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Evaluation of new cell culture inhibitors of protease-resistant prion protein against scrapie infection in mice

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Transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative diseases that affect mammals. Examples of TSEs include sheep scrapie, bovine spongiform encephalopathy, chronic wasting disease of cervids and Creutzfeldt–Jakob disease (CJD) in humans. TSEs are associated with the accumulation of an abnormal and protease-resistant aggregate of prion protein (PrP) known as PrP-res or PrPSc (Caughey & Lansbury, 2003). The exact makeup of the infectious particle of the TSEs is not clear, but infectious preparations are composed mainly of PrP-res, which accumulates primarily in the brains of affected individuals. All TSEs are fatal and there is no known cure for these diseases.

Because PrP-res is associated with disease and infectivity, it has been a target of therapeutic intervention for TSEs (Aguzzi et al., 2001; Dormont, 2003; Brown, 2002). Murine N2a cells chronically infected with the RML (Chandler) strain of scrapie (Race et al., 1988) have been used widely to test compounds for their ability to inhibit PrP-res formation (Caughey et al., 1999; Beranger et al., 2001; Kocisko et al., 2003). Many compounds that inhibit PrP-res in cell culture have also delayed the onset of TSEs in animal models, but none has been curative. These compounds include porphyrins and phthalocyanins (Caughey et al., 1998; Priola et al., 2000), polyene antibiotics (Dormont, 2003), Congo red (Caughey & Race, 1992; Ingrosso et al., 1995), suramin (Gilch et al., 2001), sulfated glycans and other polyanions (Ehlers & Diringer, 1984; Kimberlin & Walker, 1986; Farquhar & Dickinson, 1986; Caughey & Raymond, 1993; Birkett et al., 2001; Schonberger et al., 2003; Gabizon et al., 1993). We recently used N2a cell cultures infected with either the RML or the 22L strains of scrapie to screen PrP-res inhibitors from a library of 2000 drugs and natural products (Kocisko et al., 2003).

In this study we tested a number of the most potent of these new cell culture PrP-res inhibitors against scrapie infection in transgenic mice (Tg7). Tg7 mice produce no mouse PrP, but express approximately 4- to 8-fold higher levels of hamster PrP than do hamsters. They have a short disease incubation period of ~45–50 days after intracerebral (i.c.) inoculation with a high dose of the 263K strain of hamster scrapie (Race et al., 2000; Priola et al., 2000).

Compounds were administered either to treat an established infection or to test for prophylaxis. To test for activity against an established infection, compound administration started 2 weeks after i.c. scrapie inoculation and continued for 5–6 weeks. The 2-week period after i.c. inoculation...
allowed time for the disease to progress before the compound was administered. To test for prophylaxis, administration of a compound began 2 weeks before and continued for 4 weeks after intraperitoneal (i.p.) scrapie inoculation. The rationale was to have a compound approaching a steady-state level in the mouse, enabling it to block a peripheral inoculation of scrapie infectivity from being established in the brain. The treatment following inoculation would allow time for the animal potentially to clear infectious material while the compound prevented further formation of PrP-res.

Compounds were administered either as an i.p. injection or in the drinking water. For i.p. injections, compounds were dissolved or suspended in an appropriate buffer and the dose volume was 10 ml kg$^{-1}$. Injections were given three times per week, on Monday, Wednesday and Friday. Solutions of compounds in drinking water were made to yield the desired dose based on the mean daily consumption of water by mice, 15 ml (100 g body wt)$^{-1}$. A solution of compound in the drinking water was the sole source of water for the mice during the dosing period. All 263K scrapie brain homogenates made up for inoculation in these studies were in physiological buffer supplemented with 2% fetal bovine serum. Different control groups are presented because testing was not done all at once and mice were inoculated with different homogenate preparations. In these studies, Tg7 mice were euthanized when clinical signs of scrapie were present, which included ruffled fur, lethargy, ataxia and weight loss. All procedures were approved by the Institution’s Animal Care and Use Committee and were designed to minimize the animals’ pain and distress. Animals that died from causes other than scrapie, such as from inoculation, dosing and anaesthetizing procedures, have been excluded from the data.

Compounds evaluated in animals had IC$_{50}$ (concentration of a compound inhibiting half of the production of PrP-res) values of £1 μM against both the RML and the 22L scrapie strains in cell culture. Since the in vivo testing involved hamster 263K scrapie, it was felt that compounds that inhibited multiple strains of mouse scrapie had a better chance of showing efficacy against PrP from another species. The inhibitors tested had been identified previously (Kocisko et al., 2003) except for polyphenolic extracts of grape seed and pine bark (data not shown). In addition to their history of use in humans, the anti-psychotic drugs thioridazine, thiothixene and trifluoperazine were also selected for testing because they are known to cross the blood–brain barrier of humans. Amodiaquine is an inexpensive anti-malarial drug that has been used extensively in humans. The polyphenol tannic acid, which is contained in many foods, was the most potent inhibitor in our test set with an IC$_{50}$ of 100 nM in both the scrapie-infected neuroblastoma cells and a solid-phase cell-free hamster 263K conversion assay (Kocisko et al., 2003). A tea extract containing 55% epigallocatechin monogallate and other polyphenols was also tried because of its relatively low toxicity and use as a human food. Finally, tetrandrine, a Chinese herbal medicine with anti-malarial activity, was tested. Generally, the highest known tolerated dose of a compound in mice was given to maximize the chance of seeing an effect. For instance, 5 mg thioridazine kg$^{-1}$ i.p. was used in this trial because 10 mg kg$^{-1}$ i.p. is not tolerated (Burke et al., 1990). In our experiments, 10 mg trifluoperazine kg$^{-1}$ was mildly toxic but was tolerated, and 4500 mg tannic acid kg$^{-1}$ per day was not tolerated but 3000 mg kg$^{-1}$ per day had no apparent toxicity.

Table 1 contains the incubation period of each individual Tg7 mouse after i.c. inoculation of 263K scrapie brain homogenate and administration of compounds. No compound used as a treatment against established infection after i.c. inoculation significantly extended incubation periods. Nor was any compound protective when administered for a week prior to i.c. inoculation.

Prophylaxis tests with a number of compounds were also done on animals infected by i.p. inoculation to test for inhibition of the spread of infection from the periphery, where most natural infections initiate. A lack of effect against i.c. inoculation may be due to low brain penetration for some compounds such as tannic acid, epigallocatechin monogallate in tea, and other naturally occurring polyphenols from pine bark or grape seed. In addition, one of the anti-psychotic drugs that does cross the blood–brain barrier, trifluoperazine, was also tried in this type of test to see if it would perform better against an i.p. inoculation compared with an i.c. inoculation. None of these drug treatments showed any efficacy against an i.p. inoculation (Table 2).

In addition to being a potent PrP-res inhibitor in vitro, tannic acid is appealing as a potential drug because of its relatively low oral toxicity and low cost. However, with a molecular mass of 1700 Da, tannic acid would be unlikely to cross the blood–brain barrier in significant quantity and its bioavailability via the oral route in the mouse may not be high enough to be effective. In agreement with this, previously mentioned prophylaxis and therapeutic tests in scrapie-infected Tg7 mice with orally administered tannic acid demonstrated no benefit. Therefore, we tried direct incubation of tannic acid with infectious brain homogenate. Solutions of tannic acid at 10 mM, 1 mM and 10 μM in the presence of 5% scrapie-infected brain homogenate were tested for their ability to reduce infectivity. After incubation at 37 °C for 2 h, the solutions were diluted 5000-fold to 0.001% brain homogenate and then inoculated i.c. into Tg7 mice to assess infectivity. The results in Table 3 indicate that incubation of infectious material with tannic acid had no significant effect on scrapie incubation period.

There are many reasons why a given compound that is effective in vitro might not show efficacy in an in vivo test against scrapie infection. The pharmacokinetics and metabolism of these compounds in mice might be unfavourable. The drug concentration attained at an active site using the highest tolerated dose may not be high enough for long
Table 1. Treatment of Tg7 mice inoculated with 263K scrapie

M, Monday; W, Wednesday; F, Friday; inoc., inoculation; homog., homogenate; wk, week.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg kg(^{-1}))</th>
<th>Dosing regimen</th>
<th>Scrapie inoculation</th>
<th>Incubation periods (days)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>50 i.p.</td>
<td>M, W, F for 5 wks starting 2 wks after inoc.</td>
<td>50 µl 0.01 % brain homog. i.c.</td>
<td>59, 63, 63, 63, 56, 63, 64</td>
<td>61·8 ± 2·8</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>50 i.p.</td>
<td>M, W, F for 5 wks starting 2 wks after inoc.</td>
<td>50 µl 0.01 % brain homog. i.c.</td>
<td>56, 63, 59, 60, 63</td>
<td>60·2 ± 2·9</td>
</tr>
<tr>
<td>Thoridazine</td>
<td>5 i.p.</td>
<td>M, W, F for 5 wks starting 2 wks after inoc.</td>
<td>50 µl 0.01 % brain homog. i.c.</td>
<td>59, 63, 63, 58, 56, 59, 63, 59</td>
<td>60·3 ± 2·7</td>
</tr>
<tr>
<td>Thiothixene</td>
<td>5 i.p.</td>
<td>M, W, F for 5 wks starting 2 wks after inoc.</td>
<td>50 µl 0.01 % brain homog. i.c.</td>
<td>58, 56, 58, 58, 66, 58, 63, 65, 58, 56, 63, 59</td>
<td>60·2 ± 3·6</td>
</tr>
<tr>
<td>Triluoperazine</td>
<td>10 i.p.</td>
<td>M, W, F for 5 wks starting 2 wks after inoc.</td>
<td>50 µl 0.01 % brain homog. i.c.</td>
<td>65, 63, 65, 72, 59, 63, 63, 63, 64</td>
<td>64·0 ± 3·3</td>
</tr>
<tr>
<td>None</td>
<td>50 µl 0·001 % brain homog. i.c.</td>
<td>50 µl 0·001 % brain homog. i.c.</td>
<td>62, 69, 65, 66, 69, 71, 66, 71, 65, 62, 62</td>
<td>66·4 ± 3·4</td>
<td></td>
</tr>
<tr>
<td>Tetrandrine</td>
<td>50 i.p.</td>
<td>M, W, F for 6 wks starting 2 wks after inoc.</td>
<td>50 µl 0·001 % brain homog. i.c.</td>
<td>64, 77, 69, 72, 64, 61</td>
<td>67·8 ± 6·0</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>1500 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks after inoc.</td>
<td>50 µl 0·001 % brain homog. i.c.</td>
<td>66, 63, 64, 68, 63, 64, 62, 64</td>
<td>64·3 ± 1·9</td>
</tr>
<tr>
<td>Polyphenolic tea extract</td>
<td>1500 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks after inoc.</td>
<td>50 µl 0·001 % brain homog. i.c.</td>
<td>76, 79, 66, 73, 69, 57</td>
<td>70·0 ± 7·9</td>
</tr>
<tr>
<td>None</td>
<td>50 µl 0·01 % brain homog. i.c.</td>
<td>71, 71, 71, 77, 71, 71, 79, 71</td>
<td>72·8 ± 3·3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>50 i.p.</td>
<td>M, W, F for 6 wks starting 1 wk prior to inoc.</td>
<td>50 µl 0·01 % brain homog. i.c.</td>
<td>74, 77, 74, 77, 81, 79, 74</td>
<td>76·3 ± 2·7</td>
</tr>
<tr>
<td>Thoridazine</td>
<td>5 i.p.</td>
<td>M, W, F for 6 wks starting 1 wk prior to inoc.</td>
<td>50 µl 0·01 % brain homog. i.c.</td>
<td>74, 77, 71, 71, 77, 74, 71, 71</td>
<td>73·3 ± 2·7</td>
</tr>
<tr>
<td>Thiothixene</td>
<td>5 i.p.</td>
<td>M, W, F for 6 wks starting 1 wk prior to inoc.</td>
<td>50 µl 0·01 % brain homog. i.c.</td>
<td>84, 77, 71, 74, 84, 71, 80</td>
<td>77·3 ± 5·6</td>
</tr>
<tr>
<td>Triluoperazine</td>
<td>10 i.p.</td>
<td>M, W, F for 6 wks starting 1 wk prior to inoc.</td>
<td>50 µl 0·01 % brain homog. i.c.</td>
<td>74, 71, 71, 77, 84, 77, 71</td>
<td>75·0 ± 4·8</td>
</tr>
</tbody>
</table>

Table 2. Prophylaxis of Tg7 mice against 263K scrapie

All mice were infected with scrapie by i.p. inoculation with 50 µl 1 % brain homogenate. M, Monday; W, Wednesday; F, Friday; wk, week.

<table>
<thead>
<tr>
<th>Test compound/preparation</th>
<th>Dose (mg kg(^{-1}))</th>
<th>Dosing regimen</th>
<th>Incubation periods (days)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>101, 81, 127, 88, 97, 83, 88, 99</td>
<td>101, 81, 127, 88, 97, 83, 88, 99</td>
<td>95·5 ± 14·7</td>
<td></td>
</tr>
<tr>
<td>Tannic acid</td>
<td>1500 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks prior to inoc.</td>
<td>89, 94, 87, 90, 89, 90, 96, 73</td>
<td>88·5 ± 6·9</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>3000 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks prior to inoc.</td>
<td>81, 102, 87, 92, 88, 93</td>
<td>90·5 ± 7·1</td>
</tr>
<tr>
<td>Polyphenolic tea extract</td>
<td>1500 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks prior to inoc.</td>
<td>77, 105, 90, 77, 96, 77, 81, 75</td>
<td>84·8 ± 11·0</td>
</tr>
<tr>
<td>Polyphenolic grape seed extract</td>
<td>2250 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks prior to inoc.</td>
<td>80, 91, 100, 95, 73, 108, 97, 98, 90, 71</td>
<td>90·3 ± 12·1</td>
</tr>
<tr>
<td>Polyphenolic pine bark extract</td>
<td>2250 per day in drinking water</td>
<td>Continuously for 6 wks starting 2 wks prior to inoc.</td>
<td>90, 87, 95, 91, 87, 71, 73, 91, 90, 83</td>
<td>85·8 ± 7·9</td>
</tr>
<tr>
<td>Triluoperazine</td>
<td>10 i.p.</td>
<td>M, W, F for 6 wks starting 2 wks prior to inoc.</td>
<td>90, 91, 104, 99, 101, 103</td>
<td>96·9 ± 6·3</td>
</tr>
</tbody>
</table>
enough to be efficacious. Also, the mechanism by which these particular molecules inhibit PrP-res formation in infected N2a cells is unclear and may not be replicated sufficiently in vivo. These results showed that inhibition in the infected N2a cells may not always correlate with anti-scrapie activity in vivo. Nonetheless, the scrapie-infected N2a assay remains a valuable initial screen for potential drugs because numerous compounds that have been identified as PrP-res inhibitors in scrapie-infected cells have proven to have at least some anti-scrapie activity in vivo.

Since many potent inhibitors of PrP-res formation in vitro are not efficacious against scrapie in animals, it is important to consider animal testing of inhibitors prior to clinical trials, especially in cases in which there might be negative side effects for the patient. Quinacrine, an anti-malarial drug used extensively in humans, was found to inhibit side effects for the patient. Quinacrine, an anti-malarial trials, especially in cases in which there might be negative to consider animal testing of inhibitors prior to clinical are not efficacious against scrapie in animals, it is important that some anti-scrapie activity in vivo.

When testing the efficacy of compounds against scrapie infection in an in vivo system there are many complex variables. Some of these include the dose, the vehicle, the dosing regimen, when to initiate and terminate treatment, the routes of compound administration and scrapie inoculation, the animal model and the TSE strain. Part of the difficulty in deciding on these variables is a lack of understanding of TSE pathogenesis. We have tried to select reasonable options from among these variables, but many others might be considered. Although none of the inhibitors tested herein was effective in our in vivo tests, we report the results of these expensive and time-consuming experiments in the hope that future work with potential anti-TSE therapeutics and prophylactics can advance beyond our particular approaches rather than repeat them.

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References


