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Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses

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Abstract

Many viruses, including flaviviruses, display affinity for cell surface heparan sulfate (HS) proteoglycans with biological relevance in virus attachment/entry. This raises the possibility of the application of HS mimetics in antiviral therapy. We have evaluated the antiviral effect of the sulfated polysaccharides, suramin, pentosan polysulfate (PPS) and PI-88, which are currently approved or in trial for clinical use, against dengue virus (DEN) and the encephalitic flaviviruses, Japanese encephalitis virus, West Nile virus, and Murray Valley encephalitis virus. A flow cytometry-based method for the measurement of inhibition of virus infectivity was developed, which showed the in vitro antiviral activity of the three compounds, albeit with differences in efficiency which were virus-dependent. The 50% effective concentration (EC_{50}) values for DEN inhibition were in the order: PPS < suramin < PI-88, and for Japanese encephalitis virus, PPS < PI-88 ≤ suramin. Heparin inhibited the DEN infectivity 30-fold more efficiently than the best of the test compounds, which was not the case for encephalitic flaviviruses. The in vitro anti-flaviviral effectiveness of the HS mimetics did not reliably predict their in vivo therapeutic activity. In mouse models for DEN and flaviviral encephalitis, only PI-88 demonstrated a significant beneficial effect in disease outcome.

Keywords: Flavivirus; Heparan sulfate mimetic; Japanese encephalitis virus; West Nile virus

1. Introduction

The flaviviruses are mainly a group of arthropod-transmitted viruses of global medical significance. Many members of the flavivirus genus inflict disease in humans, which can range from febrile illness to fatal hepatitis, hemorrhagic fever, or encephalitis. Dengue virus (DEN) is the most important flavivirus in terms of disease frequency, causing >50 million cases of dengue fever, annually, in tropical and subtropical countries, with ∼25,000 deaths as a result of dengue hemorrhagic fever; it has been estimated that 2.5 billion people are at risk of DEN infection (reviewed in Gubler, 2002; Mackenzie et al., 2004). Japanese encephalitis virus (JEV) is the most important causative agent of viral encephalitis in humans; 30–50,000 cases of Japanese encephalitis occur annually, particularly among children in south-east Asian countries, with ∼35% mortality and frequent life-long neurological impairment among survivors (reviewed in Monath, 2002; Solomon and Vaughn, 2002). Murray Valley encephalitis (MVE) and West Nile (WNV) viruses are genetically closely related to JEV. MVE is endemic in northern Australia where it gives rise to a small number of human cases of sometimes fatal encephalitis in most years (Mackenzie et al., 1994). In contrast, WNV is considered one of the most important emerging viruses following recent epidemics of human and veterinary disease in central and eastern Europe and first appearance and spread of the virus in the USA, Canada and Mexico during the last 6 years (reviewed in Mackenzie et al., 2004). Current vaccines against the mosquito-borne flaviviruses licensed for human immunisation are those against yellow fever virus and JEV (reviewed in Burke and Monath, 2001). So far, there are no vaccines licensed for use in humans against DEN or WNV, and no therapeutic agents are available for the treatment of patients hospitalised with dengue or flaviviral encephalitis (reviewed in De Clercq, 2004; Shi, 2002).

The in vitro antiviral activity of sulfated polysaccharides against DNA and RNA viruses has been known for many years (Baba et al., 1988). For flaviviruses, Chen et al. (1997) demonstrated that DEN infection of Vero cells could be inhibited by the highly sulfated glycosaminoglycan (GAG), heparin, and the sulfated polyanion, suramin. Molecular size and level of sulfation

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correlated with the inhibitory activity of a series of polyanionic compounds (Chen et al., 1997; Marks et al., 2001); however, the therapeutic value of these compounds was not tested in animal models for DEN. In other studies, the anti-flaviviral activity of dextran sulfate (Neyts et al., 1996), sulfated polysaccharides extracted from seaweeds (Talarico et al., 2005) and sulfated galactomannans (Ono et al., 2003) were demonstrated, where the latter showed protection from lethal yellow fever virus challenge in mice when the sulfated polysaccharide and virus were simultaneously injected.

The involvement of cell surface GAG in attachment/entry and the inhibitory effect of heparin on infectivity of an encephalitic flavivirus was first shown by us in a study on host cell adaptation of MVE (Lee and Lobigs, 2000). Although GAG-binding variants of MVE and other flaviviruses were significantly more sensitive to inhibition of infectivity by heparin, parental strains of MVE, JEV and WNV were also subject to inhibition by the GAG (Lee et al., 2004; Lee and Lobigs, 2000, 2002). The enhanced affinity for GAG of the host cell-adapted variants correlated with reduced virulence in mice. Similar results were obtained by adaptation of tick-borne encephalitis virus to growth in BHK cells (Goto et al., 2003; Mandl et al., 2001). Accordingly, it appears that natural flavivirus isolates display a balance between the beneficial effect of GAG affinity in attachment and infection of target cells, and the detrimental effect of strong but non-productive binding to other surfaces enriched in GAG, such as extracellular matrices, endothelial cells and hepatocytes (Bernfield et al., 1999; Lyon et al., 1994), given that the latter results in rapid virus clearance from the bloodstream and the poor ability to spread in an infected host (Lee et al., 2004; Lee and Lobigs, 2002). Accordingly, studies on the antiviral activity of sulfated polysaccharides using flaviviral laboratory strains, which often have an extensive cell culture passage history, may not truly reflect the inhibitory potency of the compounds with respect to natural virus isolates.

We have now evaluated, both in vitro and in vivo, the anti-flaviviral activity of three HS mimetics which differ in size, structure and sulfation content, and which are approved for clinical use or currently undergoing clinical trial. We show that the in vitro anti-flavivirus activity of these compounds does not strictly correlate with the in vivo protective value, and that only one compound, PI-88 (Parish et al., 1999), significantly increased survival in mouse models of DEN and encephalitic flavivirus disease.

2. Materials and methods

2.1. Virus and cells

The MVE-1-51 (French, 1952) and JEV-Nakayama (Lobigs et al., 1986) prototype strains have been passaged 15 and 45 times in mouse brain, respectively. WNV strain NY-99 (Lanciotti et al., 1999) was obtained from the Center of Disease Control, Fort Collins. MVE and JEV working stocks were suckling mouse brain homogenates (10% in Hanks' balanced salt solution containing 0.2% bovine serum albumin and 20 mM Hepes (pH 8.0); this medium is referred to as HBSS-BSA), and WNV working stocks were infected Vero cell supernatants. DEN-2 strain New Guinea C obtained from Dr P. J. Wright, Monash University, Australia, was amplified once in C6/36 *Aedes albopictus* mosquito cells to generate working stocks. BHK-21 (baby hamster kidney) cells were obtained from the American Type Culture Collection and grown in Eagle's minimal essential medium plus nonessential amino acids (MEM) supplemented with 5% fetal bovine serum (FBS).

2.2. Inhibitors

Heparin, suramin, and pentosan polysulfate (PPS) were obtained from Sigma-Aldrich, and PI-88 was provided by Progen Industries (Brisbane, Australia). PI-88 was prepared as previously described (Parish et al., 1999; Yu et al., 2002) by the sulfation of phosphomanno-oligosaccharides derived from the phosphomannan produced by the yeast, *Pichia holsti*. The compounds were suspended in PBS or normal saline at a concentration of 20 mg/ml and stored at −20 °C.

2.3. Flow-cytometric assay for evaluation of the effect of HS mimetics on virus infectivity

BHK cell monolayers (~5×10^5 cells) in 6-well tissue culture plates (Nunc) were pre-incubated with 0.2 ml HBSS-BSA containing inhibitors at the concentrations given in Fig. 1 for 15 min in a 37 °C CO_2 incubator. Concurrently, virus inocula (0.2 ml) diluted with HBSS-BSA to give a multiplicity of infection (MOI) of ~0.5, and containing the corresponding concentrations of inhibitor, were incubated in Eppendorf tubes for 15 min at RT (20–25 °C) prior to addition to the cell monolayers. Infection was for 1 h in the presence of inhibitor or mock-treatment. Subsequently, the virus inoculum was aspirated and cells washed twice with HBSS-BSA, followed by the addition of growth medium. At 16 or 20 h post-infection (p.i.) with one of the encephalitic flaviviruses or DEN, respectively, cells were harvested using trypsin, pelleted by centrifugation, and fixed by the addition of ice-cold 75% ethanol in H_2O for 30 min at 4 °C. Permeabilised cells were suspended in 200 μl of MEM/5% FBS containing virus envelope (E) or NS1 protein-specific antibody: for MVE and JEV infected cells the E protein-specific monoclonal antibody (mAb) M2-8E7 (Hall et al., 1990) was used, for WNV infected cells the E protein-specific mAb 2B2 was used, and for DEN-2 infected cells the E protein-specific mAb 2H2 was used. Alternatively, the NS1-specific and flavivirus cross-reactive mAb 4G4 were used. Following incubation at 4 °C for 1–2 h, cells were washed twice with MEM/5% FBS and stained with FITC sheep anti-mouse Ig (Selenius Lab., Victoria, Australia) for 0.5–1 h at 4 °C. Cells were washed twice with MEM/5% FBS and once with PBS, and fluorescence was examined by a Becton Dickinson FACS scan flow-cytometer. Percent inhibition of virus infectivity was calculated by the formula: [(% infected cells in mock-treated sample − % infected cells in treated sample)/% infected cells in mock-treated sample] × 100%, and % residual infectivity was calculated by the formula: (% infected cells in treated sample/% infected cells in mock-treated sample) × 100.

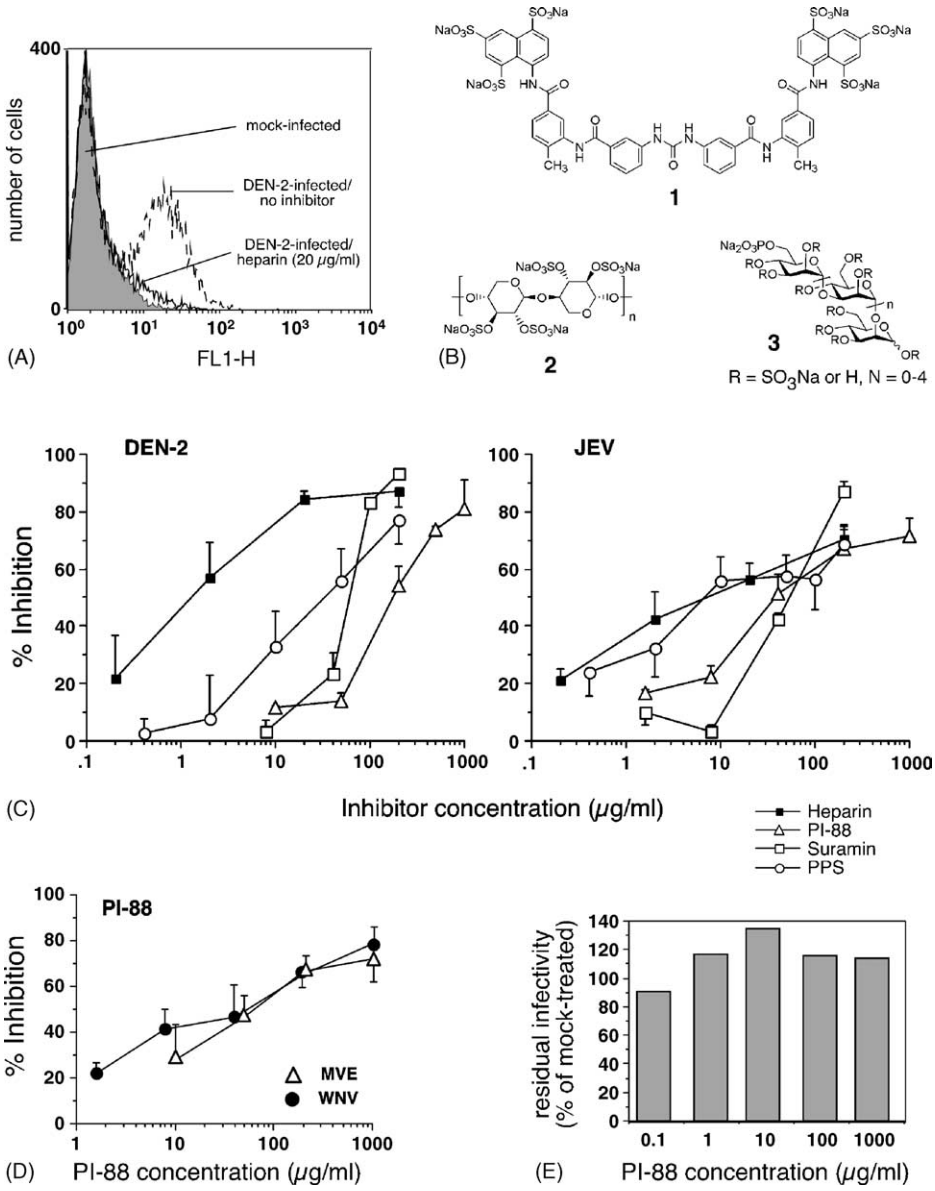

Fig. 1. Inhibition of flavivirus infectivity in BHK cells by HS mimetics: (A) the effect of heparin on DEN-2 infectivity in BHK cells was determined at 20-h p.i. at MOI ~0.5 using E protein-specific staining of permeabilised cells followed by flow-cytometric analysis. Heparin treatment (20 μg/ml) of virus inoculum and cells was for 15 min prior to infection. The filled histogram shows fluorescence of mock-infected cells, and the solid and dotted lines that of cells in the presence or absence of heparin treatment, respectively; (B) structures of suramin (1), pentosan polysulfate (2), and PI-88 (3); (C) the effect of a concentration range of heparin and three HS mimetics on infectivity of DEN-2 and JEV was measured by flow cytometry, as described above. Mean values from three independent determinations and S.E.M. are shown (up or down error bars for clarity); (D) PI-88-mediated inhibition of infectivity of MVE prototype (MVE-1-51) and WNV (NY-99 strain) on BHK cells; mean values (±S.E.M.) from three independent determinations are shown; (E) pre-incubation of BHK cells with a range of concentrations of PI-88 for 15 min in the absence of treatment of the virus inoculum does not inhibit virus infectivity. Following PI-88 treatment, cells were washed and infected with WNV at MOI ~0.5. The percentage of infected cells in the presence and absence of PI-88 pre-treatment was determined by flow cytometry and used to determine residual infectivity calculated as the ratio of percent infected cells in treated and untreated samples.

2.4. Mice, in vivo inhibitor treatment, and virus challenge

C57Bl/6, and type I and II interferon (IFN) receptor-deficient (IFN-α/γ-$R^{-/-}$) mice (129 background) (van den Broek et al., 1995) were bred under specific pathogen-free conditions and supplied by the animal breeding facility at the John Curtin School of Medical Research, Canberra. Six-week-old male or female mice were used. The average weight of 6-week-old C57Bl/6 mice was 20.5 g and ranged from 18 g–23.5 g. Inoculations of sulfated polysaccharide compounds, diluted in normal saline, was by the i.p. route, using 0.1 ml/dose. Treatment regimens were as shown in Figs. 2 and 3. Virus challenge was by intravenous (i.v.) injection of 0.1 ml of HBSS/BSA containing 10^8 PFU of MVE or 10^7–10^8 PFU of JEV, using the tail vein, or i.p. injection of 10^6 PFU of DEN-2.

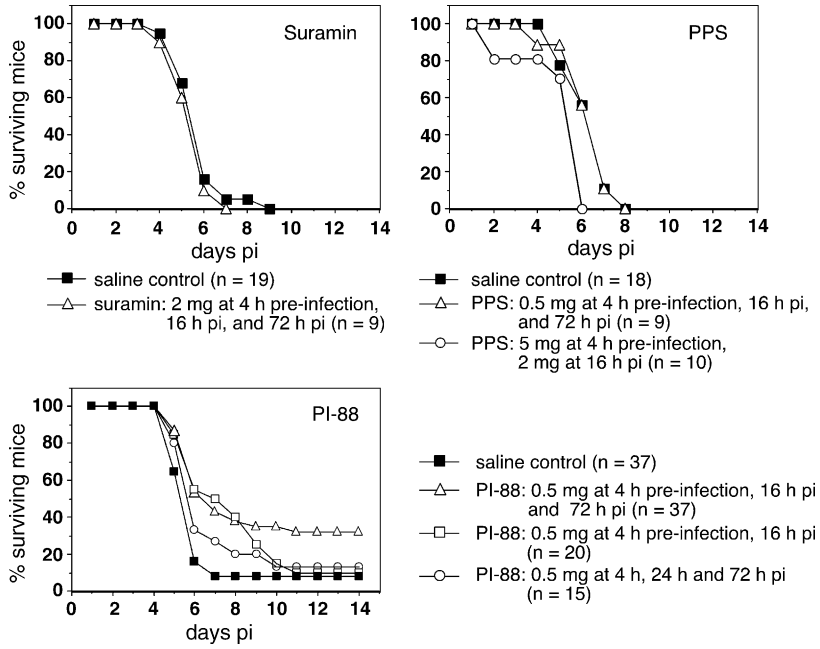

Fig. 2. In vivo anti-JEV activity of HS mimetics. Groups of 6-week-old C57Bl/6 mice were infected with 10^7–10^8 PFU, i.v., of JEV in the presence or absence of suramin, PPS, or PI-88 treatment regimens, as shown in the figure. Mortality was recorded daily and surviving mice were monitored for 21 days.

2.5. Statistics

Differences in the survival ratios for mouse challenge experiments were assessed using Fisher's exact test, and differences in average time to death (ATD) were analysed for significance using the Mann–Whitney test.

3. Results and discussion

3.1. Inhibition of flavivirus infectivity in BHK cells by HS mimetics

An infectivity rather than cytopathology-based assay for the evaluation of the antiviral activity of HS mimetics was developed, given the slow replication rate of some of the flaviviruses tested which prevented the clear distinction between late virus-induced cytopathology and cell detachment due to overcrowding. Viral infectivity was quantitated by flow-cytometry using virus envelope (E) or NS1 protein-specific staining of cells infected for 16 h with JEV, MVE, or WNV, or 20 h with DEN-2 at MOI ∼0.5. Fig. 1A shows a representative flow-cytometry histogram of BHK cells infected with DEN-2 in the presence or absence of pre-treatment for 15 min of virus and cells with heparin (20 μg/ml), a highly sulfated GAG reference standard. In the absence of inhibitor, 43% of cells were infected on the basis of the population of cells showing E protein-specific fluorescence intensity exceeding that of the uninfected cells. Heparin treatment resulted in a ∼7-fold reduction of virus infectivity (6% of cells infected).

Three sulfated polysaccharides, suramin, PPS, and PI-88 (Fig. 1B), were chosen for evaluation of their in vitro anti-flaviviral activity in view of their diverse size, structure, and level of sulfation, and their past and present clinical use or current clinical trial. Suramin (MW ∼1400), a symmetrical

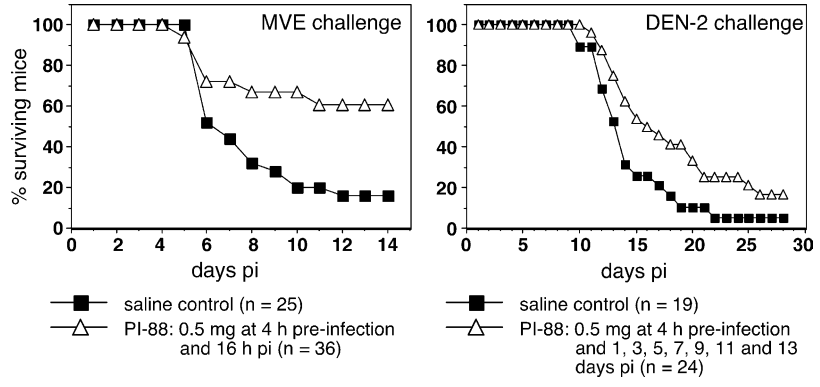

Fig. 3. Therapeutic effect of PI-88 in MVE and DEN-2 infections. Groups of 6-week-old C57Bl/6 or IFN-α/β-R$^{-/-}$ mice were infected with 10^8 PFU, i.v., of MVE or 10^6 PFU, i.p., of DEN-2, respectively, in the presence or absence of PI-88 treatment as shown in the figure. Mortality was recorded daily and surviving mice were monitored for 21 days in case of MVE infections or 60 days in case of DEN-2 infections.

polysulfonated naphthylamine derived from urea, has been used extensively for the treatment of human trypanosomiasis, and has been shown to exert anti-tumor and antiviral effects (reviewed in Voogd et al., 1993), including in vitro inhibitory activity against DEN-2 (Chen et al., 1997; Marks et al., 2001). PPS (MW ~5700) is a semi-synthetic highly sulfated polysaccharide (degree of sulfation = 1.9, which is higher than that of heparin [~1.4]) composed of β-D-xylopyranose residues and has been used for the prevention of post-operative thromboembolism and treatment of interstitial cystitis (Maffrand et al., 1991). PI-88 (MW = 1400–3100) is a mixture of highly sulfated mannose-containing di- to hexasaccharides (predominantly pentasaccharides [~60%] and tetrasaccharides [~30%]; degree of sulfation = 3.0) (Ferro et al., 2002; Yu et al., 2002). The compound is undergoing clinical trials for its anti-angiogenic and anti-metastatic effects (Joyce et al., 2005; Parish et al., 1999) and was also shown to inhibit both infectivity and cell-to-cell spread of herpes simplex virus (Nyberg et al., 2004). The inhibitory effect of these sulfated polysaccharides on the infectivity of DEN-2 (NGC) and JEV (Nakayama) was examined. Fig. 1C shows that at high concentrations (≥100 μg/ml) infectivity of both viruses was inhibited by 70–90%, whilst in the low concentration range (1–10 μg/ml) only PPS in the case of JEV and heparin for DEN-2 and JEV gave significant inhibition (≥50% reduction of infectivity). Thus, the 50% effective concentrations (EC_{50}) for DEN-2 and JEV, respectively, were as follows: heparin, ~1 μg/ml and ~10 μg/ml; PI-88, ~200 μg/ml and ~40 μg/ml; suramin, ~60 μg/ml and ~50 μg/ml; and PPS, ~30 μg/ml and ~7 μg/ml. Accordingly, heparin (for DEN-2 and JEV) and PPS (for JEV) displayed anti-flaviviral properties superior to those of suramin and PI-88 in the in vitro assay. The greater molecular size of heparin and PPS may have contributed to their better in vitro antiviral properties than those of suramin and PI-88. The EC_{50} determinations of antiviral activity of heparin against DEN-2 based on infection inhibition measured by flow cytometry were similar to those reported by others (0.3–3 μg/ml), who used assays measuring inhibition of cytotoxicity, virus yield, or recombinant E protein binding to cells (Chen et al., 1997; Lin et al., 2002; Talarico et al., 2005).

In view of the in vivo antiviral properties of PI-88 (see below), its inhibitory effect on the infectivity in BHK cells of two additional medically important flaviviruses was tested: a highly virulent WNV strain isolated during the 1999 WNV outbreak in New York and the prototype strain of MVE. The PI-88 concentration-dependent inhibition curves for WNV and MVE closely resembled that for JEV with EC_{50} values of ~50 μg/ml (Fig. 1D). Inhibition of WNV and MVE infectivity by heparin was 5- to 10-fold greater than that mediated by PI-88 (data not shown).

The WNV NY-99 strain has undergone only a small number of laboratory passages in contrast to the prototype JEV and MVE strains, which have been passaged numerous times in suckling mice. Virus passage in mouse brain did not apparently select for virus variants with enhanced affinity for cell surface GAG, according to the inhibition data shown in Fig. 1, and in contrast to the increased GAG affinity sometimes associated with virus passage in tissue culture cells (Lee et al., 2004; Lee and Lobigs, 2000, 2002).

To test whether the inhibitory activity of the HS mimetics on flavivirus infectivity is due to the interaction of the compounds with virion particles or the host cell, BHK cells were incubated with PI-88 at concentrations ranging from 0.1–1000 μg/ml for 15 min at 37 °C. The cells were washed prior to infection with an inoculum of WNV, which had not been treated with the inhibitor. Treatment of cells only with PI-88 did not inhibit WNV infectivity, given that the number of infected cells following PI-88 treatment was not significantly reduced relative to mock-treated cells when infected with a WNV inoculum that had not been incubated with the inhibitor (Fig. 1E). A comparable result was obtained when heparin was used in the same concentration range for pre-treatment of cells (data not shown). Accordingly, the inhibitory activity of PI-88 and presumably other HS mimetics is most likely due to their association with GAG-binding domains on the flavivirus E protein, as previously proposed (Chen et al., 1997). This conclusion is also supported by our finding, and that of others, showing a correlation of enhanced sensitivity of flavivirus variants to the inhibitory effect of sulfated (negatively charged) GAG with 'gain-of-positive-charge' mutations on the E protein (Lee et al., 2004; Lee and Lobigs, 2000, 2002; Mandl et al., 2001).

WNV infectivity was slightly increased at concentrations of 1–1000 μg/ml of PI-88 used for cell treatment prior to infection (Fig. 1E); the occasional enhancement of infectivity by sulfated GAG has been noted by others to occur dependent on virus strain, cell type and GAG examined (Bartolini et al., 2003). The mechanism for this phenomenon is not understood.

3.2. Anti-flavivirus activity of HS mimetics, in vivo

The in vitro inhibitory activity of the HS mimetics against a number of medically important flaviviruses raised the possibility of a therapeutic value of these compounds. We used a high virus dose (10^7–10^8 PFU) i.v. challenge model in 6-week-old C57Bl/6 mice (Licon Luna et al., 2002; Wang et al., 2003) to test the antiviral activity of suramin, PPS, and PI-88 against JEV. In this model the mortality in groups of saline-treated control mice was >90%. Administration of virus and test compounds were by different routes (i.v. and i.p., respectively) as a more stringent approach to minimize the probability of a very rapid interaction of inhibitor and virus in the blood expected to occur had both injections been by the i.v. route. Treatment of mice with suramin (three doses, i.p., of 2 mg/dose at 4-h pre-infection and 16- and 72-h p.i.) had no beneficial effect on the survival rate or average time to death (ATD) (Fig. 2). Uninfected mice did not show signs of drug-mediated toxicity when injected with four doses of 2 mg of suramin at daily intervals. Treatment of mice with PPS also failed to improve disease outcome following JEV challenge (Fig. 2). When mice were injected with 0.5 mg of PPS at 4-h pre-infection and 16- and 72-h p.i., the mortality curve closely followed that of mice in the control group. Increasing the dose of PPS to 5 mg given at 4-h pre-infection and 2 mg given at 16-h p.i. again did not show a therapeutic effect against JEV. Dose-limiting toxicity of PPS was apparent in the latter treatment regimen, reflected in the death of 2 out of 10 mice on day 2 p.i.

For PI-88 treatment a dose of 0.5 mg/injection, i.p., was chosen as the highest acceptable dose in terms of the toxicity of the compound in 6-week-old mice; it caused occasional mortality (<5%) on days 1 and 2 following commencement of treatment. In contrast to suramin and PPS, PI-88 treatment ameliorated disease outcome in JEV infection. The results in Fig. 2 show that pre-treatment was most important for the beneficial effect of PI-88, and that inoculation of PI-88 on day 3 in addition to day 1 post-challenge significantly improved survival. Thus, mortality of saline-treated control mice infected with JEV was 92%, whilst treatment with PI-88 at 4-h pre-challenge and 16- and 72-h post-challenge reduced mortality to 68% ($P = 0.0019$). The ATD was significantly increased from 5.7 days in the control group to 6.4 days in the group of mice that had received three doses of PI-88 ($P = 0.034$; surviving mice are not included in the calculation). Two doses of PI-88 given at 4-h pre- and 16-h post-challenge with JEV did not improve disease outcome in terms of survival (90% mortality), but had a significant beneficial effect in terms of prolonging ATD to 7.3 days ($P = 0.004$ relative to control group). In contrast, when the pre-treatment with PI-88 was omitted and a group of mice was treated with PI-88 at 4-, 24-, and 72-h post-challenge with JEV, survival (87% mortality) and ATD (6.3 days) were not significantly different from that of the control group (Fig. 2). Given the importance of prophylactic treatment for improvement of disease outcome, the mechanism for mortality reduction and prolongation of survival time mediated by PI-88 most likely involved the reduction of viral load in blood and/or tissues. Since JEV does not produce detectable viremia or virus titers in tissues, this issue could not be addressed. Nevertheless it cannot be excluded that PI-88 has a so far unknown immune-modulating effect that is beneficial for recovery from flavivirus infection, in view of reports that GAG can trigger a toll-like receptor and modulate dendritic cell, macrophage, and B-cell functions (Johnson et al., 2002, 2004; Wrenshall et al., 1999).

The therapeutic effect of PI-88 was also apparent in a second mouse model for flaviviral encephalitis and in DEN-2 infection (Fig. 3). Two doses of PI-88 given at 4-h pre- and 16-h post-challenge with 10^8 PFU of MVE significantly reduced mortality from 84% in a group of control mice to 39% in PI-88-treated mice ($P = 0.0006$). The ATD in the groups of saline- and PI-88-treated mice was comparable (7.2 and 6.9, respectively). This is consistent with the view that the high dose of MVE mostly results in rapid entry of virus into the brain, independent of virus growth in extraneural tissues (Licon Luna et al., 2002; Wang et al., 2003), and that the antiviral activity of PI-88 can partially prevent this process.

Interferon-α and -γ receptor knock-out (IFN-α/γ-$R^{-/-}$) mice were used for testing the in vivo antiviral activity of PI-88 against DEN-2. Extraneural inoculation of these mice with mouse-adapted DEN-2 gave rise to virus infection of the CNS and uniform mortality (Johnson and Roehrig, 1999). Groups of 6-week-old IFN-α/γ-$R^{-/-}$ mice were treated, i.p., with PI-88 (0.5 mg/dose) at 4-h pre-infection (i.v.) with 10^6 PFU of DEN-2, followed by PI-88 injections at 2-days intervals from 1 to 13 days p.i., or mock-treated with PBS (Fig. 3). Mortality of mice in the control group occurred between days 10 and 22 p.i. with one mouse surviving until day 32 p.i.; the average survival time was 15 days. PI-88 treatment significantly increased the average survival time to 22 days ($P = 0.004$), although the survival rate did not differ from that of the control group, since four mice that survived past 28 days p.i. died on days 32, 37, 47, and 60 p.i. Thus, it appears that the adaptive immune response, thought to be functional in IFN-α/γ-$R^{-/-}$ mice (van den Broek et al., 1995), fails to clear the DEN-2 infection when the IFN responses are defective. Given that PI-88 treatment was terminated at day 13 p.i., and in view of the exquisite sensitivity of the IFN-α/γ-$R^{-/-}$ mouse model to DEN-2 infection, it is not surprising that the antiviral compound did not improve the survival ratio.

4. Conclusion

This study provides (i) 'proof-of-principle' that HS mimetics can ameliorate disease outcome in animal models of flaviviral encephalitis and dengue, and (ii) raises a cautionary note on the predictive accuracy of an in vitro assay for antiviral activity of model compounds in terms of in vivo therapeutic value. The mechanism by which HS mimetics reduce virus infectivity is most likely by steric hindrance of virus attachment following binding of the compounds to regions enriched in basic residues on the viral surface. Heparin-binding motifs for DEN are predicted to reside predominantly on domain III (Chen et al., 1997; Thullier et al., 2001), the putative receptor-binding domain in the crystal structure of the flavivirus E protein (Lobigs et al., 1990; Modis et al., 2003; Rey et al., 1995). This is consistent with the interpretation that heparin and HS mimetics are attachment inhibitors. Whilst in the in vitro assays the affinity of HS mimetics for the flavivirus E protein will determine their antiviral activity, additional factors, such as bioavailability, will be critical for their inhibitory activity in vivo. The latter may explain the discrepancy seen between a therapeutic value of PI-88 only, and in vitro EC_{50} determinations showing comparable or greater virion-binding affinity of PPS and suramin relative to PI-88. Binding of heparin and heparin-like compounds to plasma proteins significantly contributes to loss of bioavailability, and the smaller size of PI-88 relative to other HS mimetics tested may account for a longer half-life of in vivo biological activity. In addition to bioavailability, the dose-limiting effect of toxicity of HS mimetics due to their anti-coagulant activity is a major factor restricting their use as antivirals. Thus in terms of anti-flavivirus application of HS mimetics it will be critical to identify compounds that demonstrate low coagulopathy and prolonged bioavailability in addition to effective in vitro inhibition of infectivity.

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