Doxorubicin and Congo Red Effectiveness on Prion Infectivity in Golden Syrian Hamster

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Abstract. *The effect of doxorubicin and Congo Red on prion protein (PrP) infectivity in experimental scrapie was studied to better understand the effect of these compounds in prion diseases and to establish whether a dose–response correlation exists for Congo Red. This was performed in order to test the effectiveness of compounds that may easily be used in human prion diseases. Brain homogenate containing membrane bound PrPSc monomers was used as inoculum and was previously incubated with doxorubicin 10^{-3} M and with increasing concentrations of Congo Red ranging from 10^{-7} to 10^{-2} M. This study shows for the first time that doxorubicin, and confirms that Congo Red, may interact with pathological PrP monomers modifying their infectious properties. Pre-incubation of infected brain homogenate with Congo Red resulted in prolonged incubation time and survival, independently of Congo Red concentration ($p<0.05$). Doxorubicin and Congo Red effects do not depend upon interaction with PrP amyloid material.*

Prion diseases (PD), also called transmissible spongiform encephalopathies, are progressive and invariably fatal neurodegenerative disorders of humans and animals. The most common human form is Creutzfeldt-Jakob disease (CJD), which accounts for almost 90% of PD in humans. CJD, a disease of middle-advanced age, is a dementing condition associated with myoclonus and variable involvement of cerebellar, pyramidal, extrapyramidal and brain stem structures. Compared to the other neurodegenerative causes of dementia, CJD has a relatively rapid course, usually leading to death within a few months. Like other neurodegenerative disorders, CJD may present in both sporadic and familial forms, both clinically very similar. However, CJD is uniquely characterized for its transmissibility to experimental animals by intracerebral inoculation of infected brain tissue. This feature led to several cases of iatrogenic CJD due to contamination of instruments during neurosurgical procedures or administration of cadaveric-derived pituitary hormones. The transmissibility of PD is preserved in iatrogenic, sporadic and many familial forms. Prions are self proteins (prion protein, or PrP) which during PD undergo a conformational change which transforms them into pathogenic proteins (1). In PD, the normal protease-sensitive isoform (PrPC, or cellular PrP) is converted into the pathological isoform (called PrPSc, from scrapie, the PD of sheep) (2). PrPSc and PrPC share identical sequence and post-translational modifications, but differ in their secondary structure and biochemical properties. In particular, PrPSc has a high content of beta-sheet, whereas PrPC contains 40% of alpha-helix structure (3-5).

Many drugs have been tested as potential therapeutic agents for PD, but none of them has been demonstrated to be effective in blocking or slowing the disease progression if administered after the onset of clinical symptoms (6). At present, no effective therapy is available but therapeutic agents for transmissible spongiform encephalopathies are emerging (7). Amphotericin B has been shown to delay disease onset in experimental animals if treatment is started around the inoculation date (8). MS-8209, a derivative of amphotericin B with low toxicity, has proven effective both in early and late treatments, but only when administered before clinical onset (9). Quinacrine and chlorpromazine, two drugs commonly used as therapeutic agents for different human disorders, have been showed effective in reducing the

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production of PrPSc *in vitro*, but their effect in human PD remains uncertain (10). Anti-PrP antibodies represent a promising approach, even if therapy started after clinical onset shows at present no effectiveness (11). Congo Red belongs to a class of compounds characterized by the ability to interact with amyloid. Congo Red can bind amyloid fibrils made of pathological PrP and modifies their infectivity *in vitro* and *in vivo* (12-14). Iododoxorubicin binds different classes of amyloid fibrils, reduces amyloid deposition in several types of amyloidosis and prolongs incubation time in scrapie hamster (15, 16). A number of other compounds are in the process of being evaluated for therapeutic use in PD: phosphorothioate DNA, statins also associated with platelet-activating factor, pentosan polysulfate, inhibitors of dehydrocholesterol reductase and vaccination against PrP. Some of these show promising partial effectiveness (17-20).

The aim of this work was to test the capacity of Congo Red to interact with native membrane-bound PrPSc in the inoculum and to reduce infectivity, and to test whether Congo Red shows a dose-dependent effect. Also whether doxorubicin, like iododoxorubicin, has an anti-prion effect when added to native membrane-bound PrPSc in the inoculum was tested.

Materials and Methods

For the experimental plan, 44 Female Golden Syrian hamsters (Charles River Italia, Lecco, Italy), weight 70-80 g (Table I) were used. All animals were maintained in a controlled environment in which light was on for 12 h per day (8:00-20:00 h). Hamsters were housed in plastic boxes in groups of two. The animals were inoculated intracerebrally with 30 μl of various inocula. For the inoculation procedure, the animals were anesthetized by ether and the solution injected by a insulin syringe in the right hemisphere. The animals were observed daily and kept with free access to water and food. The disease stage was evaluated upon clinical criteria: stage 0 (healthy), stage 1 (hyper-reactivity), stage 2 (ataxia), stage 3 (complete paralysis, typical of the terminal stage of the disease). All animals were sacrificed during stage 3 to prevent spontaneous death with the consequent risk of tissue degradation.

The inocula were prepared by using brain homogenates from terminally ill Golden Syrian hamsters infected with the 263K scrapie strain and diagnosed as affected by PD. The brain (approx. 1 g weight) was homogenized in cold PBS (10% w/v) and centrifuged at 3000 *g* for 10 minutes at 4°C. The resulting pellet was re-homogenized in the same volume and centrifuged under the same conditions as in the first step. The supernatants obtained from both centrifugation steps were then mixed, thus obtaining a 5% brain homogenate. The inocula were prepared by incubating for 60 minutes at RT a 1% dilution of infected brain homogenate with different concentrations of Congo Red (Sigma-Aldrich Italia, Milan, Italy), ranging from 3×10^{-2} M to 3×10^{-7} M, or with doxorubicin 10^{-3} M (Table I). The rate of infectivity was evaluated according to Prusiner *et al*.

Means±SE of different inoculated groups were analyzed by the analysis of variance (ANOVA) and Fisher's test for multiple comparisons. Cox's regression analysis was used to assay the presence of a relationship between incubation times, ataxia, survival and the different Congo Red concentrations used in our experiments. All statistical analyses were carried out with Graph Prism 3.0 statistical program (Prism Software Corporation, CA, USA). In all analyses, the null hypothesis was rejected at the 0.05 level.

Table I. *Experimental plan*.

Group	Number	Inoculum	Treatment
A	8	1% Brain homogenate	None
B	8	1% Brain homogenate	Congo Red 3×10^{-2}
C	8	1% Brain homogenate	Congo Red 3×10^{-3}
D	8	1% Brain homogenate	Congo Red 3×10^{-5}
E	8	1% Brain homogenate	Congo Red 3×10^{-7}
F	4	1% Brain homogenate	Doxorubicin 3×10^{-3}
Total	44		

Results

One hamster died 48 hours after the inoculation, probably because of the mechanical trauma due to intracerebral inoculation. All remaining 43 hamsters developed classical clinical signs of PD. The time interval from the inoculation to the onset of the three different stages of the disease and of death is reported.

As reported in Figure 1, the incubation period was longer in the groups treated with Congo Red homogenates or doxorubicin with respect the controls ($p<0.005$). In particular, an increased incubation period was always observed between treatments and controls. The highest difference resulted from the comparison between controls *vs*. Congo Red 3×10^{-5} ($p<0.001$) and controls *vs*. doxorubicin 3×10^{-5} ($p<0.001$) (Figure 1) since it was prolonged from a mean of 76 to 110 and 128 days, respectively.

Ataxia duration during experimental scrapie was increased by the preincubation with Congo Red and doxorubicin ($p<0.05$). The animals infected with homogenate treated with Congo Red 3×10^{-5} and doxorubicin 3×10^{-3} showed the greatest delay (on average, 33 and 48 days compared to controls, respectively) for the appearance of ataxia ($p<0.001$) (Figure 2).

In general, the time between inoculum and late disease stage was significantly longer in treated animals than in controls ($p<0.05$). Also in this case, the treatment with Congo Red 3×10^{-5} and doxorubicin 3×10^{-3} showed the maximum effectiveness ($p<0.001$) (Figure 3) since animal survival shifted on average from 89 to 125 and 140 days, respectively.

The time between disease onset and death (disease duration) was longer in all groups of treatment with respect to controls ($p<0.005$) with no inter-group differences (Figure 4).

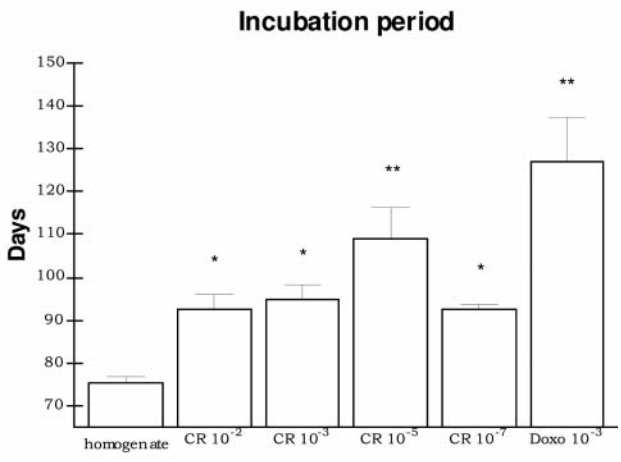


Figure 1. *Interval between inoculation and disease onset. *p<0.05, **p<0.001.*

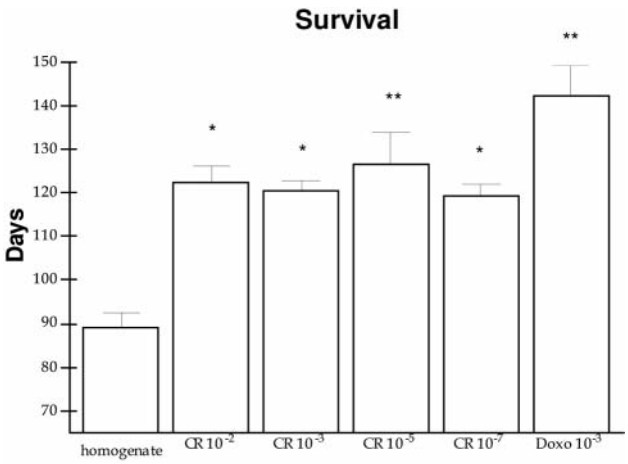


Figure 3. *The time between inoculum and late stage of the disease (phase 1 to 3) is reported. Duration was longer in all groups of treatment than in controls. *p<0.05, **p<0.001.*

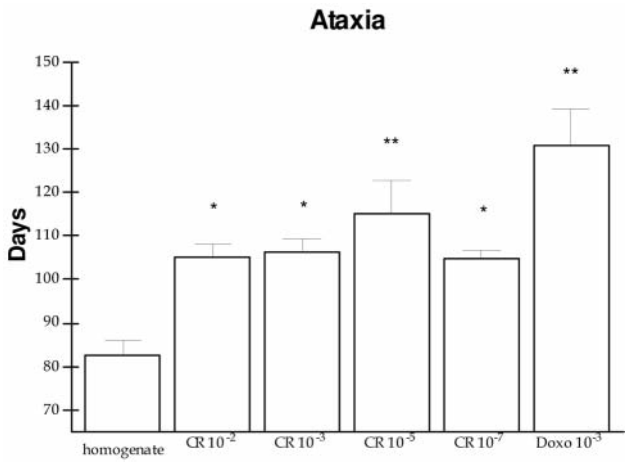


Figure 2. *Duration of ataxia in the treatment groups is shown. *p<0.05, **p<0.001.*

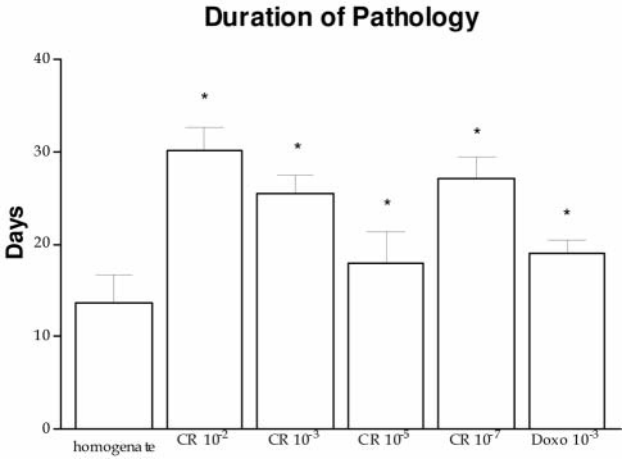
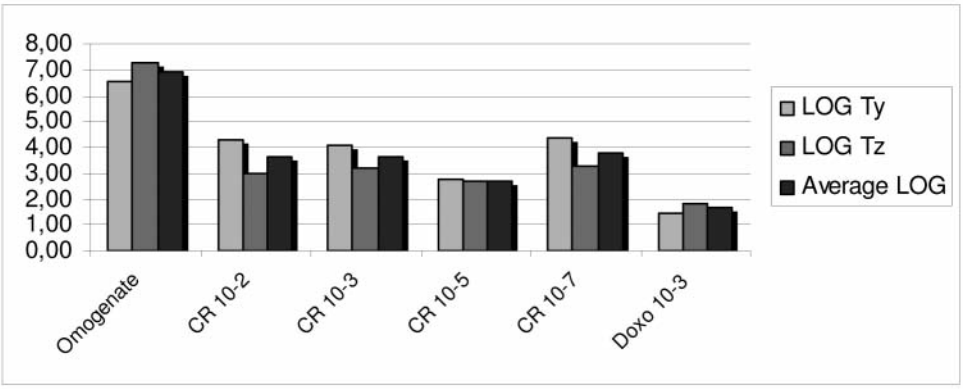


Figure 4. *The period between the onset of disease and death was longer in all groups of treatment. *p<0.05.*

Preincubation of brain homogenate with Congo Red resulted in a marked decrease of infectivity titer. The infectivity titer of the inocula were reduced 1,000 to 10,000 times in the five different preincubation treatments (Figure 5). The disease progression was also compared in the four groups inoculated with different concentrations of Congo Red to verify whether a dose–response relationship exists. Despite variance analysis revealing a slight difference in incubation times between the four groups ($p<0.05$), time intervals from onset to the development of ataxia and of the terminal stage resulted in no significant difference. Moreover, Cox regression analysis excluded a dose–response relationship.

Discussion

Congo Red and other compounds, such as iododoxorubicin, are able to bind amyloid fibrils and have good prospects for systemic amyloidosis treatment (15, 22, 23). The experiments that show the effectiveness of Congo Red and other compounds in PD were carried out by adding these compounds to a PrP27-30 preparation used as inoculum to infect hamster or mouse except for the data reported by Tagliavini *et al*. regarding iododoxorubicin (24). Thus Congo Red and iodoxorubicin effectiveness in PD was attributed to their interaction with PrP27-30 amyloid fibrils or to PrPSc



GROUP	LOG Ty	LOG Tz	Average LOG	SD
Omogenate	6.54	7.28	6.91	0.52
CR 10^{2}	4.32	3.02	3.67	0.92
CR 10^{3}	4.07	3.21	3.64	0.60
CR 10^{5}	2.78	2.66	2.72	0.09
CR 10^{7}	4.33	3.29	3.81	0.73
Doxo 10^{-3}	1.47	1.83	1.65	0.26

Figure 5. *Infectivity Titer. The infectivity titer is calculated by Prusiner's equation and is reduced in all groups infected whit homogenate treated with Congo Red (CR) and in the group treated with Doxorubicin (Doxo).*

aggregates (12, 13, 22, 25, 26). These compounds interact directly with amyloid fibrils and PrPSc aggregates and might disturb their geometry and recruitment of new molecules (27). Iododoxorubicin has been shown to be able to bind to amyloid, to hamper further aggregation and to induce amyloid solubilization. Doxorubicin shows no similar effect.

Nowadays, most attempts to treat PD assume that PrPSc production from PrPC is the crucial event (28) and that PrPSc monomer inside the cell constitutes the key point for disease induction (29).

This work provides additional evidence for the protective action of Congo Red and doxorubicin in PD. These experiments show that Congo Red and doxorubicin significantly interact with PrPSc contained in the infected brain homogenate of the inoculum.

There is evidence that in scrapie-infected cells Congo Red inhibits the replication of the infectious agent and the accumulation of the protease-resistant form of PrP (12).

The mechanism of Congo Red interaction with membrane-bound monomeric PrPSc is unknown but this interaction is able to significantly reduce the infectivity titer of the inoculum, as shown in these experiments. In fact, a pre-incubation of the scrapie-infected brain homogenate with Congo Red and doxorubicin in the present experimental conditions reduced infectivity in the inoculum between 1,000 to 10,000 times with respect to the pure homogenate. Moreover, a slowing down of disease progression with increased disease duration has been demonstrated in the animals injected with those inocula.

It should be stressed that brain homogenate from scrapie-infected animals does not contain amyloid fibrils. In fact, PrPSc tends to accumulate in neurons and to remain membrane-bound. Thus, the effect of Congo Red and doxorubicin must be attributed to their capacity to interact with membrane-bound monomeric form of PrPSc. The effectiveness of doxorubicin in decreasing infectivity in these experimental conditions confirms that the mechanism implicated is different from the capacity to bind amyloid fibrils, because, unlike iododoxirubucin, doxorubicin does not bind to amyloid. Congo Red and doxorubicin significantly interact with PrPSc contained in infected brain homogenates, thus modifying its molecular features and resulting in a decrease of infectivity. The mechanism underlying the effect of Congo Red is not known, but both overstabilization of PrPSc and promotion of its clearance from the brain in the injected animal, and interaction with PrPC preventing its conversion to PrPSc have been hypothesized (12, 30, 31).

In order to evaluate whether the effectiveness of Congo Red was dose-dependent, the inoculum was incubated with increasing concentrations of Congo Red. The data show that there is no dose/response correlation and that a major response is reached also with low doses. In fact, the application of a Cox regression model excluded a linear correlation between drug increased concentration in the inoculum and the observed effect. The dose/response curve may imply that the effect of Congo Red reaches a plateau, beyond which a further increase in its relative concentration produces no effective improvement.

Congo Red has also shown an *in vivo* anti-scrapie effect in hamster (31). In fact, hamsters infected with scrapie agent were treated with intraperitoneal injection of Congo Red before, during and after inoculum and the incubation period was evaluated (31). The data showed that an *in vivo* treatment with Congo Red immediately after inoculum can prolong the incubation period, but cannot slow disease progression. Unfortunately the data show that this compound is effective only when it is administered at the time of infection.

The presented experiments show that Congo Red and doxorubicin produce their effect when added to the inoculum, in the absence of significant amounts of amyloid, through an interaction with monomeric membrane-bound PrPSc. These data are experimental evidence that some compounds can interfere with the interaction of PrPC and PrPSc. This interaction is responsible for the conversion of PrPC to its protease-resistant form which is the basis of prion disease pathogenesis. These data suggest that Congo Red and doxorubicin may be used in the treatment of PD, but Congo Red effectiveness is limited and cannot be further increased by dose augmentation. Despite doxorubicin never having been tested as anti-prion drug in an *in vivo* experiment, it is commonly used for treating different types of cancer and its safety profile is well known. Thus, the evaluation of the anti-prion effect of doxorubicin when administered after disease onset might be an interesting perspective of this study. Different compounds with different action-mechanisms may be combined to increase overall effectiveness in the treatment of PD.

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References

1 Prusiner SB: Molecular biology of prion diseases. Science *252*: 1515-1522, 1991.

2 Prusiner SB, Scott MR, DeArmond SJ and Cohen FE: Prion protein biology. Cell *93*: 337-348, 1998.

3 Caughey BW, Dong A, Bhat KS, Ernst D, Hayes SF and Caughey WS: Secondary structure analysis of the scrapie-associated protein PrP 27-30 in water by infrared spectroscopy. Biochemistry *30*: 7672-7680, 1991.

4 Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, Mehlhorn I, Huang Z, Fletterick RJ, Cohenu FE and Prusiner SB: Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA *90*: 10962-10966, 1993.

5 Safar J, Roller PP, Gajdusek DC and Gibbs CJ Jr: Conformational transitions, dissociation, and unfolding of scrapie amyloid (prion) protein. J Biol Chem *268*: 20276-20284, 1993.

6 Dormont D: Approaches to prophylaxis and therapy. Br Med Bull *66*: 281-292, 2003.

7 Koster T, Singh K, Zimmermann M and Gruys E: Emerging therapeutic agents for transmissible spongiform encephalopathies: a review. J Vet Pharmacol Ther *26*: 315-326, 2003.

8 Adjou KT, Privat N, Demart S, Deslys JP, Seman M, Hauw JJ and Dormont D: MS-8209, an amphotericin B analogue, delays the appearance of spongiosis, astrogliosis and PrPres accumulation in the brain of scrapie-infected hamsters. J Comp Pathol *122*: 3-8, 2000.

9 Beringue V, Adjou KT, Lamoury F, Maignien T, Deslys JP, Race R and Dormont D: Opposite effects of dextran sulfate 500, the polyene antibiotic MS-8209, and Congo red on accumulation of the protease-resistant isoform of PrP in the spleens of mice inoculated intraperitoneally with the scrapie agent. J Virol *74*: 5432-5440, 2000.

10 Barret A, Tagliavini F, Forloni G, Bate C, Salmona M, Colombo L, De Luigi A, Limido L, Suardi S, Rossi G, Auvré F, Adjou KT, Salès N, Williams A, Lasmézas C and Deslys JP: Evaluation of quinacrine treatment for prion diseases. J Virol *77*: 8462-8469, 2003.

11 Korth C and Peters PJ: Emerging pharmacotherapies for Creutzfeldt-Jakob disease. Arch Neurol *63*: 497-501, 2006.

12 Caughey B, Ernst D and Race RE: Congo red inhibition of scrapie agent replication. J Virol *67*: 6270-6272, 1993.

13 Caughey B, Brown K, Raymond GJ, Katzenstein GE and Thresher W: Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red corrected. J Virol *68*: 2135-2141, 1994.

14 Rudyk H, Vasiljevic S, Hennion RM, Birkett CR, Hope J and Gilbert IH: Screening Congo Red and its analogues for their ability to prevent the formation of PrP-res in scrapie-infected cells. J Gen Virol *81*: 1155-1164, 2000.

15 Merlini G, Ascari E, Amboldi N, Bellotti V, Arbustini E, Perfetti V, Ferrari M, Zorzoli I, Marinone MG, Garini P, Diegoli M, Trizio D, and Ballarini D: Interaction of the anthracycline 4'-iodo-4'-deoxydoxorubicin with amyloid fibrils: inhibition of amyloidogenesis. Proc Natl Acad Sci USA *92*: 2959-2963, 1995.

16 Palha JA, Ballinari D, Amboldi N, Cardoso I, Fernandes R, Bellotti V, Merlini G and Saraiva MJ: 4'-Iodo-4'-deoxydoxorubicin disrupts the fibrillar structure of transthyretin amyloid. Am J Pathol *156*: 1919-1925, 2000.

17 Ludewigs H, Zuber C, Vana K, Nikles D, Zerr I and Weiss S: Therapeutic approaches for prion disorders. Expert Rev Anti Infect Ther *5*: 613-630, 2007.

18 Vana K, Zuber C, Nikles D and Weiss S: Novel aspects of prions, their receptor molecules, and innovative approaches for TSE therapy. Cell Mol Neurobiol *27*: 107-128, 2007.

19 Rainov NG, Tsuboi Y, Krolak-Salmon P, Vighetto A and Doh-Ura K: Experimental treatments for human transmissible spongiform encephalopathies: is there a role for pentosan polysulfate? Expert Opin Biol Ther *7*: 713-726, 2007.

20 Bade S and Frey A: Potential of active and passive immunizations for the prevention and therapy of transmissible spongiform encephalopathies. Expert Rev Vaccines *6*: 153-168, 2007.

21 Prusiner SB, Cochran SP, Groth DF, Downey DE, Bowman KA and Martinez HM: Measurement of the scrapie agent using an incubation time interval assay. Ann Neurol *11*: 353-8, 1982.

22 Caughey B and Race RE: Potent inhibition of scrapie-associated PrP accumulation by Congo Red. J Neurochem *59*: 768-771, 1992.

23 Poli G, Martino PA, Villa S, Carcassola G, Giannino ML, Dall'Ara P, Pollera C, Iussich S, Tranquillo VM, Bareggi S, Mantegazza P and Ponti W: Evaluation of anti-prion activity of Congo Red and its derivatives in experimentally infected hamsters. Arzneimittelforschung *54*: 406-415, 2004.

24 Tagliavini F, McArthur RA, Canciani B, Giaccone G, Porro M, Bugiani M, Lievens PM, Bugiani O, Peri E, Dall'Ara P, Rocchi M, Poli G, Forloni G, Bandiera T, Varasi M, Suarato A, Cassutti P, Cervini MA, Lansen J, Salmona M and Post C: Effectiveness of anthracycline against experimental prion disease in Syrian hamsters. Science *276*: 1119-1122, 1997.

25 Caspi S, Halimi M, Yanai A, Sasson SB, Taraboulos A and Gabizon R: The anti-prion activity of Congo Red. Putative mechanism. J Biol Chem *273*: 3484-3489, 1998.

26 Tagliavini F, Forloni G, Colombo L, Rossi G, Girola L, Canciani B, Angeretti N, Giampaolo L, Peressini E, Awan T, De Gioia L, Ragg E, Bugiani O and Salmona M: Tetracycline affects abnormal properties of synthetic PrP peptides and PrP(Sc) *in vitro*. J Mol Biol *300*: 1309-1322, 2000.

27 Aguzzi A, Glatzel M, Montrasio F, Prinz M and Heppner FL: Interventional strategies against prion diseases. Nat Rev Neurosci *2*: 745-749, 2001.

28 Brandner S, Isenmann S, Raeber A, Fischer M, Sailer A, Kobayashi Y, Marino S, Weissmann C and Aguzzi A: Normal host prion protein necessary for scrapie-induced neurotoxicity. Nature *379*: 339-343, 1996.

29 Brandner S, Raeber A, Sailer A, Blättler T, Fischer M, Weissmann C and Aguzzi A: Normal host prion protein (PrPC) is required for scrapie spread within the central nervous system. Proc Natl Acad Sci USA *93*: 13148-13151, 1996.

30 Ladogana A, Casaccia P, Ingrosso L, Cibati M, Salvatore M, Xi YG, Masullo C and Pocchiari M: Sulphate polyanions prolong the incubation period of scrapie-infected hamsters. J Gen Virol *73(Pt 3)*: 661-665, 1992.

31 Ingrosso L, Ladogana A and Pocchiari M: Congo Red prolongs the incubation period in scrapie-infected hamsters. J Virol *69*: 506-508, 1995.

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