Chapter 4

Antiviral agents—II

Amantadine hydrochloride and methisazone

AMANTADINE HYDROCHLORIDE

Amantadine is the generic name of 1-adamantanamine. It is also referred to in the literature as 1-aminoadamantane. It is a primary amine of a saturated hydrocarbon which has a cage structure (Figure 4.1). It is used as the hydrochloride, which is a stable white crystalline powder soluble in water to the extent of 40%. The compound is distinctly volatile (Vernier *et al.*, 1969).

In addition to amantadine its derivative Rimantadine (α -methyl-1-adamantanemethylamine hydrochloride) and also cyclooctylamine have been successful in clinical trials. They have as yet not been brought into general use, however, and will not be discussed further in this section.

Biological actions

In early work on the effects of amantadine in the whole animal it was found that intravenous doses of 5–20 mg/kg caused slowing of the heart rate and extra systoles in rabbits. Doses of 30–50 mg/kg caused severe dysrhythmias, such as ventricular flutter, ventricular fibrillation and irreversible conduction block. Prolongation of the P–Q interval and duration of the QRS complex occurred with doses of 10 mg/kg and higher (Kaji *et al.*, 1966). Wesemann and Zilliken (1967) found that amantadine hydrochloride in doses higher than 10 μ M sensitized the rat fundus strip preparation to the motor effect of 5-hydroxytryptamine by a factor of 10–100. With higher concentrations the ability of the strip to contract in response to 5-hydroxytryptamine was lost and could not be restored by washing. The authors suggested that amantadine interacts with 5-hydroxytryptamine receptors in some way.

The chance observation that amantadine hydrochloride is effective in the treatment of parkinsonism (Schwab *et al.*, 1969) has led to an examination of its pharmacological properties in greater detail.

An increase in synthesis and release of dopamine was observed in slices of corpus striatum from rats injected subcutaneously with amantadine hydrochloride in a dose of 40 mg/kg 2 hours previously (Scatton *et al.*, 1970). A relationship with dopamine was also observed by Brelak *et al.* (1970). In dogs given a priming dose of 0.1 mg/kg dopamine intravenously the administration of amantadine hydrochloride 6–8 minutes later caused a dose-related rise in blood pressure, observable with a dose as low as 0.08 mg/kg. Amantadine hydrochloride alone had no pressor effect, except in very high doses. These results indicated that amantadine was releasing dopamine or other catecholamines from neuronal storage sites, and the authors suggested that this action might account for its effect in the treatment of parkinsonism. Strömberg *et al.* (1970) came to the same conclusion, and suggested further that amantadine hydrochloride might act predominantly on the central neurones. It also inhibited the rigidity and hyperactivity produced by oxotremorine in rats, a finding which is consistent with the view that it abolishes rigidity in parkinsonism by activation of cerebral dopaminergic function (Jurna *et al.*, 1972). Amantadine hydrochloride was found to potentiate the increase in motor activity produced in mice by L-dopa, a result which provides an explanation for the clinical observation that the administration of amantadine hydrochloride in parkinsonism allows the dose of L-dopa to be reduced (Svensson and Strömberg, 1970).

Other actions have been reported for amantadine hydrochloride which are unrelated to its interaction with catecholamines. Rawls *et al.* (1967) found that concentrations of 25 µg/ml or higher inhibited the transformation of human lymphocytes by *Phaseolus vulgaris* phytohaemagglutinin but did not affect their ability to be agglutinated. This observation suggests that amantadine hydrochloride interacts with the cell surface, and is in accordance with the finding, described in the section on mode of action, that the adsorption of influenza virus to the cell surface takes place in the presence of amantadine hydrochloride, but penetration into the cell interior is delayed. Bredt and Mardiney (1969) were unable to confirm this inhibitory effect on transformation by phytohaemagglutinin, but observed complete inhibition of transformation when allogeneic lymphocytes were used as the transforming agent.

Amantadine hydrochloride has a general inhibitory effect on cell growth. Balode and Gibadulin (1970) found that a concentration of 25 µg/ml reduced DNA synthesis in chick embryo fibroblasts by 24%. The generation time of the cells was lengthened from 32 to 108 hours, and the duration of DNA synthesis from 5.3–9.5 hours to 12.4–13.5 hours.

Antiviral activity and mode of action

The mode of action of amantadine hydrochloride is intimately connected with its activity against influenza virus, and the two topics will therefore be reviewed together. The mechanism of its pharmacological activities will not be considered further, although they need to be borne in mind when using amantadine hydrochloride as an antiviral agent.

The antiviral activity of amantadine hydrochloride was first observed by Davies *et al.* (1964), who found that it was active against certain members of the ortho- and paramyxovirus groups. In tissue culture it inhibited the multiplication of four strains of influenza A, 1 of A1, 3 of A2, 1 of influenza C, and also Sendai virus (a paramyxovirus). Other strains were unaffected, including two strains of influenza B, Newcastle disease virus, mumps, and parainfluenza 2 and 3. The compound was inactive against a general range of RNA and DNA viruses. In chick embryo fibroblasts strains sensitive to amantadine hydrochloride were maximally inhibited at a concentration of 25 µg/ml. In single-cycle experiments the maximum reduction in yield attained was 1 log unit (90%). No inactivation occurred when virus suspensions were exposed to concentrations of 25 µg/ml for 24 hours at 37 °C, but there was some inactivation at a concentration of 100 µg/ml. Amantadine hydrochloride did not prevent the uptake of virus by cells, or its release at the end of the replication cycle.

The concentration of amantadine hydrochloride used in the test in which it was found inactive against parainfluenza 2 and 3 was not specified. It may have been too low, since Cochran *et al.* (1965) found activity against these viruses in HeLa and LLC-MK2 cells when a concentration of 62.5 µg/ml was used.

Antiviral activity could also be demonstrated in fertile eggs and mice. The multiplication of A/PR8 (HON1) in the allantoic cavity was inhibited when 500 µg of amantadine hydrochloride was injected into the yolk sac. In mice infected intranasally with A/swine, A/WS and A2 strains some reduction in mortality and prolongation of survival time was obtained when amantadine hydrochloride was given in doses of 40 mg/kg by intraperitoneal, subcutaneous or oral routes, starting 30 minutes before infection and continuing every 4 hours for 48 hours.

The antiviral activity of amantadine hydrochloride was confirmed by Schild and Sutton (1965), who also noted a considerable variation in activity against different strains. There was little effect against the NWS, A/PR8 and A1 FM1 strains, whereas A2/Scot/49/57 and 442/63, a more recent A2 strain, were completely inhibited. Variation in sensitivity was also seen in five strains of animal origin, the most sensitive being A/equine/Miami/63.

Influenza virus undergoes the phenomena of antigenic shift and antigenic drift, but the strains now prevalent are still sensitive to the action of

amantadine. In 1972 the new variant strain A/England /42/72 emerged from the prevailing A/Hong Kong/68 and caused widespread influenza in the winter of 1972-73. Tests in monkey kidney cells by McLaren and Potter (1973) showed that the new variant was as sensitive to amantadine hydrochloride as the Hong Kong strain, the mean inhibitory concentration ranging from 0.05 to 0.15 µg/ml (mean 0.10 µg/ml), compared with 0.03-0.10 µg/ml (mean 0.06 µg/ml) for the latter.

The activity of amantadine hydrochloride in mice described in early reports has been studied further. Some protective effect was seen in mice infected with the A2/Sing/4/57 strain intranasally and given amantadine hydrochloride intraperitoneally in a dose of 70 µg/kg daily for 5 days (Schild and Sutton, 1965). Grunert *et al.* (1965) infected mice intranasally with 3-6 LD₅₀ of the A/swine/S15 strain of influenza virus and treated them with amantadine hydrochloride given intraperitoneally every 4 hours, beginning 4 hours before infection and continuing up to 43½ hours afterwards. In comparison with untreated control animals there was some increase in the percentage of survivors, which increased with increasing dose. The dose which increased the percentage survival to half-way between the control value and 100% (the corrected ED₅₀) was 7 mg/kg. Amantadine hydrochloride was also effective when given by the oral route; a single dose given 1 hour before infection was effective, and the effect was not increased by continuing the treatment. No protective effect was observed if treatment was delayed until 24 hours after infection, or a single dose was given 9 hours before infection. In further work with the same strain of virus it was found that treatment with amantadine hydrochloride given intraperitoneally in a dose of 20 mg/kg every 4 hours, beginning 1 hour before infection, would reduce the virus content of the lungs by 1 log unit or so (Davies *et al.*, 1966). McGahen and Hoffmann (1968) observed an apparent therapeutic effect in mice. In preliminary work they noted that the rate of water consumption of mice infected with the A2/Bethesda/10/63 and A2/AA/2/60 strains decreased 32 hours after infection, and this was considered to be an indication of the onset of clinical illness. When 0.5 mg/ml amantadine hydrochloride was added to the drinking water from the 48th to the 240th hour after an infection the proportion of survivors rose from 14.3% to 42.3% in a group of 78 animals. There was also an increase in the mean survival time and a reduction in the severity of illness. However, this effect was probably not antiviral in nature, since the virus would probably have attained maximum titre in the lungs by the time treatment was begun.

Resistance to amantadine hydrochloride can be readily produced by passage in the presence of the compound. Cochran *et al.* (1965) found that the A2/Japan/305 strain became completely resistant after one passage in calf kidney cells in the presence of an unstated concentration. Resistance can also be induced in mice (Oxford *et al.*, 1970). The A2/Singapore/1/57 strain was passaged in mice which were given 50 mg/kg amantadine

hydrochloride intraperitoneally 30 minutes before infection and maintained on drinking water containing 1 mg/ml. The sensitivity of the virus was determined at each passage by determining the concentration which would produce 50% inhibition of haemadsorption in BSC-1 cells. The initial virus had sensitivity values of 0.04–0.43 µg/ml (mean 0.3 µg/ml), and this had risen to 10–50 µg/ml (mean 25.3 µg/ml) after six passages. The resistance was not lost after four passages in eggs in the absence of amantadine hydrochloride.

The relation of dose to the induction of resistance was investigated by Oxford and Potter (1970). When passaged in mice treated with 1.5 and 15 mg/kg amantadine hydrochloride daily the A2/Singapore/1/57 strain remained sensitive, but resistant variants were readily obtained when the dose was increased to 150 mg/kg. In some cases a 500-fold increase in resistance was obtained. The resistant variants showed no change from normal in buoyant density, morphology and serology. The authors point out that the dose required to produce resistance was very much higher than the recommended human dose of 200 mg daily.

Goedemans and de Bock (1970) investigated some of the biological properties of a resistant strain of A/swine virus. Resistance was produced by serial passage of this strain in the allantoic cavity in the presence of 200 µg of amantadine hydrochloride. Adsorption on to chick embryo fibroblasts was retarded by a concentration of 40 µg/ml in the usual way in tests with the virus before passage and up to the third passage, but at the sixth passage adsorption was no longer affected. In a study of the penetration phase of the growth cycle the virus was adsorbed on to chick embryo fibroblasts, which were then exposed to a concentration of 40 µg/ml from 15 minutes to 3 hours after infection, and the amount of virus which had penetrated to the interior of the cell was determined by sonication and titration of haemagglutinin content. Entry into the cell was completely inhibited at the third passage, but resistance appeared at the sixth passage, and by the tenth passage the amount of virus gaining entry to the cell was only 1 log unit lower than in the case of the initial virus. Resistance was still retained after four passages in the absence of amantadine. Development of resistance also occurred *in vivo*. The A2/Japan strain was passaged in mice which were given 0.5 mM/kg amantadine base by mouth each day and tested for resistance in tissue culture at each passage level. Resistance to the action of amantadine hydrochloride during the penetration phase of the growth cycle appeared after six passages, but was still incomplete after ten. The resistance disappeared after three passages in eggs in the absence of the compound, and thus differed from the resistance which arose in egg passage. The authors calculated that 80 passages in the presence of the compound were required for the development of resistance. Resistance is thus unlikely to appear when amantadine hydrochloride is used clinically as an antiviral agent.

Amantadine hydrochloride is active against rubella, a virus which is unrelated to the myxoviruses and regarded by many as a member of the togavirus group. The activity was first reported by Maassab and Cochran (1964). Cultures of the LLC MK2 continuous line of monkey kidney cells were exposed to amantadine hydrochloride in a concentration of $31.2 \mu\text{g/ml}$ and infected with the WM strain of rubella virus 5 minutes later. After an adsorption period of 5 hours the residual virus inoculum was removed and replaced with medium containing the compound in the same concentration. The virus content of the supernatant was determined after 3 and 6 days by a method based upon the interference effect between rubella and ECHO 11 virus. There was a reduction in yield of 2 log units in comparison with untreated cultures. Amantadine hydrochloride was effective when present during the first 3 hours after infection, but when the time of addition was delayed to 5 hours after infection no inhibition of virus growth was obtained.

The activity against rubella was studied further by Oxford and Schild (1965). In experiments with five strains of rubella virus growing in RK-13 cells amantadine hydrochloride produced some suppression of cytopathic effect in comparison with untreated cultures. No suppression occurred with concentrations lower than $10 \mu\text{M}$, and for complete suppression a concentration of $40 \mu\text{M}$ was required. A study of the growth cycle of rubella virus showed that infective virus began to appear 18 hours after infection, and attained a maximum titre at 120 hours. In the presence of amantadine hydrochloride infective virus appeared after the same interval, but the maximum titre attained was about 2 log units lower, and no alteration in the relative proportions of extracellular and intracellular virus could be detected.

Attempts to demonstrate an antiviral effect against rubella in animals have been unsuccessful. Rhesus monkeys infected with rubella virus parenterally or intranasally develop viraemia, shed virus from the pharynx and develop antibody, and this subclinical infection can be used as a model for testing antiviral activity *in vivo* (Stephenson *et al.*, 1965). Amantadine hydrochloride was given by mouth in doses of 75 mg/kg daily for 4–11 days to 13 monkeys, which were infected intravenously or intranasally with $2.75\text{--}4 \log \text{TCD}_{50}$ of virus; 13 other monkeys were infected and left untreated as controls. No differences could be found in the three criteria of assessment between the treatment and control groups, and amantadine hydrochloride was thus ineffective against rubella *in vivo*. Oxford and Schild (1967 a) were unable to find any antiviral effect in hamsters and rabbits.

Amantadine hydrochloride has been found to inhibit a number of other unrelated viruses. There is a single unconfirmed report of activity against pseudorabies (Neumayer *et al.*, 1965). This is a virus of the herpes group and is the only DNA virus reported to be sensitive to amantadine hydrochloride. Activity against the arenavirus group is better documented.

Monolayers of BHK-21 cells were infected with lymphocytic choriomeningitis virus, a member of this group, and treated with amantadine hydrochloride from 2 hours before infection until 24 hours after. The yield of intra- and extracellular virus at the end of the growth cycle was reduced by concentrations of amantadine hydrochloride higher than 10 µg/ml, and at 50 µg/ml a 100-fold reduction was obtained (Welsh *et al.*, 1971). Similar results were obtained by Pfau *et al.* (1972), who found that amantadine hydrochloride inhibited the multiplication of Amapari, Parana, Tacaribe and Tamiami viruses, which are also members of the arenavirus group.

Amantadine hydrochloride inhibits the multiplication of certain tumour viruses. Wallbank *et al.* (1966) found that the development of foci of tumour cells was inhibited in monolayers of chick embryo fibroblasts pretreated with amantadine hydrochloride, infected with Rous sarcoma virus and incubated in the presence of an overlay containing the compound. A similar result was obtained with Esh sarcoma virus. Neumayer *et al.* (1965) had previously found that amantadine hydrochloride was ineffective against Rous sarcoma virus, but in their test system the virus was inoculated on to the chorioallantoic membrane of fertile eggs and the compound was inoculated into the yolk sac, in which case the surface of the infected cells could not have been accessible to the compound. Essentially similar results were obtained by Oker-Blom and Andersen (1966). The spectrum of inhibition has also been extended to murine sarcoma virus (Rhim *et al.*, 1972) and fowl leukosis virus (Oker-Blom and Andersen, 1967).

The basis of the mode of action of amantadine hydrochloride resides in the fact that it is a primary amine, and thus a derivative of ammonia. It had been known since 1961 that ammonium ions would inhibit the multiplication of influenza virus in tissue culture (Jensen *et al.*, 1961). This resulted from the chance observation that the cytopathic effect produced by A/PR8 virus in dog kidney cells was inhibited in cultures which were alkaline. Further work showed that ammonium ion was the active agent, and inhibition could be obtained by the addition of ammonium chloride to the cultures. The minimum inhibitory concentration was 16 µg/ml, and at 40 µg/ml the yield of infective virus was reduced by 1.3 log units. Ammonium ions had no direct virucidal effect, since on incubation of the virus with 100 µg/ml ammonium chloride at 37 °C no inactivation occurred over a period of 5 hours. Ammonium chloride also did not interfere with the adsorption of the virus to the cells. In further work inhibition was observed in other tissue culture systems, and also with A1 and A2 strains. No protective effect could be obtained in mice infected with A/PR8 virus intranasally, presumably due to metabolism to urea preventing the access of ammonium ions to the infected cells.

Ammonium ions did not inhibit other myxoviruses (mumps, Newcastle disease virus and parainfluenza 3), or a range of other unrelated viruses

(Jensen and Liu, 1961). The antiviral activity of ammonium ions was reported independently by Eaton and Scala (1961), who found that concentrations of 5–13 $\mu\text{g/ml}$ inhibited the growth of A/PR8 and Newcastle disease viruses in ascites tumour cells. They made the additional observation that there was little or no inhibitory effect when the addition of ammonium ions was delayed until 1–4 hours after infection. Jensen and Liu (1963) further showed that similar inhibition could be obtained with aliphatic amines, which may be regarded as substituted derivatives of ammonia. In cultures of dog kidney cells infected with A/PR8 inhibition of cytopathic effect and reduction in the yield of virus was obtained with primary aliphatic amines of chain lengths increasing from methylamine to butylamine. The minimum inhibitory concentration fell with increasing chain length, and a similar but less marked effect was obtained with secondary and tertiary amines. In a time of addition study carried out with propylamine the inhibitory effect was lost when addition was delayed until 1 hour or more after infection.

The discovery of the antiviral effect of amantadine hydrochloride by Davies *et al.* (1964) would appear to be a logical extension of the foregoing work on ammonia and amines. The mode of action was evidently the same, since the compound did not inactivate the virus on contact, did not affect adsorption, and only exerted a maximum inhibitory effect when added from 5 minutes to 1 hour after infection. In a study of the penetration of virus into chick embryo fibroblasts carried out with the A2/Japan/305 strain it was found that infected cultures treated with specific antibody $1\frac{3}{4}$ hours after infection produced essentially as much virus on further incubation as cultures not treated with antibody, a result which showed that the virus had already penetrated the cells by $1\frac{3}{4}$ hours and was therefore not accessible to neutralization by antibody. In the presence of amantadine hydrochloride, however, addition of antibody after $1\frac{3}{4}$ hours caused a reduction in virus yield of around 1 log unit. In the presence of the compound, therefore, much of the virus was still present at the cell surface and was neutralized by antibody. These observations were confirmed by Hoffmann *et al.* (1965) in experiments with the A2/AA/2/60 strain. Fletcher *et al.* (1965) observed a similar effect with ammonium ions and primary aliphatic amines. In cultures of monkey kidney cells infected with the A/PR8 strain the adsorbed virus became inaccessible to neutralization by 45 minutes, but in the presence of 250 $\mu\text{g/ml}$ ammonium chloride the virus remained neutralizable for up to $3\frac{3}{4}$ hours. Similar results were obtained with propylamine, di- and triethylamine and other amines, and indicated that penetration of virus into the cell was being inhibited. The action of ammonium ions and amines thus resembled that of amantadine hydrochloride in an additional respect, and the authors concluded that all these substances had a common mode of action.

The mechanism of antiviral action against rubella appears to be the same

as against influenza. Oxford and Schild (1965, 1967 b) showed that amantadine hydrochloride did not inactivate rubella virus *in vitro* and did not affect the rate of adsorption on to RK-13 cells; the multiplication of the virus was also inhibited by ammonium ions, and the virus was similarly not inactivated when incubated with 200 µg/ml ammonium acetate for 4 hours at 37 °C.

There is some evidence that the antiviral action of amantadine hydrochloride cannot be entirely explained by its reported effect in blocking or retarding penetration. The fever produced in rabbits by the intravenous injection of the A2/Singapore/1/57 strain of influenza virus was not prevented by an injection of 60 mg of amantadine hydrochloride given from 10 minutes to 24 hours previously. This result implies that the compound does not block the penetration of virus into lymphocytes, which then causes the liberation of the pyrogen (Grossgebauer and Langmaack, 1970). Kato and Eggers (1969) devised a method for studying the uncoating phase of the growth cycle in chick embryo cells infected with fowl plague virus. The virus was labelled with Neutral Red by carrying out five passages in cultures containing the dye. This procedure rendered the virus susceptible to photoinactivation. In cells infected with the labelled virus 90% of the inoculum became resistant to photoinactivation by 1 hour as a result of uncoating, but in the presence of 25 µg/ml amantadine hydrochloride this proportion was reduced to 30%. Amantadine hydrochloride thus had the additional action of inhibiting the uncoating process. Long and Olusanya (1972) studied some aspects of the molecular biology of the growth cycle of fowl plague virus. The synthesis of virus-directed RNA as measured by the uptake of tritiated uridine was inhibited by 25 µg/ml amantadine hydrochloride. It had previously been observed by Long and Burke (1969) that cycloheximide inhibited an early event in the growth cycle, probably the synthesis of RNA polymerase coded for in the RNA of the infecting particle. When infected cultures were exposed to cycloheximide, washed, and then exposed to amantadine hydrochloride, the multiplication of the virus was not inhibited. The amantadine-sensitive step had therefore taken place during the period of contact with cycloheximide. These findings agree with the work of Kato and Eggers, and localize the site of action of amantadine hydrochloride to the processes of uncoating and release of virus RNA.

Further information on the mode of action of amantadine hydrochloride has come from studies of its effect on arenaviruses. Welsh *et al.* (1971) found that the mechanism of action against lymphocytic choriomeningitis virus was essentially the same as for the myxoviruses and rubella, in that the compound did not inactivate the virus on contact, did not inhibit adsorption to cells, and delayed penetration, but they also observed that it appeared to act at two stages in the growth cycle, since the yield of virus was still reduced when the addition of amantadine hydrochloride was

delayed for as long as 20 hours after infection. This could be explained by the observation that the compound becomes attached to the plasma membrane of the cell (Greenhalgh and Gaush, 1970), from which the virus particles emerge by budding (Dalton *et al.*, 1970). A two-stage mechanism of action was also observed by Pfau *et al.* (1972) in studies with other members of the arenavirus group.

Toxicity

The acute LD50 of amantadine hydrochloride for mice was reported by Davies *et al.* (1964) as 233 mg/kg intravenously and 1080 mg/kg by the oral route. Grunert *et al.* (1965) obtained values of 97 mg/kg and 700 mg/kg for the two routes respectively, and 205 mg/kg by intraperitoneal and 271 mg/kg by subcutaneous injection. When 12 doses were given with intervals of 4 hours the LD50 was similar by the various routes, and ranged from 147 mg/kg to 171 mg/kg. In rabbits the acute LD50 by intravenous injection was 30–40 mg/kg (Kaji *et al.*, 1966). Prokhorova and Solov'ev (1967) found that a single dose of 800 mg/kg given to mice by mouth caused vasomotor collapse and lesions in the liver parenchyma and mucosa of the small intestine. No pathological changes were seen in the internal organs after a single dose of 320 mg/kg or repeated doses of 128 mg/kg given by aerosol over a period of 10–14 days.

The chronic toxicity of amantadine hydrochloride was studied by Vernier *et al.* (1969). It was well tolerated when given to dogs by mouth over a period of 6–24 months in doses 13–33 times greater than those proposed for clinical use, and there were no pathological changes in the organs. Doses of 93 mg/kg caused stimulation of the central nervous system, with tremor, myoclonus, chronic convulsions, salivation, loss of light reflex, mydriasis and vomiting. Schwab *et al.* (1969) observed side effects in 36 (22%) of 163 patients with parkinsonism treated with amantadine hydrochloride, mainly insomnia, abdominal discomfort, anorexia, dizziness and depression. The effects disappeared within 36 hours of ending treatment. Amantadine hydrochloride also potentiated the effect of atropine-like drugs given in the treatment of parkinsonism, causing confusion and hallucinations.

The toxic effects of amantadine hydrochloride have mostly been associated with the use of doses considerably higher than that recommended for clinical use, which is 200 mg daily. At this dose level Jackson *et al.* (1967) found that side effects occurred in less than 20% of healthy volunteers. When the dose was increased to 400 mg daily side effects occurred in 40%, and with still higher doses nearly all subjects experienced adverse reactions. The usual effects reported were alterations in emotional state and cerebration, such as depression and inability to concentrate, and occasionally feelings of depersonalization and alterations in body image

accompanied by anxiety. The symptoms appeared 3 hours after taking the drug, and usually passed off after 6 hours if the dose was not repeated. In a trial of amantadine hydrochloride carried out among elderly patients in a hospital for chronic diseases there was an increase in mortality in comparison with other patients. There were 26 deaths among 147 patients who took 200 mg a day for 11½ weeks, giving a weekly mortality of 1.5%. The incidence among 600 other patients during the 4 weeks preceding the trial was 1.1%. During the trial period there were 11 deaths (0.3%) among 289 given placebo and eight (1.2%) among 60 left untreated. The mean control incidence was 0.5%, and differed significantly from the incidence among treated patients. The degree of comparability of the various groups was uncertain, but the authors nevertheless consider that the use of amantadine hydrochloride may carry a higher risk among elderly patients.

Tyrrell *et al.* (1965) reported the occurrence of troublesome insomnia among an unstated number of volunteers who took a daily dose of 400 mg, which made it necessary to reduce the dose to 200 mg. Information on the incidence of side effects is given in a number of reports of prophylactic and therapeutic trials, and is summarized in Table 4.1. In many cases the authors merely state that they observed no untoward reactions attributable to the drug. In several trials the incidence of side effects was specifically recorded, and it is evident that they appeared with similar frequencies in both treatment and placebo groups. The most frequently recorded symptoms were insomnia, headache, dyspepsia, nausea, vomiting and diarrhoea. One would expect to find symptoms of this nature in any large group of persons observed over a fair period of time. In therapeutic trials, in which patients were treated with amantadine hydrochloride when they were already suffering from influenza, it is difficult to distinguish between side effects and the usual symptoms of the illness. It may be concluded that amantadine hydrochloride can be given over extended periods without troublesome side effects if the recommended daily dose of 200 mg is not exceeded.

Metabolism

The metabolism of amantadine hydrochloride varies considerably in different species (Bleidner *et al.*, 1965). When single oral doses ranging from 1 mg/kg to 100 mg/kg were given to mice, 51–75% of the dose was recovered in the urine unchanged. Some of the remainder was probably excreted as metabolites, since additional peaks could be found by gas chromatography, and some degree of metabolism was also implied by the incompleteness of recovery of the amount administered. Only small amounts were present in the faeces. In rats the recovery in the urine was only 16–18%. The recovery was also low in dogs, and *N*-methylamantadine was found as a metabolite, in amounts not exceeding 10% of the amount

Table 4.1 Reported side effects of amantadine hydrochloride

Reference	Dose (mg/day)	No. of subjects		No. with side effects		Side effects
		Treated	Placebo	Treated	Placebo	
Wendel <i>et al.</i> (1966)	200	469	380	8 (1.7%)	7 (1.8%)	Insomnia
Togo <i>et al.</i> (1968)	200	29	29	0	0	—
Wingfield <i>et al.</i> (1969)	100	23	48	0	0	—
Galbraith <i>et al.</i> (1969 a)	200	94	82	2	0	Headache
Galbraith <i>et al.</i> (1969 b)	200	102	100	3	0	Insomnia
Hornick <i>et al.</i> (1969)*	200	94	103	0	0	—
Knight <i>et al.</i> (1970)*	200	13	16	0	0	—
Togo <i>et al.</i> (1970)	200	54	48	0	0	—
Smorodintsev <i>et al.</i> (1970 a)	100, 200	206	198	0	0	—
Smorodintsev <i>et al.</i> (1970 b)	100	1313	512	94 (7.1%)	26 (5.2%)	Insomnia
Oker-Blom <i>et al.</i> (1970)	200	192	199	— (8.7%)	— (3.4%)	Dyspepsia Headache Vertigo Insomnia
Kitamoto <i>et al.</i> (1970)*	200	182	173	18 (9.9%) 9 (4.9%) 5 (2.7%)	21 (12.1%) 9 (5.2%) 6 (8.5%)	Malaise Nausea Vomiting Diarrhoea Insomnia
O'Donoghue <i>et al.</i> (1973)	200	50	61	0	0	—

* Therapeutic trial. Some reported side effects may have been symptoms of influenza

excreted as unchanged compound. In African green monkeys the recovery was 54%. In human volunteers 86% of a single oral dose was recovered unchanged in the urine; acetylated and methylated derivatives could not be detected, and there were no peaks indicative of metabolites in the gas chromatogram. Amantadine hydrochloride is thus readily absorbed from the intestines and is excreted without undergoing metabolic change.

Clinical pharmacology

The distribution of amantadine hydrochloride in mice was investigated by Uchiyama and Shibuya (1969), in experiments using compound labelled with tritium. It was given orally in a single dose of 1.6 mg/kg, corresponding to the dose in man recommended for clinical use. The compound attained maximum concentration in the internal organs 30 minutes after administration; the heart contained 0.3%, lungs 2.0%, liver 12.4%, kidney 5.2% and the spleen 0.7% of the dose given. Amantadine hydrochloride could be detected in the faeces and urine by 1 hour; by 12 hours 1.8% and 61.8% of the dose administered was excreted by the two routes respectively, and excretion continued up to 100 hours, when the total amounts were 7.5% and 86.7% respectively.

The blood level was 176 ng/ml at 15 minutes, 18.5 ng/ml at 30 minutes, 87 ng/ml at 1 hour and 45 ng/ml at 2 hours. Some compound was still present at 48 hours (14 ng/ml), but it could no longer be detected at 100 hours.

In three volunteers given single oral doses of 2–7 mg/kg Bleidner *et al.* (1965) found that the half-lives based on excretion were 9, 13 and 15 hours. After a dose of 5 mg/kg the maximum blood level was 0.6 µg/ml, and was attained 1–4 hours after administration. Biantrate *et al.* (1972) found blood levels ranging from 0.25 µg/ml to 1.01 µg/ml in four patients who were given 200–300 mg daily.

In volunteer studies carried out by Geuens and Stephens (1967) it was found that the rate of excretion of amantadine hydrochloride was highly dependent on the pH of the urine. The rate was very low when the urine was made alkaline by the administration of sodium bicarbonate, and increased rapidly when it was made acid by giving ammonium chloride. The body level of amantadine hydrochloride in a treated patient could thus vary widely, and a dosage sufficient for one patient might be insufficient for another. Toxic effects could be expected more frequently in patients who excreted the drug slowly. The authors suggested that combined treatment with sodium bicarbonate and amantadine hydrochloride would allow an effective level to be attained in 1 day, which could then be maintained on a reduced daily dose. They estimated that a loading dose of three times the daily maintenance dose would give a stable body level which would be three times as high if 4.2 g of sodium bicarbonate were given three times a day.

Combined treatment was not recommended for prophylaxis, but the authors saw no contraindication for using it in a 7-day course of treatment during the acute phase of influenza.

Assay methods

Amantadine hydrochloride is determined in blood, urine and tissue samples by utilizing its properties as an organic base, which enable it to be extracted into toluene and determined by gas chromatography. The method of Bleidner *et al.* (1965) is usually used, and amounts down to 0.1 µg can be determined. For the determination in blood samples 5 ml of oxalated whole blood is mixed with 20 ml of 5 N sodium hydroxide solution and 12.5 ml of benzene and shaken for 5 minutes. This procedure converts the amantadine hydrochloride to the free base which then passes into the benzene. One ml of 1 N hydrochloric acid is shaken with 10 ml of the benzene extract and the mixture is centrifuged to separate the aqueous phase, which now contains the amantadine as the hydrochloride. A volume of 0.75 ml of the aqueous phase, representing 3 ml of the original blood sample, is made alkaline with 1.5 ml of 1 N sodium hydroxide solution and the free base is extracted into 1 ml of benzene; 5 µl of this are injected into a gas chromatograph. The amount contained in it is determined by reference to standards prepared by adding known amounts of amantadine hydrochloride to blood and carrying out the same extraction procedure.

With urine samples the concentrations are higher and interfering substances are usually not present, so that the hydrochloric acid step may be omitted. The sample is mixed with 8 N sodium hydroxide solution and benzene; ratios of 4:4:2, 4:4:1 or 10:5:1 are suitable. The benzene extract is then used directly for gas chromatography. If interfering substances are present the method is modified by conversion of amantadine to its acetamido derivative. This is done by mixing 0.5 ml of benzene extract with 0.5 ml of acetic anhydride. The mixture is left overnight in a stoppered vessel. This is then chilled, and excess acetic anhydride is destroyed by the cautious addition of 2 ml of 8 N sodium hydroxide solution. An aliquot of the benzene phase is then analysed. Standards are prepared from known amounts of amantadine hydrochloride carried through the same procedure.

Samples of tissue are homogenized in five parts of 5 N sodium hydroxide solution and extracted into a measured volume of benzene. An aliquot of the benzene phase is then analysed. A similar method can be used for faeces.

Other workers have converted amantadine into a derivative as a standard procedure. Biantrate *et al.* (1972) used the trichloroacetyl derivative. In their method 1 ml of plasma is mixed with 1 ml of N sodium hydroxide solution and extracted with 5 ml of toluene, which is then extracted back with 2 ml of N hydrochloric acid. The aqueous phase is rebaseified with

0.5 ml of 6 N sodium hydroxide solution and extracted with 2 ml of toluene; 20 µl of 2% trichloroacetyl chloride in toluene is added to 1.5 ml of the toluene extract and the mixture is heated at 70 °C for 30 minutes. Determination is carried out by gas chromatography of a volume of 1–3 µl. The method is linear over the range of 25–1000 ng/ml plasma. In a similar method Tsubouci *et al.* (1970) used the 1-monochloroacetamido derivative, and gave the sensitivity as 10 ng. They also developed a method based on a colour reaction which does not require gas chromatography. Amantadine reacts with *p*-nitrobenzaldehyde to give the 1-*p*-nitrobenzylidene derivative. This can be determined spectrometrically by its absorption at 292 nm. In biological fluids the method can be used down to 1 µg/ml.

Clinical use

Amantadine hydrochloride is used in the prophylaxis and treatment of influenza infections caused by A2 strains, and has been reported to shorten the duration of pain in zoster. It is indicated for the prophylaxis of influenza in persons who are at particular risk, such as infants and the elderly and patients suffering from chronic debilitating diseases, such as cardiovascular, renal and metabolic disorders.

Contraindications

Amantadine hydrochloride is contraindicated in patients with a history of epilepsy, elderly persons with cerebral arteriosclerosis and patients treated with stimulants of the central nervous system. It may enhance the effects of benzhexol, benzotropine and orphenadrine, and if it is used it may be necessary to reduce the doses of these drugs. In view of its slight inhibitory effect on DNA synthesis it should not be used in pregnancy, although there are no reports of teratogenic effects.

It is not known whether amantadine hydrochloride passes into the milk, but it would be advisable to avoid its use during lactation.

Preparations

Amantadine hydrochloride capsules (USNF) 100 mg
Amantadine hydrochloride syrup (USNF) 50 mg in 5 ml
Symmetrel (Geigy); capsules of 100 mg.

CHANGE OF NOMENCLATURE

The literature on amantadine hydrochloride is indexed in *Chemical Abstracts* under 1-aminoadamantane as far as 1972. From this time onwards the literature should be looked for under the entry tricyclo[3,3,1,^{3,7}]-decan-1-amine.

GENERAL READING

Hoffmann, C. E. (1973). Amantadine HCl and related compounds. In: W. A. Carter (ed.) *Selective Inhibitors of Viral Functions* (Cleveland, Ohio: Chemical Rubber Co. Press)

METHISAZONE

Methisazone (Figure 4.1) is the generic name of 1-methyl-1*H*-indole-2,3-dione 3-thiosemicarbazone. It is also referred to in the literature as 1-methylisatin 3-thiosemicarbazone and N-methylisatin β -thiosemicarbazone. It is the 1-methyl derivative of isatin 3-thiosemicarbazone (isatin β -thiosemicarbazone, IBT), and much of the work on the antiviral activity and mode of action has been carried out with the parent compound, and will be referred to in the sections which follow.

Figure 4.1 Structural formulae of (I) amantadine hydrochloride and (II) methisazone

Methisazone is a yellow crystalline solid. It is soluble in water at room temperature to the extent of 0.006%. As a result of this low solubility methisazone can only be given by mouth. A stable 1% microcrystalline suspension can be prepared by mixing 20 ml of a 5% solution of methisazone in dimethylformamide with 80 ml of water. A solution for use in tissue culture can be prepared by dissolving 23 mg of methisazone in 0.5 ml of dimethylformamide and adding rapidly to 900 ml of distilled water. The compound precipitates immediately in finely divided form, but will go back into solution after autoclaving for 10 minutes at 10 lbs pressure. Addition of 100 ml of a 10× concentrated solution of tissue culture salts gives 1000 ml of a 100 μ M solution. This can be diluted appropriately to give the concentration required. The solution should be

stored at 37 °C in the dark, but the compound will eventually crystallize out. Methisazone is soluble to the extent of at least 20% in dimethylsulphoxide, but the solution rapidly begins to deposit crystals and eventually solidifies. This is probably due to the formation of an adduct with the solvent, and the crystalline product is devoid of antiviral activity. Methisazone is therefore not suitable for local application in this vehicle.

Biological actions

The biological actions of methisazone, and perhaps also its antiviral action, are dependent to a considerable extent upon its chemical properties. The compound can exist in two isomeric forms, depending upon the configuration of the side-chain (Barz and Fritz, 1970). In the solid state it exists as the *syn*-isomer, with the oxygen atom in the 2- position forming a hydrogen bond with the hydrogen atom on the second nitrogen atom of the side-chain. In solution the compound assumes the *anti*-configuration, with the side-chain directed away from the remainder of the molecules. In this form the molecule acts as a bidentate ligand, and can form complexes with copper and zinc ions, which are co-ordinated with the two nitrogen atoms in the side-chain, and iron, cobalt and zinc co-ordinated with the first nitrogen and the sulphur atoms. The molecule can further assume a thiolate configuration, in which the hydrogen on the first nitrogen atom migrates to the sulphur atom. In this configuration the molecule can act as a tridentate ligand and form a divalent copper complex co-ordinated with the first nitrogen, oxygen and sulphur atoms.

In view of this chelating ability it is not surprising to find that methisazone is an inhibitor of certain metalloenzymes. Influenza A and influenza B contain an RNA-dependent RNA polymerase, which is associated with the core of the virion. The RNA polymerases of certain bacteria are known to be metalloenzymes containing zinc, and Oxford and Perrin (1974) were therefore led to study the effect of a number of chelating agents upon the influenza enzyme. In experiments with the RNA polymerase of the B/Lee strain a 50% reduction in activity was obtained with isatin 3-thiosemicarbazone in a concentration of 30 μM , and methisazone had the same inhibitory effect at 100 μM . When the sulphur atom is replaced by oxygen (isatin 3-semicarbazone) or an -NH group (isatin 3-amidinohydrazone) the chelating activity is much reduced, and the 50% inhibitory concentrations for these two compounds were above 500 μM . These observations led the authors to conclude that the RNA polymerase of influenza virus was probably a zinc metalloenzyme. Inhibition was also observed with the RNA polymerase of a strain of type A influenza (A/R1-5⁺), but not with polymerases from *Escherichia coli* or *Micrococcus lysodeikticus*.

Haase and Levinson (1973) observed that methisazone inhibited the RNA-dependent DNA polymerase (reverse transcriptase) of the RNA-

containing slow viruses visna, maedi and progressive pneumonia virus. A concentration of 40 μM caused an 85% reduction in enzyme activity. Levinson *et al.* (1973 b) observed a similar effect with the RNA-dependent DNA polymerase of Rous sarcoma virus. Methisazone in a concentration of 40 μM caused a 98% reduction in the activity of the enzyme. It also caused a 99% reduction in the transforming ability of the virus, as measured by counts of foci of transformed cells in chick embryo cell cultures. Concentrations of 1 μM reduced the respective activities to 42% and 50% of the control values, and to 1% in both cases when copper sulphate was also present in the same molar concentration. Cupric ions and methisazone were therefore acting in synergism. The authors suggest that the antiviral activity of methisazone and other thiosemicarbazones is a function of their ability to act as ligands for metallic ions. Steric and other factors must also be involved, however, since not all thiosemicarbazones possess antiviral activity.

In further work (Levinson *et al.*, 1973 a) it was found that 40 μM methisazone would inhibit the RNA-dependent DNA polymerases of mouse sarcoma, mouse leukaemia and mouse mammary tumour viruses. Translation from single-stranded RNA was unaffected, however, since methisazone did not inhibit polypeptide synthesis from the messenger RNA of rat hepatoma cells.

Webb *et al.* (1965) found that the cyclic AMP content of human peripheral blood lymphocytes was increased 4-fold by incubation in 10% dimethylsulphoxide containing 200 μM methisazone. This was found to be due to inhibition of phosphodiesterase, the enzyme which is responsible for the degradation of cyclic AMP. Methisazone did not cause any inhibition of phosphodiesterase activity in human lymphocytic cell lines, HeLa cells, or a cell line derived from a mouse lymphoma. The inhibition was therefore specific for normal human lymphocytes, and might be expected to have some influence upon immune responses. An effect of this nature was demonstrated by McNeill *et al.* (1972), who found that daily intraperitoneal doses of 23 μg caused a reduction in the antibody-forming cell response to injected sheep red cells in mice. The titre of haemolytic antibody was also greatly reduced. The authors concluded that methisazone has a marked immunosuppressive activity. In further work (McNeill, 1972) the effect on colony formation from suspensions of mouse spleen cells was studied. The number of colonies formed during a 7-day period of incubation was reduced when methisazone was incorporated into the medium. The extent of reduction was linearly related to the concentration of the compound, and 50% inhibition occurred at a concentration of 13 μM (3 $\mu\text{g}/\text{ml}$). The inhibitory activity of methisazone was of the same order as that of 6-mercaptopurine (12 $\mu\text{g}/\text{ml}$) and rifampin (10 $\mu\text{g}/\text{ml}$), but did not approach the activity of daunorubicin (6 ng/ml). Exposure of the cells to 10 $\mu\text{g}/\text{ml}$ methisazone for 1 hour at 37 °C did not affect their

ability to form colonies, and the reduction in colony formation was thus not due to a short-term cytotoxic action. In further experiments the cell suspensions were exposed to methisazone after an initial delay ranging from 3 to 48 hours. The degree of inhibition observed fell off rapidly when the addition was delayed until 24 hours or longer, and methisazone thus exerted its effect at an early stage in colony formation. Methisazone also inhibited the formation of colonies by suspensions of cells from mouse and human bone marrow (McNeill, 1973).

A further biological action of methisazone is its ability to inactivate certain viruses on direct contact with the virion. This was first observed by Levinson *et al.* (1971) in work done mainly with the 1-ethyl analogue. When Rous sarcoma virus in tissue culture medium was exposed to 40 μ M 1-ethylisatin 3-thiosemicarbazone for 15 minutes at 37 °C, more than 90% of the infectivity was lost. The effect was apparently specific, and no inactivation was observed in similar experiments with a representative selection of both RNA and DNA viruses, including herpes, but the compound inactivated herpes when the virus was suspended in phosphate-buffered saline. Methisazone was equally effective, but no inactivation was obtained with the parent compound isatin 3-thiosemicarbazone.

In further work Levinson *et al.* (1973) observed that Rous sarcoma virus inactivated by methisazone would still attach to chick fibroblasts, and lack of attachment was therefore not the explanation of the loss of transforming activity brought about by methisazone. They also reported that methisazone would inhibit plaque formation by type 1 and type 2 herpes viruses, but plaque formation by polyoma virus was unaffected. In their work on the slow viruses and thiosemicarbazones reported above, Haase and Levinson (1973) found that exposure to 40 μ M methisazone over a period of 30 minutes at 37 °C reduced the infectivity of visna virus by 85%.

Inactivation of lymphocytic choriomeningitis virus was reported by Logan *et al.* (1975), and they also found that methisazone inactivated Parana and Pichinde viruses, which are also members of the arenavirus group.

Antiviral activity

The discovery of methisazone resulted from the original observation of Brownlee and Hamre (1951) that *p*-aminobenzaldehyde thiosemicarbazone inhibited the multiplication of vaccinia virus. This was the first antiviral compound to be discovered. Thompson *et al.* (1953) investigated thiosemicarbazones based on other ring systems, and found particularly high activity with isatin 3-thiosemicarbazone. Groups of mice were infected intracerebrally with approximately 1, 10 and 100 LD₅₀ of vaccinia virus and given a diet containing isatin 3-thiosemicarbazone in concentrations of 0.08–0.16%. There was a reduction in mortality of 2 log units in com-

parison with untreated control groups infected with the same dose levels of virus. These results were confirmed by Bauer (1955), who observed a reduction in mortality of 4 log units in mice given the compound subcutaneously in repeated doses of 1.25 and 2.5 mg/kg.

The introduction of continuous cell lines into virology made it possible to examine the antiviral activity of isatin 3-thiosemicarbazone in a more sensitive system. Sheffield *et al.* (1960) demonstrated antiviral activity in tube cultures of HeLa cells infected with decimal dilutions of vaccinia virus and supplied with medium containing isatin 3-thiosemicarbazone in concentrations ranging from 0.4 μ M to 40 μ M. The infectivity titre as determined by the appearance of cytopathic effect was reduced by 4.7 log units in comparison with infected control tubes incubated in the absence of the compound. This observation did more than confirm the antiviral activity previously found in mice, since it showed that the active agent was the compound itself and not a metabolite produced by enzymatic degradation in the tissues.

Bauer and Sadler (1960 a) examined a number of derivatives of isatin 3-thiosemicarbazone with the object of finding compounds with greater antiviral activity. Most substitutions reduced or abolished activity, but alkylation in the 1- position increased activity, with the 1-methyl and 1-ethyl derivatives respectively having 2 and 3 times the activity of the parent compound. The 1-methyl derivative, methisazone, was selected for further development.

Methisazone inhibits the multiplication of other members of the pox-virus group. In early work with isatin 3-thiosemicarbazone Bock (1957) could find no evidence of antiviral effect in mice infected with ectromelia virus intranasally or by injection into the footpad and treated with the compound subcutaneously in doses ranging from 25 to 250 mg/kg. Bauer and Sadler (1960 a) also found no evidence of activity in mice infected with ectromelia virus intracerebrally, but Sheffield *et al.* (1960) had no difficulty in demonstrating antiviral activity in tissue culture. In tube titrations of ectromelia virus carried out in HeLa cells a concentration of 40 μ M reduced the titre of virus by 2.8 log units. It is evident that isatin 3-thiosemicarbazone inhibits the multiplication of ectromelia virus, but the activity is not great enough to be detectable in animal models.

At the time when isatin 3-thiosemicarbazone was considered to be inactive against ectromelia it was considered of interest to investigate its activity against a wider range of pox viruses. In titrations of rabbitpox virus carried out by intracerebral infections with mice it was found that treatment with the compound in doses of 100 mg/kg given twice daily by the subcutaneous route conferred protection against 100 000 LD₅₀ of virus. Rabbitpox was therefore as sensitive as vaccinia (Bauer and Sheffield, 1959). In further work Bauer (1961) obtained dose-response lines based on the mean reciprocal survival times of mice infected intracerebrally with

rabbitpox, vaccinia, cowpox and its white variant and treated with isatin 3-thiosemicarbazone in a range of dose levels. The results showed that the sensitivities of these pox viruses covered a wide range, with rabbitpox being the most sensitive, followed by vaccinia and white cowpox with similar sensitivities, and cowpox being the least sensitive, with an ED₅₀ about 1000 times greater than that of rabbitpox.

The most important member of the poxvirus group is smallpox. This exists in two variants, alastrim and variola major. The 1-ethyl derivative of isatin 3-thiosemicarbazone was shown by Bauer and Sadler (1960 b) to be active against alastrim. This virus produces a fatal encephalitis on intracerebral injection in infant mice up to 6 days of age, and subcutaneous treatment with the compound in doses ranging from 10 to 250 mg/kg gave complete protection against infection with 100 LD₅₀ of virus; the 50% protection level was around 0.25 mg/kg. In similar experiments with variola major Bauer *et al.* (1962) found that subcutaneous injection of isatin 3-thiosemicarbazone in doses of 25 to 100 mg/kg would confer complete protection against intracerebral infection with 1000–10 000 LD₅₀ of virus; the 50% protective dose was around 5 mg/kg. Methisazone was more effective, with a 50% protective dose around 2.5 mg/kg. These findings show that variola major is somewhat less sensitive than alastrim.

Methisazone has antiviral activity against other DNA viruses. Bauer and Sadler (1960 a) found no evidence of activity against herpes in mice infected intracerebrally and treated with 100 mg/kg methisazone by the subcutaneous route, but it is uncertain whether the compound passes the blood–brain barrier. Rapp (1964) found that concentrations of 4.8 µM in the overlay did not reduce plaque formation by herpes, but this cannot be accepted as a negative result since the concentration is very low. Caunt (1967) found that methisazone would reduce the yield of herpes virus in cultures of primary human thyroid cells, with inhibition approaching 100% with a concentration of 20 µM. This was confirmed by Herrmann (1968), who obtained zones of inhibition in plaque inhibition tests carried out in HeLa cells. Methisazone is thus active against herpes, but the activity is too low to be of clinical use.

Rapp (1964) found that methisazone did not inhibit plaque formation by varicella-zoster virus in monolayers of human embryo lung cells, but he was using the same low concentration which was ineffective against herpes virus. In experiments similar to those carried out with herpes virus Caunt (1967) found that methisazone in a concentration of 20 µM reduced the yield of varicella-zoster virus by 90%.

Methisazone is considerably more active against infectious bovine rhinotracheitis, which is also a member of the herpesvirus group. Munro and Sabina (1970) found that methisazone in a concentration of 20 µg/ml (82 µM) reduced the single-cycle yield of the virus in bovine kidney cells by 4–5 log units.

Methisazone will inhibit the multiplication of several types of adenovirus (Bauer and Apostolov, 1966). The production of adenovirus type 11 in HeLa cells was completely inhibited by a concentration of 40 μ M, and similar results were obtained with types 3, 7, 9, 14, 16, 17, 21 and 28, and also the simian adenovirus SV15.

Methisazone is also active against a wide range of RNA viruses. No activity was detected in early work, which was carried out by the intracerebral infection of mice. Isatin 3-thiosemicarbazone was reported to be inactive against Rift Valley fever (Minton *et al.*, 1953) and Ilhéus, Wyeomyia, Zika, California, Ntaya, Semliki Forest, dengue 1, Anopheles A and Anopheles B viruses (Bauer and Sadler, 1960). These findings do not exclude activity, however, since it is not known whether the compound can pass the blood-brain barrier.

Activity against rhinoviruses can be readily demonstrated in tissue culture. Bauer *et al.* (1970) found that methisazone reduced the single-cycle yield of Bunyamwera and Semliki Forest viruses in HeLa cells: the reduction was dose-dependent, and attained 3 log units at a concentration of 20 μ M. The inhibition observed was not due to toxicity, since the multiplication of Sindbis virus in the same cell system was unaffected by methisazone in concentrations up to 40 μ M. In similar experiments methisazone inhibited the multiplication of echovirus types 7, 11, 12 and 13, reovirus 3, the A/England/66 strain of influenza and the Sendai strain of parainfluenza 1.

Lwoff and Lwoff (1964) found that the single-cycle yield of type 1 poliomyelitis virus was reduced by isatin 3-thiosemicarbazone in a concentration of 40 μ M, and Bauer *et al.* (1970) found that methisazone was active against all three types by the plaque inhibition method.

In an investigation of the effect of various compounds on the multiplication of type A foot-and-mouth disease virus in calf kidney cells Polatnick (1965) found that 100 μ g/ml isatin 3-thiosemicarbazone reduced the single-cycle yield by 1.5 log unit; methisazone was more active, and reduced the yield by 4.4 log units in a concentration of 50 μ g/ml. Isatin 3-thiosemicarbazone and methisazone have also been reported active against rhinoviruses (Gladych *et al.*, 1969).

The antiviral spectrum of methisazone is given in Table 4.2.

Resistance can be induced in pox viruses by serial passage in the presence of methisazone and its analogues. Appleyard and Way (1966) passaged rabbitpox virus intranasally in mice which were treated with 0.5 or 1 mg of methisazone daily. At each passage the virus was recovered from the lungs and dose-response lines against isatin 3-thiosemicarbazone were obtained by plaque reduction. With successive passages the virus became less sensitive, as indicated by a shift of the dose-response lines towards higher concentrations of the compound. Resistance was also demonstrated in tissue culture by passage in the presence of concentrations of isatin

3-thiosemicarbazone rising from 0.05 to 20 µg/ml. In plaque reduction tests the initial virus was almost completely inhibited by a concentration of 0.1 µg/ml, but after 15 passages a concentration of 10 µg/ml inhibited plaque production by only 50%. The virus had thus become 100 times less sensitive. Katz *et al.* (1973 b) isolated resistant strains of vaccinia virus by passage in the presence of isatin 3-thiosemicarbazone, and also strains which were dependent upon the compound and would not grow in its absence.

Table 4.2 Antiviral spectrum of methisazone (Bauer *et al.*, 1970)

<i>Nucleic acid</i>	<i>Group</i>	<i>Virus</i>
DNA	Poxvirus	Vaccinia Smallpox Cowpox
	Adenovirus	Adenovirus
	Herpesvirus	Varicella-zoster Herpes? Infectious bovine rhinotracheitis
RNA	Picornavirus	Poliomyelitis Echovirus Rhinovirus Foot-and-mouth disease
	Reovirus	Reovirus 3
	Arbovirus	Rift Valley fever Bunyamwera Semliki
	Myxovirus	Influenza A, B
	Paramyxovirus	Parainfluenza 1

Rabbitpox virus also shows another type of resistance which is dependent upon the type of cell in which it is multiplying. Appleyard *et al.* (1965) found that it was less sensitive to isatin 3-thiosemicarbazone in RK13 and L cells than in HeLa cells, and they considered that the compound was 100 times less effective in the first two cell lines.

Mode of action

Although isatin 3-thiosemicarbazone inactivates a number of viruses on direct contact it has no effect on pox viruses, and does not prevent their adsorption to cells (Sheffield *et al.*, 1960). The site of action is thus intracellular. Easterbrook (1962) found that the production of infectious vaccinia virus in KB cells was completely inhibited, but the production of the virus proteins, as observed by immunofluorescence, was unaffected. The virus grew to the normal extent in cultures which had been exposed to isatin 3-thiosemicarbazone for 18 hours and then washed before infection. The antiviral effect was therefore not due to toxicity or the induction of interferon.

In experiments with radioactive thymidine and valine Bach and Magee (1962) showed that isatin 3-thiosemicarbazone did not inhibit the synthesis of DNA and protein in infected HeLa cells. These results are in accordance with the observations of Easterbrook, and indicate that virus components are produced in the presence of isatin 3-thiosemicarbazone but are not assembled into infectious virus particles. Magee and Bach (1965) found that vaccinia virus DNA made in the presence of isatin 3-thiosemicarbazone was incorporated into infective virus particles when the compound was removed, and it was therefore unlikely that any direct interaction between isatin 3-thiosemicarbazone and DNA had occurred.

Appleyard *et al.* (1965) investigated the effect of time of addition of isatin 3-thiosemicarbazone on the yield of rabbitpox virus in HeLa cells. Virus growth was still completely inhibited when treatment was delayed until 3 hours after infection, and was much reduced when the interval was extended to 4 hours. In the absence of treatment infective virus appeared after 5 hours, and it was therefore concluded that isatin 3-thiosemicarbazone exerted its action at a late stage in the growth cycle. However, when the compound was added at the time of infection and removed 2 hours later the final yield of infectious virus was reduced to one-quarter, and isatin 3-thiosemicarbazone must therefore affect an early stage in virus multiplication as well.

The authors also studied the effect of isatin 3-thiosemicarbazone on the formation of virus antigen. Cultures were infected and incubated with the compound for various periods of time, and extracts of the cells were then prepared and examined by immunodiffusion against rabbitpox antiserum. Formation of virus antigen proceeded normally for 4 hours, but after this time the additional lines found with extracts of untreated cultures failed to appear.

In further experiments it was observed that isatin 3-thiosemicarbazone in a concentration of 0.1 µg/ml reduced the 24-hour yield of virus by 95%, but if actinomycin D was present in the culture in a concentration of 0.04 µg/ml the antiviral effect of isatin 3-thiosemicarbazone was almost

entirely abolished. The authors interpreted this effect as indicating that DNA-dependent synthesis of RNA must be free to occur if the compound is to manifest its antiviral activity, and they postulated that the presence of isatin 3-thiosemicarbazone brought about the synthesis of a new species of messenger RNA, which was then translated into a protein which was the true antiviral agent.

Woodson and Joklik (1965) applied the methods of molecular biology to a study of the mechanism of action of isatin 3-thiosemicarbazone. In the presence of the compound they found that the amount of virus DNA resistant to DNase through being coated with virus protein was reduced by 75%. This implies that the synthesis of virus protein is reduced, and is in agreement with the inhibition of late virus antigens observed by Appleyard *et al.* (1965). Reduction in the synthesis of virus proteins was observed in experiments in which the uptake of labelled amino acids was studied. In the presence of 15 μ M isatin 3-thiosemicarbazone virus protein was formed at the normal rate for the first 3 hours, but the amount formed then fell to 20% of normal at 4 hours and 10% at 6 hours. The synthesis of virus messenger RNA was unaffected, and the inhibition of protein synthesis must therefore occur at the stage of translation. This was found to proceed normally for the first 4 hours, but the polyribosomes then became unstable and fell away from the messenger RNA strands, which became broken down into shorter lengths. The formation of the late virus proteins was therefore brought to an end. The effect on the messenger RNA could conceivably be due to the action of an abnormal nuclease which is formed as a result of the action of the compound, a step which cannot take place in the presence of actinomycin D.

These results are not in agreement with the observations of Easterbrook (1962), who found that the production of virus proteins was unaffected. Katz *et al.* (1973 a) studied the formation of vaccinia virus polypeptides by polyacrylamide gel electrophoresis, and found that the formation of both early and late virus proteins was unaffected by isatin 3-thiosemicarbazone. They pointed out, however, that the vaccinia virus genome is large enough to code for several hundred polypeptides, and it is therefore possible that the absence of some might not be detectable. They also postulated that the breakdown of polyribosomes and decline in protein synthesis were secondary effects of the compound, resulting from the accumulation of un-assembled structural components of the virus.

It is clear from the above that the precise mode of action has not been established, and it is likely that the formation of metal chelates plays some part.

Toxicity

There have been a number of reports on the cytotoxic concentration of methisazone in tissue culture systems. Owing to the low solubility of the

compound it is doubtful whether it remains for long in true solution at concentrations above 100 μM (23.4 $\mu\text{g/ml}$), and some reports have referred to concentrations higher than this. Bauer and Apostolov (1966) found that a concentration of 40 μM was not toxic for HeLa cells, but Munro and Sabina (1970) found that the multiplication of a line of bovine kidney cells was arrested after the third subculture in the presence of a concentration of 20 $\mu\text{g/ml}$. The compound has some toxic effect on the DNA pathway, since Magee and Bach (1965) found that the incorporation of labelled thymidine by HeLa cells was inhibited to the extent of 65% by a concentration of 50 $\mu\text{g/ml}$.

The acute oral LD₅₀ of methisazone in mice, rats and rabbits is in excess of 2000 mg/kg. In chronic toxicity studies in which rhesus monkeys were given 250 mg/kg daily by mouth for 1 month there was some evidence of liver damage, but the liver was unaffected at the end of similar treatment in rats and dogs.

Methisazone has an embryotoxic effect in very high doses. In mated rat does given 2000 mg/kg daily by mouth for 12 days there were no implantations in the majority of cases, and animals treated later in pregnancy showed an increase in resorptions in comparison with untreated controls. Similar effects have been noted in rabbits, and fetal malformations are occasionally produced.

The duration of hypnosis after pentobarbitone sodium is greatly prolonged in mice given 25 mg/kg methisazone, probably due to competition for the detoxifying systems in the liver. In rats with unrestricted access to food and water the stomach emptying time is greatly prolonged by the oral administration of 500 mg/kg methisazone. The delay in emptying was much less in fasted rats, and methisazone should therefore be given on an empty stomach.

Metabolism

The metabolism of methisazone has been studied in volunteers and also in contacts of smallpox patients treated for prophylactic reasons. Unaltered drug does not appear in the urine, and five or more metabolites can be detected, including 1-methylisatin, 1-methylisatin 3-thiosemicarbazone, isatin 3-semicarbazone, isatin 3-thiosemicarbazone and 6-hydroxy-1-methylisatin. There is also a possibility that metabolites in the 4- and 7-positions may appear. The hydroxylated derivatives are probably excreted as conjugates with glucuronic and sulphuric acids, and excretion in this form is probably considerably increased in patients in whom the activity of the conjugating systems has been increased by treatment with barbiturates. The metabolites impart a brown colour to the urine, which appears 2–3 hours after taking the compound by mouth and persists for several hours. Methisazone probably interacts with the systems which metabolize ethanol,

since it frequently induces a marked intolerance to alcohol. The metabolism of methisazone thus follows a number of pathways, which involve detachment of the methyl group, replacement of sulphur by oxygen in the side-chain and hydroxylation in the aromatic ring. Of the known metabolites only isatin 3-thiosemicarbazone possesses antiviral activity, amounting to one-half that of methisazone.

Clinical pharmacology

Methisazone is poorly absorbed from the gastrointestinal tract. The extent of absorption is greatly influenced by particle size. When given to rats in the form of a suspension in sucrose syrup of micronized particles of mean diameter 3 μm , between 40 and 50% of the dose can be recovered in the faeces. With 100 μm particles the recovery approaches 80% (Axon, 1972).

Methisazone can be detected in the plasma after oral administration, but there is a wide variation in the amounts reported. Turner *et al.* (1962) found a concentration of 8 μM (1.90 $\mu\text{g/ml}$) in a child aged 4½ months who had been given 250 mg 6-hourly, a concentration which is known to be inhibitory in tissue culture experiments. Kempe *et al.* (1965) found that plasma levels attained a maximum between 4 and 8 hours after administration. In an infant with progressive vaccinia a single oral dose of 100 mg/kg gave a peak level of 51.3 μM (12 $\mu\text{g/ml}$). With 200 mg/kg the maximum level was 85.5 μM (20 $\mu\text{g/ml}$). In an adult with eczema vaccinatum a single dose of 3 g gave a maximum level of 27.0 μM (6.3 $\mu\text{g/ml}$). Methisazone had mostly disappeared from the plasma after 12 hours, and repeated administration did not lead to any cumulative rise in plasma concentration. Much lower values were found by Gomez and Sandeman (1966) in one normal subject and six patients with malignant conditions treated with methisazone in doses of 40 and 80 mg/kg. Measurable levels ranging from 1.3 μM (0.3 $\mu\text{g/ml}$) to 3.4 μM (0.8 $\mu\text{g/ml}$) were found in three and trace amounts in two; in one patient methisazone could not be detected in the serum. No information is available on the concentrations of methisazone attained in the tissues.

Assay method

Methisazone readily dissolves in alkali to form a yellow solution with an absorption maximum at 400 nm. In the method developed by Turner *et al.* (1962) 1 ml of heparinized blood is acidified with 0.1 ml of 2 N hydrochloric acid and extracted into 1 ml of toluene. After centrifugation for 20 minutes at 2500 g 0.5 ml of the toluene layer is basified with 0.1 ml of 0.05 M methylbenzethonium hydroxide in ethanol and the optical density is read at a wavelength of 400 nm. The concentration of methisazone is then determined from a standard line obtained by the treatment of solutions of

known concentration. The determination may be carried out more conveniently by extraction of the acidified sample into benzene and re-extraction of the benzene solution into 1 N sodium hydroxide solution. Readings should be made without delay, as the colour of the alkaline solution fades rapidly.

Clinical use

Methisazone is indicated for the prophylaxis of smallpox in contacts and for the treatment of infections of the skin with vaccinia virus. It is also used to cover primary smallpox vaccination when it must be carried out in the presence of contraindications, and it has been reported to have some effect in the treatment of varicellar pneumonia.

Contraindications

Methisazone is contraindicated in patients with liver dysfunction unless they are contacts of smallpox or are suffering from a severe complication of smallpox vaccination. The decision whether or not to use it in pregnancy should be weighed against the known severe course of smallpox in pregnant women and the risk to the fetus.

Preparations

Marboran suspension (Wellcome Foundation Limited). Sachets containing 15 ml of a 20% suspension of Marboran in sucrose syrup (3 g per sachet).

GENERAL READING

- Hoffmann, C. E. (1973). Amantadine HCl and related compounds. In: W. A. Carter (ed.) *Selective Inhibitors of Viral Functions*, pp. 199–211, (Cleveland Ohio: Chemical Rubber Co. Press)
- Bauer, D. J. (1972). Thiosemicarbazones. In: D. J. Bauer (ed.) *Chemotherapy of Virus Diseases*, vol. 1, 35–113. (Oxford: Pergamon Press)