



Human rabies encephalitis prevention and treatment: progress since Pasteur's discovery

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TITLE

Human Rabies Encephalitis Prevention and Treatment: Progress Since Pasteur's

Discovery

RUNNING TITLE

Prevention and Treatment of Human Rabies

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ABSTRACT

Rabies remains one of the most ancient and deadly of human infectious diseases. This viral zoonosis is transmitted principally by the saliva of infected dogs, inducing a form of encephalomyelitis that is almost invariably fatal. Since the first implementation, by Louis Pasteur in 1885, of an efficient preventive post-exposure treatment, more effective protocols and safer products have been developed, providing almost 100% protection if administered early enough. However, this disease still represents a major, but neglected public health problem, with an estimated 50,000 human deaths due to rabies reported each year, mostly in Africa and Asia. Once the first clinical signs appear, there is no effective treatment. A ray of hope emerged in 2004, with the report of a patient recovering from rabies after aggressive, innovative treatment. However, this case was not clearly reproduced and the identification of targets for antiviral treatment in cases of rabies infection remains a major challenge.

In this context, this review presents the state-of-the art in the prevention and curative treatment of human rabies. We begin by describing the viral etiological agent and the disease it causes, to provide an essential background to rabies. An overview of the post-exposure prophylaxis of rabies in humans is then given, from its initial implementation to possible future developments. Finally, an analysis of the various antiviral compounds tested in rabies *in vitro*, in animal models or in humans is presented, focusing in particular on potential new strategies.

KEYWORDS

Antiviral drugs, encephalitis, immunoglobulins, lyssavirus, Milwaukee protocol, post-exposure prophylaxis, rabies virus, vaccine

MAIN TEXT

Introduction

Rabies is an acute, almost invariably fatal form of viral encephalomyelitis in humans. The rabies virus (RABV) — one of the 11 species of the genus *Lyssavirus* in the family *Rhabdoviridae*— is the main etiological agent. It is acquired from the saliva of infected animals by bites, scratches and mucous membrane exposure and, more rarely, by aerosol exposure and tissue/organ transplantation. Rabies is the infectious disease with the highest case-fatality ratio in humans. An extraordinary leap forward was made with the work of Louis Pasteur at the end of the 19th Century, with the development of the first efficient post-exposure treatment able to prevent rabies in exposed patients. Unfortunately, the original vaccine was derived from brain and nerve tissues and was associated with neurological complications [1]. These complications eventually led to the replacement of such vaccines by tissue-cultured vaccines. This disease remains a major but neglected public health concern throughout the world, with an estimated 50,000 cases of rabies still reported in humans each year, mostly in rural area of Africa and Asia, and with a particularly high incidence in young children (under the age of 15 years) [2]. These figures, although high, must be considered an underestimate, as there is undoubtedly a high frequency of misdiagnosis and underreporting [3-4]. Dogs are the main source of exposure for several billion people worldwide, and remain responsible for almost 99% of all human deaths from rabies [2]. Other mammal species also serve as natural vectors and/or reservoirs of rabies, including various carnivores and several bat species. Even though this zoonotic disease was known and described in the Classical Era, our knowledge of its physiopathological mechanisms remains limited, except for detailed descriptions of the clinical symptoms. Rabies virus, like other lyssaviruses, is a neurotropic agent that is transported via the peripheral nerves from the site of inoculation to the brain, where it replicates massively, subsequently spreading centrifugally to the salivary glands and

other peripheral innervated tissues [5]. The major neurological signs appear at least five to six days after the virus has reached the central nervous system (CNS) [6-7]. They include the classic encephalitic (or furious) form with hydrophobia and hyperactivity, but also a paralytic (or dumb) form, occurring in about 30% of cases [8]. The basic molecular mechanisms of this disease and its associated clinical signs remain unknown. However, electrophysiological studies of the nerve and muscle in paralytic rabies patients have shown that different neural structures are involved in encephalitic and paralytic rabies in humans. Peripheral nerve dysfunction (axon- and myelinopathy) underlies the weakness observed in paralytic rabies [7, 9]. Subclinical anterior horn cell dysfunction is evident only in furious rabies [7]. Rabies-infected dogs are a relevant model for studies of human rabies [10]. Little or no apparent histopathological modification is observed in infected brains and neurons. The virus appears to induce neuronal dysfunctions, but these remain to be elucidated. In this context, effective antiviral targets remain to be identified, and there is currently no effective treatment for rabies once the clinical signs have appeared. Nevertheless, various compounds have been studied *in vitro* and in animal models, and several protocols have also been attempted in individual cases of human rabies.

In this review, we aim to present the state-of-the-art in the preventive and curative treatment of human rabies. We begin by considering general aspects of rabies and then describe the pre- and post-exposure prophylaxis (PrEP and PEP, respectively) currently available for rabies. Finally, we present an analysis of the various antiviral compounds tested for the treatment of RABV infection *in vitro*, in animal models or in humans, focusing in particular on potential new developments.

1 General considerations relating to rabies and lyssaviruses

1.1 Virus presentation

Lyssaviruses are enveloped bullet-shaped particles, rounded on one side and flat on the other, approximately 180 to 200 nm long and 75 nm in diameter. Their genome is composed of a single-strand, negative-sense, non-segmented RNA molecule almost 12,000 nucleotides in length [11]. The extremities (leader and trailer region at the 3' and 5' ends, respectively) of the genome are conserved and have inverted complementary sequences. The genome contains five monocistronic genes, encoding the five viral proteins, which are, in sequential order from the 3' to the 5' end: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the large protein (L) carrying all the enzymatic activities required to fulfill the function of an RNA-dependent RNA polymerase [12]. Each gene is surrounded by initiation and termination sequences. All the encoded proteins are encapsidated in the viral particle. The RNA genome is tightly associated with several copies of N protein in the viral helical nucleocapsid, the structure of which has recently been elucidated [13-14]. The nucleocapsid is associated with smaller amounts of P and L proteins. These two proteins form the polymerase complex and are responsible for the RNA polymerase activity associated with the virions, in association with the N-RNA complex. The M protein condenses the nucleocapsid and provides the virion with its bullet-shaped appearance. The structure of the full-length M protein has been determined, leading to the description of a model for the polymerization of this protein, potentially involving the insertion of the N-terminal part in the membrane and association with the nucleocapsid [15]. This model was recently confirmed and improved, with the results of cryo-electron microscopy for vesiculovirus, another rhabdovirus [14]. The viral envelope consists of a lipid bilayer with spike-like projections composed of trimers of G protein, a transmembrane protein with a very short cytoplasmic domain [16]. The lipids of the envelope are derived from the host cell membrane, from which

the virus buds. The G protein is responsible for virus attachment to and penetration into the cell.

1.2 Viral replication cycle

Lyssaviruses have a broad cellular tropism *in vitro* after adaptation to cell culture. These viruses use the exposed region of the glycoprotein to target different surface components, facilitating their entry into host cells. The surface components targeted include specific peripheral membrane receptors, such as the α subunit of the nicotinic acetylcholine receptor (nAChR) on muscle cells, and also neuronal membrane receptors, such as nAChR, the neural cell-adhesion molecule (NCAM or CD56) and the low-affinity nerve growth factor receptor (p75NTR) [17-19]. The significance of these non peripheral receptors remains unclear. Other molecules, such as phospholipids and sialylated gangliosides, may also be used, in a non specific manner, for viral entry [20-23]. Following attachment, RABV enters the cell, mostly via the endocytic pathway, and releases its ribonucleocapsid into the cytoplasm after membrane fusion mediated by a pH-dependent conformational modification of the glycoprotein. The viral genome is transcribed by the polymerase complex, to produce capped and polyadenylated monocistronic messenger RNAs corresponding to each of the five viral genes. Transcription starts at the 3' end (leader region) of the genome and continues to the other end, with a gradient in the amount of each transcript as a function of its order and distance from the 3' end, regulating the relative abundance of the various viral proteins. These mRNAs are translated by the cellular protein synthesis machinery. There is then a switch from transcription to replication, regulated by a subtle mechanism dependent on the quantity of N protein produced. This leads to the generation of intermediate RNAs (antigenome), which act as templates for progeny genomes, and genome molecules then become predominant. Once sufficient quantities of viral proteins and RNA genomes have accumulated in the infected cell, ribonucleoprotein complexes form and virus particles are assembled near

the plasma membrane, from which mature virions are released after budding.

1.3 Taxonomy and epidemiology of lyssaviruses

1.3.1 Taxonomy

Rabies virus is the prototype species of the genus *Lyssavirus*, which belongs to the family *Rhabdoviridae*, order *Mononegavirales* [24]. Lyssaviruses constitute a distinct monophyletic clade less divergent than other genera in this virus family [25-27]. The classification of these viruses is based on a combination of knowledge from serological studies and phylogenetic analysis. Serotypes and species (formerly genotypes) were initially used to separate lyssaviruses. Species classification is based on the similarity of the N gene sequence. The cutoff point for species separation was initially set at 80 to 82% nucleotide identity [28]. Based on this cutoff point, 11 different species of lyssaviruses have been recognized by the International Committee on Taxonomy of Viruses (ICTV) (Table 1) [24] (Figure 1). They include the classical rabies virus (RABV) species (formerly genotype 1) and rabies-related lyssavirus species, including the three African lyssaviruses Lagos bat virus (LBV), Mokola virus (MOKV) and Duvenhage virus (DUVV), the two European bat lyssaviruses type 1 (EBLV-1) and type 2 (EBLV-2), and the Australian bat lyssavirus (ABLV) (formerly genotypes 2 to 7, respectively). Four new lyssavirus species have also been recognized: the Irkut (IRKV), Aravan (ARAV), Khujand (KHUV) and West Caucasian bat (WCBV) viruses, each isolated once from insectivorous bats in Eurasia [29-30]. Another two bat lyssaviruses have recently been identified: Ozernoe, which was identified in a fatal case of human rabies, probably contracted after a bat bite nearly one month before the onset of symptoms and in the Far East of Russia during 2007 [31], and Shimoni bat virus (SHIBV), which was isolated from an insectivorous bat in Kenya in 2009 [32].

Species definition criteria based solely on N gene nucleotide sequence have been shown to be insufficiently powerful to discriminate between closely-related lyssaviruses. Indeed, as the number of new and/or full-length genome sequences available increases, rigorous phylogenetic analysis based on these complete sequences is likely to provide the highest level of discriminatory power for species identification [11]. For example, the results of such analyses have suggested that viruses currently classified as LBV species (may actually correspond to several different species [11].

1.3.2 Epidemiology

Rabies is widely distributed throughout the world and is present on all continents, with the exception of certain specific regions, such as Antarctica and some islands (Table 1) [5, 33]. RABV has the broadest geographic distribution of the 11 species of lyssaviruses identified to date, and the widest spectrum of vectors or reservoirs within the orders *Carnivora* and *Chiroptera*. Moreover, RABV has been isolated from almost all the orders of terrestrial mammals. The dog remains the main reservoir and vector of RABV, and is responsible for almost all rabies infections in humans, particularly in Asia and Africa [2]. However, many other species of carnivores are involved in the maintenance and transmission of RABV worldwide. These species include other species from the family *Canidae*, such as red foxes and raccoon dogs (particularly in Europe), but also arctic foxes in polar regions, gray foxes and coyotes in North America and jackals in Africa. Skunks (family *Mephitidae*) and raccoons (family *Procyonidae*) also represent major terrestrial reservoirs in North America and rabies virus circulation has been observed in mongooses (family *Herpestidae*) in Africa and in the Caribbean. Many other terrestrial mammal species are susceptible to rabies but do not transmit the disease further, acting as epidemiological dead-end hosts (including livestock species, such as cattle and horses). The dog has been identified as the probable main vector involved in inter-species RABV transmission [34]. Bats have been identified as an active

reservoir and vector for RABV only in the Americas, where the chief species involved are insectivorous bat species in North and Latin America and vampire bats in South America [8]. All species of lyssaviruses other than RABV species have been isolated from bats, which are thought to be the sole reservoir of these species, with the exception of MOKV, which has been isolated, in Africa only, from shrews, cats, dogs and a rodent, but for which the reservoir species remains to be identified [35]. LBV and DUVV have been isolated from frugivorous and insectivorous bats, respectively, in Africa [35]. Lyssaviruses EBLV-1 and EBLV-2 circulate in specific species of insectivorous chiropterans in Europe [36] and ABLV has been isolated from insectivorous and frugivorous bats in Australia [37]. The various new lyssavirus species identified more recently — ARAV, IRKV, KHUV and WCBV, and the tentative SHIBV and Ozernoe species — were all isolated from bats, suggesting that there may be more, as yet undiscovered species of lyssavirus in the order *Chiroptera* (Table 1) [29-32, 38]. Moreover, the considerable diversity of the viruses circulating in bats has led to suggestions that RABV may have originated from chiropteran lyssaviruses [39]. Spill-over transmissions of bat lyssaviruses (EBLV-1, LBV) to carnivores occur, as demonstrated by a number of reported cases, but without successful adaptation or propagation [35, 40].

From a public health standpoint, the complex epidemiological context of rabies poses a continual challenge and threat. Effective mass vaccination campaigns have greatly decreased the incidence of rabies in dogs and have proved efficient for rabies control in developed countries. However, canine rabies remains an increasing problem in Asia and Africa. Successful rabies eradication within wild reservoirs is also possible, as illustrated by the various oral vaccination campaigns in red fox carried out in Western Europe [41]. However, it may be difficult to transpose these achievements to large geographic regions containing multiple wild reservoirs. Finally, new reservoirs may emerge in rabies-free areas, in Western Europe for example, as already observed for the red fox and raccoon dog, or may be identified

for the first time, particularly for bats. Furthermore, the reintroduction of rabies through the transport of uncontrolled domestic carnivores (particularly dogs) or through the migration of wild rabid reservoirs across borders (such as the red fox in Italy) poses a real threat [41-42].

1.4 Pathogenesis

Rabies is transmitted by inoculation with infected material (mostly saliva) through the skin, into muscle and subcutaneous tissues (mostly after bite wounds). Inoculation may also occur through scratches or the licking of mucous membranes or broken skin. The efficacy of virus transmission depends on several factors, including the characteristics of the inoculation process (severity, location and number of bites, for example), the characteristics and dose of the virus, and the susceptibility of the host [5, 33, 43-44]. Other routes of transmission have been described but have a limited impact on rabies epidemiology in humans. They include aerosol contamination [45-47], contact with and the preparation of infected meat or carcasses [48] and human-to-human transmission through the transplantation of various solid organs and tissues, including the cornea and vascular conduits [49-51]. The transplacental transmission of rabies seems to be rare, as most of the infants born to rabid mothers are healthy [52]. After inoculation, RABV may reach the peripheral nerves directly, via the nAChR present at the neuromuscular junction. However, an initial period of virus multiplication may be observed, in the muscle, as in the skunk animal model [53], or in fibroblasts and epithelial cells in the dermis, as in experimental studies of RABV isolates of bat origin [54]. Direct inoculation of the nerve with the virus is also possible, as in the case of severe bites at the brachial plexus [55]. Having gained access to the peripheral nerves, RABV is transported to the CNS via the fast retrograde axoplasmic flux (experimentally estimated at 50-100 mm/day) within motor axons and, possibly, via the anterograde axoplasmic flux within sensory axons [56]. There is experimental evidence to suggest that viral phosphoprotein or glycoprotein is involved in this axonal transport, through interaction with

the light chain LC8 protein or after binding to p75NTR, respectively [57-59]. During this transport, the virus may replicate again in the dorsal-root ganglia and anterior-horn cells [43]. The virus acts as a specific neurotropic agent *in vivo*, replicating rapidly in infected neuronal cells. The transneuronal migration of the virus along neuroanatomical pathways is not well defined, but *trans*-synaptic spread dependent on the presence of the viral glycoprotein has been suggested [60]. Once the virus has gained access to the brain, it disseminates rapidly, particularly in specific anatomical areas such as the brainstem, thalamus, basal ganglia and spinal cord [61]. In the terminal phase of infection, the virus spreads, by axonal anterograde transport, to peripheral sites, including the salivary glands (necessary for the transfer of the virus through saliva and for propagation of the infectious cycle in a natural reservoir) and other innervated tissues [62-63].

Despite the severe clinical neurological signs observed in rabies cases, no major histological change can be observed in the brains of rabid patients, and cellular abnormalities are limited in infected neurons. It has been suggested that major dysfunctions in infected and uninfected neurons are the main cause of the neurological symptoms. Several hypotheses have been tested, but no clear or definitive conclusion has been drawn. The hypotheses tested include abnormalities of neurotransmitter functions affecting serotonin, opioids, gamma-aminobutyric acid (GABA), acetylcholine, the involvement of neuronal membrane ion channels, excitatory amino acids, apoptosis, nitric oxide, electrophysiological alteration or cellular RNA and protein synthesis [5, 43-44]. However, peripheral nerve dysfunction has been implicated in the weakness observed in paralytic rabies, whereas anterior horn cell dysfunction may be associated with the furious form of rabies [64].

1.5 Clinical presentation of rabies encephalitis

1.5.1 Human rabies

Rabies is an acute, almost invariably fatal form of viral encephalomyelitis, but is preventable provided that correct prophylaxis is administered soon after exposure (see section 2.2.2). Only a very small number of patients developing symptoms have survived (see section 3.2.1). In addition to RABV, viruses from all the other known lyssavirus species have either been shown or are predicted to cause rabies encephalitis in humans. Classically, the clinical features of the disease can be divided into five stages, including the incubation or asymptomatic period, the prodrome, the acute neurological phase, coma and death [43, 65]. The mean incubation period is typically 20 to 60 days, but incubation periods of less than seven days or up to several years have been reported [43, 66]. Short incubation periods have been associated with the direct inoculation of nerve tissue, including the brain, in particular. Post-exposure prophylaxis is effective only during the incubation period, and should be administered as soon as possible after exposure (see section 2.2.2). The non specific prodromal symptoms last for one to 10 days and may include fever, headache, anxiety and irritability [43, 66-67]. Pain, paresthesia or pruritus close to the site of viral inoculation is commonly described in rabid patients. It has been suggested that that the prodromal clinical signs are related to the centripetal migration of the virus from the peripheral nerves to the dorsal-root ganglia and, finally, to the CNS [43]. Once the virus reaches the CNS, it may cause one of two distinct forms of the disease: classical or encephalitic (furious) rabies or paralytic (dumb) rabies, which affect about 70% and 30% of infected patients, respectively. The encephalitic form of rabies usually includes fluctuating consciousness with intermittent episodes of confusion, hallucinations, agitation or aggressive behavior [43, 66]. Signs of autonomic dysfunction are also observed, with hypersalivation, excessive sweating, dilated pupils, piloerection or priapism. Inspiratory or phobic spasms occur mostly in the encephalitic

form, and include hydrophobia (in about 50 to 80% of patients), a characteristic and unique manifestation of rabies, which may occur in association with aerophobia, although these two manifestations may occur independently. Fever and dysphagia are generally associated with encephalitic rabies, and seizures may also be observed. These signs classically persist for one to four days, subsequently progressing to severe flaccid paralysis, coma and multiple organ failure. Patients generally die after paralysis of the cardiorespiratory system, which occurs a mean of five days after onset of symptoms, in the absence of intensive medical care [43, 66]. The paralytic form of rabies remains difficult to diagnose and may be confused with Guillain-Barré syndrome. Flaccid muscle weakness develops early in the course of the disease and may be associated with sphincter involvement, myoedema and phobic spasm, including hydrophobia. Patients presenting paralytic rabies may survive for almost two weeks, with death resulting from respiratory paralysis [43, 65].

1.5.2 Animal rabies

Natural rabies infection causes an acute neurological illness in almost all mammalian species. As in humans, the initial signs of rabies are non specific, but both clinical forms of rabies can be observed in infected animals. Indeed, aggressiveness and hyperactivity are frequently observed in carnivores with encephalitic rabies. Paralytic forms have also been described, and both clinical forms may occur alternately in the same infected animal. Dramatic changes in behavior, such as wild animals losing their fear of humans, may be an indication of a rabies infection. Death generally occurs within two weeks of the onset of symptoms, whatever the clinical form observed. However, asymptomatic episodes and survival have been observed or suggested in various species, following the detection of rabies antibodies or viral RNA in apparently healthy animals, including mongooses, skunks, raccoons, dogs, foxes, hyenas, jackals, frugivorous and insectivorous bats [5, 33].

1.6 Diagnosis

There is currently no test for the diagnosis of rabies infection before the onset of clinical disease. The clinical diagnosis of rabies therefore remains difficult and unreliable. In humans, signs of hydrophobia or aerophobia, when present, may be indicative of rabies infection. However, rabies diagnosis must be confirmed by laboratory techniques, using approved and validated methods [68]. Post-mortem diagnosis in animals with suspected rabies is based on methods for detecting viral antigens with the gold standard fluorescent antibody test (FAT) [69], ELISA [70-71], the recently developed direct rapid immunohistochemical test (dRIT) [72] and the rapid immunodiagnostic test (RIDT) [73], or viral isolation techniques using the rapid tissue culture infection test (RTCIT) (which should replace the mouse inoculation test (MIT)) [68, 74]. Post-mortem diagnosis in humans can be achieved by applying the same techniques to brain biopsy specimens. However, biopsy is not always feasible and the detection of viral RNA in skin biopsy specimens may be an interesting alternative for the diagnosis of rabies encephalitis [66]. Several biological samples can also be used for the diagnosis of rabies in living human patients, with lyssavirus RNA detection with molecular diagnostic tools, including RT-PCR, real-time PCR or other techniques, such as nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) [75-77]. The samples suitable for such analyses include saliva, cerebral spinal fluid (CSF), skin biopsy specimens containing hair follicles collected at the nape of the neck, extracted hair follicles, tears and urine [66, 77]. In a recent study, a sensitivity of more than 98% was demonstrated for tests on single skin biopsy samples, regardless of the time at which the sample was collected (i.e., from 1 day after the onset of symptoms to just after death), with a sensitivity of 100% reported when at least three successive saliva samples per patient were analyzed [66]. When possible, the positive detection of viral RNA in samples should be confirmed by sequencing, to exclude false-positive results [75]. This also makes it possible to

genotype viral strains, which is useful for epidemiological surveillance. Viral antigens can also be detected in skin biopsy specimens by FAT, and it is possible to isolate the virus from several types of bodily fluid sample, including saliva and CSF. Antibodies against rabies can also be detected in living patients, by the reference techniques, the rapid fluorescent focus inhibition test (RFFIT) [78] and the fluorescent antibody virus neutralization test (FAVN) [79], but also with ELISA [80-82]. However, it is rather difficult to determine rabies antibody titers from serum until eight days of illness have elapsed, and detection does not become possible until even later in the CSF (lower titers than for serum). In cases of infection with dog variant RABV, as in Thailand, CSF antibody remains undetectable regardless of the time after onset [75, 77]. In bat variant RABV cases, it is possible to detect antibody after eight days.

2 Post-exposure rabies prophylaxis: from history to future development

2.1 Historical development of postexposure prophylaxis

2.1.1 The preliminary works of Louis Pasteur

Pasteur's work on a vaccine for rabies occurred in the context of intense research on rabies in France. Pasteur reported the first successful transmission of rabies in rabbits to the Academy of Sciences in 1881. This transmission was achieved by inoculating the CNS tissues and spinal fluid, demonstrating that the causal agent was present not only in the saliva, but also in the tissues of the nervous system. Pasteur also observed that intracerebral inoculation with infected brain material in dogs, an innovation of his associate, Roux, shortened the usual incubation period to 1 to 2 weeks, making this system a useful laboratory model for the disease [83-84]. The following year, Pasteur described a small number of cases of animals recovering from rabies after experimental inoculation and that were subsequently immune to the virus [85].

By 1885, Pasteur had formulated the fundamental tenets of his vaccination strategy [86]. Through a series of passages in rabbits by intracerebral inoculation, Pasteur was able to create a form of rabies with enhanced virulence, decreasing the incubation time and “fixing” it at seven days. The virus populated the entire length of the spinal cord in rabbits, and became progressively more attenuated when infected tissue was suspended in dry air. The tissue lost all virulence after 15 days of desiccation. Dogs repeatedly inoculated with a series of rabbit cord suspensions of increasing virulence, or tissues that had been dried for shorter periods, displayed resistance to rabies, even when challenged via the intracerebral route, presumably due to the development of strong immune responses. Pasteur used 50 dogs for these experiments, a large number even by current standards. The animals were generally challenged with rabies only after the series of vaccinations [87].

2.1.2 The first human vaccinations against rabies

Pasteur recognized that, given the unpredictable occurrence and clinical biology of rabies, a vaccine would be most beneficial if it could be given after a bite had occurred and if it induced protection over a time period shorter than the clinical incubation period of the disease. In modern terms, he was describing post-exposure vaccine prophylaxis for the prevention of symptomatic disease. Human use of the vaccine was associated with significant risk and controversy, but Pasteur justified such risks based on the invariably fatal outcome of rabies infections.

The first real opportunity for Pasteur to test his novel vaccine “treatment” for human rabies occurred in July 1885 and involved nine-year-old Joseph Meister, from Alsace, who had received numerous, severe bites from a rabid dog but had not yet developed symptoms of rabies. Pasteur, who was not medically qualified, arranged for the boy to be examined by two physicians [88]. Treatment started about 60 h after bites, an acceptable delay for post-exposure prophylaxis by today’s standards. The boy was inoculated in the skin of the right

upper abdomen, with a suspension of 15-day-old desiccated spinal cord from an infected rabbit, corresponding to the most attenuated viruses in the regimen. He received another 12 inoculations over the next 10 days, each less attenuated than the last and hence containing more virulent rabies virus. The last spinal cord preparation was dried for only one day [86]. Meister returned home, feeling well, after three weeks in Paris.

The Meister episode was a major accomplishment, but it did not attract the attention of the world to the subsequent use of rabies vaccines in humans by Pasteur. Later in 1885, a 15-year-old shepherd from a small rural village in the Jura, Jean-Baptiste Jupille, was sent to Pasteur after being bitten several times on his left hand by a rabid dog. Again, the risk of rabies was high, but the treatment was successful. This time, the success of the vaccination was reported worldwide. In no time, Pasteur was treating potentially infected people from many different nations. By the end of 1886, more than 2,000 people had received Pasteur's rabies vaccine regimen [89] and only a few rare failures were reported. In the 1898 annual report of the Institut Pasteur, 96 deaths were reported among 20,166 treated patients, a mortality rate of only 0.5% whereas that for unvaccinated individuals was 16% [90].

2.1.3 Further developments of Pasteur's treatment

Over the next 50 years, the vaccine underwent various modifications. Early on, Pasteur's colleagues began experimenting with variations on the number and timing of inoculations, and of the method for preparing rabbit spinal cords, based on their increasing experience and the individual history of exposure of each case. Many colleagues and students of Louis Pasteur exported this methodology to other parts of the world, in which new Pasteur Institutes opened.

Early in the 20th Century, methods involving the inactivation of the virus with phenol were introduced, to minimize the risk of vaccine-induced disease [91]. This approach was sporadically associated with incomplete inactivation and subsequent vaccine-induced

paralysis or serious adverse events related to hypersensitivity to nervous tissues in the vaccine, but nonetheless eventually replaced the method of Pasteur, continuing to be used into the 1950s.

2.1.4 The development of safer products

Vaccines derived from nervous system tissues were known to be associated with allergic encephalomyelitis, triggered by hypersensitivity to residual myelin basic protein in the preparations, causing severe demyelination in some recipients [92]. This led to the discontinuation of Pasteur's method of vaccine preparation in 1953. At the end of the 1950s, Fuenzalida and coworkers introduced the first myelin-free inactivated vaccines against RABV, prepared from neonatal mouse brains. These vaccines are still used in some parts of the developing world, due to their low cost, but their production and use are not recommended by the World Health Organization (WHO) [1, 93]. The recommended approaches are instead based on tissue culture methods developed in the 1950s and 1960s subsequently improved by other technical modifications. The duck embryo vaccine (DEV) was prepared from virus propagated in embryonated duck eggs [94]. The Swiss Serum and Vaccine Institute later improved the DEV by using density gradient ultracentrifugation to generate a purified DEV (PDEV). The PDEV is still produced today and used for human vaccination worldwide [91]. In the early 1960s, Hilary Koprowski and his colleagues at the Wistar Institute, Philadelphia, selected the human diploid cell line WI-38, to avoid the problems inherent in the use of primary tissue cultures [95-97]. They adapted the Pitman–Moore strain of rabies virus to WI-38 cells and the free virus was inactivated with β -propiolactone and then concentrated by ultracentrifugation [98]. This human diploid cell vaccine (HDCV), now prepared from the MRC-5 human diploid cell line, induced much stronger immune responses in animals and humans than any of the other vaccines known at the time. Another vaccine, the purified chick embryo cell vaccine (PCECV), was prepared

from the RABV strain Flury low-egg passage (LEP), grown in primary cultures of chick embryo fibroblasts [99-100]. The virus was inactivated with β -propiolactone, then purified and concentrated by zonal centrifugation in a sucrose gradient [101-102]. The use of this vaccine is essentially restricted to the Americas and Asia. The last vaccine, developed at the end of the 1980s, was the purified Vero cell rabies vaccine (PVRV), based on the Pitman–Moore strain of the RABV produced in the Vero cell line, a continuous African green monkey kidney cell line [103]. This vaccine is inactivated with β -propiolactone and concentrated and purified by zonal centrifugation and ultrafiltration. PVRV is licensed for use in humans in Europe and in many countries of the developing world. PCECV and PVRV have safety and efficacy records equivalent to those of HDCV and are cheaper [99-100, 104]. These purified cell culture and embryonated egg-based rabies vaccines are designed for both PrEP and PEP and have been administered to millions of people worldwide.

2.1.5 Societal and economic problems encountered

About 15 million people receive post-exposure vaccination worldwide each year [105]. In industrialized countries, animal vaccination, developed in parallel with that for humans, has almost eliminated rabies as a significant problem. Unfortunately, in developing parts of the world, rabies remains a global public health threat. In these countries, many people who are exposed to rabies do not seek PEP, because they are not even aware of the risk of contracting this deadly disease, or because they live in rural areas, too far away from rabies prevention centers, which are generally located in large cities. Instead, traditional healers are frequently consulted and various types of wound care applied. When subjects at risk of contracting rabies do consult, it is often too late. Furthermore, the cost of rabies prevention or PEP is often too high [106-107]. Vaccines and immunoglobulins remain relatively expensive for much of the target population. The mean cost of the rabies vaccine alone for PEP is \$45 in Africa and Asia (where the average salary is \$2 person-day).

2.2 State-of-the art for rabies prophylaxis

2.2.1 Pre-exposure prophylaxis

PrEP is recommended for people frequently or continuously at risk of exposure to rabies, such as laboratory workers dealing with rabies, veterinary surgeons and animal handlers in countries in which rabies is enzootic. It is strongly recommended for travelers with extensive outdoor exposure in these countries, when access to appropriate medical care is limited. Young children visiting or living in areas in which the disease is enzootic should also be vaccinated, as they are more frequently exposed than adults and may not report potential exposure to their parents [93]. Two protocols, by the intramuscular (IM) and intradermal (ID) routes, are recommended by the WHO [93]. IM doses of 0.5 or 1 ml (depending on the type of vaccine) are administered on days 0, 7, 21 or 28, in the deltoid area (adults and children >2 years old) or the anterolateral area of the thigh (children < 2 years old). ID administrations are performed on the same days, but with only 0.1 ml. Booster injections are given only to those at regular risk of exposure [93]. Monitoring of the titer of rabies antibody may be useful for limiting the number of injections. A booster injection is required when titers fall below 0.5 IU/ml.

2.2.2 Post-exposure prophylaxis

Decisions regarding prophylaxis are complex. The indication for PEP depends on the type of contact with the suspected rabid animal, as defined by the WHO [93] (Table 2), but other factors should also be taken into account when deciding whether to initiate PEP. These factors include the epidemiological situation of the country in which exposure occurred, the circumstances in which exposure occurred, the clinical features of the animal, if known, and the availability of the animal for veterinary observation. For cases in countries in which rabies is enzootic, PEP, if required according to WHO criteria, should not be delayed on the basis of

epidemiological aspects or the clinical features or vaccination status of the animal. However, PEP may be discontinued if the animal remains healthy after the observation period, starting from the day of the bite (10 days according to the WHO and 15 days according to the legislation in force in some countries). For exposure categories II and III, thorough (~15 minutes) washing of all bite wounds and scratches, which should be flushed with soap/detergent and copious amounts of water, should be carried out immediately, or as soon as possible.

Data concerning the efficacy of prophylaxis are provided by experiments in animals and clinical trials. The PEP schedules recommended by the WHO are safe and highly effective. PEP consists of three primary elements: wound care, infiltration of rabies immune globulin into the wound and vaccine administration. These three components are reviewed below. Other measures, such as the use of tetanus toxin or antibiotics, are applied as needed. Decisions are urgent, because delays may affect the outcome of treatment [108]. When completion of PEP with the same cell culture or embryonated egg-based rabies vaccine is not possible, another vaccine of a similar category may be used instead.

2.2.2.1 IM administration

The schedule of PEP vaccination consists of either four or five doses of vaccine [93] (Figure 2). A dose of 1 or 0.5 ml is administered, depending on the type of vaccine used. Doses are delivered by the IM route, to the deltoid muscle (or the antero-lateral thigh in children < 2 years of age).

2.2.2.2 ID administration

Many studies have demonstrated the safety and efficacy of the IM delivery method, but modern vaccines are too expensive for many developing countries [106-107, 109]. ID vaccine regimens, which use smaller amounts of vaccine and are less costly, therefore play an

important part in strategies to reduce the incidence of rabies. Initial studies demonstrated the safety and dose-sparing benefits of this route. Successful experience in Thailand has encouraged other countries to implement rabies PEP via the ID route and has led vaccine producers to validate the use of these ID regimens with their new vaccines [110-111]. A two-site regimen, with the injection of 0.1 ml at 2 sites (deltoid and thigh) on days 0, 3, 7 and 28, is currently recommended by the WHO [93]. ID delivery methods for rabies vaccine have been thoroughly investigated in humans and subjected to expert panel review by the WHO, but a number of precautions are required when using the ID technique, including staff training, attention to the conditions and duration of vaccine storage after reconstitution and the use of appropriate 1 ml syringes and short hypodermic needles.

2.2.2.3 PEP in previously vaccinated individuals

One IM or ID dose of vaccine on days 0 and 3 or four ID doses (equally distributed over the left and right deltoid or prescapular areas) at a single visit should be administered as a booster in patients exposed to rabies that have previously received preventive vaccination or a complete course of PEP with a cell culture or embryonated egg-based rabies vaccine [93].

2.2.2.4 Passive immunization against rabies

According to WHO guidelines, rabies exposure requires post-exposure vaccination, and rabies immunoglobulins (RIGs) in cases of severe (category III) exposure [93]. The life-saving benefit of adding specific RIG to post-exposure treatments of subjects exposed to rabies has been clearly established, particularly in cases of severe wounds. The underlying rationale is that many studies and observations have confirmed the importance of immediate administration of RIG to inhibit viral spread during the first seven to 14 days, before the immune response to the vaccine becomes sufficiently strong. Unfortunately, in many developing countries, only limited quantities of RIGs may be available via the private sector

and most of those at high risk of rabies cannot afford them. There are two types of product on the market: human rabies immune globulin (HRIG) and equine rabies immune globulin (ERIG). Crude equine sera have gradually been replaced by ERIGs of various degrees of purity.

Modern commercial preparations of HRIG are much safer than heterologous sera. They may contain antibodies against other agents and may inhibit immune responses to viral vaccines that have not been inactivated. Interference depends on the amount of specific antibody present. The administration of vaccines against other diseases, such as measles and varicella, should be delayed for at least four months after PEP, to allow the degradation of HRIG. Interest has been growing, in developing countries in which rabies is endemic, in the use of purified ERIG in PEP for rabies, because HRIG is unavailable or unaffordable. Purified ERIG has been reported to generate adverse reactions in about 1% of patients. Life-threatening reactions are very rare. Serum sickness-like reactions to purified ERIG are also rare (less than 0.05%) in patients under the age of 10 years [112-116]. In this context, the use of highly-purified ERIG represents a safe and effective alternative. Some of these preparations are digested with pepsin (to remove the Fc part of the immunoglobulin molecules, which might be responsible for some adverse reactions), yielding $F(ab')_2$ fragments, corresponding to the antigen-binding part of the molecule. These preparations are effective and safe [112].

2.2.2.5 Management of immunocompromised patients

Immunocompromised patients should receive the full course of PEP vaccination by the IM route and the vaccination should be completed by passive immunization for both category II and category III exposures [93]. When feasible, the rabies virus neutralizing antibody titer of the patient should be checked two to four weeks after the last dose of vaccine, to assess the possible need for an additional dose of vaccine.

2.2.2.6 Adverse effects and contraindications

The purified cell culture and embryonated egg-based rabies vaccines are safe and well tolerated. However, transient pain, redness and swelling at the site of injection occur in 35 to 45% of vaccinated patients, particularly after ID injections. Occasionally, more severe reactions may occur, such as transient fever, headache, dizziness and gastrointestinal symptoms. There is no contraindication for vaccination, because rabies infection is fatal. Individuals taking chloroquine for malaria treatment may have a weaker response to rabies vaccination, in which case, the IM regimen of PEP is recommended. If a previous severe reaction is documented, the use of a different rabies vaccine is recommended.

2.3 Future developments

Several new approaches to vaccine production have been developed: the production of rabies antigens in baculovirus [117] and the development of edible vaccines through the expression of rabies antigens in plants [118-121]. Recombinant poxvirus vaccines are used extensively in animals and have been evaluated for human use. A canarypox vector encoding the rabies G protein has been prepared and tested in humans [122]. Canarypoxvirus, unlike vaccinia virus, cannot replicate in humans and is therefore unlikely to elicit any serious side effects. Recombinant adenoviruses encoding the G protein have also been tested in mice and dogs [123]. Furthermore, recombinant DNA vaccines are currently being developed for use in humans and in dogs [124-128]. However, none of these approaches is likely to graduate to use in clinical settings in the near future.

Several technological approaches currently being used to develop human monoclonal antibody mixtures have yielded promising results. Human monoclonal antibodies directed against non overlapping epitopes of the G protein have been shown to mediate the broad neutralization *in vitro* of a large panel of field isolates of RABV from various animal species,

and to ensure *in vivo* protection in a Syrian hamster rabies challenge model; they have also been demonstrated to be effective in phase I trials in humans [129-133]. The successful development of such antibodies would help to ensure the supply of life-saving biological drugs to people exposed to rabies, at a reasonable cost.

3 In search of an effective antiviral treatment for human rabies: where are we now?

During the progress in our understanding of RABV infection, the various steps of the viral cycle have been considered as potential antiviral targets for preventing, limiting or stopping the infection. With this goal in mind, the antiviral activities of various drugs thought to affect early events in the cycle of RABV replication (including virus entry and release into the cytoplasm of infected cells) and transcription/replication steps, have been evaluated *in vitro* or in animal models. In parallel, well characterized broad-spectrum antiviral compounds, such as interferons and inducers of interferon, and molecules with no suspected antiviral activity have been assessed for use against rabies infection. Some of these compounds were also administered, alone or in combination, in cases of clinical human rabies. However, although some studies have reported interesting preliminary results *in vitro*, no drug has been shown to be effective, with convincing and reproducible activity against infection in cases in which clinical signs are already present, in animal models or in humans. The successful outcome achieved in 2004, in which a rabid patient treated aggressively subsequently recovered restored some hope. Unfortunately, the efficacy of this protocol was not subsequently confirmed, as all published attempts to reproduce these findings have been unsuccessful. The quest to identify an effective and potent antiviral treatment against human rabies therefore continues.

In this section, we present an overview of the various drug evaluations performed *in vitro* and in animal models (described in Table 3), followed by the major drug treatments administered in cases of human rabies, including the case of the Milwaukee protocol and subsequent

unsuccessful attempts at replication (described in Table 4). This section ends with a brief description of potential future advances in the field of antirabies drug discovery.

3.1 Overview of drugs evaluated for activity against rabies virus *in vitro* and in animal models

3.1.1 Inhibitors of viral entry into the cell

The attachment of viruses on target cells and their penetration into those cells are of great interest when considering an antiviral strategy. Indeed, blocking or disturbing these early steps in the cycle of virus replication may decrease or abolish infection. With this aim in mind, viral proteins involved in cell entry (such as viral glycoproteins) and the binding domains of the cellular receptors are considered potential antiviral targets. Several different compounds have been tested in cultured cells infected with rabies. Some of these compounds, including drugs targeting the nicotinic acetylcholine receptor (nAChR) and various lipid molecules and molecules with high affinity for lipids, have been found to have potential antiviral effects.

3.1.1.1 Antibodies against rabies glycoprotein and rabies vaccines

The antiviral activities of rabies antibodies targeting the viral glycoprotein have been clearly demonstrated *in vitro* and in animal models. The efficacy of these compounds is now used, in combination with rabies vaccines, in PEP, to prevent rabies infection (see section 2.2). However, the use of antibodies remains of limited value against clinical rabies infection, for which no efficacy has been demonstrated (see section 3.2.2.2.4).

Live attenuated rabies vaccines have been investigated for the treatment of symptomatic rabies encephalitis in various animal models, including dogs, monkeys and mice. No convincing results have been obtained, despite the potential induction of neutralizing antibodies in the CNS and/or CSF after intrathecal or intracerebral administration [134-136].

Indeed, although promising results were obtained in a preliminary experiment in dogs, in which three symptomatic animals (of 12) treated intrathecally with a live attenuated ERA-derived rabies vaccine appeared to recover, a second experiment based on the same treatment resulted only in the probable prolongation of the survival period, with all the symptomatic dogs eventually dying [134]. In another study conducted in monkeys, a live attenuated Flury LEP-derived rabies vaccine was also administrated intrathecally at the clinical stage, but without success, as all symptomatic animals (n=2) succumbed with no detectable prolongation of the survival period [136]. A highly attenuated live recombinant RABV (SPBAANGAS-GAS-GAS) derived from the SAD B19 strain and expressing three mutated G genes (mutation R333G), has been evaluated for the prevention of rabies infection (for PrEP or PEP) in the mouse model. Interesting results have been obtained in the preexposure context, but the complete protection of mice was observed only if attenuated recombinant RABV was administered intramuscularly or intracerebrally shortly (4 hours) after infection. When administered at a time (4 days post-infection) close to the predicted onset of the first symptoms (5-6 days post-infection in this model), survival rates fell to 50% and 30% after intracerebral and intramuscular route of administration of the live attenuated vaccine, respectively. Although potentially promising, effective treatments of human clinical rabies based on such strategies appear to be some way off. Moreover, another major obstacle to the use of live attenuated rabies vaccines in humans is the safety of these vaccines, which must be extremely high, particularly for rabies and taking into account the existence of an effective and safe, albeit complex and costly vaccine [137].

3.1.1.2 Receptor-specific inhibitors

Alpha-bungarotoxin (α -Btx) is a high-affinity irreversible competitive antagonist of AChR that interacts irreversibly with the ACh binding site on the 40 kDa subunit of this receptor.

This drug is a snake venom neurotoxin, obtained from the Taiwanese banded krait, *Bungarus multicinctus*, which inhibits rabies infection in various types of cultured cells, including chick embryo muscle cells [17], cultured primary rat myotubes [138] and IMR-32 human neuroblastoma cells (continuous nerve cell line expressing neuronal nicotinic acetylcholine receptors) [139]. Competition between α -Btx and RABV was observed shortly before infection (from 2 h to 30 min), with a dose-dependent response (from 10^{-5} to 10^{-7} M in cultured primary rat myotubes) [17, 138-139]. However, the antiviral effect of α -Btx was not observed when cells were treated later, 15 minutes before infection, at the time of infection or a few hours later [17, 138, 140]. No virucidal effect was identified [17]. In a single study, an inhibitory effect was also demonstrated with d-tubocurarine (dTC), a cholinergic antagonist isolated from the South American plant *Chondrodendron tomentosum*, when cells were treated before infection [17]. Like α -Btx, this toxin interacts with the ACh binding site of the AChR and reversibly and competitively inhibits the binding of α -Btx. However, the prevention of rabies infection by this drug was not replicated in other studies [139-140].

These toxins probably act by inhibiting the attachment step, as demonstrated for α -Btx with A/J (H-2) mouse neuroblastoma cells [141]. These observations, together with experiments aiming to demonstrate the colocalization of rabies virus and AChR, led to the identification of AChR as a rabies virus receptor. Moreover, the amino-acid sequences of the toxic loop of α -Btx and the G protein of rabies virus have been shown to be very similar [142] and synthetic peptides of the major neurotoxin determinant on the $\alpha 1$ subunit of AChR inhibit infection [139]. Both then compete for attachment sites on the AChR in *Torpedo* sp. [142-144]. However, the potential value of this drug as an antiviral agent for treating rabies infection remains limited, because α -Btx acts as an inhibitor only at the viral binding step, in preventive action, and has not been shown to be virucidal. Moreover, AChR is not an obligate receptor for susceptibility to rabies infection in different cell types [138, 140].

Ligands of the NCAM receptor, which has been identified as another possible cellular receptor for RABV, also inhibit rabies infection *in vitro*. They include heparan sulfate, one of the natural ligands of this receptor, and antibodies targeting NCAM receptors [18]. In this same study, a reduction of virus infectivity was also observed after the incubation of rabies virus with soluble NCAM protein. However, drugs targeting this receptor appear to be of limited value, because NCAM receptor-deficient mice are not resistant to rabies infection [145]. Antiviral effects of two neurotrophin molecules (nerve growth factor and neurotrophin-3) have been evaluated on rabies-infected mouse dorsal root ganglia cell cultures [146]. The inhibition of viral infection has been suggested when these drugs were added at the time of infection or later.

3.1.1.3 Lipid-based inhibitors

Early in the 1980s, evidence was obtained that lipids, together with gangliosides and phospholipids, might also contribute to the binding of lyssaviruses to cell membranes [20-22]. A dose-dependent inhibition of viral infection was observed *in vitro* with chick embryo-related (CER) cells when RABV was incubated with gangliosides before infection. By contrast, the addition of gangliosides before infection did not modify infectivity. This observation suggests that highly sialylated gangliosides are part of the cellular membrane receptor structure for the attachment of infective rabies virus [20]. These results also suggest that competition occurs between RABV and gangliosides for binding to the cell surface, or possibly to aggregated formations, with the entrapment of virions, thereby preventing the infection of cells. An inhibition of viral attachment was also observed with phospholipids or phospholipase [20, 22]. Indeed, viral inhibition was clearly demonstrated with RABV in the same CER cell line treated with phospholipase A2 before infection, and, to a lesser extent, with various other phospholipases (phospholipase C, D and shingomyelinase). The prior

incubation of RABV with various phospholipids also exerted an inhibitory effect that was stronger than that observed when these compounds were added during infection (for L- α -phosphatidylserine and, to a lesser extent, L- α -phosphatidylethanolamine and L- α -phosphatidylinositol). However, targeting or using gangliosides and phospholipids for antiviral strategies against rabies infection appears to be of little value, as the inhibitory effects of such treatment are limited to the viral binding step. Indeed, the addition of these molecules before or after infection did not modify infection rates [20-22]. Moreover, these receptors are not specific and consist of multicomponent structures, the composition of which may differ between cell types.

3.1.1.4 Lectins and neuraminidases

Some studies have demonstrated an inhibitory activity of lectins — sugar-binding proteins that are highly specific for their sugar moieties — in rabies infections of CER cells [23, 147]. Concanavalin A, a lectin extracted from the jack-bean *Canavalia ensiformis*, which specifically binds to α -D-mannopyranosyl and α -D-glucopyranosyl residues of carbohydrate-containing components on the cell surface, has been shown to have a direct inhibitory effect on virion infectivity [23]. This inhibitory effect was observed when cells were treated before infection or after the viral binding step, and could be reversed by α -D-methylmannopyranoside. In a more recent study, the ability of 11 lectins of various origins to inhibit RABV infection was assessed [147]. Five of these lectins seemed to prevent rabies infection in CER cells: the agglutinins of *Ulex europaeus* (UEA), *Limulus polyphemus* (LPA), *Narcissus pseudonarcissus* (NPA), wheat germ (WGA) and *Bauhinia purpurea* (BPA). A dose-dependent effect was observed for LPA, NPA, WGA and BPA. It was suggested that LPA and NPA prevent virus attachment, whereas WGA and BPA inhibit infection at a later stage. Only two of these four lectins (NPA and BPA) have potentially useful selectivity indices [147].

Neuraminidases, which are also known as sialidases, are glycoside hydrolases that cleave the glycoside bonds of neuraminic acids. The activity of various bacterial neuraminidases, including those of *Clostridium perfringens* (type V and X) and *Vibrio cholerae*, was evaluated *in vitro* in RABV infection [20-21]. A dose-dependent inhibition of viral attachment was observed only with target cells that had previously been treated for one hour with the neuraminidase of *C. perfringens*. The inhibition mediated by neuraminidase treatment persisted for 3 h after removal of the enzyme, the cells gradually recovering their susceptibility to rabies infection thereafter [20].

Like other drugs targeting the first steps of the viral replication cycle (attachment and penetration into cells), the use of lectins or neuraminidases for the treatment of rabies infection appears to be of little value, as the inhibitory effects of such treatment are not selected, and are transitory and limited to the viral binding step. Moreover, the potential antiviral effects of these molecules have never been evaluated in animal models of rabies infection.

3.1.2 Analog and metabolic inhibitors

It has long been demonstrated that antimetabolic agents affecting DNA synthesis (such as mitomycin C, fluorodeoxyuridine and bromodeoxyuridine) or DNA transcription by RNA polymerase (such as actinomycin D) have no effect on RABV replication *in vitro*, leading to the classification of RABV as an RNA-containing virus [148-150]. Further therapeutic evaluations of these agents (including mitomycin C, actinomycin D, fluorodeoxyuridine and other antimetabolic drugs and analog inhibitors) provided no evidence of a virucidal effect, inhibition of viral replication or protective effect in mouse models [151-153].

However, cytarabin (also known as cytosine arabinoside or ara-C) is a potent inhibitor of DNA synthesis that has surprisingly been shown to be effective *in vitro* against the replication of laboratory-adapted RABV strains [153-155]. It has been suggested that this inhibition occurs soon after infection (3 hours post-infection) [153-154] and is specific to this molecule, as other pyrimidine nucleoside analogs or derivatives (such as 2-thio and 5-iodo derivatives of ara-C and uracil arabinoside) have no inhibitory effect [154]. Inhibition was overcome by adding high concentrations of both deoxycytidine and cytidine, without concomitant reversal of the inhibition of all DNA synthesis [153]. It has been suggested that the inhibition of RABV by ara-C requires the induction of a cellular protein, as the addition of antimetabolic agents acting on DNA synthesis partially or completely reverses this inhibition [154]. This *in vitro* inhibitory effect of ara-C on virus replication was partly reproduced in another study, in which viral replication was only slightly decreased, even in conditions completely shutting down host DNA synthesis [152]. Another pyrimidine analog inhibitor, 6-azauridine (6-azu), was shown to have an antiviral effect on laboratory-adapted RABV strain replication *in vitro* [155] that was unlikely to be due to a direct virucidal effect [151]. However, neither of these drugs was effective at reducing the mortality rate in mice challenged with non adapted virus strains [155]. Only one study suggested beneficial effects of ara-C for rabies prevention in mice, together with iododeoxyuridine, actinomycin D, rifampicin or azathioprine [156]. However, this result remains difficult to interpret, due to the complexity and lack of standardization of the various experimental protocols performed in the study concerned.

Purine analogs with antiviral activities against RNA and DNA viruses have also been extensively studied in RABV infection, including adenosine analogs, such as vidarabine (arabinosyl adenosine or ara-A) and dihydroxypropyladenine ((*S*)-DHPA) [157]. A dose-dependent inhibitory effect of vidarabine on laboratory-adapted RABV strain replication has

been observed *in vitro*, but was not further confirmed in the mouse model [155, 158]. This molecule was not virucidal and it was suggested that it acts by inhibiting cell and viral RNA synthesis and viral protein synthesis [158]. Four different acyclic derivatives of adenosine (including (*S*)-DHPA), together with one carbocyclic substance, 3-deazaadenosine (CDZ), have been shown to inhibit RABV production by 90% in single-growth cycle conditions [158-159]. This inhibition was dose-independent over a large range of concentrations (5 to 500 µg/ml). However, a strong antimitogenic effect was observed with these drugs. No direct virucidal effect was observed, particularly with (*S*)-DHPA [158-160]. It has been suggested that the inhibitory effects of (*S*)-DHPA on viral multiplication result from the inhibition of viral RNA synthesis [158]. The therapeutic effects of this molecule were assessed against rabies infection in mice [161]. Despite the use of several different doses, beginning one day after viral challenge and using different routes of administration, no effect was demonstrated, by contrast to reports from a previous study [160]. Foxes were treated orally with (*S*)-DHPA at a dose of 30 mg/kg, on the day of infection. Despite the limited number of animals tested, the time of death was found to be only slightly delayed by treatment [161]. Ribavirin is another purine analog that has been shown to have a dose-dependent effect on RABV production, within the 5 to 500 µg/ml range of concentrations, in single-growth cycle conditions, with 90% inhibition of infectious virion release observed at a concentration of 25 µg/ml, and 99.9% inhibition at concentrations above 50 µg/ml [158-159]. It has been suggested that this drug exerts its antiviral effects by inhibiting cellular and viral RNA synthesis, together with viral protein synthesis [158]. No direct virucidal effect was observed, but a strong antimitogenic effect was observed at a concentration of 25 µg/ml. Once again, the antiviral effect was not replicated in mice experimentally infected with rabies virus, and two related molecules (including a lipophilic analog with RTA and a structurally and functionally related drug with selenazofurin) had only a slight effect [161]. It has been suggested that

another purine-related molecule, guanine 7-N-oxide (G-7-Ox), isolated from *Streptomyces* sp., has broadly inhibitory effects against several RNA viruses *in vitro*, including RABV [162]. However, these preliminary results were not confirmed in subsequent studies.

No conclusive or strongly demonstrative result was obtained in any of these studies based on the use of analogs and metabolic inhibitors in rabies infection. This suggests that either these drugs are not suitable for rabies treatment or that the right inhibitor has yet to be identified.

3.1.3 Lysosomotropic agents

As described above, RABV is an enveloped virus that makes use of the endocytic pathway as its main route of entry into target cells (see section 1.2). Following the delivery of the virus to an endosomal compartment, membrane fusion occurs, mediated by the viral glycoprotein in a pH-dependent manner and leading to the release of the virus into the cytoplasm. This mechanism has been demonstrated with various lysosomotropic agents (which may selectively enter the lysosomes of certain cell types), including ammonium chloride, chloroquine and quinine, suggesting that it may be possible to target new viral steps in rabies infection. These drugs interfered *in vitro* with the replication of RABV, by raising the pH of endosomes and lysosomes and then preventing the fusion of the viral envelope with endosomal membranes. An inhibition of infection was observed in IMR-32 human neuroblastoma cells [139], CER cells [20] and other cells (including NIE-115 murine neuroblastoma cells, NS20 cell lines, dissociated neuronal cells and cultured rat myotubes) [163]. Antiviral effects occurred only when drugs were applied shortly (a few hours) after the viral attachment step, with a potentially dose-dependent relationship, at least for chloroquine [20, 139, 163]. Two other lysosomotropic molecules, methylamine and monensin, displayed dose-dependent virus inhibition *in vitro*, acting after the virus adsorption step [164]. None of these molecules had any effect on viral attachment or virucidal activity. Moreover, the

antiviral activity of lysosomotropic agents against rabies infection has not yet been evaluated in animal models.

3.1.4 *Drugs acting on microfilaments and the microtubule cytoskeleton*

In an effort to understand the cellular mechanisms of RABV replication and maturation, several drugs acting on the cellular organization of microfilaments or calmodulin processes have been tested, including cytochalasin B, which acts directly on actin microfilaments [165-167], anti-calmodulin drugs, including trifluoperazine and chlorpromazine [167], and inhibitors of Ca^{2+} -dependent processes, such as EGTA and nifedipine (reversible L-type Ca^{2+} channel antagonist) or the Ca^{2+} -specific ionophore A23187 [167]. However, no inhibitory effect on viral multiplication was observed, indicating that the actin-based cytoskeleton is not a target for RABV inhibition, as viral maturation was independent of the integrity of microfilament structures and calmodulin-dependent processes in neuronal cells. Only a single study has suggested a potential antiviral effect of cytochalasin B and trifluoperazine in IMR-32 and CER cells, but this finding was not subsequently confirmed [164]. An absence of antiviral effects on established rabies infection was also demonstrated *in vitro* with drugs affecting microtubules, such as vinblastine, colchicine and colcemid [165-166]. Moreover, no virucidal effect on RABV was observed with colchicine or vinblastine [168-169]. By contrast, pharmacological impairment of microtubulin and microfilament function prevented virus transport by axonal flow to differentiated rat embryonic dorsal root ganglion cells and a compartmentalized cell culture system [166]. Local administration of colchicine or vinblastine to the sciatic nerve of mice was demonstrated to prevent rabies infection, even if given up to 6 to 24 h after viral inoculation [169]. This preventive effect of colchicine was confirmed in an adult rat model in which this drug was administered to the sciatic nerve through a silicone cuff, 4 to 48 hours before viral challenge [168]. These results suggest that the retrograde transfer of RABV, from the periphery to the neuronal soma, requires the

integrity of tubulin- and actin-containing structures. However, the use of these drugs in rabies infection appears to be of little value, particularly if antiviral activity is obtained only after local administration.

3.1.5 Interferons and interferon-inducing molecules

Since the discovery of interferon (IFN) and its potent antiviral effect, several experiments have been carried out *in vitro* and in various animal models, to evaluate the potential use of this molecule for combating rabies infection. These studies were also prompted by failures of PEP with antirabies vaccines developed in the 1970s and 1980s, as a possible way to increase the level of protection provided [170-171].

Treatment with exogenous IFN was rapidly shown to prevent the replication of RABV in cell cultures [172-175]. This antiviral property was not related to a virucidal effect [176]. Complete inhibition was obtained when IFN was administered before (12-24 h) or at the time of infection, but only partial inhibition was observed when IFN was administered shortly after infection (12-24 h) [172-173].

This antiviral effect was rapidly confirmed in animal models. Preliminary studies were performed in rabbits, hamsters and mice infected with IFN-inducing viruses, such as live vaccinia virus [177-178], Newcastle disease virus (NDV) [179-180] or bovine parainfluenza 3 virus [181]. Effective protection was observed in all species other than mice. Interferon injection in mice, induced by the intraperitoneal injection of statolon, a fermentation product of a mold, *Penicillium stoloniferum*, provided no protection [182].

Treatment with exogenous homologous or heterologous IFN [175, 183-186] or inducers of IFN, such as polyriboinosinic-polyribocytidylic acid (poly I:C) [187-189], its derivatives containing kanamycin and CaCl₂ (PICKCa) [190] or other structural molecules [191] also completely or partly prevented rabies infection in animal models including mice, rabbits and

monkeys. Various protocols have been tested, including modifications of the following parameters: area of administration (same or different sites of inoculation), route of administration (intramuscular, intravenous, intralumbar, intraperitoneal, subcutaneous, intracarotid or intracerebral), time of drug administration (before, at the time of viral challenge or after viral challenge), number of doses and amount administered per dose, quantity and strain of virus used, route of inoculation, immunological status of the challenged animals. These experiments showed that efficacy was achieved essentially when large doses of IFN or IFN-inducers were administered before or shortly after viral challenge, close to the site of inoculation. Indeed, treatment administered at later time points after infection offered weaker protection, although the incubation period was prolonged in some treated animals [176, 183]. Combinations of drugs, including combinations of IFN-inducers, such as poly I:C, and chlorite-oxidized amylose (COAM), a small inducer of interferon [192], or combinations of rabies vaccines and IFN or IFN inducers [193-195] were also effective only when administered close to the time of viral challenge, and the protective effects obtained were variable. Finally, neither clinical improvement nor extension of the incubation period was observed when IFN-based therapy was initiated in a primate model at the onset of clinical symptoms [183, 196].

3.1.6 Ketamine and dizocilpine (MK-801)

In preliminary studies on experimental rabies infection, brain dysfunctions were described, suggesting that functional alterations might play an important role in rabies pathogenesis [197-199]. The inhibitory effects of two dissociative anesthetic agents, ketamine and dizocilpine (also known as MK-801) against RABV infection have been evaluated [200-202]. These molecules are non competitive antagonists of the *N*-methyl-D-aspartate (NMDA) receptor and were initially demonstrated to protect against the neurotoxicity induced by excitatory amino acids, such as L-glutamate and L-aspartate [203].

In a preliminary study, dizocilpine was shown to inhibit rabies infection in rat primary cortical neurons, when present after inoculation, for the duration of the experiment [202]. This inhibitory effect was shown to be dose-dependent only at millimolar concentrations (0-2 mM) and was selective for RABV, because no effect was observed with other viruses, such as herpes simplex virus, vesicular stomatitis virus, poliovirus and human immunodeficiency virus. This molecule was found not to be virucidal and its effect was highly selective for the class of non competitive NMDA receptor antagonists binding to the phencyclidine site, because an inhibitory effect was also observed with ketamine and phencyclidine derivatives, but not with AP5, a competitive antagonist. Its effect appeared to be independent of a high-affinity NMDA-binding mechanism [202]. The effects of ketamine on the infectivity of RABV in neuronal culture systems and *in vivo* have also been investigated [201]. This molecule was demonstrated to have a dose-dependent effect (0.2-2 mM) on virus multiplication in infected neuronal cell cultures (for both rat primary cortical neurons and human neuroblastoma cells). However, efficient antiviral effects were achieved only with high concentrations of drug, with a decrease in viral production by a factor of 100 to 1000 observed with 1 to 2 mM ketamine. The antiviral effect of ketamine was confirmed in rats, following intraperitoneal administration (15 mg, twice daily for four days) after the stereotaxic inoculation of the right striatum with RABV [201]. A decrease in rabies infection was observed four days after infection in certain regions of the brain, including the thalamus, cortex and hippocampus. In a complementary report, studies of the effects of ketamine and dizocilpine on rat brain and neuronal cell culture (rat cortical neurons) showed that these drugs probably acted specifically on transcription of the RABV genome, inhibiting viral protein synthesis [200]. This inhibition was incomplete and displayed time-dependent reversibility, as a recovery of viral transcription and protein synthesis was observed over time.

Further evidence of the inhibitory activity of ketamine (3 mM) was subsequently obtained *in vitro*, in McCoy cells, with the PV strain of RABV [204]. However, in a more recent study, neither ketamine nor dizocilpine was observed to protect against rabies infection in the mouse model [205]. The absence of a neuroprotective effect was demonstrated in primary cultures of mouse cortical and hippocampus neurons, for 125 μ M ketamine and 50 μ M dizocilpine. The lack of a therapeutic effect of ketamine was confirmed *in vivo*, in infected mice (following peripheral or intracerebral inoculation with the CVS virus strain) treated with ketamine at a dose similar to that used in rats (60 mg/kg every 12 h intraperitoneally on days 3 to 6 post-infection) [205].

The conflicting results obtained with ketamine and dizocilpine, particularly for *in vivo* experiments, raise the question of the choice of the most relevant animal model to be used. Indeed, different species (rat and mouse) have been used in different studies and it would be interesting to evaluate these drugs in similar studies using both these species and possibly even other species.

3.1.7 Amantadine

The antiviral effects of amantadine have long been demonstrated, particularly for the prevention of influenza infection. Interesting results have also been obtained with vesicular stomatitis virus, a member of the family *Rhabdoviridae*, with this drug inhibiting virus uptake into the cell and endocytosis [206-208]. The effect of this lipophilic amine on RABV replication has been tested *in vitro* with various laboratory-adapted virus strains; it has been shown to have a dose-dependent antiviral effect [159, 208]. In particular, viral replication was shown to be inhibited when amantadine was added shortly after viral inoculation (during the first 2 h), with a minimum incubation of 30 minutes required for the detection of antiviral

activity [208]. No virucidal effect was detected in either of these studies. It has been hypothesized that amantadine affects the uncoating of the virus, as no activity is observed during viral attachment or entry into the cell [208]. Superti and coworkers suggested that the antiviral effect of this lipophilic drug on rhabdovirus infections might be related to its capacity to cross the cell membranes and to increase the natural pH of lysosomes, hindering the uncoating of rhabdoviruses. However, an evaluation of therapeutic effect of this drug against rabies infection in mice demonstrated an absence of beneficial effects, even if treatment was initiated only one day after intramuscular infection and despite the application of several protocols involving different dose schedules (30 or 67 mg/kg/day) or routes of administration [161].

3.1.8 *Isoprinosine*

Isoprinosine, an alkylamino-alcohol complex of inosine, also known as inosine pranobex, has long been recognized as a broad-spectrum antiviral compound. The potential antiviral effect of this drug was evaluated in a large coordinated study of several viruses and animal models, including rabies in mice [209]. The daily intraperitoneal administration of 300 mg/kg isoprinosine, initiated one day before infection and continuing for up to five days, had no protective effect in this model following intracerebral or intramuscular inoculation with RABV. In two other studies conducted *in vitro*, a slight decrease in the titer of laboratory-adapted virus strains was observed [204, 210].

3.1.9 *Corticosteroids*

The effect of corticosteroid treatment on RABV infection has been investigated in various rodent models, including rats, hamsters and guinea pigs [211] and mice [212]. Hydrocortisone acetate and prednisolone are the most frequently investigated treatments. In most experiments, the mortality rates of infected animals (with vaccinal, attenuated or virulent

virus strains) were slightly higher for the treated than for the untreated animals [211-212]. The biological mechanisms underlying this increase in mortality remain unknown. However, these results have not been confirmed by other groups or in other studies.

3.1.10 Heteropolyanions

Heteropolyanion (HPA)-based molecules have been tested as antiviral drugs in RABV infection. In particular, *in vitro* dose-dependent inhibition has been demonstrated with ammonium-5-tungsto-2-antimoniate heteropolyanion (HPA-23), in tests with the Flury HEP vaccinal strain [213]. Inhibition rates of 50% were obtained with 4.5 µg/ml HPA-23, and complete inhibition was obtained with 12.5 µg/ml HPA-23. However, inhibitory effects were observed only when treatment was initiated early, ideally within 18 to 24 hours after inoculation, with no further inhibition observed beyond 48 hours post infection [213]. The antiviral effect of HPA-23 on RABV multiplication was further confirmed in single-growth cycle conditions, with CVS, and similar effects were observed with six other HPAs: HPA-39, -56, -40, -51, -52 and -57 [158-159]. A dose-dependent effect was observed, with almost complete inhibition at concentrations exceeding 50 µg/ml [158-159]. No virucidal effect of these drugs was observed in any of these studies, even at high concentrations [158-159, 213]. The antiviral activity of HPA-23 and -39 against RABV was thought to target viral RNA synthesis and cellular protein synthesis [158]. These molecules have been evaluated in animal models, including foxes and mice [161, 214]. In total, 14 different HPA derivatives have been used at various concentrations and with different protocols of administration in mice, with assessment of their antiviral activity against rabies infection. Only HPA-23 and -39 were found to have a slightly protective effect, essentially when treatment was both initiated soon after virus inoculation (1 day post-infection) and administered intramuscularly at the site of virus inoculation [161]. Late treatment with HPA-39 was not protective even when initiated only five days after inoculation. A slight dose-dependent protective effect was also observed

with HPA-23 in rabies-infected foxes when this molecule was administered intramuscularly at the time of inoculation; a decrease in the incubation period appeared to occur when the treatment was administered one to two days after viral challenge [161, 214]. Moreover, a hypothetical therapeutic effect of HPA-39 has been described when this molecule is administered shortly after the onset of clinical signs [161]. However, high mortality rates were observed in both these studies, due to high toxicity of these drugs in foxes, particularly after intraperitoneal, subcutaneous or intramuscular administration, for doses ranging from 50 to 150 mg/kg. The small numbers of animals studied therefore render interpretation difficult, even if a very small number of animals seem to have survived after treatment (2/7 or 2/15 with HPA-23 and 1/1 with HPA-39) [161, 214]. From these results, and given the high toxicity and the low antiviral activity of HPA-based molecules, the use of these compounds in the treatment or even the prevention of animal (including humans) rabies infection remains rather unlikely.

3.1.11 Ascorbic acid (vitamin C)

Surprisingly, a treatment based on the intramuscular injection of ascorbic acid has been proposed as a means of preventing rabies infection in a guinea pig model [215]. Treatment was initiated 6 hours after intramuscular inoculation with RABV and was based on the administration of a dose of 100 mg/kg of body weight twice daily for seven days. Mortality rates were lower in treated animals than in control animals, with 17 of 48 (35.4%) and 35 of 50 (70%) animals, respectively, dying. However, the results were variable between groups of animals and lower doses of vitamin C were less effective. Moreover, this result was not confirmed by further studies conducted in animal models. Only one recent study has suggested that a solution of ascorbic acid (0.5 mg/ml) and copper sulfate (5 µg/ml) can completely inactivate RABV *in vitro* after 72 hours of incubation [216].

3.1.12 *β-phenylserine*

Partial prophylactic protection of rats against RABV was achieved by daily intraperitoneal administration of β -phenylserine, a competitive inhibitor of phenylalanine hydroxylase [217]. This protection was obtained when animals were treated for three days before inoculation, and treatment administered 24 hours after infection was also protective. No virucidal effect of β -phenylserine was demonstrated. The inhibition of rabies infection was abolished if DL-phenylalanine or DL-tyrosine was administered in combination with this drug. However, the antiviral effect of β -phenylserine was restricted to the rat strain used in this experiment, limiting the potential utility of this molecule [217]. A protective effect of this drug was also suggested in rabies-infected mice, but the results could not be uniformly replicated and the differences observed were not statistically significant [151].

3.1.13 *Minocycline*

Minocycline, a derivative of tetracycline with antiapoptotic and anti-inflammatory properties, has been evaluated in experimental models of RABV infection using a cell-adapted rabies virus strain [218]. Only minor effects on neuroprotection or viral multiplication were demonstrated *in vitro* in mouse cortical and hippocampal primary neurons, even if the drug was added within an hour of inoculation with the virus. Detrimental effects were observed in rabies-infected newborn mice, with higher mortality rates and an earlier onset of neurological symptoms than observed in mock-infected mice, jeopardizing the possible future use of this molecule in the prevention or treatment of rabies infection.

3.1.14 *Phosprenyl*

It has been claimed that phosprenyl, a molecule obtained by the chemical phosphorylation of polyprenols (natural long-chain isoprenoid alcohols), has broad-spectrum antiviral activity,

with effects on rabies [219]. Significant results were reported *in vivo*, but were not confirmed by subsequent studies.

3.1.15 Other drug derivatives from plants or fungi

The inhibitory effects of an antibiotic substance, cinnabarin, extracted from the fungus *Pycnoporus sanguineus*, against RABV replication *in vitro* have been evaluated [220]. At non cytotoxic doses (0.155 to 0.31 mg/ml), it has been suggested that cinnabarin has antiviral effects, leading to decreases in viral titer by a factor of two to four (evaluations with the PV strain). These effects were observed after prior incubation of the drug with the viral suspension, and maintenance of the drug in the cell culture medium for 24 h. No tests on animal models or analyses of antiviral activity were carried out. In another study, antiviral activities of exudate fluids from small red beans (*Vigna angularis*) have been evaluated *in vitro* against rabies infection with BHK- 21 cells [221]. Virucidal and dose-dependent virus inhibition effects have been suggested ($IC_{50\%}=0.48\%$ dose), especially during the early phase of infection (0-6 h p.i.). Lastly, in another study, four different extracts and fractions from South American plants (*Alamanda schottii*, *Passiflora edulis* and *Sloanea guianensis*) were evaluated to assess their potential *in vitro* antiviral activity against RABV [222]. Based on determination of the selectivity index (SI), which is the ratio of the 50% cytotoxic concentration ($CC_{50\%}$) to the 50% inhibitory concentration ($IC_{50\%}$), only one extract (obtained from *Alamanda schottii*) had potent antiviral activity ($SI > 5$). These extracts and fractions were not studied further in animal models of rabies infection, and their molecular composition was not determined.

3.1.16 RNA-interference

RNA interference-based antiviral approaches have been evaluated *in vitro* for rabies infection. With short cDNA, short-interfering RNAs (siRNAs) or artificial microRNAs (amiRNAs)

targeting viral nucleoprotein or phosphoprotein mRNAs, partial inhibition of RABV multiplication was obtained [223-225]. However, the direct application of such an approach to the treatment of rabies is not yet realistic, and several obstacles remain, including the induction of viral resistance or escape, due to natural genetic variability of the nucleotide target, drug delivery in the CNS, drug efficiency in animal models, etc.

3.1.17 Antiviral peptides

Strategies have been proposed for antiviral peptide discovery in the field of rabies infection, based on genetically encoded combinatorial peptide libraries of intrinsically constrained peptides screened with the viral phosphoprotein [226]. This approach led to the identification of peptides with efficient antiviral activities *in vitro* against RABV. However, these peptides were generated by transfecting host cells with expression vectors before virus infection. No further study based on the direct use of these peptides was carried out. In another study, two peptides covering the first 42 and 60 residues of the N-terminal region of the RABV phosphoprotein were shown to have antiviral activity against rabies infection *in vitro* [227]. The viral infection of neuroblastoma cells was found to be inhibited by the delivery of synthetic peptides shortly after virus inoculation. However, the antiviral activities of these peptides has not been confirmed in animal models of rabies infection.

3.1.18 Multiple-drug screening

Over a number of years, various groups have performed large drug screening studies *in vitro* or in animal models, to identify compounds effective against RABV infection. For example, the viral inhibitory activity of 21 molecules was tested *in vitro* (in CER cells) [159]. Eight — ribavirin and seven heteropolyanions — displayed dose-dependent inhibition, whereas the others — four acyclic derivatives of adenosine and carbocyclic 3-deazaadeonisine — displayed 90% inhibition at all doses used, as previously indicated (see sections 3.1.2 and

3.1.10). In another study with the IMR-32 human neuroblastoma cell line, Lentz and coworkers screened 25 chemical agents, assessing their ability to inhibit rabies infection in a fluorescent focus assay [139]. Inhibitory effects were observed for agents with the potential to inhibit the attachment of the virus to the cell surface (with antibodies against the viral glycoprotein, gangliosides, a synthetic peptide of the neurotoxin-binding site of the *Torpedo* acetylcholine receptor $\alpha 1$ subunit and α -bungarotoxin) and lysosomotropic agents, as previously indicated (see sections 3.1.1.2 and 3.1.3). Antiviral activities of 14 natural and semisynthetic polymeric carbohydrates were evaluated *in vitro* using CER cells and the CVS strain of rabies virus [228]. Most were able to inhibit infection by interfering with the virus adsorption step, and this was particularly true for the following polysaccharides: scleroglucan, Keltrol[®], alginate acid and two sulfated tamarind gums (also known as glyloid sulfates). Finally, the potential antiviral activity of 24 synthetic phenolic compounds against RABV was recently evaluated in McCoy cells with the PV strain [204]. Only four of these compounds — catechin, quercetin, 3,4,5-trimethoxyacetophenone and 3,4,5-trimethoxybenzoic acid ethyl ester — displayed potent antiviral effects against rabies.

Enright and coworkers used the mouse model to test up to 65 different drugs in PEP [151]. These compounds were classified on the basis of their chemical structure or biological activity and included antimetabolites and antibiotics, proteins, nucleic acids and related compounds and miscellaneous substances, including solvents and detergents. Treatments were administered at the site of inoculation (virus entry), and were systematic or blocking (injection of the treatment compound between the site of viral inoculation and the CNS). They followed several different schedules and doses, depending on the compounds tested. The most active compounds were solvents or detergents (probably affecting viral lipids). None of the other protein compounds, enzymes, antimetabolites or antibiotics tested had a significant chemotherapeutic effect. In another study in mice, 17 immunosuppressive, cytostatic or

antiviral agents were assessed for rabies prevention [156]. It was suggested that iododeoxyuridine (IUdR) (intraperitoneal, 20 mg/kg), actinomycin D (intraperitoneal, 0.5-1.0 mg/kg) and ara-C (subcutaneous, 20 mg/kg) inhibited intracerebral infection, and that rifampicin (subcutaneous, 100 mg/kg) and azathioprine (intravenous, 2.5 mg/kg) inhibited intramuscular infection (see section 3.1.2). However, these results remain difficult to interpret, due to a lack of standardization, as studies were carried out in different conditions (drug doses, routes of administration, rabies strains used for inoculation, etc.). In total, 40 different compounds were evaluated for their potential protective activity against rabies in mice and/or in foxes, using different doses, time and schedules of administration [161]. Only five HPA molecules (HPA-23, -39, -46, -51 and -56) were identified as effective preventive drugs.

3.1.19 Conclusion

From these numerous studies evaluating the antiviral activities against rabies infection of a large range of molecules (from well characterized broad-spectrum antiviral compounds, such as interferons, to more unexpected compounds, such as ascorbic acid), we can conclude that no drug has been shown to be effective *in vivo*, particularly in cases in which clinical signs are already present. These results suggest that other rabies virus targets in the viral replication cycle must be identified, and more specific antiviral molecules designed. Moreover, a key issue is the identification of effective drugs able to cross the blood brain barrier, a natural obstacle blocking access to the CNS, the specific target tissue of RABV.

3.2 The history of drug treatment for human rabies

3.2.1 *The hope: the existence of rare cases of human recovery after rabies encephalitis*

Human rabies is an almost always preventable provided that effective PEP is administered to exposed individuals early enough. However, this acute viral encephalitis is also almost invariably fatal once the first clinical signs have appeared. Nonetheless, a few cases of survival have been reported, raising hopes that it may be possible to cure this infection and to survive rabies. Five well documented cases of prolonged survival or recovery from rabies were specifically associated with the administration of PEP before the onset of symptoms. However, a presumptive case of abortive rabies was described in 2009, in a patient who recovered from rabies encephalitis despite the absence of PEP before the illness. No other specific antiviral treatment was administered in any of these cases. With the exception of the last case, early, aggressive, intensive support therapy was given to anticipate the problems suffered by many patients, including severe complications. Interestingly, it was not possible to isolate RABV or to detect rabies antigen in any of these patients. Two other less documented cases of human recovery have been also reported in rabid patients given treatment in combination with PEP and aggressive care [229] (described in sections 3.2.2.2.2 and 3.2.2.2.3).

3.2.1.1 Case 1

The first reported case of recovery from rabies was that of a six-year-old boy in November 1970, in Ohio, USA [230]. The child developed rabies 20 days after he was bitten on the left thumb by a big brown bat (*Eptesicus fuscus*) and two days after the completion of a 14-day course of PEP without antirabies serum (multidose therapy with DEV). The bat was confirmed as rabid four days after the bite, leading to vaccination. The patient presented neck pain at the onset of symptoms, extending to the arms, legs and head a few days later.

Fluctuating consciousness occurred, with neurological abnormalities, and the patient became increasingly lethargic and comatose. Biological analysis of a brain biopsy specimen and a CSF sample confirmed encephalitis. Cardiac and pulmonary complications occurred during the hospitalization of this patient. He later presented focal seizures, which were treated with diphenylhydantoin therapy. Almost 25 days after the onset of symptoms, the patient gradually improved and he was discharged two months later [230]. No detectable psychological or neurological abnormality was observed after a few months of follow-up. A high titer of specific antirabies antibodies was detected in serum and CSF. In particular, antibody levels increased strongly in the patient's serum and CSF during hospitalization, consistent with rabies infection rather than the effects of vaccination. However, RABV was not isolated from any of the samples tested, including a brain biopsy specimen, probably due to the presence of high levels of neutralizing antibodies.

3.2.1.2 Case 2

In 1972, another case of recovery was described in a 45-year-old female patient from Mendoza, Argentina, who was severely bitten, on the left arm, by a dog with suspected rabies and who developed neurological signs 21 days later [231]. As in the first case, this patient received a 14-day course of post-exposure treatment, based on suckling mouse brain vaccine administered 10 days after the bite, with two booster doses, without antirabies antiserum. The encephalitic syndrome persisted for 75 days, and the patient made an almost complete recovery 13 months later. Onset of the symptoms began at the time of the 12th dose of vaccine, with paresthesia of the bitten limb, extending to the other arm and accompanied by generalized pain. A severe cerebellar striatal syndrome occurred. Rabies antigens (on corneal smear) were not detected and it was not possible to isolate the virus (from saliva and CSF samples). However, neutralizing antibodies were detected in CSF and serum, the titer increasing with time of infection to a magnitude never observed after vaccination with the

vaccine used. Chemotherapeutic treatment was based on vitamin B1 and B12, diphenahydramine, diazepam, biperiden, dihyphenylhydantoin, betamethasone and ACTH for a period of almost 10 days. After a period of improvement, leading to the discharge of the patient, neurological symptoms reappeared shortly after the first booster dose of vaccine and began to worsen with the second booster dose, with generalized seizures, neurological abnormalities, fluctuating consciousness and tetraparesis, hypertonia, hyperreflexia and Babinski's sign. Clinical improvement then occurred progressively. The patient was discharged a few months later and made an almost complete recovery from rabies 13 months after the onset of symptoms. Rabies diagnosis was based on clinical, epidemiological and laboratory data, supporting the conclusion of a non fatal case of rabies in a human clinical presentation [231]. However, the hypotheses of encephalomyelitis or a post-vaccine accident after treatment with suckling mouse brain vaccine, particularly after the administration of rabies vaccine booster doses, could not be formally rejected [65].

3.2.1.3 Case 3

In 1977, a case of partial recovery from rabies was identified in a 32-year-old male technician working in a rabies laboratory in New York, USA [47, 232]. This patient was pre-immunized and was probably exposed to high concentrations of aerosolized modified live RABV strains (derived from the attenuated SAD virus strain) two weeks before the beginning of the illness [47]. The initial symptoms were not specific, with malaise and headache, followed by chills, fever and nausea on the next few days. The patient then became lethargic, with fluctuating consciousness and fell into a deep coma after hospitalization. Two weeks later, he began to show gradual improvement. Three months after the onset of clinical signs, he recovered motor function and was ambulatory but conserved sequelae, with occasional aphasic periods of agitation and spasticity [232]. Rabies was diagnosed purely on the basis of the increase in

antirabies antibody titer in the serum and CSF, and corneal impression and neck skin biopsy testing by FAT gave negative results.

3.2.1.4 Case 4

A nine-year old Mexican boy partially recovered from rabies after being bitten on the face by a rabid dog in August, 1992 [233]. PEP was administered the day after the bite, according to a modified five-dose regimen of cell-cultured rabies vaccines (PVRV and HDCV), without rabies antiserum or immunoglobulin. Symptoms began 18 days after exposure, with fever and dysphagia, followed by neurological abnormalities and encephalitic signs. Cranial hypertension was observed, leading to coma a few days later. Progressive but limited improvement was then observed, with severe neurological sequelae [233]. The patient died less than four years after the onset of symptoms [65]. No rabies antigens were detected on corneal impression and nuchal skin biopsy, and the virus could not be isolated from saliva. High levels of neutralizing antibodies were observed in the CSF and serum, and the intrathecal production of rabies antibodies was demonstrated.

3.2.1.5 Case 5

In 2001, in Bangalore, India, a six-year old girl developed rabies 20 days after she had been bitten by a dog on the face and hands [234]. Incomplete PEP was given (three doses of PCECV cell vaccine on day 0, 3 and 7 days), without local wound treatment or antirabies immunoglobulin. The symptoms at onset of illness included fever, swallowing difficulties, photophobia, hallucinations and sensory alterations, followed by fluctuating consciousness. The patient became comatose, with focal motor seizures and excessive salivation and sweating. Methylprednisolone, antibiotics and intravenous fluids were administered, together with an additional dose of rabies vaccine (HDCV). High titers of rabies antibodies were detected in serum and CSF, with a significant increase in titers not associated with rabies

vaccination, as in the other cases. No rabies antigen was detected on corneal smear and nuchal skin biopsy and it was not possible to isolate the virus from the CSF. Intrathecal synthesis of rabies antibodies was demonstrated. The child remained comatose for three months with the maintenance of supportive care. Steroid doses were tapered and discontinued. The patient's condition gradually improved, but she did not recover completely, retaining severe neurological sequelae [234]. She died about two years after the beginning of the disease [65].

3.2.1.6 Case 6

The first presumptive case of abortive human rabies was described in a 17 year-old female patient in Texas, USA, in 2009 [235]. Almost two months after contact with bats, this patient developed symptoms, including headache, followed by photophobia, paresthesia of the upper extremities, neck pain, transient disorientation, fever and severe headache over the next two weeks, requiring hospitalization. Hypercytosis was demonstrated in the CSF. After a short period of recovery, clinical and biological signs of encephalitis recurred and intensified, leading to a second hospitalization with a diagnosis of suspected infectious encephalitis. After a period of weakness and emesis followed by agitation, rabies was suspected. Diagnosis was based purely on the detection of RABV antibodies in CSF and serum samples by indirect fluorescence antibody tests, as neither viral antigen nor viral RNA was detected in saliva or a nuchal skin biopsy specimen. Moreover, neutralizing rabies antibodies were detected in the serum by RFFIT only after the administration of single doses of rabies immune globulin (HRIG, 1500 IU) and rabies vaccine, almost one month after the onset of symptoms. The patient was discharged after the resolution of symptoms, after almost 17 days of hospitalization, but suffered two other episodes of severe headache and potential increase in intracranial pressure. The current clinical status of the patient is unknown [235]. In this report, the authors considered this patient to constitute a presumptive case of abortive rabies. However, the biological confirmation of rabies infection remains limited and debatable.

Clinical presentation was nonspecific and unusual, with strong alternation between periods of illness and recovery. Additional information is required to provide a full description of this potential case of recovery.

3.2.2 Description of the first trials of treatment for human rabies

The treatment of the rare cases of rabies recovery described to date was specifically designed to maximize the chances of recovery, by preventing all treatable complications of rabies infection through aggressive supportive care [230]. Such rare reports have encouraged physicians to set up rigorous intensive measures to prolong life in patients with rabies, in the hope that the virus may be cleared in due course, with partial or complete recovery. Various antiviral therapies have been used in parallel, following the discovery and evaluation of new antiviral drugs *in vitro* or in animal models, effective particularly against rabies infection.

3.2.2.1 Interferon (IFN)

In the 1970s and 1980s, several attempts were made to treat clinical cases of rabies with human leukocyte IFN associated with medical intensive care in France and the Americas, but without success [236]. The first two cases were French children, a 10-year-old girl and a five-year-old boy hospitalized in Paris, France, after being bitten by rabid dogs in Africa one and a half months previously [236]. Neither of these patients received PrEP or PEP. IFN treatment was administered just after admission, corresponding to two to three days and five days after the onset of symptoms, respectively. The protocol of human leukocyte IFN-based treatment was as follows: intravenous injection of 3×10^6 U on a continuous basis for 12 days and intrathecal administration of 1×10^6 U every other day for six days for the girl, and administration of 1×10^6 U intramuscularly for 12 days and 0.5 to 2×10^6 U intrathecally every other day for six days for the boy. Both died of rabies, 28 and 23 days after the onset of symptoms, respectively.

A few years later, between 1981 and 1983, three American cases of human rabies were also treated with IFN [236]. All received human leukocyte IFN according to a similar protocol, including the intramuscular injection of 10×10^6 U twice daily (12 h apart) and the intraventricular administration of 5×10^6 U once daily into a Rickham reservoir, connected by a cannula to a lateral ventricle of the brain. The first case was a 16-year old Brazilian girl who developed rabies after being licked by a rabid dog. She received no PrEP or PEP. Treatment was initiated 14 days after the onset of symptoms and continued until the death of the patient, 10 days later (with a total of 250×10^6 U IFN administered) [236]. The other two cases of human rabies treated with IFN occurred in the US. In 1981, a 40-year-old man who had been bitten two months previously by a dog with suspected rabies in Mexico, was treated [236-237]. This patient received no PEP until his hospitalization for probable rabies, on the sixth day of illness. He was given a single dose of rabies vaccine (HDCV) and of HRIG, and human leukocyte IFN treatment was initiated 10 days after the onset of symptoms. The patient died 14 days later, after 14 days of treatment (total of 350×10^6 U IFN given). The other case of human rabies in the USA was treated with human leukocyte IFN, initiated seven days after the onset of symptoms (one day after the biological confirmation of rabies infection) [236, 238] and continued for 17 days. This patient, who did not receive PEP, died 28 days after the onset of illness, after receiving a total dose of 425×10^6 U of IFN.

An open trial of intensive care and chemotherapy with lymphoblastoid α -IFN was later conducted in Thailand, on three patients with confirmed rabies infection (from 1.5 to 7 days of illness before treatment) [239]. High doses of α -IFN were administered intravenously and intraventricularly and concentration was monitored. The following protocol was used: loading dose of 50×10^6 U/m² body surface area over 6 h, repeated 18 h later and followed by a daily continuous intravenous infusion. The first dose of intrathecal IFN was administered by the lumbar route, and subsequent doses were administered to the cerebral ventricle via an

Ommaya reservoir. A loading dose of 2×10^6 U/m² body surface area was repeated after 6 h and then daily. Despite the high concentrations of α -IFN measured in serum and cerebrospinal fluid, the patients died after 5.5 and 12.5 days of treatment, with no evidence of virostatic or clinical benefits, suggesting that IFN is not effective against rabies encephalitis.

Combined drug therapy including IFN or IFN-inducers and other drugs was also attempted early in the history of rabies treatment. In a retrospective study over a period of 17 years of the management and follow-up of rabid patients in India, 17 of the 54 patients studied received cytarabine, nucleic acid lipoprotein complex or IFN-inducer, in combination with intensive care management [240]. All patients died of rabies despite this treatment. In another study in 1985, a combination of IFN (10×10^6 U, administered daily, intramuscularly and intraventricularly), rabies immunoglobulin (1 and 5 ml doses given intramuscularly and intrathecally) and vidarabine (350 mg to 700 mg infused intravenously over 24 h) was given to a 25-year-old Canadian patient who presented clinical signs of rabies, probably contracted after exposure to a bat three months earlier [241-242]. This patient died almost one month after the onset of symptoms (25 days after admission to hospital), and a generalized necrosis of the brain was observed post mortem.

3.2.2.2 Antimetabolic agents

3.2.2.2.1 Ribavirin

In 1984, an experimental treatment with ribavirin was administered to a 12-year-old girl in Houston, Texas, USA, presenting the paralytic form of rabies [243]. This treatment was given after biological confirmation of rabies infection, obtained almost 16 days after the onset of symptoms. Little information is available concerning this case, but the patient is known to have died 27 days after the onset of the first clinical signs. In another study, clinical (furious) rabies was treated with ribavirin in a Thai patient in the early stage of infection (3 days after

the onset of symptoms), in association with intensive care clinical management [239]. The drug was given intravenously as a loading dose of 2 g (30 mg/kg) over 20 min, followed by 1 g every 6 h (60 mg/kg/day) for 4 days and 0.5 g every 8 h (25 mg/kg/day) daily thereafter. In parallel, intraventricular injections of 100 mg ribavirin (2 mg/kg) were administered daily through an Ommaya reservoir. The patient died of rabies after a nine-day period of treatment, indicating an absence of antiviral or beneficial effects of ribavirin [239]. Later, through a retrospective analysis of 64 potential cases of human rabies admitted to the You-An Infectious Disease Hospital in Beijing, China, over a 15-year period (1974 to 1989), 16 patients were found to have received intravenous doses of 16 to 400 mg ribavirin [244]. No significant effect on survival was noted.

3.2.2.2.2 Other antimetabolic agents

As previously indicated, combined drug treatment including the systemic and intrathecal administration of IFN, rabies immunoglobulin and vidarabine was given to a Canadian rabid patient, without success [241-242].

Intensive treatment for human rabies was attempted in seven Indian patients in the 1970s [229]. The measures used included aggressive treatment with cytosine arabinoside (acting as an antiviral agent), in association with Freund's emulsion (used for stimulating antibody formation). In parallel, intensive and critical care was administered, with intermittent positive pressure facilitated by muscle relaxants and sedatives to prevent hypoxia, maintenance of nutrition, correction of electrolyte concentration and blood pH and intensive nursing care. However, none of the patients survived, although survival was prolonged in some, to up to 17 days after the onset of symptoms [229]. The same group subsequently carried out a retrospective study over a period of 17 years including 54 rabid Indian patients treated with the same intensive care regimen but different drugs [240]. Seventeen of these patients

received cytarabine, nucleic acid lipoprotein complex or IFN-inducer, with no beneficial effect on survival period (see paragraph 3.2.2.1).

3.2.2.2.3 Other drugs

In the same retrospective study conducted by Gode and colleagues in 1976 [229], 37 of the 54 patients were treated with diphenylhydantoin and vitamin C. It was suggested that these patients survived for longer. However, all but two of these patients died from rabies. The two survivors were still alive and normal after 28 and 13 months, at the time of communication [240]. These two patients received PEP with a course of Semple's vaccine without antiserum, which may have accounted for the favorable outcome independently of the drugs administered. Furthermore, only three of the 54 patients had biologically confirmed rabies, with diagnosis purely on clinical symptoms for the others.

3.2.2.2.4 Vaccines and rabies immune globulins

Other therapeutic interventions after the onset of clinical illness, based on intensive supportive care and the administration of rabies immune globulins (given intravenously, subcutaneously, intrathecally or intraventricularly), alone or in combination with antirabies vaccines, have been performed in multiple cases of human rabies. Unfortunately, no beneficial effect was observed in any of these clinical cases [245-249].

3.2.2.2.5 Quinine and urea hydrochloride

In 1913, a 73-year-old patient with suspected rabies was given quinine and urea hydrochloride intravenously for two days after admission to hospital in Saint Louis, USA [250]. This treatment was initiated four to five days after the onset of symptoms and the patient recovered four days after hospitalization, eight to nine days after the onset of the illness. However, this case remains inconclusive and dubious as the diagnosis of rabies was based purely on clinical (principally hydrophobia) and epidemiological (dog bite 5-6 weeks before) grounds. Moreover, no further follow-up of this patient was performed.

3.2.3 *New directions, moving towards combination therapy for human rabies*

3.2.3.1 Expert recommendations for the treatment of human rabies in 2003

Recommendations concerning the management of rabies in humans were presented in 2003 by a group of physicians and researchers with expertise in rabies [251]. This group concluded that the routine management of patients with rabies should be palliative, with the anticipation of disease complications, and use of appropriate treatments, such as sedatives, narcotic analgesics, neuromuscular blockers and antiepileptic drugs. However, in some circumstances, an aggressive approach to treatment may be attempted, particularly in previously healthy patients presenting at a very early stage of disease, vaccinated before the onset of symptoms and with access to adequate medical resources and facilities. In these cases, a combination of treatments was suggested, including rabies vaccines (particularly by the multiple-site intradermal route), rabies immune globulin (given intramuscularly, as in PEP, for example), ribavirin (administered intravenously and intraventricularly through an Ommaya reservoir), α -IFN (administered in the same way as ribavirin), monoclonal antibodies (in the future and after clinical evaluation) and ketamine (administered intravenously in a continuous infusion). The administration of corticosteroids for rabies treatment should be avoided [251].

In line with these recommendations, a combined therapeutic approach including rabies vaccine, rabies immune globulin, ribavirin and α -IFN was administered to a 66-year-old patient on admission to hospital, almost two weeks after the onset of symptoms [252]. However, this patient, who had been bitten by a bat five weeks previously, died seven days after admission.

3.2.3.2 The unexpected successful case of rabies recovery in 2004 and the Milwaukee protocol

An unexpected and instructive case of survival was reported in a 15-year-old girl who developed encephalitic rabies after being bitten by a bat one month earlier [253]. During the clinical illness, she was treated with an experimental protocol combining antiexcitatory and antiviral drugs, therapeutic coma and supportive intense care. This patient never received rabies vaccine or antirabies immunoglobulins. The rationale of this protocol was based on previous clinical cases suggesting that death from rabies results from a regional imbalance of neurotransmitters or neuromodulators and autonomic failure, and that supportive care could potentially be successful [230, 240, 254-255]. Agonists of gamma-aminobutyric acid (GABA) receptor, such as benzodiazepines (midazolam or diazepam) and barbiturates (phenobarbital) were used to induce a therapeutic coma, to produce a burst-suppression pattern on electroencephalogram. In parallel, attempts were made to reduce excitotoxicity, brain metabolism and autonomic reactivity by NMDA-receptor antagonism with amantadine and ketamine. Both these drugs also have antiviral activity, reportedly specific against rabies *in vitro* and in rat models for ketamine (see section 3.1.6). Antiviral therapy was also implemented, with the administration of ribavirin. After 31 days of isolation, the patient was removed from intensive care. She was discharged home after 76 days. The patient survived with mild to moderate neurological sequelae, which seem to have improved with time [253, 256]. However, the efficacy and utility of this protocol remains debatable, as it is unclear whether it played a significant role in recovery, or whether survival resulted from an effective endogenous antirabies immune response developed in this patient, in combination with the quality of critical care implemented during hospitalization [257-258]. This patient had antirabies neutralizing antibodies in her CSF and serum shortly after the onset of symptoms, but RABV remained undetectable by classical diagnosis tests (including viral isolation,

antigen or RNA detection in saliva, CSF and nuchal skin biopsy). This unusual biological presentation was also observed in the other case of survival after rabies that had been vaccinated [230]. It is therefore possible that both these patients managed to mount an unusually effective early immune response to the infection that contributed partially or fully to recovery. However, we also cannot exclude the possibility that survival was due to an unusual variant of RABV that was less pathogenic (a bat-related virus in both cases) or a particular polymorphism in these hosts rendering them less susceptible.

3.2.3.3 Experimental attempts to reproduce the Milwaukee protocol

The recent case of recovery from human rabies in 2004 and its promotion have led to the repetition of this protocol in a number of individual cases. Various attempts have been made to use the original or closely related protocols. According to the special rabies registry website dedicated to the promotion of this protocol, it has been repeated at least 25 times [259]. Regardless of the precise protocol used, most of these trials remained unsuccessful. A brief description of the best described attempts to use this protocol, in chronological order, is provided below.

The first repetition was carried out in multiple cases of rabies transmission via transplanted solid organs from a single infected donor, which occurred in Germany in 2004 [50]. This repetition followed a similar report several months previously in the USA, in which four recipients received solid-organ transplants and vascular grafts from a donor with undetected rabies [51]. In the German report, six patients received organs (corneas in two cases, kidney, lung, liver, and kidney with pancreas for the others) from the German donor, who was retrospectively diagnosed with rabies 45 days after transplantation. At this time, three of the recipients (who had received lung, liver or kidney with pancreas) developed clinical symptoms and aggressive treatment was initiated, based on the recently described Milwaukee

protocol. This included deep sedation (with ketamine and midazolam, with or without phenobarbital) and the administration of potentially antiviral drugs (with ribavirin, α -interferon and amantadine). PEP was also started immediately, including antirabies vaccination (five-dose regimen) and the administration of several HRIG. However, this treatment was unsuccessful, as all these patients developed rabies and died within days of the onset of symptomatic disease. The other three recipients were also given PEP and did not develop clinical signs. Transplanted corneas were removed in two of these cases and tested negative for rabies virus RNA. The third patient, who received a liver transplantation, was treated with ribavirin and α -IFN for three weeks, in addition to PEP. This patient did not develop infection, probably due to a previous antirabies vaccination, 20 years previously.

In 2006, two similar unsuccessful therapeutic approaches were attempted. The first was performed in a male Thai patient in 2006 who presented furious rabies [260]. An aggressive approach was adopted, with treatment with diazepam and thiopental for a 46 h, in addition to ribavirin (enteral) and ketamine (intravenous), but this patient died after eight days of hospitalization. The second of these cases, which was also not confirmed, was treated with the Milwaukee protocol and concerned a 17-year-old male patient in Texas, USA, possibly contaminated by a bat [261]. However, little information is available concerning this case.

At least four other reports of unsuccessful treatment based on the Milwaukee protocol were assessed in 2007. One of these reports concerned two patients in the US [262]. The first case was a female patient probably contaminated a few months previously by an infected bat at home (Indiana, USA). She received the full protocol (including antiviral agents, antiexcitatory therapy and supplementation) but died after 23 days of treatment. The second case in this report was a male patient probably bitten by a rabid dog two years previously in the

Philippines. Sedative and antiviral molecules were administered, but the patient died after 27 days of treatment [262]. The second report concerned a German case treated with a modified version of the protocol, including a vaccination strategy based on classical PEP with two additional vaccinations with live attenuated rabies virus vaccine injected intradermally [263]. The aim of this strategy was to induce an early antibody response, in the hope of improving antigen presentation and response in the skin, via the intradermal route. Ribavirin was not given, due to reports of a lack of antirabies activity and the potential of this drug to delay the cellular response [239]. However, this strategy failed. The third report concerned a Dutch patient infected with Duvenhage virus (lyssavirus, species 4), after contact with a suspected rabid bat in Kenya a few weeks earlier [264-265]. Antiviral and sedative treatment was given, with PEP and aggressive supportive care. The patient died after 20 days of hospitalization. The last report concerned a patient treated with the Milwaukee protocol in Equatorial Guinea [266]. The authors claimed possible success for this treatment, as some semblance of neurological recovery was observed during the course of hospitalization. The patient nonetheless died and the authors suggested that this was due to complications of malnutrition. However, this interpretation is highly debatable. Moreover, it should be stressed that the environment for testing this protocol did not appear to be satisfactory and that the patient was not correctly managed.

During 2008 alone, several other treatments were carried out in different part of the world. A modified Milwaukee protocol was tested in a Canadian patient [267]. This patient presented clinical signs of rabies after being bitten by a bat six months earlier. On day 15 after the onset of symptoms, a therapeutic coma was induced in this patient, with infusions of ketamine, midazolam and propofol. Administration of ribavirin and amantadine was initiated, together with metabolic supplementation (with tetrahydrobiopterin and L-arginine) and intramuscular

injection of HRIG (1200 IU). The therapeutic coma was stopped after three weeks of treatment but the patient remained comatose for the next four weeks, subsequently undergoing brain death. In Northern Ireland, a modified Milwaukee protocol was used on a female patient 14 days after the onset of symptoms. It included the administration of ketamine and midazolam for coma induction, antiviral treatment with amantadine and ribavirin, and metabolic supplementation with co-enzyme Q, tetrahydrobiopterin and vitamin C [268]. Neither rabies vaccine nor rabies immunoglobulin was administered. The patient died after 25 days of treatment. Finally, two other reports of Milwaukee treatment given to patients with clinical rabies have been described in brief. One concerned a nine- or 12-year-old boy from the Goias region of Brazil who contracted rabies from a bat bite. He was treated with antiviral drugs and by coma induction, but died after cardiorespiratory arrest [269-270]. The other case was an eight-year-old Colombian girl who was not vaccinated against rabies (not published). She died one month after the initiation of treatment, about one month after being bitten by a cat. However, very little information is available for either of these two cases.

In the same year, a short description of the potentially successful treatment based on the Milwaukee protocol of a 15-year-old Brazilian boy presenting clinical rabies was published. This patient developed rabies almost 29 days after being bitten by a hematophagous bat [271-272]. According to the few data available, the treatment of this patient, hospitalized in the region of Pernambuco, included the administration of ketamine, midazolan and amantadine, without ribavirin or EEG monitoring. This treatment was initiated seven days after the onset of symptoms and continued for 28 days. Partial PEP was given four days after the bat bite (almost 25 days before the onset of symptoms), including four doses of vaccine without the administration of rabies immunoglobulin. A 5th dose of vaccine was given after the onset of the clinical symptoms. Viral RNA was detected by skin biopsy and an increase in neutralizing

antibody levels was measured in the CSF and blood. This patient was still alive at least three months after the first published reports [273-274]. He was conscious and speaking (with difficulty), with good cognitive function but with motor limitations (difficulty walking). This case may be interpreted as a failure of PEP, and remains similar to other cases of vaccination failure. Indeed, the survival of children receiving treatment with rabies immune serum or immunoglobulin before the onset of symptoms, associated with intensive, aggressive care is well recognized (see paragraph 3.2.1). More details of this case are required.

3.2.4 *Conclusions*

All attempts at antiviral treatment for cases of clinical human rabies to date have failed. Retrospectively, these disappointing results are not particularly surprising, given the absence of convincing and reproducible activity against rabies infection in animal models for most of the compounds tested in human rabies. The role of the Milwaukee protocol in the successful and unexpected recovery from encephalitic rabies in one case in 2004 remains unclear, as all published attempts to reproduce these findings have been unsuccessful. Palliative therapy remains of paramount importance in this fatal disease.

3.3 **The future development of antiviral treatment for human rabies disease**

Despite its long history, human rabies remains an almost invariably fatal disease once the clinical signs have appeared. No curative drug therapy is currently available. Several molecules have been tested *in vitro* and in animal models, without convincing results. Antiviral activity against rabies infection has been tested individually for drugs, and the few studies based on large-scale drug screening carried out were too broad and not specific enough. Novel and innovative strategies of antiviral drug identification for use in rabies are clearly required, bearing in mind that such drugs must be actively delivered to the CNS, crossing the blood brain barrier. However, searches for such compounds are limited by the

lack of knowledge on rabies pathogenesis, particularly at the cellular level. The molecular mechanisms leading to the appearance of clinical signs and the neuronal dysfunction observed in rabies remain unsolved. Integrative research programs, including a basic and comprehensive analysis of the biology of RABV (and lyssaviruses in general), together with the use of advanced technology to identify novel effective antiviral compounds, constitute a promising approach. The VIZIER program, an EU-funded FP6 program, provides an interesting example of such a strategy (www.vizier-europe.org). This program was based on the identification of potential new drug targets in RNA viruses, including rhabdoviruses, through comprehensive structural characterization of the replication machinery [12]. This approach is now being pursued through the SILVER program. Until new effective therapeutic molecules against rabies can be identified, careful evaluation of treatments for human rabies is required. The recent case of recovery from human rabies in 2004, following an experimental protocol combining antiexcitatory and antiviral drugs, therapeutic coma and supportive intense care, has been a source of much hope and has attracted considerable attention. However, multiple repetitions of this protocol have been unsuccessful, suggesting that this protocol probably played only a limited role in the favorable outcome of this patient [258]. These findings also demonstrate that the application and promotion of a therapeutic protocol that has not been validated with robust and reproducible antiviral studies *in vitro* and in animal models may lead to unproductive, disappointing results.

Conclusion

With an estimated 55,000 human deaths from rabies occurring each year worldwide, this disease is the infectious disease with the highest mortality case ratio, affecting mostly young children from rural regions of developing countries. Despite the lack of an effective antiviral treatment once the disease is established, tools for decreasing and eliminating human rabies

viruses are well known, validated and generally available. These tools combine coordinated public health and veterinary programs. In particular, established public health systems and networks of surveillance, education of the population about rabies and widespread access to medical care for the administration of PEP are essential. In parallel, canine rabies elimination is a key step towards decreasing the burden of disease, with mass vaccination campaigns and management of the dog population, associated with a national program of rabies surveillance. The use of oral vaccination in free-ranging carnivore hosts has demonstrated the feasibility of rabies elimination in wildlife. This approach has proved effective in many countries, including those of Europe in particular.

In parallel, there is clearly an urgent need to increase basic knowledge on rabies virus and the physiopathological mechanisms involved in rabies encephalitis, for the ultimate development of effective antiviral drugs for treating rabies infection.

Table 1: Lyssavirus classification and associated epidemiological characteristics

Name	Species	Geographic distribution	Reservoirs and/or vectors	Other infected animals	Human cases	Vaccine protection
Rabies virus	RABV	Worldwide except Antarctica, Australia, Western Europe, United Kingdom, part of Scandinavia and some islands	Dog, wild carnivores, bats (solely for American continent)	Human, wild and domestic carnivores, herbivores	55,000/year (99% due to dogs, <1% due to bats)	Yes
Lagos bat virus	LBV	Africa: Nigeria, Central African Republic, South Africa, Senegal, Ethiopia, Guinea, Zimbabwe	Frugivorous bats (species <i>Eidolon</i> , <i>Epomophorus</i> , <i>Rousettus</i> , <i>Micropteropus</i>)	Insectivorous bats (species <i>Nycteris</i>), cats, dogs, aquatic mongoose	None	No
Mokola virus	MOKV	Africa: Nigeria, Central African Republic, Zimbabwe, Cameroon, Ethiopia, South Africa	Undetermined	Human, shrew, cats, dogs, rodent	1 confirmed case (Nigeria, 1971), 1 suspected (Nigeria, 1969)	No
Duvenhage	DUVV	Africa: South Africa, Zimbabwe	Insectivorous bats (species <i>Miniopterus</i> , <i>Nycteris</i>)	Human	3 (South Africa, 1971, 2006; Netherlands via Kenya, 2007)	Partial
European bat lyssavirus type 1, (subtype a or b)	EBLV-1	Europe	Insectivorous bats (mainly species <i>Eptesicus</i>)	Human, others insectivorous bats (?), cats, sheep, stone marten	1 confirmed and 2 suspected (Russia, 1985)	Partial
European bat lyssavirus type 2	EBLV-2	Europe	Insectivorous bats (mainly species <i>Myotis</i>)	Human	2 (Finland, 1985, Scotland, 2002)	Partial
Australian bat lyssavirus	ABLV	Australia	Frugivorous bats (species <i>Pteropus</i>) and insectivorous bats (mainly species <i>Saccolaimus</i>)	Human	2 (Australia, 1996, 1998)	Partial
Aravan virus	ARAV	Central Asia (Kirghizistan)	Insectivorous bats (species <i>Myotis</i>) (isolated once in 1991)	?	None	Partial
Khujand virus	KHUV	Central Asia (Tadjikistan)	Insectivorous bats (species <i>Myotis</i>) (isolated once in 2001)	?	None	Partial
Irkut virus	IRKV	East Siberia	Insectivorous bats (species <i>Murina</i>) (isolated once in 2002)	?	None	Partial
West Caucasian	WCBV	Caucasian region	Insectivorous bats (species	?	None	No

bat virus			<i>Miniopterus</i>) (isolated once in 2003)			
Ozernoe lyssavirus	To be assigned, closely related to Irkut virus	Far East Russia	Bats (?)	Human	1 human case (2007)	?
Shimoni bat virus (SHIBV)	To be assigned	Africa: Kenya	Bats (species <i>Hipposideros commersoni</i>)	?	None	?

Table 2: Modalities of rabies post-exposure prophylaxis (PEP) according to WHO recommendations [93]

Categories of exposure	Description of exposure	Prophylaxis
Category I	touching or feeding animals, licks on intact skin (that is, no exposure)	no prophylaxis
Category II	nibbling of uncovered skin, minor scratches or abrasions without bleeding	immediate vaccination ^a
Category III	single or multiple transdermal bites or scratches, contamination of mucous membrane with saliva from licks, licks on broken skin, exposures to bats	immediate vaccination and administration of rabies immunoglobulin ^a

^a For categories II and III, all bite wounds and scratches should be thoroughly washed and flushed (for about 15 minutes if possible) with soap/detergent and copious amounts of water immediately, or as soon as possible.

Table 3: Overall description of the main drugs tested for antiviral activities against rabies infection *in vitro* and in animal models

Molecule	Test ^a	Treatment (dose, route of administration, starting time, incubation period or duration of treatment, other)	Virus challenge (strain, dose, route of inoculation)	Evaluation of antiviral activities	Main results (best results: number of survivors /total number)	Reference
Inhibitors of viral entry						
Antibodies against rabies glycoprotein and rabies vaccines						
Live-attenuated ERA-derived rabies vaccine (produced on BHK cells)	<i>In vivo</i> (dogs, 2 successive experiments)	2 ml (titer = $10^{6.5}$ i.c.LD ₅₀ /0.03 ml in mice); i.t.; At the time of clinical signs of rabies	Fox strain; 5.92×10^6 - 5.92×10^4 i.c.LD ₅₀ (mice) (1st exp.) or 5.92×10^4 i.c.LD ₅₀ (mice) (2 nd exp.); i.ma. for both	Mortality rate; Virus detection and isolation (also intra-vitam for 2 nd exp.); Level of NA (2 nd exp. only)	1 st exp.: suggested therapeutic effect (recovery of 3/12 dogs), but no <i>intra-vitam</i> confirmation of rabies infection and no use of control animal group; 2 nd exp.: no effect (only 4/7 challenged dogs became symptomatic and all died), suggested increase in survival period for 2 animals; No convincing therapeutic activity in clinical rabies	[134]
Live-attenuated Flury HEP derived rabies vaccine (RV 675 strain, produced on BHK-21 cells)	<i>In vivo</i> (monkeys)	1 ml (titer = $10^{8.3}$ i.c.LD ₅₀ /ml in mice); i.t.; At onset of symptoms	Dog strain (MR strain); i.m.	Mortality rate; Virus detection and isolation (intra-vitam and post- mortem); Level of NA	No therapeutic effect or increase in survival period (only 2/12 challenged monkeys were symptomatic and receive the vaccine, and both died)	[136]
Live-attenuated recombinant SAD B19- derived rabies vaccine (SPBAANGAS- GAS-GAS)	<i>In vivo</i> (mice)	10^7 FFU for i.c. or 10^8 FFU for i.ma. i.t.; T4h to T5d p.i.	Dog strain (DOG4 strain); 10 i.m.LD ₅₀ i.m. (10^6 FFU)	Mortality rate	Complete and effective protection only when given i.c. or i.ma. shortly after infection (4 h p.i.); Number of (healthy) survivors lower when given >4 h p.i. (50% or 30% at 4d p.i., after	[135]

	strain expressing 3 mutated (R333G) G genes, produced in BSR cells)					i.c. or i.ma. administration, respectively); No evaluation in symptomatic animals	
Receptor-specific inhibitors							
	α -bungarotoxin (α -Btx), d-tubocurarine (d-TC)	<i>In vitro</i> (cultured chick myotubes)	10^{-5} and 10^{-6} M (α -Btx), 10^{-3} M (d-TC); T(-2)h (for both) to T3 d p.i. (for α -Btx; incub. up to 6 d; For α -Btx: directly with virus suspension, at the binding step	Lab. strain (CVS), 1820B strain; 2×10^6 LD ₅₀	Detection of infected cells; Virus titration	Inhibition of virus infection and production (α -Btx=99.9% with 10^{-5} M, d-TC=99.7% with 10^{-3} M); Restricted to the virus binding step; No virucidal effect	[17]
	α -bungarotoxin (α -Btx), d-tubocurarine (d-TC)	<i>In vitro</i> (CER, BHK-21, NA-1300, C6/36, L8 and L8CL3U cell lines)	10^{-5} - 10^{-8} M (α -Btx), up to 5×10^{-4} M (d-TC); T(-15) min p.i. or during infection (incub. 1-5 d)	Lab. strain (CVS); moi=10 PFU/cell	Counts of infected cells; Virus titration	No inhibition of virus infection; No decrease in virus production	[140]
	α -bungarotoxin (α -Btx)	<i>In vitro</i> (primary cultured rat myotubes)	10^{-5} M or 10^{-10} M; T(-2)h p.i. or T2h p.i.	Lab. strain (CVS); Dilutions of suspension of 10^7 - 10^8 PFU/ml	Counts and observation of infected cells	Dose-dependent virus inhibition only at T(-2) h p.i., with 10^{-5} - 10^{-7} M	[138]
	α -bungarotoxin (α -Btx)	<i>In vitro</i> (mouse neuroblastoma cells)	1 μ g; incub. o.v. p.i.	Lab. strain (CVS unlabeled and H ³ labeled)	Measurement of radioactivity at different times p.i.	Virus inhibition (specific inhibition of virus binding)	[141]
	α -bungarotoxin (α -Btx), d-tubocurarine (d-TC)	<i>In vitro</i> (IMR-32)	Determination of the IC ₅₀ ; 10^{-4} - 10^{-9} M (α -Btx) and 10^{-4} M (d-TC, conc. max.); T(-0.5) h p.i. (incub with virus or with cells), T0 p.i. and incub. 1-3 d or just during binding step	Lab. strain (CVS); Dilutions of suspension of 5×10^5 - 5×10^7 FFU/ml	Counts of infected cells	Dose-dependent virus inhibition only with α -Btx: IC _{50%} = 5.6×10^{-6} M; Inhibition of virus binding	[139]

	Heparan sulfate, anti-NCAM antibodies, soluble NCAM protein	<i>In vitro</i> (BSR and N2a cells)	10 µg/ml (heparan sulfate), 5-10 µg/ml (anti-NCAM antibodies); T(-0.5) h p.i.; incub. 30 min; for both compounds; 0.7-1 µg (soluble NCAM protein); T0 p.i. (with virus); incub. 40 min	Lab. strain (CVS); Serial threefold dilutions or 13 µg of virus (with soluble NCAM protein only)	Counts of infected cells; Virus titration	Virus inhibition (heparan sulfate and anti-NCAM antibodies), at the virus attachment step; Dose-dependent virus inhibition with soluble NCAM protein (virucidal effect) (up to 100% with 1 µg) and virus production (3 log reduction)	[18]
	Neurotrophins (NGF, NT-3-)	<i>In vitro</i> (dorsal root ganglion cells)	NGF: 2 and 2 ng/ml; NT-3: 1 and 5 ng/ml; T<0 p.i., incub. 4 d; T≥0 p.i., incub 1, 6 or 24 h	Lab. strain (CVS); moi=0.025 or 0.1	Counts of infected cells; Detection of viral RNA	Potential virus inhibition (T≥0 p.i., best results= 15.2-17.8% inhibition)	[146]
	Gangliosides (from bovine brain material)	<i>In vitro</i> (CER cells)	0.048-30 µg/ml or 0.15-3.75 mg/ml; T-2 h p.i. (incub with virus or with cells), T0 p.i.	Lab. strain (CVS); moi= 1.5 and 0.15 PFU/cell	Counts of infected cells	Dose-dependent virus inhibition with pretreated cells and when mixed with virus suspension (probably at the virus binding step)	[21]
	Gangliosides	<i>In vitro</i> (IMR-32)	Determination of the IC ₅₀ ; T(-0.5) h p.i. (incub with virus or with cells), T0 p.i. and incub. 1-3 d or just during binding step	Lab. strain (CVS); Dilution 5 x 10 ⁵ -5 x 10 ⁷ FFU/ml	Counts of infected cells	Virus inhibition (IC ₅₀ =1:25); Acting at the virus binding step	[139]
	Phospholipids and phospholipases (9 compounds)	<i>In vitro</i> (CER cells)	Various concentrations (phospholipases) and 500 µg/ml for all phospholipids (with also a range of dilutions); Before (incub. with cells or virus), at the time of inoculation or after infection	Lab. strain (PV, CVS); moi= 1.5 and 0.15 PFU/cell	Counts of infected cells	Virus inhibition (depending on the nature and conc. of drugs, time of incub.); Stronger effect when prior incub. phospholipids with virus; Dose-dependent virus inhibition with phospholipase A2	[20, 22]
	Concanavalin A	<i>In vitro</i> (CER cells)	62.5-1000 µg/ml; Before or after infection, just after viral binding step or directly added to virus suspension	Lab. strain (CVS); moi=1.5 or 0.2 PFU/cell	Counts of infected cells	Dose-dependent virus inhibition (50-100% with 200 µg/ml) when added at about the time of viral attachment; Virucidal effect	[23]
	Lectins	<i>In vitro</i>	various concentrations;	Lab. strain	Counts of	Virus inhibition suggested for	[147]

	(5 compounds)	(CER cells)	during the adsorption step or/and incub. 1 h or 24-48 h	(CVS)	infected cells	all, with dose-dependent effect for 4, and relevant SI for only 2	
	Neuraminidases (from <i>C. perfringens</i> , and <i>V. cholerae</i>)	<i>In vitro</i> (CER cells)	2.5-20 U/ml; T(-1) h p.i.	Lab. strain (CVS); moi=1.5 and 0.15 PFU/cell	Counts of infected cells	Virus inhibition only after prior treatment of cells (100% and 80-90% with 20 U/ml and 10 U/ml of neuraminidases from <i>C. perfringens</i> , respectively)	[20-21]
Metabolic and nucleotide analog inhibitors							
Metabolic inhibitors							
	Actinomycin D	<i>In vitro</i> (BHK-21)	2-250 µg/ml; T(-0.5) h p.i.; incub. 30h	Lab. strain (CVS); moi = 5 PFU/cell	Virus titration	No virus inhibition; Drug cytotoxicity	[152]
	Several drugs including actinomycin D, puromycin, nogalamycin, cycloheximide, and mitomycin C	<i>In vitro</i> (BHK-21)	actinomycin D (0.25 µg/ml), nogalamycin (0.25 µg/mL), cycloheximide (5 µg/ml), puromycin (5 µg/ml), mitomycin C (2 µg/ml); T(-2) h p.i.; incub. ≤26h; +/- ara-C	Lab. strain (PM); 5-10 mouse LD ₅₀	Counts of infected cells; Virus titration	Potential virus inhibition with puromycin and cycloheximide	[154]
Pyrimidine nucleoside analogues							
	Ara-C	<i>In vitro</i> (BHK-21)	2-250 µg/ml; T(-0.5) h; Incub. 30h	Lab. strain (CVS); moi = 5 pfu/cell	Virus titration	No virus inhibition; Drug cytotoxicity	[152]
	ara-C, ara-U and 5- and 3-iodo derivatives, MADU	<i>In vitro</i> (BHK-21 cells and various other cells for ara-C)	Ara-C: 10-500 µg/ml, T(-24) h to T3 h p.i., incub. ≤26 h; Other drugs: 50 µg/ml, T0 p.i., incub. 24 h	Lab. strain (PM, CVS, HEP, PV for ara-C); 5-10 mouse LD ₅₀ /cell	Counts of infected cells	Virus inhibition with ara-C at T0-T3h p.i.	[154]
	Ara-C, 6-azu	<i>In vitro</i> (BHK-21 cells)	25-200 µg/ml; T1 h p.i.; Incub. 72 h	Lab. strain (HEP); moi=3 x 10 ⁻³ PFU/cell	Virus titration	Dose- and time-dependent virus inhibition; Drug cytotoxicity	[155]

		<i>In vivo</i> (mice)	ara-C: 20-80 mg/kg (twice a day for 7 d), 6-azu: 12.5-100 mg/kg (single dose for 7 d); i.p. for both; T2 h p.i.	Skunk strain (with ara-C), bobcat strain (with 6-azu); 1 i.m.LD ₅₀ for both; i.m.	Mortality rate	No protection; Drug toxicity	
Purine nucleoside analogs							
	Ara-A	<i>In vitro</i> (BHK-21 cells)	25-200 µg/ml; T1 h p.i.; Incub. 72 h	Lab. strain (HEP); moi=3 x 10 ⁻³ PFU/cell	Virus titration	Dose- and time-dependent virus inhibition; Drug cytotoxicity	[155]
		<i>In vivo</i> (mice)	100-600 mg/kg (twice a day for 7 d); i.p.; T3 h p.i.	Skunk strain 1 i.m.LD ₅₀ ; i.m.	Mortality rate	No protection; Drug toxicity	
	Ara-C	<i>In vivo</i> (mice)	20 mg/kg; s.c.; T(-1) d to T3 d p.i.	Rodent strain; i.m. or i.c.;	Mortality rate; Virus titration	No protective effect Results remain difficult to interpret	[156]
	(S)-DHPA	<i>In vivo</i> (mice)	1-10 mg/kg; single (subdivided or not) or multiple doses; p.o. or i.m. T(-2) d to T2 d p.i.	Lab. strain (CVS), fox and rodent strains; 3-36 LD ₅₀ ; i.m. or i.c.	Mortality rate; Level of NA	Virus inhibition and partial protective effect; No virucidal effect; Results remain difficult to interpret	[160]
	(S)-DHPA	<i>In vivo</i> (mice and foxes)	Mice: 0.5 mg/kg for i.c., 118 or 150 mg/kg/d for i.m.; T1-5 d p.i.; up to 5 d; Foxes: 30 mg/kg; p.o.; T0 p.i.	Fox strains; 10 ² i.m.LD ₅₀ (mice), 40-9000 LD ₅₀ (foxes); i.m. for both species	Mortality rate; Level of NA	No protective effect	[161]
	(S)-DHPA and other derivatives	<i>In vitro</i> (CER cells)	5-500 µg/ml; T40 min p.i. or direct	Lab. strain (CVS);	Virus titration, protein and	Dose-independent virus inhibition (up to 90%);	[158-159]

	of adenosine (CDZ and 3 acyclic derivatives)		incubation with virus suspension; Incub. up to 40 h	moi=10 PFU/cell	RNA synthesis, electron microscopy	No virucidal effect	
	Ribavirin	<i>In vitro</i> (CER cells)	5-500 µg/ml; T40min p.i. or direct incubation with virus suspension; Incub. up to 40 h	Lab. strain (CVS); moi=10 PFU/cell	Virus titration, protein and RNA synthesis, electron microscopy	Dose-dependent virus inhibition (50% and 99.9% with 6.25 and >50 µg/ml, respectively, at ≤ 8h p.i.); No virucidal effect	[158-159]
	Ribavirin and two analogs (RTA and selenazofurin)	<i>In vivo</i> (mice)	i.m. route: T1-5d p.i.; up to 5 d; 59 or 100 mg/kg/d (ribavirin), 50 or 100 mg/kg/d (RTA), 100, 143 or 286 mg/kg/d (selenazofurin); i.c. route:	Fox strains; 102 i.m.LD ₅₀ (mice); i.m.	Mortality rate, level of NA	No protective effect	[161]
	G-7-Ox	<i>In vitro</i> (BHK-21 cells)	N.Av.	Lab. strain (HEP)	Counts of infected cells; Determination of IC ₅₀	Virus inhibition: IC ₅₀ less than 3 µg/ml	[162]
Lysosomotropic agents							
	Chloroquine, quinine and NH ₄ Cl	<i>In vitro</i> (IMR-32 cells)	Determination of the IC ₅₀ ; T(-0.5)h p.i. (incub with virus or with cells), T0 p.i. and incub. 1-3 d or just during binding step	Dilutions of suspension of 5 x 10 ⁵ -5 x 10 ⁷ FFU/ml	Counts of infected cells	Dose-dependent virus inhibition (IC ₅₀ = 2.4 x 10 ⁻⁵ M for chloroquine; 2.2 x 10 ⁻⁵ M for quinine and 1.5 x 10 ⁻³ M for NH ₄ Cl; Acting after virus attachment step	[139]
	Chloroquine, NH ₄ Cl	<i>In vitro</i> (mouse neuroblastoma cell lines and primary rat cells)	20 mM (NH ₄ Cl), 0.1 mM or 5 x 10 ⁻⁴ -5 x 10 ⁻⁹ M (chloroquine); Add at different times with various incub.	Lab. strain (CVS); moi=1.5 and 0.15 PFU/cell	Count of infected cells	100% virus inhibition with chloroquine (0.1 mM) and NH ₄ Cl (20 mM) at T0 or nearly time and after incub. 24 h; No virucidal effect	[20, 163{]}
	Methylamine, monensin	<i>In vitro</i> (IMR-32 and CER cells)	Methylamine: 2 x 10 ² -100 x 10 ² µM; Monensin: 0.05-10 µM;	Lab. strain (CVS); moi = 5-10 PFU/cell	Detection of infected cells	Dose-dependent virus inhibition; Acting after virus adsorption step (T≥1 h p.i.);	[164]

			T(-1) h p.i. (with cells), T0 (at virus attachment step) or T(1-5) h p.i. (after virus adsorption step) Incub. 1-24 h			No virucidal effect	
Drugs acting on the cytoskeleton							
Drugs targeting actin microfilaments or calmodulin processes							
	Cytochalasin B	<i>In vitro</i> ^b (rat embryonic DRG cells)	0.1 mM, +/- vinblastine; T0 or T4-24 h p.i.; incub. 72 h	Lab. strain (CVS); 5 x 10 ^{6.85} PFU	Count of infected cells, virus titration	No virus inhibition after established infection (24 h p.i.)	[166]
	Cytochalasin B	<i>In vitro</i> (BHK-21 and CER cells)	13-208 µM; immediately p.i.; incub. 32 h	Lab. strain (CVS); moi = 10 PFU/cell	Virus titration	No virus inhibition	[165]
	Cytochalasin B and D, trifluoperazine	<i>In vitro</i> (IMR-32 and CER cells)	Cytochalasin B and D: 0.128-16 µM; Cytochalasin B: 0.128-16 µM; Trifluoperazine (1-20 µM); T(-1) h p.i. (with cells), T0 (at virus attachment step) or T(1-5) h p.i. (after virus adsorption step) Incub. 1-24 h	Lab. strain (CVS); moi = 5-10 PFU/cell	Detection of infected cells	Dose-dependent virus inhibition; Acting after virus adsorption step (T≥1 h p.i.); No virucidal effect	[164]
	Cytochalasin B, EGTA, A23187, chlorpromazine, trifluoperazine, nifedipine	<i>In vitro</i> (rat primary cortical neurons, IMR-32, BHK-21 and CER cells)	Cytochalasin B: 1-20 µg/ml; EGTA (0.5-0.37 mM), A23187 (0.05-1.0 µM), chlorpromazine (1-30 µM), trifluoperazine (1-20 µM), nifedipine (1-10 µM); T1 h p.i.; Incub. 48 h (up to 4 d with EGTA)	Lab. strain (CVS); 4.6 x 10 ⁶ FFU	Virus titration; Detection of infected cells	No virus inhibition	[167]
Drugs targeting microtubules							
	Vinblastine, colchicine	<i>In vitro</i> ** (rat embryonic DRG cells)	Vinblastine: 0.1 mM, (+/- cytochalasin); T0 or T4-24 h p.i.; Colchicine: 0.1 mM; T(-1-4) h, T0 or T(1-4) h p.i.;	Lab. strain (CVS); 5 x 10 ^{6.85} PFU	Counts of infected cells, virus titration	No virus inhibition after established infection (24 h p.i. for vinblastine, 4 h p.i. for colchicine)	[166]

			Incub. 72 h for both				
	Vinblastine, colchicine	<i>In vitro</i> (IMR-32 and CER cells)	Vinblastine: 1-10 μ M; Colchicine: 10-100 μ M; T(-1) h p.i. (with cells), T0 (at virus attachment step) or T1 h p.i. (after virus adsorption step) Incub. 24 h	Lab. strain (CVS); moi = 5-10 PFU/cell	Counts of infected cells	No virus inhibition; No virucidal effect	[164]
	Colchicine, colcemid	<i>In vitro</i> (BHK-21 and CER cells)	Colchicine: 16-250 μ M, colcemid 17-336 μ M; immediately p.i.; incub. 32 h	Lab. strain (CVS); moi = 10 PFU/cell	Virus titration	No virus inhibition	[165]
	Colchicine	<i>In vivo</i> (rats)	Solution of 2, 5 or 10% colchicine, in the sciatic nerve (delimited with a silicone cuff); T-(4-48) h p.i.	Lab. strain (CVS), fox strain; i.m. for both	Mortality rate; level of NA; antigen detection and virus isolation (on sciatic nerve sections)	Dose-dependent virus inhibition; Protective effect (up to 100% or 6/6 with 5% colchicine solution, T-(24) h p.i.) No virucidal effect	[168]
	Vinblastine, colchicine	<i>In vivo</i> (mice)	26-64 μ g (colchicine), 9-12 μ g (vinblastine), given locally on sciatic nerves using impregnated blotting papers); T0 or T(2-60) h p.i.	Lab. strain (CVS, $10^{7.3}$ i.c.LD ₅₀), fox strain ($10^{5.3}$ i.c.LD ₅₀); i.m. for both	Mortality rate; Level of NA	Protective effect (up to 100% or 10/10 at T0 p.i. for both with CSV strain); Protective effect up to 6 h or 24 h with CVS or fox strain, respectively	[169]
IFN and IFN-inducers							
IFN							
	Exogenous (mice origin)	<i>In vitro</i> (mouse cells)	10-20 x 10^3 U; T(-24) h to T24 h p.i.; incub. up to 96 h	Lab. strain; 650LD ₅₀	Counts of infected cells	Dose- and time-dependent virus inhibition	[172]
	Exogenous (dog origin)	<i>In vitro</i> (dog cells)	Fixed quantity, different batches; T(-24-1)) h p.i.	Lab. strain (PV); 10^{-5} (MIT titration)	Virus titration	Decrease in virus production (T(-24) h p.i.)	[173]
	Exogenous (human or hamster origin)	<i>In vitro</i> (BHK cells, primary human)	Overnight incubation (18 h) with 100 IU/2 ml before infection	Lab. strain (ERA); moi=5 PFU/cell	Counts of infected cells; Virus titration	Decrease in virus infectivity (80-97-%) and production (nearly 1 to 3 log)	[174]

		fibroblasts)					
	Exogenous (human origin)	<i>In vitro</i> (MCK cells)	25-6,400 U/ml; T0 p.i.; Incub. 1 d	Lab. strain (LEP); 10 ⁴ mouse LD ₅₀	Virus titration	Decrease in virus production	[183]
	Endogenous (inactivated vaccinia virus)	<i>In vivo</i> (rabbits)	Multiple doses; i.c., i.ca., i.d., i.p., i.v., s.c.; T(-24) h or from T24 h p.i.;	Dog strain; 10 ^{-5.31} (MIT titration); i.d.	Mortality rate	100% protection with i.d. s.s.i. (5/5); Partially protective with i.c. and i.ca.	[185-186, 275] [275]
	Exogenous (rabbit origin)	<i>In vivo</i> (rabbits)	1.6-6.4 x 10 ⁶ U (total dose); i.v. and/or i.m.; T(0-24) h p.i.	Fox strain; 80 rabbit i.m.LD ₅₀ ; i.m.	Mortality rate	Total to partial protection (6/6, i.m., T0 p.i. highest dose); No virucidal effect	[176]
	Exogenous (rabbit origin)	<i>In vivo</i> (rabbits)	1-10 x 10 ⁶ U (total dose), multiple doses, up to 3 we. i.v. or i.v. and i.m. (in combination); T(0-21) p.i.	Fox strain; ≥ 28DL ₅₀ ; i.m.	Mortality rate	Partial protection with i.v. and i.m. route (2/3)	[276]
	Exogenous (mouse origin)	<i>In vivo</i> (mice: immuno- competent, - depressed or – deficient)	10 ⁵ U/d, up to 30 d i.p.; T1 h p.i.	Fox strain; 10 mouseLD ₅₀ ; i.m.	Mortality rate, Titration of NA	Partial protection with nude (7/8) and immunocompetent mice (7/14); Virucidal	[175]
	Exogenous (human or mouse origin)	<i>In vivo</i> (mice and rhesus monkeys)	0.03 ml (mice), 1 x 10 ⁶ U (monkeys) (multiple doses); i.m.; T24 h p.i. (both) and T(-6) h p.i. (monkeys); +/- rabies vaccine	Fox and skunk strain; 5-30 mouse peripheral LD ₅₀ (mice); i.m. for both	Mortality rate, level of IFN and NA	Partial protection (monkeys= 7/8, T6h p.i., + rabies vaccine) (mice= 41/50, T0 p.i., s.s.i.)	[194]
	Exogenous (human origin)	<i>In vivo</i> (<i>Cynomolgus</i> monkeys)	1-10 x 10 ⁵ U/d; i.m. and/or i.l.; T(1-3) d p.i.; up to 7 d	NYC strain; 10 ^{5.7} mouseLD 50; i.m.	Mortality rate; Level of NA	Partial protection (8/10 with i.m. and i.l., T24 h p.i. up to 13 d)	[184]
	Exogenous (human origin)	<i>In vivo</i> (<i>Cynomolgus</i> monkeys)	1-11 x 10 ⁶ U (total dose) (multiple doses and/or days); i.m. and/or i.l.; T(-4) h to T11 d p.i., and after first clinical signs	NYC strain; 10 ^{5.7-6} . (mouse)/monk ey LD ₅₀ ; i.m.	Mortality rate; Level of IFN and NA	Partial protection (5/9 for i.m. or 8/10 for i.m.+i.l. at T1d p.i. with multiple doses); No effect when given after appearance of clinical signs	[183, 196]

IFN-inducers							
	Poly I:C or/and COAM	<i>In vivo</i> (mice)	poly I:C: 0.1 mg (1 or 2 doses); i.m. s.s.i.; COAM: 0.3 mg; i.p.; T3h p.i. for both	Skunk strain; i.m.	Mortality rate; Level of IFN and NA	Partial protection with poly I:C alone (30/32) or in combination with COAM (31/32); No protection of COAM alone (15/33)	[192]
	Poly I:C	<i>In vivo</i> (rabbits and mice)	Rabbits: 0.5-1 mg (multiple doses); i.m. or i.v.; T(-1) d to T1 d p.i. Mice: 0.26 -33 µg (i.m.) or 100 µg (i.p.) (single or multiple doses); T0-T91 h p.i.	Lab. strain (CVS, 1 rabbit LD ₅₀); Skunk strain (1 mouse LD ₇₅ , 1 rabbit LD ₁₀₀); i.m. for both	Mortality rate, level of IFN and NA	Total to partial protection with poly I:C at highest dose, given around time inoculation (up to 100% and 94% in rabbits and mice, respectively)	[189]
	Poly I:C, poly I, poly C	<i>In vivo</i> (rabbits)	0.04-1 mg/kg (single dose); i.v., i.m. or i.ma.; T0 p.i.	Fox strain; 5-3125 LD ₅₀ ; i.m. or i.ma.	Mortality rate, Level of IFN	Total to partial protection, only with poly I:C (up to 100% or 6/6)	[188]
	Poly I:C	<i>In vivo</i> (rabbits)	1 mg (single dose); i.v.; T-1 d to T1 d p.i.	Fox strain; 25-30 rabbit DL ₅₀ ; i.ma.	Mortality rate; Level of IFN and NA	Total to partial protection (up to 100% until T3 h p.i.)	[187]
	Poly I:C, NDV	<i>In vivo</i> (rhesus monkeys)	Poly I:C: 15 mg/kg. i.v.; NDV: 2.5 x 10 ⁸ PFU per animal; T(5-8) h p.i. +/- rabies vaccines	NYC strain; 10 ⁵ i.c. mouse LD ₅₀ ; i.m.	Mortality rate; Level of IFN and NA	Partial protection with rabies vaccines (poly I:C = 5/10 and NDV = 2/10); No improvement of protection over rabies vaccines alone	[195]
	Statolon	<i>In vivo</i> (mice)	i.p.; T(-(24-18)) h p.i.	i.m. or i.c.	Mortality rate	No protection	[182]
	NDV (infectious or inactivated)	<i>In vivo</i> (hamsters, mice, rabbits)	i.v. (mice), i.p. (rabbit and hamster); T(-24) h, T0 or T24 h p.i.	Lab. strains (CSV, VP10 or 11); i.m. (hamsters and mice), i.c. (rabbits)	Mortality rate Level of IFN	No protection in mice; Partial protection in hamster (35/51 to 40/57) and rabbits (5/8)	[179]
	Poly I:C	<i>In vivo</i> (mice)	100 µg (per dose) (single or multiple doses); i.m.;	bobcat and skunk strains; nearly 1 LD ₅₀ ; i.d.	Mortality rate; Level of IFN	Partial protection at s.s.i, alone (83/132) or combined with rabies vaccines (130/150)	[193]

			Starting T3 h p.i.; +/- rabies vaccines				
	PICKCa (derivative of poly I:C)	<i>In vivo</i> (mice)	25-50 µg (per dose) (single or multiple doses); T6 h or T3 d p.i., up to 7 s.c. at s.s.i. +/- rabies vaccines	Lab. strain (CVS); Fox strain; 1.5-4 x 10 ⁶ , 1.5-4 x 10 ⁷ or 1 x 10 ⁸ i.c.LD ₅₀ ; s.c.	Mortality rate, Level of IFN and NA	Complete to partial protection (at s.s.i., T ≤6 h p.i., up to 100% or 20/20); Comparable protective effect with poly I:C; Stronger protective effect in combination with rabies vaccines	[194]
	Poly ICLC	<i>In vivo</i> (mice and rhesus monkeys)	0.06 mg equi. poly I:C (per dose) (mice), 2 mg poly ICLC (per dose) (monkeys), multiple doses; T24 h p.i. (both) and T6 h p.i. (monkeys); +/- rabies vaccine	Fox and skunk strain; 5-30 mouse peripheral LD ₅₀ (mice); i.m. for both	Mortality rate, level of IFN and NA	Partial protection (monkeys= 8/8, T6 h p.i., + rabies vaccine) (mice= 47/50, T0 p.i., s.s.i.)	[194]
Ketamine and dizocilpine (MK-801)							
	Dizocilpine and AP5	<i>In vitro</i> (rat primary cortical neurons)	0-2 mM; T1h p.i.; Incub. 4 d	Lab. strain (CVS); 10 ⁶ UFF/ml	Virus titration	Specific dose-dependent virus inhibition (range 0.5-2 mM); No effect with AP5; effective at high doses; No virucidal effect	[202]
	Ketamine and dizocilpine	<i>In vitro</i> (rat primary cortical neurons)	Ketamine: 1-1.5 mM, dizocilpine: 1 mM; After infection for both; Incub. 48 h	Lab. strain (CVS); 10 ⁶ UFF/ml	Count of infected cells; Viral titration; Radiolabeling; Northern blot	Virus inhibition; 100-fold decrease of virus production	[200]
	Ketamine and other competitive or non-competitive NMDA receptors	<i>In vitro</i> (rat primary cortical neurons, BHK-21 and IMR-32 cells) <i>In vivo</i> (rats, only with ketamine)	<i>In vitro</i> : ketamine: 0.2-2 mM, after virus infection, incub. 1- 4 d <i>In vivo</i> : i.p.: 15 mg, twice daily for 4 d	Lab. strain (CVS) <i>In vitro</i> : 4 x 10 ⁶ UFF/ml <i>In vivo</i> : 10 ⁵ mouse i.c.LD ₅₀ , stereotaxic inoculation (right striatum)	Virus titration, counts of infected cells (<i>in vitro</i>); Mortality rate (<i>in vivo</i>)	Dose-dependent virus inhibition (<i>in vitro</i>); 100-1000-fold decrease in virus production (1-2 mM, <i>in vitro</i>); No virucidal effect; Potential virus inhibition in certain parts of the CNS (<i>in vivo</i>)	[201]

	Ketamine and dizocilpine	<i>In vitro</i> (mice primary cortical and hippocampus cells) <i>In vivo</i> (mice, only with ketamine)	<i>In vitro</i> : 125 μ M (ketamine), 60 μ M (dizocilpine); incub. 3 d for both <i>In vivo</i> : 60 mg/kg (twice daily); i.p.; T3d p.i., for 3 d	Lab. strain (CVS); i.c. or i.m.	Cellular viability (<i>in vitro</i>); Mortality rate (<i>in vivo</i>)	No (neuro)protection	[205]
	Ketamine	<i>In vitro</i> (McCoy cells)	3 mM; T0 p.i.; incub 96 h	Lab. strain (PV); moi=1	IC ₅₀ and S.I. determination (inhibition of viral cytopathic effect)	Virus inhibition (S.I. > 3.3)	[204]
Amantadine							
		<i>In vitro</i> (CER cells)	0.032 -200 μ M/ml; Before (incubation with cell) or at the time of infection, after attachment step, incub. up to 2 d	Lab. strains (PV, CVS, ERA); moi=1.5 PFU/cell	Counts of infected cells	Dose-dependent virus inhibition (> 4 mM), only effective <2 h after infection, with \geq 30 min incubation; No virucidal effect	[208]
		<i>In vitro</i> (CER cells)	5-500 μ g/ml; T40 min p.i. or direct incubation with virus suspension incub. up to 40 h	Lab. strains (CVS); moi=10 PFU/cells	Virus titration (single-growth cycle conditions)	Dose-dependent virus inhibition, from 50 (20%) to 250 μ g/ml (99.9%); No virucidal effect	[159]
		<i>In vivo</i> (mice)	30 or 67 mg/kg/d; i.m.; T1-5 d p.i.; for up to 5 d	Fox strains; Mice: 10 ² i.m. LD ₅₀ ; i.m.	Mortality rate; Virus isolation; Level of NA	No protective effect	[161]
Isoprinosine							
		<i>In vitro</i> (BHK-21 cells)	150 μ g/ml/d; T1h p.i.; incub. up to 2 d	Lab. strain (ERA), V319 strain; moi=1-2 PFU/cell	Virus titration; Counts of infected cells; Electron microscopy	Weak virus inhibition (<2 log fold decrease)	[210]
		<i>In vitro</i>	1.8 mM.;	Lab. strain	IC ₅₀ and S.I.	Virus inhibition (S.I. > 5.2)	[204]

	(McCoy cells)	T0 p.i.; Incub. 96 h	(PV); moi=1	determination (inhibition of viral cytopathic effect)			
	<i>In vivo</i> (mice)	300 mg/kg/d for 3-5 d; i.p.; T(-24)h to T5d p.i.	Bobcat strain; i.c. and i.m.	Mortality rate	No protective effect	[209]	
Corticoids							
	Prednisolone, hydrocortisone acetate	<i>In vivo</i> (rats, hamsters, guinea pigs)	Total dose = 4-6 mg i.m. (prednisolone) or 2-7.5 mg s.c. (hydrocortisone,); Single or several doses; At different times; T(-(10-11d)), T0 or T(1-4d) p.i.	Lab. strain (LEP, HEP), bat strain; 1 LD ₅₀ or 1 LD ₁₀₀ ;; i.m. or i.c.	Mortality rate	No protective effect; Increase in mortality rate	[211]
	Prednisolone, hydrocortisone acetate	<i>In vivo</i> (mice)	Total dose = 1-6 mg; 1-2 mg per dose; single or several doses; at different times; T(-(10-11d)), T-4 h, T0 or T(1-7 d) p.i.; i.m. (prednisolone) or s.c. (hydrocortisone); +/- rabies vaccine	Lab. strain (LEP, HEP, CVS), bat and coyote strains; low (0-29%), intermediate (30-70%) or high (71- 100%) mortality doses; i.m. or i.c.	Mortality rate	No protective effect; Increase in mortality rate when given as soon as T1 d p.i.	[212]
Heteropolyanions (HPA)							
	HPA-23	<i>In vitro</i> (BHK-21 cells)	2.5-50 µg/ml; T(-24) h or T0; Incub. up to 72 h	Lab. strain (HEP, PV); moi=50 PFU/cell (HEP); moi=10 PFU/cell (PV)	Virus titration (single cycle virus multiplication, plaque inhibition)	Virus inhibition (50% with 4.5 µg/ml and nearly 100% with 15 µg/ml,) when incub. 18-24 h; 2 log decrease in virus titer with 50 µg/ml; No virucidal effect	[213]
	HPA-23 and 6 other HPAs	<i>In vitro</i> (CER cells)	T40 min p.i. or direct incubation with virus	Lab. strain (CVS);	Virus titration (single cycle	Dose-dependent virus inhibition: from 6.25 µg/ml	[158-159]

	(including HPA-39)		suspension incub. up to 40 h Various doses: 5-500 µg/ml	moi=10 PFU/cell	virus multiplication) ; Analysis of protein and RNA synthesis; Electron microscopy	(50%) to >50 µg/ml (99.9%) No virucidal effect	
	HPA-23	<i>In vivo</i> (foxes)	120 mg/kg; Single dose; T0 or T(1-2d) p.i.; s.c. and i.p.	Fox strains; 3000 mouse i.c.LD ₅₀ ; i.m.	Mortality rate; Level of NA	Slight preventive effect (2/7) at T0 p.i., Suggested increase in survival at T(1-2d) p.i; Drug toxicity	[214]
	HPA-23 and 13 others HPA (including HPA-39)	<i>In vivo</i> (mice, foxes only for HPA-23 and -39)	Mice: 150-420 mg/kg; i.m. or i.c.; T(1-11)d p.i.; up to 5 d Foxes: 50-150 mk/kg; i.p., s.c. or i.m.; T0 or T(1-2)d p.i. with HPA-23, after clinical signs for HPA-39 Single to multiple doses for both species	Fox strains; Mice: 10 ² i.m. LD ₅₀ ; i.m. or i.c.; Foxes: 3000- 9000 i.m.LD ₅₀ ; i.m.	Mortality rate; Virus isolation; Level of NA	Slight preventive effect for HPA-23 and -39; Effective only when given early after infection; Drug toxicity	[161]
Ascorbic acid							
	Ascorbic acid and copper sulfate	<i>In vitro</i> (Vero cells)	0.1, 0.5 or 1 mg/ml (final), with 5 µg/ml of copper sulfate; Add to the viral suspension; incub. up to 7 d at 4°C	Lab. strain (CSV); 10 ⁷ mouse i.c.LD ₅₀	Virus titration	Virucidal effect (complete inactivation after 72 h with 0.5 mg/ml)	[216]
	Ascorbic acid	<i>In vivo</i> (guinea pigs)	25-100 mg/kg; i.m.; T-6 h p.i.; twice daily, for 7 d	Lab. strain; LD ₅₀ -LD ₉₀ ; i.m.	Mortality rate	Potential protective effect (31/48)	[215]
β-phenylserine							
		<i>In vivo</i> (rats)	10-15 mg/d, daily until death (up to 14 d); i.p.; T-4 d to T2 d p.i.	Dog strain; i.m. 0.3-30 LD ₅₀	Mortality rate; Virus titration	Weak protective effect; Effective only close to the time of infection, limited to a specific rat species;	[217]

					No virucidal effect	
Minocycline						
	<i>In vitro</i> (mouse cortical and hippocampal primary neurons) <i>In vivo</i> (mice)	<i>In vitro</i> : 5 nM; T1h p.i.; incub. 1-3 d <i>In vivo</i> : 50 mg/kg/d; s.c.; T6 h p.i., for 17 d	Lab. strain (derivative of SAD); moi=10 FFU/cell (<i>in vitro</i>), 100 FFU i.m. (<i>in vivo</i>)	<i>In vitro</i> : virus titration, cell viability <i>In vivo</i> : Mortality rate, histological and immunohistological analysis	Slight protective effect <i>in vitro</i> with decrease in virus production Not protective <i>in vivo</i> (increase in mortality rate)	[218]
Other drugs derived from plants or fungi						
Cinnabarin (from fungus <i>Pycnoporus sanguineus</i>)	<i>In vitro</i> (NA, C-1300 cells)	0.155-5 mg/ml; Added to viral suspension (incub. 30 min), then added to cells (incub. 24 h)	Lab. strain (PV)	Virus titration	Decrease in virus titer (50% with 0.155-0.31 mg/ml)	[220]
4 extracts from South American plants	<i>In vitro</i> (McCoy cells)	doses \leq 50% cytotoxic concentration; T0 p.i.; Incub. 96 h	Lab. strain (PV); moi=1 FFU/cell	Measurement of cytopathic effect	Virus inhibition for only one extract (S.I.>5).	[222]
Exudate fluids from small red beans	<i>In vitro</i> (BHK-21 cells)	Final concentrations: 0-20%; T(-1)h p.i. (with cells or virus) or T(0-24)h p.i.; Incub. 24h	Lab. strain (HEP); moi=0.01 PFU/cell	Counts of infected cells	Dose-dependent virus inhibition (IC ₅₀ =0.48% dose) observed only during early phase of infection; Virucidal effects (obtained with 3% dose)	[221]
RNA interference						
cDNA (2 short cDNA fragments targeting the nucleoprotein and phosphoprotein genes)	<i>In vitro</i> (BSR cells)	Stably transfected BSR cells (1-2 x 10 ⁴ cells/well); Incub. 1-3 d	Lab. strain (CVS); moi=0.25 or 2.5 PFU/cell	Virus antigen quantification (ELISA); Counts of infected cells	Virus inhibition (up to 100% for both cDNAs); Potential direct interactions with protein of the cDNA targeting the nucleoprotein gene (coding cDNA)	[224]
siRNAs	<i>In vitro</i>	Transfection with 20 pmols of	Lab. strain	Quantification	Slight decrease in viral titer	[223]

	(3 siRNAs targeting nucleoprotein mRNA)	(BHK-21)	each siRNA; Incub. 2-22 h p.i.	(PV)	of infection (fluorescence intensity); Virus titration		
	amiRNAs (targeting nucleoprotein mRNA)	<i>In vitro</i> (N2a)	Transfection with 1 µg of DNA; T-12 h p.i. with incub. 72 h; T12 h p.i. with incub. 24 h	Lab. strain (CVS, HEP), dog strain; 10 or 100 TCID ₅₀	Counts of infected cells; Quantification of nucleoprotein mRNA and virus genome	Reduction of viral genome, nucleoprotein mRNA and infectious cells	[225]
Antiviral peptides							
	Peptides mimicking phosphoprotein (first 42 and 60 residues of N-terminal region)	<i>In vitro</i> (BHK-21, neuronal cell lines)	Synthetic peptide: 10 µM.; T1 h p.i. Plasmid transfection: 2 µg; T(-5) h p.i.	Lab strain (PV), LBV (species 2); 90% infection, or moi=5 PFU/cell	Counts of infected cells, virus titration, minireplicon system	Reduction of virus infection	[227]
Multiple-drug screening							
	65 compounds (antimetabolites and antibiotics; proteins, nucleic acids and related compounds; miscellaneous substances with solvents and detergents)	<i>In vivo</i> (mice)	Administered at the site of inoculation, systemically or between the site of inoculation and the CNS; T5 min p.i. (+/- up to 5 d) or T1 h p.i.; i.m., i.p., s.c., p.o.	Coyote strain; \geq LD ⁵⁰	Mortality rate	Most effective drugs: solvents, detergent and antirabies serum; No therapeutic effect with proteins, enzymes, antimetabolites or antibiotics (including actinomycin D, mitomycin D and β -phenylserine	[151]
	40 compounds	<i>In vivo</i> (mice, foxes)	Early (from T1 d p.i.) or late treatments (up to T13 d p.i.), single or multiple doses; Different concentrations of drugs; i.m., i.c.	Fox strains; i.m.; 10 ² i.m.LD ₅₀ ,	Mortality rate; Virus isolation; Level of NA	Effective preventive drugs restricted to 5 HPA molecules (HPA-23, -39, -46, -51 and -56); No effect with other molecules including amantadine and nucleosides analogs	[161]
	21 compounds	<i>In vitro</i> (CER cells)	T40min p.i. or direct incubation with virus	Lab strain (CVS);	Virus titration (single-growth	Virus inhibition with 14 compounds;	[159]

			suspension incub. up to 40 h Various doses: 5-500 µg/ml	moi=10 PFU/cell	cycle conditions)	Dose-dependent inhibition with 7 HPA, ribavirin and amantadine; 90% inhibition for 5 derivatives of adenosine including (S)-DHPA; No virucidal effect	
	17 compounds (immunosuppressive, cytostatic or antiviral agents)	<i>In vivo</i> (mice)	T(-1)d to T3d p.i.; Single or multiple doses; Various concentrations; i.p., i.v., s.c. or i.m. (depending on the drug tested)	Fox and rodent strains; i.m. or i.c.	Mortality rate; Virus titration	Best protective effect obtained with iododeoxyuridine and actinomycin D (i.c.) and with 6-azacytidine and azathioprine ; Minor or no protective effects with ara-C and vinblastine; Results remain difficult to interpret	[156]
	24 synthetic phenolic compounds	<i>In vitro</i> (McCoy cells)	T0 p.i.; Incub. 96 h; 100 µl/dose of non cytotoxic concentrations	Lab. strain (PV); moi=1	IC ₅₀ and S.I. determination (inhibition of viral cytopathic effect)	4 compounds with potential antiviral effect (S.I.>3)	[204]
	14 natural and semisynthetic polymeric carbohydrates	<i>In vitro</i> (CER cells)	0.1-800 µg/ml T(-1.5) h p.i. (incub. with virus or with cells) or T0 p.i. (incub. during virus attachment step); Incub. 1.5 h for all conditions	Lab. strain (CVS); Dilution=50% infected cells	Counts of infected cells; IC ₅₀ and S.I. determination	Dose-dependent virus inhibition for most of the compounds; Acting at the virus binding step	[228]
	26 compounds	<i>In vitro</i> (IMR-32 cells)	Determination of the IC ₅₀ ; T(-0.5) h p.i. (incub with virus or with cells), T0 p.i. and incub. 1-3 d or just during binding step	Lab. strain (CVS); Dilutions of suspension of 5 x 10 ⁵ -5 x 10 ⁷ FFU/ml	Counts of infected cells	Virus inhibition with 8 compounds, including drugs targeting the virus binding step and other lysosomotropic agents	[139]

Legend:

^a Administration of drugs: *in vitro* or in animals

^b Using a compartmentalized cell culture system

6-azu: 6-azauridine; amiRNA: artificial microRNA; AP5: competitive antagonist of NMDA receptor; ara-A: adenosine arabinoside; ara-C: cytosine arabinoside; ara-U: uracyl arabinoside; BHK: baby hamster kidney cells; CDZ : carbocyclic 3-deazaadenosine; CER: chick embryo-related cells; COAM: chlorite-oxidized amylose; CVS: challenge virus strain (laboratory strain); ERA: Evelyn-Rokitnicki-Abelseth; FFU: fluorescent focus unit; G-7-Ox: guanine 7-N-oxide; HPA: heteropolyanion; HEP: high egg passage; i.c.: intracerebral; IC₅₀: inhibitory concentration 50%; i.ca.: intracarotid; i.d.: intradermal; i.l.: intralumbar; i.m.: intramuscular; i.ma.: intramasseter; incub.: incubation; IFN: interferon; i.p.: intraperitoneal; i.t.: intrathecally; IU: international unit; i.v.: intravenous; i.ve.: intraventricular; LBV: Lagos bat virus; LD: lethal dose; LEP: low egg passage; MADU: 5-methylamino-2'-deoxyuridine; MIT: mouse inoculation test; moi: multiplicity of infection; NA: neutralizing antibodies; N.Av: not available; NCAM: neural cell-adhesion molecule; NDV: Newcastle disease virus; NGF: nerve growth factor; NMDA: *N*-methyl-D-aspartate; NT-3: neurotrophin-3; o.v.: overnight; PFU : plaque-forming unit; p.i.: post-infection; poly I:C: polyribonucleic polyribocytidylic acid; poly ICLC: poly I:C containing poly-L-lysine and carboxymethylcellulose; PM: Pitman-Moore; PV: Pasteur virus; RTA: lipophilic analog of ribavirin; SAD: Street Alabama Dufferin; s.c.: subcutaneous; (*S*)-DHPA: dihydroxypropyladenine; S.I.: selectivity index (inhibitory concentration/cytotoxicity concentration); siRNA: short interfering RNA; s.s.i.: same site of virus inoculation; (T: time in days (d), weeks (we.), hours (h) or minutes (min) (after virus inoculation, beginning of treatment); U: unit

Table 4: Description of the principal attempts at the treatment of human rabies

Case description	Rabies exposure (source, time of exposure before o.s. or treatment, geographic location, year, PEP)	Onset of symptoms (o.s.)	Drug-based therapeutic treatment (time after o.s. ^a , duration, description of treatment)	Diagnosis of rabies	Outcome (time after o.s., exposure or hospitalization)	Reference
First treatments for cases of human rabies						
Interferon						
Female, 10 y	Dog bite=1.5 mo. before o.s. (shoulder, Africa) Paris, France; 1976	N.Av.	d5 before o.s.; 15 d; Human leukocyte IFN: 3 x 10 ⁶ U i.v. on a continuous basis for 12 d, 1 x 10 ⁶ U i.t. daily for 6 d (total: 42 x 10 ⁶ U); Never received PEP rabies vaccine or RIG)	Detection of rabies virus: + (saliva, postmortem brain biopsy)	Death (19 d after o.s.)	[236]
Male, 5 y	Dog bite=1.5 mo. before o.s. (buttock, Africa); Paris, France; 1976	N.Av.	d2-3; 18 d (?); Human leukocyte IFN: 1 x 10 ⁶ U i.m. for 12 d and 0.5-2 x 10 ⁶ U i.t. daily for 6 d (total maxi: 24 x 10 ⁶ U); Never received PEP rabies vaccine or RIG)	Detection of rabies virus: + (saliva, postmortem brain biopsy)	Death (28 d after o.s.)	[236]
Female, 16 y	Dog lick=several weeks before o.s.; Sao Paulo, Brazil; 1981-1983	N.Av.	d14; 10 d; Human leukocyte IFN: 10 x 10 ⁶ U twice daily i.m., 5 x 10 ⁶ U once daily i.v. into a Rickham reservoir (total: 250 x 10 ⁶ U); Never received PEP rabies vaccine or RIG)	Viral iso.: + (postmortem brain biopsy)	Death (23 d after o.s.)	[236]
Male, 40 y	Probable dog bite (right hand), 58 d before o.s.; Arizona, USA (probably infected in Mexico); 1981	Paresthesia and numbness of the right hand (near the site of the bite), then fever malaise and	d10 (for IFN treatment), d8 (?) for PEP, before o.s.; 14 d; 1 dose of HDCV and HRIG: d8 (?); Human leukocyte IFN: d10, i.m. (twice a d) and i.v. (once a d, using an Omay	FAT: + (neck biopsy, several other tissues collected at post-mortem stage, including brain biopsies); Abs: - (serum and CSF	Death (24 d after o.s.)	[236-237]

		aching in the right arm	reservoir) (total: 350×10^6 U)	collected daily); Virus iso.: + (throat swab and tracheal aspirate), - (CSF)		
Male, 30 y	Dog bite= d85 before o.s. (Nigeria); Massachussets, USA; 1983	Numbness, tingling of the right bitten site, then low back pain, fever, sore throat, anorexia and malaise	d8 after o.s. 17 d Human leukocyte IFN: 10×10^6 U i.m. (twice daily), 5×10^6 U i.v. (once daily) into a Rickham reservoir, (total: 425×10^6 U); Never received PEP rabies vaccine or RIG)	FAT: + (neck biopsy, intra-vitam brain biopsy, several postmortem samples including CNS biopsies); Abs: + positive (serum d16), - (CSF); Virus iso.: + (several specimens including CSF, sputum, nasal secretion and saliva)	Death (d28 after o.s.)	[236, 243]
Male, 56 y	Dog bite=10 we. (right big toe); Bangkok, Thailand; 1989 (?)	N.Av.	d7 before o.s.; 5.5 d; Human lymphoblastoid α IFN: i.v. = loading dose of 50×10^6 /m ² body area per 6 h, repeated over the next 18 h, then given daily in continuous infusion; i.t. = loading dose of 2×10^6 U/m ² given by the lumbar route, repeated 6 h later then daily through the cerebral ventricle by the Ommaya reservoir; No rabies vaccine	FAT: + (brain biopsies, skin biopsy from neck); virus iso.: + (brain biopsies)	Death (12.5 d after o.s.)	[239]
Male, 27 y	Dog bite=3 mo. after o.s. (right arm); Bangkok, Thailand; 1989 (?)	N.Av.	d1.5 after o.s.; 10.5 d; Human lymphoblastoid α IFN: i.v. = loading dose of 50×10^6 U/m ² body area per 6 h, repeated over the next 18 h, then given daily in continuous infusion; i.t. = loading dose of 2×10^6 U/m ² given by the i.l., repeated 6 h later then daily through the cerebral ventricle by the Ommaya reservoir; No rabies vaccine	FAT: + (brain biopsies, skin biopsy from neck); virus iso.: + (brain biopsies)	Death (12 d after o.s.)	[239]

Male, 14 y	Dog bite= 1 y (left leg); Bangkok, Thailand; 1989 (?)	N.Av.	d2 after o.s.; 12.5 d; Human lymphoblastoid α IFN: i.v. = loading dose of 50×10^6 U/m ² body area per 6 h, repeated over the next 18 h, then given daily in continuous infusion, dose halved after the first we.; and i.t. (loading dose of 2×10^6 U/m ² given by the lumbar route, repeated 6 h later then daily through the cerebral ventricle by the Ommaya reservoir, dose halved after the first we.; No rabies vaccine	FAT: + (brain biopsies, skin biopsies, from neck and leg); Virus iso.: + (brain biopsies); Abs: + (sera, CSF)	Death (14.5 d after o.s.)	[239]
54 patients (51 males, 3 females), 6-70 y (mean 21.7 y)	Dog bite (52 patient), dog lick (1), mongoose bite (1); New Delhi, India; over a 17 y period	N.Av.	Group I: Cytarabine: 0.5-2.0 mg/kg/d i.v. for 10 d, then 5.0-15.0 mg/kg/d for 8 d; Nulip (nucleic acid lipoprotein complex); 2 ml/dose i.m. every 6 h for up to 4 d; Poly I:C: 100 microg/kg/d i.v. for up to 4 d Group II: Diphenylhydantoin: 3-5 mg/kg/d Vitamin C: 0.5-1.0 mg/kg/d Both for a we., then tapered depending on patient's response	Clinical diagnosis of rabies for all Laboratory diagnosis for 3 patients (Negri body detection)	Death (52 patients); Survival (2 patients, but PEP given before treatment, at the time of exposure)	[240]
Male, 25 y	Bite or scratch by a bat=3 mo. (nearly) (left cheek); Vancouver, Canada; 1985	neck pain, chest discomfort and general malaise then swollen cervical lymph nodes, weakness and profuse sweating, fever	d7 after o.s.; 27 d; IFN: 10×10^6 U, daily, i.m. and i.v. (using an Ommaya reservoir) for 21 and 20 d, respectively; Rabies immunoglobulins: 1 and 5 ml doses, i.m. and i.v. for 27 and 26 d, respectively; Vidarabine: 300 mg for 5 d, then 700 mg for 6, infused i.v. over 24 h)	FAT: + (brain biopsies); viral iso.: + (brain biopsies, saliva), - (CSF, urine and saliva collected later); Abs: + (LCR, \geq d13), - (serum, d6 and LCR d11- 12)	Death (d33 after o.s.)	[241-242]
Antimetabolic agents						
Female, 12 y	Houston, Texas; 1984	Headache, then sore throat,	d16 (?); Ribavirin;	FAT: + (intra-vitam brain biopsies);	Death (27 d after o.s.)	[243]

		fatigue, difficulty swallowing, leg weakness	Never received PEP rabies vaccine or RIG)	Electron microscopy: + (intra-vitam brain biopsy); Abs: + (serum, d18; CSF, d15)		
Male, 28 y	No definite bite=caught stray dogs, 2 months; Bangkok, Thailand; 1989 (?)	N.Av.	d3 after o.s.; 9 d; Ribavirin: i.v. = loading dose of 2 g (30 mg/kg) over 20 min, then by 1 g every 6 h (60 mg/kg/d) for 4 d and 0.5 g every 8 h (25 mg/kg/d) daily; i.ve = injection of 100 mg (2mg/kg) given daily through an Ommaya reservoir; No rabies vaccine	FAT: + (brain biopsies, skin biopsies, from neck and leg); Virus iso.: + (brain biopsies and saliva)	Death (12 d after o.s.)	[239]
16 patients (of 64)	Dog exposure in most cases; Beijing, China; over a period of 15 y (1974 to 1989)	N.Av.	Ribavirin: 16-400 mg, i.v. dose	Clinical and epidemiological diagnosis	Death	[244]
7 male patients (indication for treatment for 5 of these patients)	Dog bite; India;1971-1976	Variable	Cytosine arabinoside; Freund's emulsion	Clinical diagnosis in all cases Histology: + (for 3 patients, on necropsy, with the detection of Negri bodies)	Death (survival period 1-17 d after o.s. vs 2- 4 d naturally)	[229]
Male, 37 y	Dog bite=51 days (lip, Gambia); Great-Britain; 1975	Tingling of the inside of the left arm	d5 after o.s.; 19 d; DEV (d3?); 4000 IU of horse antirabies serum s.c. (d3?); Then a 17-day course DEV starting at d5, associated with 11500 U and 3500 U of human antirabies serum given on d9 and d10, respectively	Abs: + (serum and CSF); FAT: - (necropsy tissues including cerebral biopsy); Histology (Sellar's test for Negri body detection): - (necropsy tissues including cerebral biopsy); Virus iso.: - (saliva, CSF, corneal smears, necropsy tissues including cerebral biopsies)	Death (34 d after o.s.)	[247]

Male, 22 y	Dog licks on scratches= 6 we. before o.s. (India); Great-Britain; 1975	Headache, aching limbs, fever	d6 after o.s.; 3 d; 3000 IU of horse antirabies serum (40 IU/kg) (d6); Rabies immune human plasma transfusion (8000 IU) together with 2 ml of DEV (d10 and 11)	Viral iso.: + (saliva); Abs: :+ (serum, ≥d16)	Death (24 d after o.s.)	[248]
Female, 26 y	Dog bite=2 mo. (right leg); Chulalongkorn, Thailand	Fever, restless with neuropathic pain in the right leg, then phobic spasm	d1 after o.s.; 3 d; HRIG: 4 doses of 150 IU/ml i.v. (2 h after admission, 12 h interval for the 2 following doses, and 24 h later); No PrEP or PEP given	Viral RNA: + (saliva, CSF); Histology (Negri body detection): + (post-mortem brain biopsy); Abs: - (CSF)	Death (15 d after o.s.)	[246]
Male, 2.5 y	Bobcat bite=17 d (severe including occipital scalp, back of the neck); San Diego, USA; 1969; Partial PEP after bite: DEV with no antirabies antibodies	Fever, hyperemic pharynx and tympanic membranes	d5 after o.s.; 15 d; 2 booster injections of DEV (d5 and d15); 50 U and 2400 IU equine rabies antiserum given i.ve. and i.t. and intrathecally, respectively (d9)	Virus iso.: - (several ante-mortem and post-mortem samples including brain biopsies); Ags: + (only for one ante-mortem brain biopsy), Abs: + (increase in Ab levels in CSF and serum)	Death (133 after o.s.)	[249]
Male, 73 y	Suspected dog bite=5-6 we. before o.s (right leg); Saint-Louis, USA; 1913	Tingling and prickling in the right leg	d4-d5 after o.s.; 2 d; Quinine and urea hydrochloride, i.v. , several times per d, during 2 d	Based only on clinical (hydrophobia) and epidemiological (dog bite 5-6 weeks later) data	Recovery (d8-d9); Rabies diagnosis doubtful, especially given the rapid clinical recovery	[250]
Treatments of human rabies based on combination therapy (following recommendations published in 2003)						
Male, 66 y	Bat bite=3 we. before o.s) (right index finger); California, USA; 2003	Mild, non specific complaints (e.g. drowsiness, chronic headache, malaise), then progressive	2 we. before o.s.; 7d; Rabies vaccine (at admission, then three d later); Rabies immune globulin; Ribavirin; αIFN; All given at admission	RT-PCR: + (saliva)	Death (3 weeks after o.s.)	[252]

		right arm pain and paresthesia followed by weakness of the right arm				
Female, 15 y	Bat bite (left index finger); 1 mo. before o.s. Wisconsin, USA; 2005	Generalized fatigue, paresthesia of the left hand	d6 after o.s. 28 d (nearly); Ketamine: 2 mg/kg/h; Midazolam: 1-3.5 mg/kg/h. Ribavirin: loading dose of 33 mg/kg then 16mg/kg/6h; Amantadine: 200 mg/day, p.o.; Addition of high dose of benzodiazepines with supplementary barbiturates Never received PEP rabies vaccine or RIG)	Abs: + (LCR, serum, \geq d6); RT-PCR: - (saliva, skin biopsy); Viral iso.: - (saliva, skin biopsy); FAT: - (saliva, skin biopsy)	Survival 6 months??	[253]
Female, 46 y	Transplantation (lung from rabid donor, bitten by a dog in India); 46 d after transplantation; Germany; 2005		d45 after transplantation Ketamine (100-125 mg/h); Midazolam (15-20 mg/h); α IFN (1.5Mio IE , .c.); Ribavirin (loading dose, 200 mg, i.v.); PEP (d45, HRIG+ vaccine) + additional 20 IU/kg dose HRIG (d47)	Positive RT-PCR: + (bronchoalveolar lavage, sputum, bronchial secretion, CSF, post-mortem brain biopsy) Viral iso.: +	Death (d49, post transplant)	[50]
Male, 72 y	Transplantation (kidney from rabid donor, bitten by a dog in India); 46 d after transplantation Germany; 2005		45 d after transplantation Ribavirin (4–8 mg/kg every 6 h after initial loading dose of 20 mg/kg); α IFN (3,000,000 IU, s.c. every 2 nd d), amantadine (200 mg/d, starting d50; PEP (d45, HRIG+ vaccine) + additional 25 IU/kg dose HRIG per d (until d51)	Positive RT-PCR: + (saliva, CSF, post-mortem brain biopsy)	Death (d52, post transplant)	[50]
Male, 47 y	Transplantation (kidney/pancreas from rabid donor, bitten by a dog in India); 46 d after transplantation Germany; 2005		d45 after transplantation PEP (d45)+ HRIG at 27 IU/kg per day until d52, then arrest and readministration on d60 (27 IU/kg every 2 days); Midazolam (4–8 mg/h, starting d47, stopped d55); Ketamine (25–100 mg/h, stopped d76);	Positive RT-PCR: + (corneal swabs, sputum, CSF, post-mortem brain biopsy); Viral iso.: + Ab: + (serum with PEP)	Death (d95 post transplant)	[50]

			Phenobarbital (8– 16 mg/h, stopped d56); αIFN (s.c., d46=180 mg, d53=5 x 10 ⁶ IU and every 3 d starting from d56=3 x 10 ⁶ IU), also d54 and d65=1.5 x 10 ⁶ IU (i.t.); Amantadine (4–16 mg/h from d53); Ribavirin (22 mg/kg, 4 times/d over 3 d, then 11 mg/kg every 8 h and then 40 mg/d (i.t.) on d52–d58 and 20 mg/d on d63, 65, 67, 70, and 77), discontinued on d59–64 and after d69			
Male, 26 y	Transplantation (liver from rabid donor, bitten by a dog in India); 46 d; Germany; 2005	No symptoms	d45 after transplantation Ribavirin (800 mg/d); Pegylated αIFN2b (100 µg per we.), 3 we. of treatment (stopped due to the onset of liver rejection); PEP (d45): single 20 IU/kg dose of HRIG and Essen regimen	Negative RT-PCR: - (corneal swabs, saliva)	Survey with no symptom; vaccinated 20 years earlier with NA titer present before PEP (0.4 IU/ml)	[50]
Male, 33 y.	Dog bite=2 mo. (Thailand, two months earlier)	Fever, burning sensation of the left hand and arm, phobic spasms	Duration of 46 h for coma induction; Ketamine (i.v., 48 mg/kg/d); Diazepam; Thiopental; Ribavirin (loading dose: 66 mg/kg, then 128 mg/kg/d for 2 d, then 48 mg/kg/d for two d p.o.) Never received PEP rabies vaccine or RIG)	Positive NASBA: + (hair follicles, saliva); Abs: - (serum, LCR) Virus iso.: + (terminal brain and spinal cord tissues)	Death (d8 after hospitalization)	[260]
Female, 10 y.	Suspicion of bat bite=3.5 mo. before o.s.; Indiana, US; 2006	Pain at the suspected bitten area, ascending pain (from arm to neck, agitation, temperature, difficulties swallowing,	d10 after o.s. (at time of rabies diagnosis); 23 d; Phenobarbital; Midazolam; Ketamine; Amantadine; Ribavirin i.v. (d16); Metabolic supplementation (coenzyme Q10, L-arginine, tetrahydrobiopterin,	Positive RT-PCR: + (saliva, skin biopsy from the nape of the neck); FAT: + (skin biopsy, terminal brain biopsy); Abs: + (serum, d13)	Death, (d34 after o.s.)	[262]

			vitamin C) Never received PEP rabies vaccine or RIG)			
Male, 11 y.	Dog bite=2 years before o.s. (Philippines); California, US; 2006	Sore throat, fatigue, inconsistent fever, then agitation, hallucination, irregular movement and aerophobia, hydrophobia, hypersalivation	d2 after o.s. 27 d; Ketamine (d2); Midazolam i.v (d2).; Ribavirin i.v (d2); Amantadine (p.o.) Metabolic supplementation (coenzyme Q10, tetrahydrobiopterin); Never received PEP rabies vaccine or RIG)	Positive FTA: + (corneal impressions, nuchal skin biopsy, terminal brain biopsy); RT-PCR: + (saliva); Abs: + (serum, d12, LCR d16)	Death (27d after o.s.)	[262]
Male, 55 y	Dog bite=6 we. (left hand); six weeks, Morocco; Germany; 2007	paresthesia, severe pain in the left hand, then fever, nausea, headache and difficulties swallowing	d2; 25 d; PEP (d2) with HRIG (20 UI/kg); Ketamine (d2); Midazolam (d6); Amantadine; Live attenuated rabies vaccine (VirBac, i.d., d8?, d15) Metabolic supplementation (vitamin C, coenzyme Q, tetrahydrobiopterine)	Positive RT-PCR: + (saliva, corneal swab, LCR), Abs: - (serum or CSF at d5, d8), + (serum d11)	Death (d31 after o.s.)	[263]
Female, 34 y.	Face contact with bat=23 d before o.s. (Kenya); Netherlands; 2007; species 4 of lyssavirus	Malaise, headache, difficulty swallowing, speech, muscle aches, dizziness, hypoesthesia, dysarthria, hypoesthesia of both cheeks and unsteady gait	d11 after o.s.; >14 days; PEP: HRIG (20 IU/mg i.m.) and rabies vaccine (4 doses); Phenobarbital (4 mg/kg/h); Midazolam (5 mg/h) Ketamine (100 mg/h, d7); Amantadine (100 mg twice per d, d8, nasogastric); Ribavirin (iv, 1g x 4 a d, d12); Metabolic supplementation (tetrahydrobiopterin and co-enzyme Q10, d15)	Abs: - (serum and CSF); RT-PCR: - (LCR, serum), + (nuchal skin biopsy, saliva); FAT: - (skin biopsy, cornea smear), + (terminal brain biopsies); Virus iso.: - (CSF, saliva)	Death (d20 after admission)	[264]
Male, 5 y	Dog bite= 5 we. before o.s. (neck);	Difficulties swallowing,	d4 after o.s.; Ketamine (20 mg, 3.12mg/kg/h) and	RT-PCR: + (saliva); FAT: + (skin biopsy);	Death, (25 after o.s.)	[266]

	Equatorial Guinea; 2007	hydrophobia, aerophobia, phonophobia, agitation, anxiety, unsteady gait and drooling	midazolam (9 mg, 1.4 mg/kg/h), same ratio; Amantadine (6 mg/kg/d, p.o., d6); Ribavirin (15 mg/kg/6h, p.o., d5, arrest at d8); Boluses of phenobarbital, propofol or diazepam, continuous infusion of thiopental 3 mg/kg/h, d3-d7 (without EEG management), arrest of thiopental at d8; nifedipine (calcium channel blocker) (0.1 mg/kg every 6 h p.o., d8?); co-enzyme Q10 d5 after hospi. Never received PEP rabies vaccine or RIG)	Viral iso.: + (skin biopsy); Abs: + (serum d17-18, CSF: d19)		
Male, 73 y	Bat bite=6 months (left shoulder); Canada; 2008	Pain in shoulder, then fever, dysphagia, muscle spasms and progressive generalized weakness	d15 (o.s.); 3 w.; 1200 IU of HRIG; Ketamine; Midazolam; Propofol; Ribavirin; Amantadine; Metabolic supplementation (tetrahydrobiopterin, L-arginine); No vaccine given	Positive RT-PCR: + (nuchal skin biopsy, saliva); FAT: + (nuchal skin biopsy and terminal brain biopsy); Abs: + (IgG and IgM detection)	Death (d65 after o.s.)	[267]
Female, 37 y	Dog bite = 24 mo., monkey bite = 9 mo.; both in South Africa; Ireland; 2009 No PEP	Sweating, leg weakness, paresthesia in finger	d10 (adm.), d14 (o.s.); 25 d; Ketamine (2 mg/kg/h) for 15 d; Midazolam (0.5-0.67 mg/kg/h), for 15 d; Amantadine (100 mg twice a d for 15 d); Ribavirin (3 g i.v. loading, then 1.2 g 4 times per d for 2 d.); Metabolic supplementation (co-enzyme Q, tetrahydrobiopterin, vitamin C); Never received PEP rabies vaccine or RIG)	Positive FAT: + (terminal brain biopsy); Virus iso.: + (CSF, saliva, brain biopsy); Ab: + (serum≥d11), CSF≥d13); RT-PCR: + (brain biopsy, CSF, saliva, skin biopsy)	Death (d39 after o.s.)	[268]

Male, 9 or 12 y	Bat bite (foot); Brazil; 2009	N.Av.	Hospitalization: 3 we. Antiviral drugs; Coma induction	Laboratory diagnosis: +	Death	[269-270]
Female, 8 y	Cat bite; Columbia; 2008	N.Av.	1 mo. (nearly); Drug therapies; Coma induction	N.Av.	Death	Not published
Male, 15 y	Hematogenous bat bite=29 d before o.s.; PEP (4-dose regimen), 4 d after exposure (25 before o.s.) Brazil; 2008	N.Av.	d8 after o.s.; 28 d; Ketamine; Midazolam; Amantadine; One additional rabies vaccine injection after treatment	RT-PCR: + (skin biopsy); Abs: + (increase NA in neutralizing CSF, serum)	Survival (at least 3 months after report, with motor limitations)	[271-273]

Legend:

^a or exposure (transplantation)

?: not confirmed; Abs: antibodies (against rabies); Ags: antigens (rabies); CSF: cerebral spinal fluid; d: day; DEV: duck embryo vaccine; FAT: fluorescent antibody test; HDCV: human diploid cell vaccine; hospi.: day of hospitalization; HRIG: human rabies immunoglobulin; IFN: interferon; ; i.l.: intralumbar; i.m.: intramuscular; i.t.: intrathecal; iso.: isolation; i.v.: intravenous; i.ve.: intraventricular; mo.: month; NA: neutralizing antibodies; NASBA: nucleic acid sequence-based amplification; N.Av.: not available; o.s.: onset of symptoms; PEP: post-exposure prophylaxis; PrEP: pre-exposure prophylaxis; p.o.: per os; RIG: rabies immunoglobulin; RT-PCR: reverse transcription-polymerase chain reaction; s.c.: subcutaneous; U: unit; IU: international unit; y: year

Figure 1: Modes of intramuscular administration of rabies post-exposure prophylaxis (PEP) [93]



Two protocols for the intramuscular administration of rabies vaccines for PEP are recommended by the WHO: the Essen protocol, consisting of five doses of vaccine (with the possibility of giving only four doses in exceptional circumstances), and the Zagreb protocol, which includes only

four doses of vaccine [93]. In cases of category III exposure, rabies immunoglobulin should be administered during the week after the first rabies vaccine injection (ideally at the same time or D0). PEP should be started (D0) immediately after rabies exposure (category II and III), or as soon as possible.

Legend:

D: day; ERIG: equine rabies immunoglobulin; HRIG: human rabies immunoglobulin, RIG: rabies immunoglobulin

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