper are focused on the explanation of the mechanism of amantadine antiviral and antiparkinsonian effects and on general use of this agent in medicine.

Keywords: influenza A; M2 protein; chemotherapy; chemoprophylaxis; Parkinson's disease

List of abbreviations: **AA** – amino acid; **AMA** – amantadine; **AMA-HCl** – amantadine hydrochloride; **AMA-S** – amantadine sulphate; **AZT** – azidothymidine; **DDI** – dideoxyinosin; **DNA** – deoxyribonucleic acid; **DOPC** – 1,2-Dioleoyl-sn-glycero-3-phosphocholine; **FTIR** – infrared spectroscopy using the Fourier transformation; **HA** – haemagglutinin; **M1** – matrix system; **NA** – neuraminidase; **NA** – nucleic acid; **NMDA** – N-methyl-D-aspartate; **NMR** – nuclear magnetic resonance; **NP** – nucleoprotein; **PD** – Parkinson's disease; **RNA** – ribonucleic acid

CONTENTS

1. INTRODUCTION

At present, viral diseases represent a considerable problem in medicine, which despite significant progress in the field of biophysics, biochemistry, molecular biology, pharmacology and medicine has not been satisfactorily coped with. Influenza, in comparison with other viral diseases such as AIDS, neoplastic diseases, hepatitis and others, is often perceived by the laic as well as professional public as a banal disease, however the opposite is true. Every year, thousands of people die of influenza or connected complications all over the world (Beran, 1999). In the history of mankind there were five particularly destructive pandemics of which the last one in 1918, known as Spanish influenza, took a toll of 20 million victims (Beran, 1999), especially from young people. At the time of epidemics, besides the health effects this disease also causes extensive economic losses.

Antibiotics, used only for prevention of subsequent potential bacterial infections, are ineffective to the viruses alone. Tricyclic amines have a great potential in the treatment and prevention of influenza A of which the most important is amantadine (AMA). This medicament has found its wide use also in other fields of human medi-

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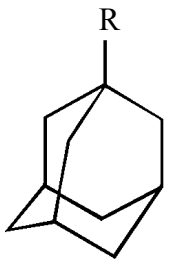
cine. Well-known is its use in the treatment of Parkinson's disease and other neurological diseases (Grelak *et al*., 1970; König *et al*., 1996). Its application to suppress the symptoms of Parkinson's disease is very frequent and very good results are achieved. Recently, amantadine has begun to be used also in veterinary medicine as an effective antiviral preparation in the treatment of influenza A in horses.

The aim of this paper is to summarise the literary data concerning the antiviral and antiparkinsonian effects of amantadine, its use in human and veterinary medicine with emphasis on the explanation of some mechanisms of these effects. The presented paper is part of the thesis dealing with the study of the mechanism of antiviral effects of amantadine on the molecular level and its interactions with biological macromolecules by the methods of Raman's spectroscopy.

2. AMANTADINE IN THE TREATMENT AND PREVENTION OF INFLUENZA A

Amantadine (Figure 1A) and its structural analogue rimantadine (Figure 1B) are applied in the chemotherapy of viral diseases. Amantadine is effective in the treatment of hepatitis C (Smith, 1996; Martin *et al*., 1999), herpes (Jáuregui *et al*., 1997), herpes zoster neuralgia (Douglas, 1990), Creutzfeldt-Jacob's disease (König *et al*., 1996), Born's disease (Hallensleben *et al*., 1997), influenza B (Douglas, 1990) and others. The studies on the therapeutical effects of amantadine on depression (Huber *et al*., 1999) and cocainism (Volchow *et al*., 1990) are also known. Both medicaments, however, are preferentially intended for the treatment and prevention of influenza A because they specifically inhibit replication of its viruses already at the micromolar concentration (Douglas, 1990).



1A: R = NH_2

1B: R = $CHNH_2CH_3$

Figure 1. The structure of amantadine and rimantadine

2.1. The use of amantadine in veterinary medicine

The viruses of influenza A are periodically transmitted from the reservoir animals – especially water birds to pigs, horses, domesticated poultry as well as to sea mammals and they cause infections. While in water birds the infection and circulating of the virus in the population have rarely clinical manifestations, transmission to domesticated poultry and mammals has usually bad consequences for a new host. There is a certain relationship between the human, swine and equine influenza, which are in the interrelation with avian influenza that is their common ancestor (Sabó, 1998). At present, the idea is supported that pigs and turkeys are mediators at the outbreaks of new epidemics or pandemic viruses of human influenza (Sabó, 1998). In 1997 in China there was an outbreak of avian influenza of the subtype H5N1 which was characterised by more specialities. Within the history of human influenza, this subtype had never occurred in man before and it was a case of direct interspecies transmission of influenza from birds (hens) to man. Overall 18 people got ill with this influenza, out of which 6 succumbed to this contagion. The disease broke out enormously fast and early administration of amantadine had a favourable therapeutic effect (Sabó, 1998). Therefore making diagnosis and monitoring the influenza disease in animals is of great importance (Sabó, 1998).

Recently the papers investigating possibilities of chemotherapy of influenza in animals, namely in horses, have appeared (Chambers *et al*., 1995a, b; Oxford, 1997; Rees *et al*., 1997). Influenza is, for example, a highly significant acute disease in horses and at an equine influenza outbreak in Hong Kong in 1989, herds were destroyed within a month with losses of almost 100 million USD (Chambers *et al*., 1994). Therefore both the therapy and chemoprophylaxis with amantadine or rimantadine could be very useful because vaccination of horses is only partially effective. Clinical symptoms of the disease could be improved by an early treatment. Infected animals can be treated with appropriate doses of these drugs and at the same time vaccinated for the stimulation of the natural immunity development. Amantadine and rimantadine are effective against all the susceptible viral strains of influenza, whereas vaccines generally protect only against the strains derived from those being involved in the vaccine (Nahata and Brady, 1986).

Until influenza A in horses was reliably and fast diagnosed, its therapy was not practised. At present, there are new, fast and simple diagnostic methods such as e.g. Directigen FLU-A test (Chambers *et al*., 1995a) and others that make the antiviral treatment possible also in horses.

Evaluation of the pharmacological aspects and side-effects of amantadine at influenza A treatment in horses can be found in the paper of Rees *et al*. (1997). In these experiments amantadine and rimantadine were tested on the viruses of equine influenza of the subtype H3N8 of Miami strain from 1963 and Chinese equine influenza from 1989 which circulated in horses also in 1991–1994 in Kentucky. *In vitro* amantadine reliably inhibited these viruses at the concentrations of 30 ng/ml and less, with the exception of the strain KY/92, which was resistant

also at the concentrations higher than 300 ng/ml (Rees *et al*., 1997). In comparison with amantadine, rimantadine is more effective. *In vivo* it was preliminarily tested at the treatment of horses at the dose of 10 mg/ml of live body weight administered intravenously, which did not induce any apparent side-effects, while the dose of 20 mg/ml l.b.w. or more caused different effects on the CNS, inclusive attacks. At the dose of 15 mg/ml l.b.w., the middle and temporary responses to the CNS in 3 out of 6 treated horses were observed. These signs appeared within 30 to 60 minutes following the drug administration. They involved stumbling, disharmonious position of limbs, dragging of hind limbs and weakness in the lower posterior muscles. Thus it has been concluded that administration of the individual dose of 15 mg/kg l.b.w. intravenously is connected with a significantly unfavourable response in horses (Rees *et al*., 1997). At the same doses administered orally, the efficacy of preparations depended on the ability of their absorption from the gastrointestinal tract, which was different in individual patients. Thus, intravenous administration of amantadine and rimantadine in the chemotherapy of equine influenza is more favourable than oral and the recommended effective dose is 10 mg/kg l.b.w. of the animal.

2.2. The use of amantadine in human medicine

Amantadine and rimantadine are suitable for the treatment of influenza A and other viral disease for many reasons:

– they are characterised by high selectivity of the effect
– they are effective in sufficiently low concentrations
– they are characterised by low toxicity to experimental cellular systems as well as to the organism – they have negligible side effects (Boreko *et al*., 1996)

In vitro doses from 0.4 µg/ml to 10 ng/ml (Douglas, 1990; Oxford, 1997) are high enough for the inhibition of most influenza viruses, specially of subtypes H3N2, H2N2, H1N1 (viral subtypes – see part 2.3.1.)

In vivo amantadine is well absorbed from the gastrointestinal tract. The maximum concentrations in the blood plasma after using a dose of 200 mg attain the values from 0.3 to 0.6 µg/ml. Almost the whole absorbed dose is excreted into urine in unchanged form. The drug is not almost metabolised. The half-time of excretion is 16 hrs, this value increases in older people and in patients with a decreased function of kidneys (Aoki and Sitar, 1988).

Amantadine is used in the form of amantadine hydrochloride salt (AMA-HCl) with the trade name Virosol, Virofral, Symadine or Symmetrel – it was registered under these names for human use in Europe and in the USA 34 years ago. In this country it is registered under the name Viregyt-K; Symmetrel and Symadine are not registered. The preparations Contenton and PK-Merz on the basis of amantadine sulphate (AMA-S) are also known. In the countries of the former USSR rimantadine is used that, however, does not have a licence in Western Europe and in the USA. It is produced as capsules and also as syrup. A daily dose for children from 1 to 9 years is from 4.4 to 8.8 mg/kg of body weight and it must not exceed 150 mg per day. For older children and adults the dose of 200 mg per day or better 100 mg twice a day is suitable. In older people and in the patients with a decreased function of kidneys the dose should be reduced to 100 mg per day. The drug should be applied during 5 days (Douglas, 1990).

Its side-effects manifest sporadically at the recommended doses, at the dose of 200 mg daily in 1–5% of patients with the normal function of kidneys some smaller neurological symptoms (insomnia and troublesome concentration) were recorded (Dolin *et al*., 1982). Excessively high doses when the amantadine concentration in the blood plasma is from 1 to 5 µg/ml are connected with the toxic effects of the drug on the central nervous system, namely nervousness, hallucinations, attacks and coma. Persons with psychiatric diseases, epilepsy or pregnant and nursing women should not be treated with amantadine (Dolin *et al*., 1982).

Preventive effects of amantadine are very significant. A high number of clinical studies proved the efficacy of amantadine in the prevention of influenza A (Dolin *et al*., 1982; Blake, 1990; Monto, 1994; Tamblyn, 1997). The efficacy of the agent in the prevention shows a frequency within the range of 70–90% (Tamblyn, 1997), which is comparable with vaccination. Its utilisation is recommended especially in various social facilities where there is a high potential of contagion spreading.

Vaccination is a preferred method in the prevention of influenza A. Unlike the use of vaccine, amantadine has an advantage that it is recommended for protection of patients of any age at the moment when the influenza incidence was proved in the given community. It is not necessary to administer it in advance before the influenza season. The treatment with amantadine should start immediately after the outbreak of influenza epidemics and continue during its persistence (usually 5–6 weeks). Since the drug does not weaken the immune response to vaccine, the vaccinated patient could discontinue it after 2 weeks (Douglas, 1990).

2.3. Mechanism of the amantadine effect on the influenza A virus

2.3.1. Characteristics of the influenza virus

The influenza virus, discovered in 1932, belonging to the family *Orthomyxoviridae*, is an enveloped RNA virus 80–120 nm in size. The virion nucleus contains a

nucleocapsid that consists of the viral genome, nucleoprotein (NP) and the complex RNA-transcriptase (Čiampor *et al*., 1998). The reaction of NP antigen with specific antibodies allows a classical division of influenza viruses into three types A, B, and C (Beran, 1999). The viral genome is formed by a single-fibrillar RNA of negative polarity and is segmented (8 segments in the virus of influenza A and B and 7 in the virus of influenza C). In the virion nucleus there is also a matrix protein M1 that stabilises a viral particle and is extraordinarily important for its maturation (Čiampor, 1998). The lipid coat of the virion contains majority glycoproteins – haemagglutinin (HA), neuraminidase (NA) and minority non-glycosyled protein M2.

Influenza viruses are characterized by a high ability to change the genetic complement of their antigens HA and NA. This change leads to different variants of one type of virus, e.g. H3N1, H2N2, H3N8 and others (Sabó, 1998; Beran, 1999). Fifteen subtypes of HA and nine subtypes of NA were described (Čiampor *et al*., 1998), which allows relatively numerous combinations of variants.

The specificity of the virus of type A is a phenomenon called "antigen shift", when a part of genetic information is supplemented stepwise – in this case there is an outbreak of the whole world pandemics. New strains and subtypes within the type A occur suddenly due to the false genetic recombination between human and animal influenza viruses, when during the mixed infection an exchange of some of 8 segments of the genome occurs (Čiampor *et al*., 1998; Beran, 1999). Contrariwise, so-called "antigen drift", which is very frequent in the type A but it was also observed in the type B, is formed by means of multiple gradual changes in the genetic information of HA and NA (Čiampor *et al*., 1998; Beran, 1999). This variability of the influenza virus of type A complicates both the treatment and prevention of the disease. The latest vaccines contain more subtypes of HA and NA; despite this they do not have to contain the very subtype that originates due to the above-mentioned alterations, which influences the success of vaccination to a great extent.

The fact that viral replication runs in several steps and can be inhibited in a certain stage is employed when searching for new possibilities of the treatment of viral disease. For example, to interrupt the synthesis of nucleic acids by means of nucleoside analogues such as azidothymidine (AZT) or dideoxyinosin (DDI) which operate as the inhibitors of replication of nucleic acids (NA) of the viruses of HIV (Kraus *et al*., 1996). Another possibility is to inhibit maturation of viruses in some other stage of replication. This inhibition can be performed by blocking so-called viroporines participating in the adjustment of pH of the environment in transport vesicles so that it would conform to viral replication (Čiampor *et al*., 1998).

2.3.2. Viroporines

Viroporines are minority proteins occurring in the virion coat of some animal viruses. Coded by viral genome they also get to the lipid bilayers of cellular membranes of host cells (Carrasco, 1995). Some viroporines (e.g. Vpu-protein) of HIV-1 virus are present in the plasma membrane of attacked cells, but they have not been found in the virion coat (Carrasco, 1995; Ewart *et al*., 1996).

Viroporines operate as ionophores and increase the membrane permeability, whereby they help release mature virions from the infected cell (Čiampor *et al*., 1992a, b; Carrasco, 1995). These properties are preserved also when they are individually cloned in other cellular systems. Viroporines are short proteins consisting of 50 to 120 amino acids (AAs) having a high content of leucine and isoleucine and a low content of glycine. They can be structural and non-structural, but they are always coded by a viral genome. In the lipid membranes they are organised as integral proteins with a tendency to form oligomers, most frequently tetramers, as hydrophilic pores transmitting ions and low-molecular hydrophilic compounds without specificity (Čiampor, 1997). The transmembrane hydrophobic region is composed of 20–22 AAs, forms an amphipathic helix and contains basic AAs (Hay *et al*., 1985; Čiampor, 1997) participating in the permeability of membranes by destabilisation of the lipid bilayer (Čiampor, 1997). The pores formed from viroporines do not play a role in the regulation, but disorganisation of the host cell membrane with aim to destroy the cell (Čiampor, 1997).

Several viroporines have been discovered until now, for example 3A protein of picornaviruses, 6K protein of togaviruses, Vpu protein of HIV-1 virus, M2 protein of influenza A virus, NB protein of influenza B virus (Carrasco, 1995; Čiampor, 1997). The viroporines could play an important role in the prevention of viral infection spread if effective blockers were found. In the case of influenza A it is sufficiently proved that the proton channel formed by viroporine M2 is blocked by amantadine and amantadine-derived compounds (Sugrue and Hay, 1991).

2.3.3. The structure and function of M2 protein

High amounts of M2 protein occur in the plasma membrane of influenza virus-infected cells, but there is only about 16–64 molecules in one virion (Lamb *et al*., 1985; Zebedee *et al*., 1985; Zebedee and Lamb, 1988; Čiampor *et al*., 1998). It is coded by segment 7 of viral RNA (Surgue and Hay, 1991; Pinto *et al*., 1992). This segment also codes the matrix protein M1, coded by almost a complete transcript of segment 7, while the smaller protein M2 is coded by a transcript originating by cutting of seg-

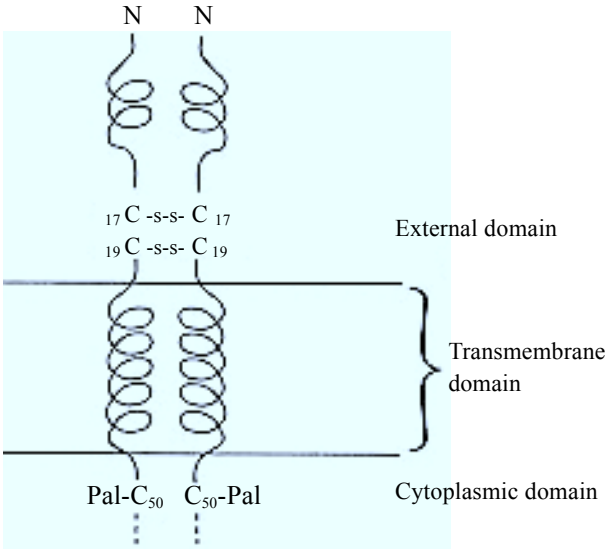


Figure 2. Arrangement of M2 protein within the membrane (Sugrue and Hay, 1991)

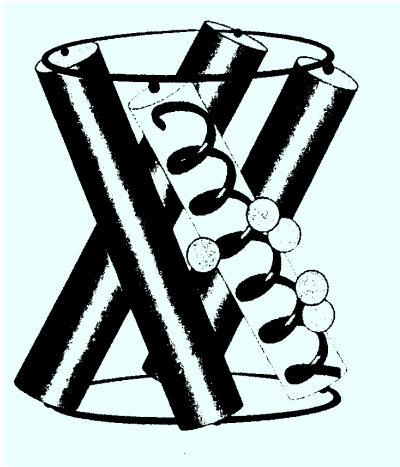


Figure 3. Three-dimensional representation of M2 protein (Kovacs and Cross, 1997)

ment 7. Both proteins co-operate in the process of viral replication (Čiampor *et al*., 1998).

Ninety-seven AAs form the primary structure of M2 protein (Hay *et al*., 1985) and its amino acids sequence is steady for all the viral strains coming from various sources (Wang *et al*., 1993). The M2 protein is an integral protein of the IIIrd type in the protein topology with uncleaved signal anchor sequence and $N_{exo}C_{cyt}$ orientation (von Heijine, 1988; Parks and Lamb, 1991). It spans the membrane once and is orientated in such a way that it has 23 N-terminal extracellular residues and a 54 residue C-terminal cytoplasmic domain. In the transmebrane region it forms homo-tetramers secondarily aligned as the α-helix (Sugrue and Hay, 1991). Tetramers are con-

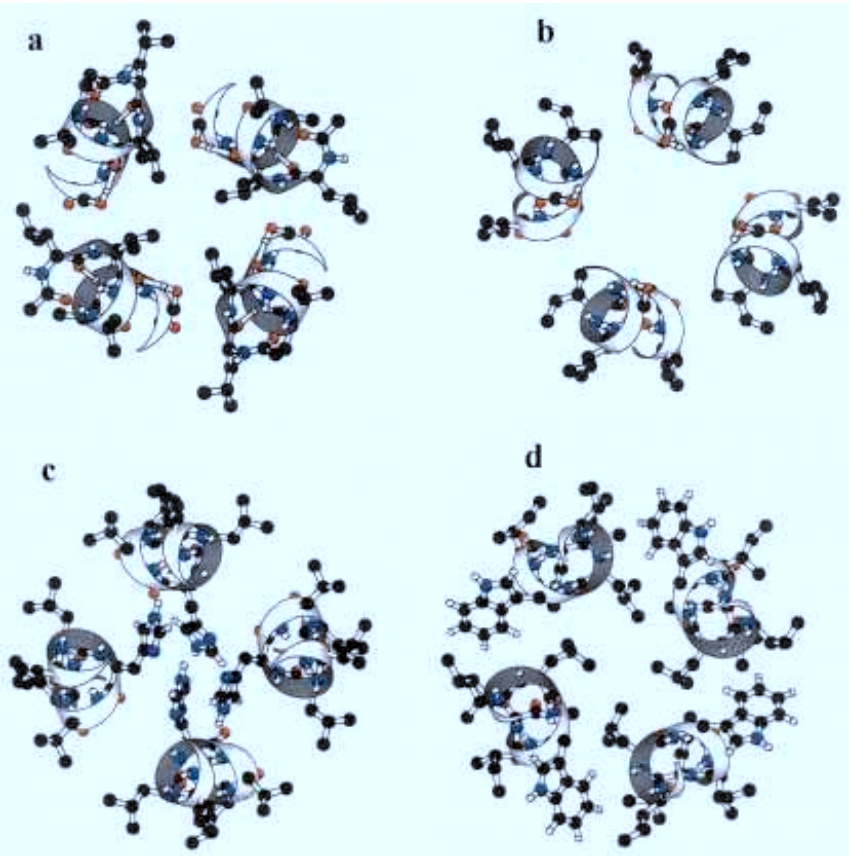


Figure 4. α-helix of the M2 transmembrane region and the conformation of side-chains a = Leu 26-Ser 31, b = Ser 31-Ile 35, c = Ile 35-Ile 39, d = Ile 39-Leu 43 (Kukol *et al*., 1999)

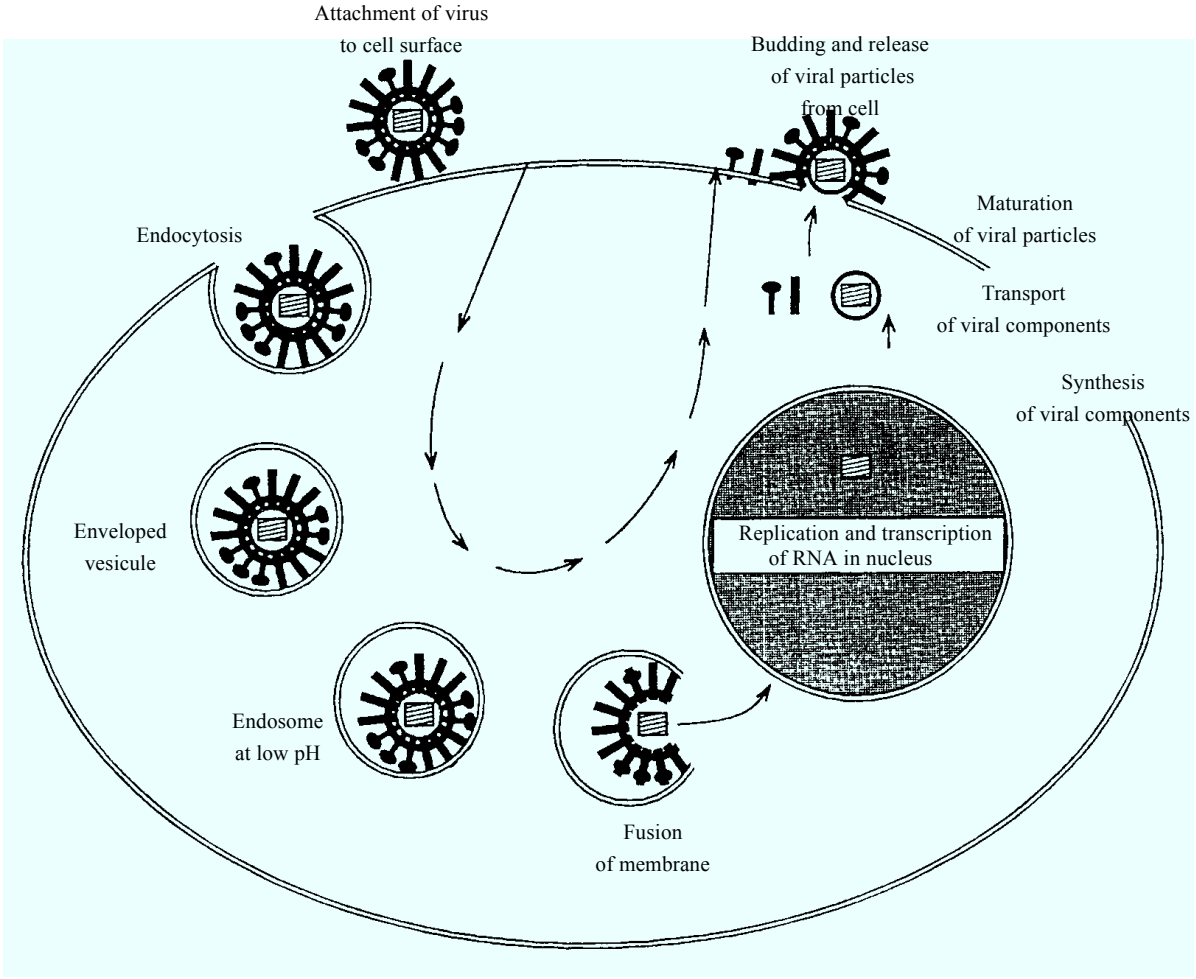


Figure 5. The replicate cycle of influenza A virus (Čiampor *et al*., 1998)

nected by disulphide bridges at the site of cysteins C_{17} and C_{19} in the extracellular region (Figure 2) (Sugrue and Hay, 1991).

The secondary and tertiary structures of M2 protein were studied in greater detail by Sugrue and Hay (1991), Duff *et al*. (1992), Kovacs and Cross (1997), Kukol *et al*. (1999). Duff *et al*. (1992) found by means of circular dichroism that the transmembrane region of M2 protein incorporated in 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes exists in the α-helix form while this structure is not altered by either higher temperature or AMA addition. The α-helix parameters in four bundles of M2 protein were determined by the NMR (Kovacs and Cross, 1997) and FTIR method (Kukol *et al*., 1999). Using the NMR method, the tilt of the helix with respect to the bilayer normal was determined to be 33° ± 3° as well as the orientation round the helix axis. These results imply that the tertiary arrangement of tetrameric protein in the membrane is a left-handed four-helix bundle (Figure 3). Only with such a large tilt angle are the hydrophilic residues aligned to the channel axis, which forms the M2 tetramer in the membrane (Kovacs and Cross, 1997).

Kukol *et al*. (1999) confirmed the helix angle described by Kovacs and Cross (1997) by the FTIR method. They found the precise position of AAs in the α-helix, and based upon this finding with respect to the lateral chains it follows that a pore is closed by His-37 residues (Figure 4).

It can bee seen in Figure 4 that the His-37 residues from four monomers play a role of the regulator that conducts protons in one direction. Protonation of one N His-37 atom from the outside of the virus could induce deprotonation of another N atom of the imidazole part of His-37, because a positive charge is distributed through the π-electron circular system. The initial status could be renewed by tautomerization or a ring flip. At the low pH value due to multiple protonation a conformation change could occur that would allow the passage of cations (Shimbo *et al*., 1996).

As it was mentioned above, the M2 protein forms an ion channel whose function is the passage of protons from

endosome to virion. Due to a decrease in pH, the matrix M1 protein is separated from the nucleocapsid and is released to the cytoplasm – this process is often called uncoating (Hay *et al*., 1985; Pinto *et al*., 1992; Hay, 1992). In the process of virus replication (Figure 5) it is so-called early effect or early permeabilization of the membrane by M2 protein. In the late stage of the replication cycle, M2 protein fulfils the function of a proton channel again when it regulates pH in the transport vesicles of HA, whereby it ensures the formation of right conformation of newly synthesized HA during its transport to the cytoplasmic membrane of the cell (Čiampor *et al*., 1992a, 1998). This process occurs shortly after the outlet of HA from the Golgi complex during the passage of HA through the trans-Golgi network (Čiampor *et al*., 1992a). The M2 protein recognises and binds itself to the cytoplasmic part of the HA trimer. Subsequently, the virion is assembled and released from the cell by budding through the plasmic membrane (Figure 5) (Čiampor *et al*., 1998). The M2 protein has the same function in both stages of viral infection, namely to regulate pH by the formation of proton channel and to create a suitable environment for viral replication.

2.3.4. Inhibition of M2 protein by amantadine

The activity of some alicyclic amines was compared with the influenza viruses from various sources *in vitro* (Table 1) (Hay *et al*., 1985). It follows from the results that the effect of the tricyclic amine AMA is highest in most viral subtypes from various sources. Cyclooctylamine exhibits activity similar to amantadine.

In the cell cultures, amantadine manifests two concentration-dependent inhibitory effects against the viral replication (Hay and Zambon, 1984) that are connected with an early and late stage of viral replication. First, it is a non-specific inhibition at the concentrations higher than 0.1 mM (Daniels *et al*., 1985). At these concentrations it has an effect on the influenza viruses of B type and on a number of other enveloped viruses, e.g. paramyxoviruses, togaviruses, retroviruses (Skehel *et al*., 1978), herpesviruses, viruses of rubella (Douglas, 1990), hepatitis C (Martín *et al*., 1999). Amantadine concentrations from 0.1 to 5 μM specifically inhibit only the influenza A virus (Hay *et al*., 1985) while the substance efficacy is different for different strains of viruses and depends on the time of administration (Table 2). The resistance of viral particles against AMA could originate. It is evident from the data in Table 2 that replication of amantadine-resistant mutants was not stopped either by the concentrations higher than 50 μM (Hay *et al*., 1985).

The non-specific inhibition of the virus replication by AMA is ascribed to the early stage of viral replication. The natural environment of endosome is acid – pH is about 5 (Ohkuma and Poole, 1981). The proton channel formed by M2 protein transports protons from the endosome inside the virion. This pumping is necessary for the interruption of macromolecular interactions which keep together the virion coat (Hay *et al*., 1985; Pinto *et al*., 1992; Wang *et al*., 1993). Amantadine behaves as a lysosomotropic substance that passes easily through the endosome membrane (Duff *et al*., 1993) and accumulates in it. As a weakly basic substance it binds protons in the endosome to itself, whereby making them impossible to flow inside the virion. In this way, both the interruption of the matrix M1 protein and release of the virion nucleus into the host cell environment are prevented (Hay *et al*., 1985; Pinto *et al*., 1992; Wang *et al*., 1993). A more detailed explanation of the lysosomotropic features of amantadine is presented in Chapter 3.1. Higher concentrations of amantadine (above 0.1 mM) are necessary for viral inhibition in this stage than for inhibition in the later stage of viral replication. Amantadine is also effective in the early stage against the other above-mentioned enveloped viruses.

At the low concentrations (from 0.1 to 5 μM, see Table 2) amantadine blocks the late stage of viral matura-

Table 1. Effect of cyclooctylamine and related compounds on virus production. Singapore, Rostock, Weybridge are virus strains (Hay *et al*., 1985)

Compound	Virus yield (% of control)				
	Singapore	Rostock		Weybridge	
	5 μM	5 μM	50 μM	0.5 μM	5 μM
Amantadine	6	7	8	3	15
Cyclooctylamine	5	5	5	10	5
Cycloheptylamine	22	30	15	35	9
Cyclohexylamine	60	70	30	67	60
Cyclopentylamine	95	100	100	90	95
Cyclooctanol	100	100	100	ND	ND
Octylamine	98	100	ND	92	ND

ND = not determined

Table 2. Concentration dependence of the inhibition of virus production by amantadine (Hay *et al*., 1985)

Amantadine concentration (μM)	Virus yield (% of control)							
	Rostock		Weybridge		Singapore		BEL^R	
	a	b	a	b	A	b	a	b
0.05	95	92	20	30	100	100	92	100
0.5	21	25	7	7	55	92	95	98
5	4	5	30	45	9	66	81	93
50	25	22	45	100	3	71	38	100
500	17	70	<2	100	<2	87	<2	100

amantadine was added: a = 30 min after infection; b = 60 min after infection
BEL^R = amantadine resistant virus strain

tion – viruses are able to bud out on the cell surface, but are not able to separate from it (Ruigrock *et al*., 1991). The M2 protein plays a role in inhibition in this stage. Due to its blocking by amantadine, reduction of pH in the transport vesicles occurs that induces a conformational change in HA (Čiampor *et al*., 1992a). So amantadine is supposed to induce a premature conformational change in HA that occurs in the trans-Golgi network during the HA transport on the cell surface. This change in HA blocks a release of virions out of the host cell (Sugrue *et al*., 1990; Čiampor *et al*., 1992b). Viral particles with conformation HA originate at pH 5, they aggregate on the cell surface after maturation and do not separate from the infected cell surface (Čiampor *et al*., 1998). The need for a hundred fold lower concentration of the drug in this stage, compared to so-called early permeabilization, results from different mechanisms of the AMA effect in both stages. At inhibition in the process of virus uncoating, the ratio of proton concentration inside the endosome and in cytoplasm is 1 : 100 because the pH in endosome is 5 and in cytoplasm 7. At non-specific inhibition the amount of amantadine has to be such (about 0.5 mM) to balance this ratio and to prevent the proton passage through the virion coat by means of M2 channel. In the second stage, however, the interior of the transport vesicle of Golgi apparatus is neutral because the M2 channel pumps protons from it into the cytoplasm. Its evidence is the fact that viral infection causes an increase in the acidity in the trans-Golgi region (Čiampor *et al*., 1993). These results confirm the contrary orientation of M2 protein together with HA. Thus, it pumps protons from a vesicle into the cytoplasm to ensure the optimal environment for obtaining the proper HA conformation. Therefore AMA does not behave as a lysosomotropic

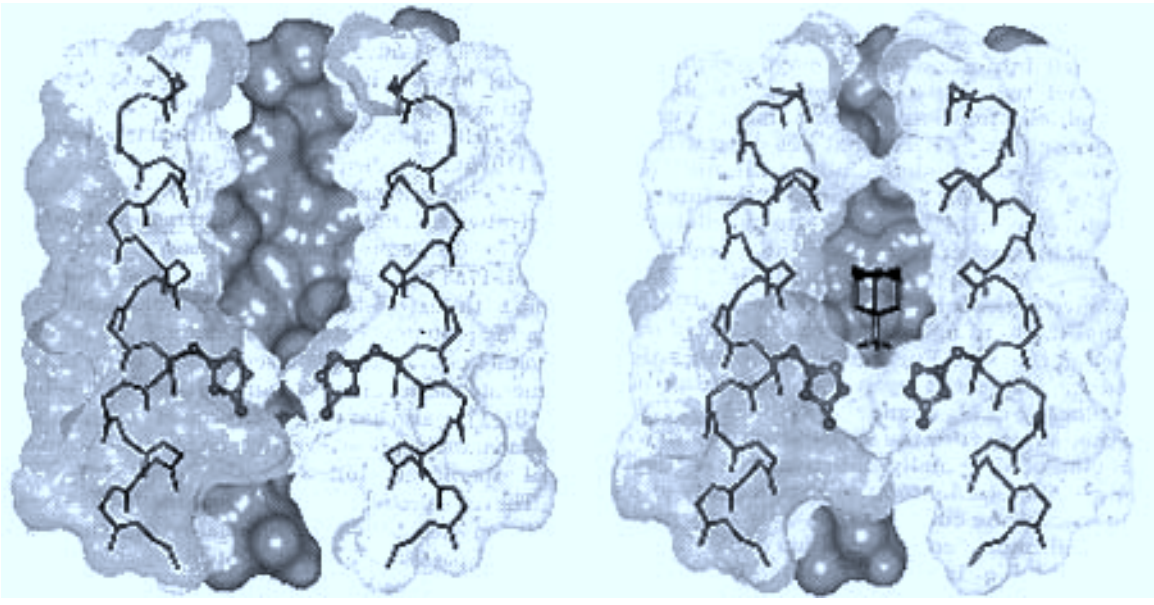


Figure 6. Location of histidine residues and possible mode of interaction of the M2 protein transmembrane region with amantadine (Gandhi *et al*., 1999)

substance in this case, but after passage into a vesicle it blocks the transmembrane channel formed by the M2 protein. It requires a 100 fold lower concentration compared with the first stage, as well as the specificity of this inhibition regarding the existence of M2 channel only in the viruses of influenza A.

A group of American scientists concentrated around L.W. Pinto and R.A. Lamb in 1999 carried out the electrophysiological measurements by which it was proved that His-37 was a decisive AA in the structure of the M2 protein with respect to its blocking. A possible way of interaction of the transmembrane region of M2 protein with AMA was suggested (Pinto *et al*., 1997; Gandhi *et al*., 1999). This model is based upon the knowledge of the M2 protein and especially on the fact that a M2 protein-formed pore is a proton-selective, water permeable channel that is disrupted at the site of His-37. Four histidine residues can act as the regulators of protons. In Figure 6 there is a transmembrane region of protein while the fourth helix is not illustrated in order that the pore interior may be visible. At Gly-34 there is a wide cavity followed by the channel closure that forms a side chain of His-37. The side chains of His-37 of each tetramer helix are packed to the alignment that can markedly slow down the flow of most ions through the channel. Thus, by alteration of protonation and deprotonation of N^{δ} and N^{ε} atoms of histidine residues the flow of protons can be regulated by the mechanism resembling the proton regulation of carbonic anhydrase (Silverman and Lindskog, 1988; Nair and Christianson, 1991). AMA-HCl interacts with a side chain of His-37. By its protonated amino group it is oriented towards the imidazole part of His-37 (Figure 6). The cavity expected in M2 involves the binding site for amantadine and for other compounds BL-1743. Amantadine-resistant viruses exhibit mutations at the sites lining the expected central pore of the channel (Gandhi *et al*., 1999). It may be supposed that the ammonium group forms stabilised H-binding interactions with lone electron pairs on the imidazole nitrogen N3 atoms of His-37 (Gandhi *et al*., 1999).

The complex amantadine-histidine was studied in water solution by the method of Raman spectroscopy to verify the presented suggestion. Molecules were found to interact mutually by means of hydrogen bond between the amino group of amantadine and N1H group of histidine imidazole (Staničová *et al*., 2001). This result can be one of the possible models for formation of the amantadine – M2 protein complex.

3. AMANTADINE – THE AGENT IN THE TREATMENT OF PARKINSON'S DISEASE

Unlike other drugs used in the treatment of Parkinson's disease (PD) the effect of amantadine on the PD symptoms were discovered randomly. In April 1968 a patient with PD cured by the neurologist R.S. Schwab was preventively taking amantadine against influenza twice a day. The PD symptoms such as akinesia, rigidity, tremor disappeared. After discontinuing amantadine the PD symptoms returned. This finding was verified many times, and so amantadine has become an effective antiparkinsonian agent (Danielczyk, 1995). It has a good effect mainly at akinetic crises and in combination with the conventional preparation L-Dopa it suppresses the main symptoms of PD if L-Dopa alone is not effective (Schwab and Poskanzer, 1972; Greulich and Fenger, 1995).

Monotherapy of PD with amantadine is indicated in the early stage of PD and in the cases of weaker symptoms (Greulich and Fenger, 1995). It is also effective after the prolonged treatment. Long-term studies on a great population of patients indicate the effects of AMA for a lot of years.

Two forms of amantadines are used in the therapy of PD. It is already mentioned AMA-HCl used in the treatment and prevention of influenza, and amantadine sulphate (AMA-S). In the Central Europe AMA-S is preferred, which is practically unknown in most parts of the western world – it was registered only in 12 countries (Danielczyk, 1995). While AMA-HCL has not been used for example in Austria, in the other parts of the world the maximal doses of 200–300 mg are used. The disadvantage of this form is that this dose cannot be elevated in the case of disease progression because the side-effects of the drug manifest to a greater extent (Danielczyk, 1995). On the other hand, AMA-S offers the possibility to increase the dose up to 600 mg daily because the level of AMA-S in the blood rises much more slowly than in AMA-HCl. Thus, the side-effects, especially those induced by circulation, are less pronounced. At the states of acute akinesia that is accompanied by aphagia, high fever persisting for several days it is appropriate to use AMA-S infusions. Series of 3 to 10 infusions are able to return the patient to the state before the crisis. In general, such patients tolerate only the smallest doses of L-Dopa or other dopaminergic drugs (Danielczyk, 1995). AMA-S infusions are also effective in later stages of the disease.

AMA-HCL and AMA-S are internally well-resorbed and remain unchanged by the urine secretion. This pharmaco-biochemical nature defines their side-effects. They are well compatible and manifestation of side-effects is low if a daily dose does not exceed 400 mg of AMA-HCl and 600–800 mg of AMA-S (Greulich and Fenger, 1995). Precautious doses are necessary in older patients. Among the side-effects of amantadine at the treatment of PD belong: insomnia, oedema, nausea, anxiety, confusion as well as pains of stomach, muscles, pruritus, livedo reticularis, halucinations, nightmares (Schwab *et al*., 1972; Zeldowicz and Huberman, 1973; Bauer and McHenry, 1974; Hayden *et al*., 1983; Duvoisin, 1991; Pfeiffer, 1996).

Therapeutical characteristics of amantadine were compared with other antiparkinsonian preparations such as Biperiden (König *et al*., 1996). The AMA effects were comparable with conventional drugs. In recent works some other side-effects of AMA have appeared, and a few reports indicate even the sudden death cases probably caused by AMA in combination with other drugs. A case of acute delirium was recorded in the patients whom AMA was discontinued after the long-term therapy of PD. After readministration of AMA, their state was satisfactory again (Factor *et al*., 1998). Amantadine and also other antiparkinsonian drugs cause antidiuretic hormonal secretion (Van Laar *et al*., 1998) and in the case of the patient treated for PD, a high concentration of AMA in the blood induced the kidney dysfunction. The next sudden case of death caused by insufficient function of kidneys was recorded at the treatment of cranio-cerebral trauma with AMA (Hartshorne *et al*., 1995).

3.1. Mechanism of amantadine effect on suppressing the symptoms of Parkinson's disease

The mechanism of the AMA effects as an antiparkinsonian drug is not precisely known, or better expressed, is less explained than in the case of influenza. It refers to the ability of the preparation to block the neuromuscular transmission, which has an influence on the synthesis and excretion of dopamine (Grelak *et al*., 1970). Amantadine likewise at influenza manifests as a blocker of these channels and blocks:

– serotonine-activated ion channel
– ion channel of nicotine-acetylcholine receptors
– ion channel of N-methyl-D-aspartate (NMDA) receptors (Kornhuber and Streifler, 1992).

There are other biochemical and pharmacokinetic characteristics of amantadine:

– increase in fluidity of cellular membranes
– increase in electrically stimulated excretion of dopamine and serotonine

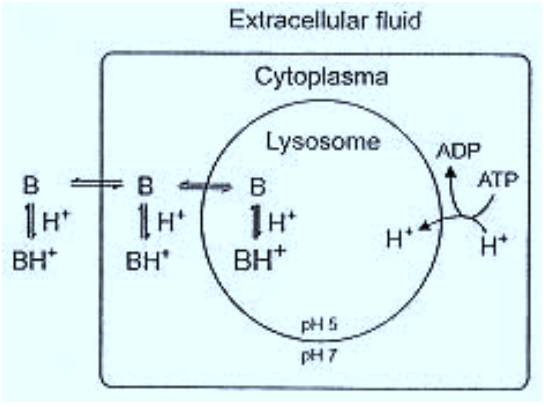


Figure 7. Schematic model of lysosomal pH maintenance and intralysosomal trapping of weak bases (B)

– inhibition of monoaminooxidase A
– loss of manifestation for a direct effect on the dopaminergic receptors (Kornhuber and Streifler, 1992) that can explain why there is no akinesia at its application (Danielczyk, 1995).

Another characteristic of AMA is lysosomotropism or also acidotropism that probably cause its high preservation in the cerebral tissue (Kornhuber *et al*., 1995). Concentration of AMA in the brain is high if referring to the CNS and blood serum – the ratio is 20 : 1. Particularly interesting is a slow accumulation of the drug in the brain. Approximately 65 mg of AMA is necessary to reach the concentration of AMA free base 217 μM in 1.5 l (the brain volume). Taking into consideration that AMA accumulates not only in the brain but also in other organs (Aoki and Sitar, 1988), and at oral treatment most AMA is excreted as non-metabolised (more than 85%), then it is clear that treatment with AMA has to persist at least for several days so that such high concentrations in the brain may be reached.

A high preservation and slow accumulation of AMA could have two reasons that follow from the nature and characteristics of the amantadine molecule: a) its high lipophility, and b) already mentioned lysosomotropism (Kornhuber *et al*., 1995). The AMA lipophility has also been confirmed by other authors investigating the interaction of AMA free base with 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) using X-ray diffraction who found that the ability of non-protonated form of AMA to penetrate through the bilayer is higher than in other compounds (Duff *et al*., 1993). A direct evidence for lysosomotropism of AMA comes from several *in vitro* studies (Okhuma and Poole, 1981; Johnson *et al*., 1981; Richman *et al*., 1981) and can be explained by a few sentences. The intracellular subcompartments such as lysosomes and endosomes have an acid nature with pH of about 5 (Okhuma and Poole, 1981). Weakly basic substances, to which a non-protonated form of AMA also belongs, have a tendency to accumulate in these compartments. While plasmic and lysosomal membranes are permeable for neutral forms of weak bases, the same membranes are impermeable for protonated forms of these substances. Weak bases are captured by protonation inside the lysosomes and accumulate there (Figure7) (Okhuma and Poole, 1981).

The ratio of intra/extra lysosomal concentrations of these substances is equal to the ratio of concentration of hydrogen ions in lysosomes and in their vicinity, i.e. 1 : 100 if we suppose that pH in lysosomes is 5 and in cytoplasm 7. The amount of the permeable form of weak base passing through the membrane depends on the substance pK_a value and pH value of solution. The higher the pK_a value, the lower the permeable form ratio. Therefore the drugs with high pK_a values similar to AMA (pK_a = 10.14 at 37°C (Perrin and Hawkins, 1972)) have a slow speed of penetration into lysosomes (de Duve *et al*., 1974).

When the concentration of the base inside the lysosome reaches isotonia, water starts to enter the lysosome osmotically, which enlarges its volume and forms a large vacuole (de Duve *et al*., 1974).

Many effects of lysosomotropic drugs on the cell function are not fully studied. These effects involve a direct inhibition of lysosomal functions by an increase in the intralysosomal pH. It may be expected that besides lysosomes the biochemical processes of all other intracellular organelles (whose normal function is conditioned by the acid environment) are also disturbed. (Goldstein *et al*., 1985; Mellman *et al*., 1986). A well-known example is the transport of monoamines into the sympathetic vesicles. The vesicle membrane contains reserpine – a sensitive transporter that can mediate an exchange of cytoplasmic amines for internal protons. The vesicular monoamine transporter non-selectively accumulates biogenic amine transmitters such as serotonine, dopamine and noradrenaline. Lysosomotropic substances such as e.g. AMA influence the intravesicular increase in pH whereby they disturb the accumulation of amines (Kornhuber *et al*., 1995).

4. SUMMARY

The aim of the present paper was to provide available information about the antiviral and antiparkinsonian drug amantadine. Here belongs the knowledge of its biological and pharmacological activities as well as information about amantadine use in medicine. Since some attempts to employ this drug also in veterinary medicine have appeared recently, the study of its properties, mechanisms of effects and interactions can be interesting also in this field.

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