are present in titin, and (iv) titin domains are connected axially, limiting their accessible conformations during refolding. Thus, "wearing-out" could still reflect the systematic increase in the number of prolines in the cis state and the consequent increased fraction of slow-to-renature domains in the molecule. Such wearing out may explain why the pre-unfolded fraction of titin was longer than that attributable to the PEVK region. Because all tethers were fully stretched one or more times before data was taken, the pre-unfolded titin could also contain unfolded Ig or FNIII domains that needed more time to renature. Indeed, single molecules recover if left relaxed for several minutes and regain some of their hysteresis behavior. Notably, wearing out and recovery has also been seen in muscle fibers (*24*).

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Inhibition of Pathogenicity of the Rice Blast Fungus by *Saccharomyces cerevisiae* α-Factor

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Magnaporthe grisea is a fungal pathogen with two mating types, *MAT1-1* and *MAT1-2*, that forms a specialized cell necessary for pathogenesis, the appressorium. *Saccharomyces cerevisiae* α-factor pheromone blocked appressorium formation in a mating type–specific manner and protected plants from infection by *MAT1-2* strains. Experiments with α-factor analogs suggest that the observed activity is due to a specific interaction of α-factor with an *M. grisea* receptor. Culture filtrates of a *MAT1-1* strain contained an activity that inhibited appressorium formation of mating type *MAT1-2* strains. These findings provide evidence that a pheromone response pathway exists in *M. grisea* that can be exploited for plant protection.

The heterothallic ascomycete *Magnaporthe grisea* is a pathogen of a wide variety of grasses but is best known as the causal agent of rice blast disease. The costs of controlling disease with fungicides and the difficulty in breeding durable and effective resistance have led to intense interest in understanding the mechanisms governing the pathogenicity of this fungus (*1*). Conidia of *M. grisea* attach to the plant host with an adhesive that is released from the tip of the conidium upon hydration (*2*). After germination, the fungus responds to contact with the host surface by producing an appressorium, a specialized cell that uses turgor pressure to aid in penetration of the host cell (*3*).

The mating behavior of *M. grisea* is determined by the mating-type locus, which contains either *MAT1-1* or *MAT1-2* DNA. One parent of each of the two mating types participates in a sexual cross. The mating-type loci of filamentous ascomycetes are thought to encode master regulators that control the expression of mating type–specific genes, such as pheromones and pheromone receptors (*4*). In *Saccharomyces cerevisiae*, α-factor and **a**-factor pheromones are produced by strains with *MAT*α and *MAT***a** mating types. Each pheromone is recognized by a corresponding heterotrimeric GTP-binding protein–coupled receptor expressed in the opposite mating type (*5*).

Appressorium formation of mating type *MAT1-2* strains of *M. grisea* is inhibited when conidia are germinated in the presence of 2% yeast extract. However, 2% peptone and 2% tryptone do not inhibit appressorium formation (*6*). We found that appressorium formation of *MAT1-1* strains was not inhibited by yeast extract to the same degree as *MAT1-2* strains (Table 1). Yeast extract contained an unidentified factor that could be partially purified by an organic extraction procedure designed for purification of small peptides (*7*). This fraction inhibited appressorium formation in *MAT1-2* strains, and the active component appears to be a polypeptide. We found 91 ± 7% appressorium formation of strain 4091-5-8 (*MAT1-2*) with proteinase K–treated extract and 1 ± 1% appressorium formation with untreated extract (*8*). The mating type–specific effect of yeast extract on appressorium formation suggested that *M. grisea* might respond to a pheromone by suppressing infection-related development.

The α-factor pheromone of *S. cerevisiae* has activity in closely related yeast species (*9*). We tested the effect of synthetic *S. cerevisiae* α-factor on *M. grisea* and found that appressorium formation was inhibited in *MAT1-2* strains (Table 1 and Fig. 1, A and B) but not in *MAT1-1* strains (Table 1 and Fig. 1C). The concentration of α-factor needed to cause >95% inhibition of appressorium formation of all *MAT1-2* strains tested was 300 μM. This is 10^4-fold higher than the concentration of α-factor required

Table 1. Mating type–specific inhibition of appressorium formation in *M. grisea*. Conidia were incubated in 50 mM potassium phosphate buffer (pH 6.5) (control), 2% yeast extract, α-factor pheromone in 50 mM potassium phosphate buffer (pH 6.5), or extracts of strain CP987 culture filtrate. Appressorium formation assays were performed as described (*6*). In each of three experiments, a minimum of 300 conidia were counted. The average number of conidia producing at least one appressorium is reported. Variation in appressorium formation between experiments in controls was ±10% or less; appressorium formation in *MAT1-1* strains in yeast extract or CP987 extracts varied by ±20% or less; for *MAT1-1* strains in α-factor, variation was ±10% or less; for *MAT1-2* strains in yeast extract, α-factor, or CP987, extract variation was ±2% or less.

Strain	Mating type	Appressorium formation (%)			
		Control	Yeast extract	α-Factor (300 μM)	CP987 extract
Guy11	1-2	92	0.0	0.8	2.2
4091-5-8	1-2	95	0.0	0.5	0.7
4360-R-12	1-2	92	0.0	0.4	2.2
4375-R-6	1-2	97	0.0	0.0	1.0
CP987	1-1	98	43	91	56
4360-17-1	1-1	96	26	94	44
4136-4-3	1-1	92	50	86	51
4375-R-26	1-1	88	47	91	44
CP2738	1-1	95	45	96	91
CP2735	1-2	93	0.2	0.2	0.2

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to induce morphological changes in *S. cerevisiae* (*10*). Because it does not contain α-factor (*7*), we conclude that yeast extract contains a distinct peptide that has a specific biological effect on M. *grisea*.

We tested segregation of mating type and sensitivity to α-factor by examining the progeny of a cross between strains CP987 and 4091-5-8. In the 31 progeny examined, inhibition of appressorium formation by α-factor cosegregated with the *MAT1-2* mating type. As a further test that the mating type–specific response was tightly linked to the mating-type locus, we examined strains in which mating types had been switched by transformation with cosmid clones containing the mating-type loci (*11*). Strain CP2738 was derived from strain 4091-5-8 (*MAT1-2*) by transformation with *MAT1-1* mating-type DNA. The genome of CP2738 does not contain *MAT1-2* DNA, but does contain *MAT1-1* DNA (*11*). Likewise, CP2735 is derived from the *MAT1-1* strain 4136-4-3. These strains are switched in mating behavior (*11*) and exhibit a corresponding switch in the response to α-factor (Table 1).

Appressorium formation is induced by cyclic adenosine 3′,5′-monophosphate (cAMP) (*12*). The inhibitory activity of α-factor toward *MAT1-2* strains can be overridden by cAMP. For strain 4091-5-8, we found 79 ± 10% appressorium formation with α-factor and 10 mM cAMP, and 0.7 ± 0.3% appressorium formation with α-factor alone. Although, the appressoria often appeared to be immature or delayed in formation (Fig. 1D), this suggests that α-factor acts at an early stage in the pathway that signals appressorium formation and that the block can be reversed or bypassed by cAMP.

We examined a series of α-factor analogs to test the specificity of α-factor in inhibiting appressorium formation (Table 2). Analogs that retained high activity toward *S. cerevisiae* also retained the ability to inhibit appressorium formation. No effect on growth or appressorium formation of either mating type of M. *grisea* was observed with the **a**-factor pheromone of *S. cerevisiae* (*13*).

To determine whether an activity could be identified from M. *grisea* that inhibited appressorium formation, we prepared extracts from culture filtrates of both mating types (*14*). The extract from the *MAT1-1* strain inhibited appressorium formation in *MAT1-2* strains but had little effect on *MAT1-1* strains (Table 1 and Fig. 1, E and F). The extract derived from strain 4091-5-8 (*MAT1-2*) did not significantly inhibit appressorium formation in any strain.

To determine whether the appressorium-inhibiting activity of *MAT1-1* culture filtrates might be a polypeptide, we tested the sensitivity of the extract to protease digestion. Because chymotrypsin recognizes bulky hydrophobic residues in polypeptides, we used chymotrypsin linked to acrylic beads (Sigma) to examine whether the appressorium-inhibiting activity of *MAT1-1* culture filtrates might be a polypeptide. Chymotrypsin eliminated the activity in *MAT1-1* culture filtrates (90 ± 4% appressorium formation for strain 4091-5-8), but heat-inactivated chymotrypsin did not affect the activity (0.5 ± 0.1% appressorium formation). Chymotrypsin treatment of α-factor destroyed activity toward strain 4091-5-8 and the ability to arrest growth in *S. cerevisiae* RC629; activity was retained when heat-treated protease was used.

As with the artificial substrates, we noted that appressorium formation by strain 4091-5-8 (*MAT1-2*) was inhibited when conidia germinated on the surface of barley leaves in the presence of 300 μM α-factor. To determine whether inhibition of appres-

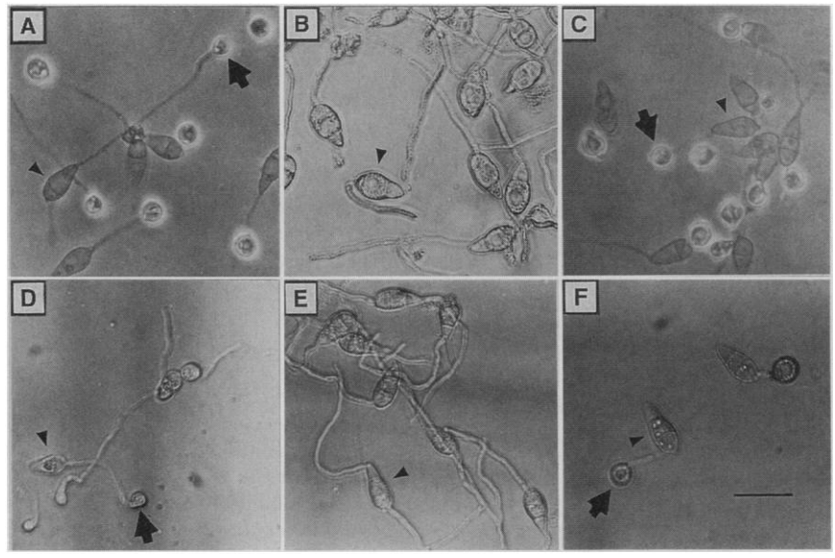

Fig. 1. Mating type–specific inhibition of appressorium formation. Conidial suspensions (1×10^4 conidia per ml) were inoculated onto Teflon film (DuPont). Conidia are indicated by arrowheads. Appressoria are spherical cells indicated by arrows. (**A**) Germlings of strain 4091-5-8 (*MAT1-2*) in 50 mM phosphate buffer. Addition of 300 μM α-factor inhibits appressorium formation of strain 4091-5-8 (**B**) but not strain CP987 (*MAT1-1*) (**C**) or strain 4091-5-8 germinated in the presence of 300 μM α-factor and 10 mM cAMP (**D**). Extract of strain CP987 culture filtrate blocks appressorium formation in strain 4091-5-8 (**E**) but not in strain CP987 (**F**). Samples were incubated for 16 hours and the Teflon sheet was placed on a microscope slide before examination by bright-field microscopy. Bar, 30 μm.

Table 2. Biological activity of α-factor analogs. Appressorium assays were performed as described in the legend of Table 1. α-Factor is H_2N-WHWLQLKPGQPMY-COOH (*17*). Asparagine substituted for glutamine at position 5 is designated [Asn^5]. The removal of tryptophan at position 1 is designated des-Trp^1. Other substitutions are norleucine (Nle), cyclohexylalanine (Cha), and 3,4-dehydro-L-proline (DHP). The previously reported (*10*) concentration of analog required to induce mating projections in 50% of yeast **a** cells (*bar1-1*) is shown for comparison.

Peptide	Appressorium formation in strain 4091-5-8 (*MAT1-2*) (%)		Appressorium formation in strain CP987 (*MAT1-1*)		*S. cerevisiae* morphological response
	Peptide (30 μM)	Peptide (300 μM)	Peptide (30 μM)	Peptide (300 μM)	(nM peptide)
α-Factor	36	0.3	97	96	1.7
[Asn^5, Arg^7]	59	0.5	96	73	1.7
[Nle^{12}]	56	0.0	90	90	1.7
[DHP^6, DHP^{11}, Nle^{12}]	37	11	93	94	1.7
[Phe^3]	16	0.2	90	90	3.4
des-Trp^1	90	91	84	79	550
des-Trp^1-[Ala^3]	95	93	93	95	>35,000
des-Trp^1-[Cha^3]	92	87	96	91	1,100
des-Trp^1-[Cha^3, Ala^9]	94	95	92	95	>35,000
des-Trp^1-[Cha^3, D-Ala^9]	95	89	90	89	1,100

sorium formation could be correlated with reduced pathogenicity, we inoculated barley plants with conidia of strain 4091-5-8 and the corresponding switched mating-type strain CP2738 in the presence or absence of α-factor with norleucine substituted at position 12 (*15*). Significant protection against the *MAT1-2* strain 4091-5-8 was observed (Fig. 2). Mean lesion densities for strain 4091-5-8 were 50 lesions per 12.5-cm leaf segment for the control and 2.3 lesions per 12.5-cm leaf segment for the α-factor–treated plants ($t = 11.16$, $P < 0.0001$, df = 22). α-Factor treatment did not significantly protect plants against strain CP2738 (*MAT1-1*) ($t = 0.82$, $P = 0.43$, df = 22). A similar result was obtained with the second switched mating-type pair. Strain 4136-4-3 (*MAT1-1*) was unaffected by α-factor, but infection of plants with strain CP2735 (*MAT1-2*) was significantly reduced by α-factor treatment.

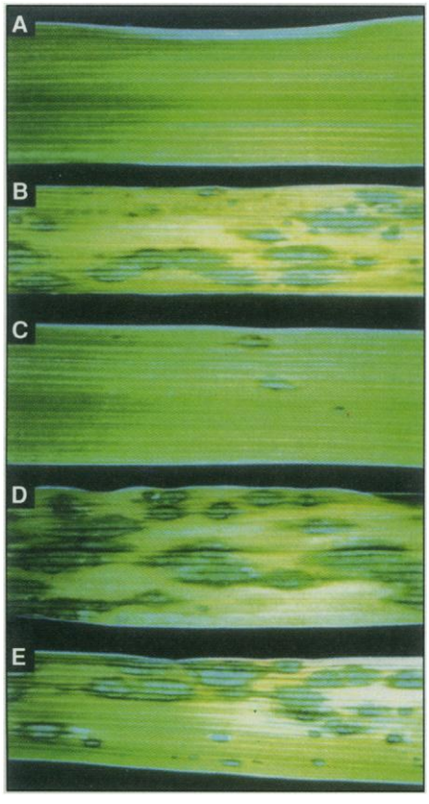

Fig. 2. Effect of α-factor pheromone on *M. grisea* pathogenicity. Fourteen-day-old barley seedlings were spray inoculated with 3 ml of 1% gelatin in 50 mM potassium phosphate buffer (pH 6.5) (**A**), 1% gelatin containing 10^4 conidia per ml of strain 4091-5-8 (**B**), strain 4091-5-8 with 300 μM α-factor (**C**), 1% gelatin containing 10^4 conidia per ml of strain CP2738 (**D**), and strain CP2739 with 300 μM α-factor (**E**). The conidial suspensions (2×10^4 conidia per ml) were mixed with equal volumes of 1% gelatin in 50 mM potassium phosphate (pH 6.5) or 1% gelatin in 50 mM potassium phosphate containing 600 μM [Nle^{12}] α-factor to give a final suspension containing 1×10^4 conidia per ml.

The ability of α-factor to block appressorium formation in *M. grisea* can be explained if α-factor is similar in sequence to an *M. grisea* pheromone and can interact with a corresponding receptor. In this model, binding of pheromone to receptor interferes with signaling of appressorium development. Because cAMP induces appressorium formation in the presence of α-factor, pheromone–receptor interaction may inhibit appressorium formation by preventing cAMP accumulation. The correlation of inhibition of appressorium formation by α-factor analogs with biological activity in yeast argues for a specific recognition of α-factor by *MAT1-2* strains. Structural and functional similarities of peptide hormones have been recognized previously. α-Factor has limited sequence similarity to gonadotropin-releasing hormone and stimulates the release of luteinizing hormone from cultured gonadotrophs, although α-factor is less active than the authentic hormone by a factor of 10^4 (*16*). α-Factor pheromones of three *Saccharomyces* species that differ from each other at four of 13 positions have similar interspecies activity in causing growth arrest (*9*).

Our results suggest that stimulation of the pheromone receptor of *M. grisea* blocks pathogenic development. Field isolates of *M. grisea* that infect rice are usually found to be infertile, although the cause of this infertility is not known. Thus, examination of the activity of α-factor against typical *MAT1-2* field isolates is needed to evaluate the use of pheromones for disease control. However, our results demonstrate the potential for fungal pheromones to control plant disease. Presumably, the authentic mating factors from *M. grisea* have biological activity at low concentrations, as is observed in the pheromone response in *S. cerevisiae*. As peptides, fungal pheromones are amenable to chemical modification to optimize their activity and to control their persistence in the environment.

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8. The yeast extract benzyl alcohol fraction was treated overnight at room temperature with proteinase K at 1 mg/ml or was left untreated as a control. *Magnaporthe grisea* conidia were mixed directly with these preparations and placed on the hydrophobic side of gel-bond film for appressorium formation assays. In experiments with chymotrypsin-linked beads, the enzyme-linked beads were added (5 mg/ml) to strain CP987 extract or to α-factor and incubated for 12 hours at 25°C. The beads were removed by centrifugation, and the extract was tested for activity. As a control, enzyme-linked beads were heat treated (95°C for 60 min) to destroy protease activity before addition to extract.
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13. **a**-Factor pheromone [C. B. Xue, G. A. Caldwell, J. M. Becker, F. Naider, *Biochem. Biophys. Res. Comm.* **162**, 253 (1989)] was dissolved in dimethyl sulfoxide to 1 mg/ml and droplets of 1, 5, 10, and 20 μl were placed on the surface of Teflon film (DuPont). The dimethyl sulfoxide was allowed to evaporate for 48 hours, and *M. grisea* conidia of strains 4091-5-8 and CP987 were placed on the dried **a**-factor to test for appressorium formation. This same preparation of **a**-factor was previously shown to elicit growth arrest in a solid agar halo assay and alter cell morphology in suspension with *S. cerevisiae* strain RC757 [S. Marcus *et al.*, *Mol. Cell. Biol.* **11**, 3603 (1991)].
14. *Magnaporthe grisea* mates and produces perithecia on the mycelial mat formed in standing cultures of oatmeal broth. A culture was coinoculated with strains CP987 and 4091-5-8 and grown until perithecia were visible (about 14 days). Separate flasks were inoculated with strain CP987 or 4091-5-8 and harvested when perithecia formed in the mated control culture. The strain CP987 and 4091-5-8 culture filtrates were harvested, and extracts were prepared as described for yeast extract. A second extract preparation from strain CP987 was used for appressorium assays with strains CP2735 and CP2738. Strains 4136-4-3 and 4091-5-8 produced 92 ± 5% and 8 ± 4% appressoria, respectively, with this second extract preparation. These strain CP987 extracts did not possess activity toward *S. cerevisiae* strain RC629 in halo assays.
15. Barley seedlings (cultivar Bonanza) were spray inoculated and incubated in plastic bags overnight. The next day, plants were removed from bags and symptoms were allowed to develop for 7 days. Lesions were scored on 12.5-cm leaf segments starting near the tip of the leaf and were analyzed with Image Pro Plus (Media Cybernetics, Silver Spring, MD). Statview (Abacus Concepts, Berkeley, CA) was used for data analysis. In a separate experiment, plants were protected by 300 μM α-factor (0.5 mg/ml) from infection by strain 4091-5-8 (*MAT1-2*). The mean lesion densities were 35 lesions per 12.5-cm leaf segment for the control and 3.1 lesions per 12.5-cm leaf segment for α-factor treatment ($t = 5.833$, $P < 0.0001$, df = 13). Diseased leaf area was reduced from 42% to 5% with pheromone treatment ($t = 6.506$, $P < 0.0001$, df = 13). Lesion size was not altered by α-factor treatment. A control peptide (CQSMSGPAGSPGLLNLIPVDLS-NH_2) (*17*) with no sequence similarity to yeast pheromone did not pro-

tect plants from infection by strain 4091-5-8 at 0.5 mg/ml.
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17. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; S, Ser; V, Val; W, Trp; and Y, Tyr.
18. Strains were generously provided by B. Valent of DuPont, Wilmington, DE, and J. Becker of the University of Tennessee, Knoxville. We thank S. McBride for technical assistance and B. Valent and J. E. Hamer for providing research materials and encouragement to work with *M. grisea*. We also thank J. Becker and D. Cook for helpful discussions. Support for this work was provided by funds from USPHS grant R29GM47977, the Texas Rice Research Foundation, the Texas Advanced Research Program (D.J.E.), and USPHS grant GM22086 (F.N).

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Effectiveness of Anthracycline Against Experimental Prion Disease in Syrian Hamsters

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Prion diseases are transmissible neurodegenerative conditions characterized by the accumulation of protease-resistant forms of the prion protein (PrP), termed PrPres, in the brain. Insoluble PrPres tends to aggregate into amyloid fibrils. The anthracycline 4′-iodo-4′-deoxy-doxorubicin (IDX) binds to amyloid fibrils and induces amyloid resorption in patients with systemic amyloidosis. To test IDX in an experimental model of prion disease, Syrian hamsters were inoculated intracerebrally either with scrapie-infected brain homogenate or with infected homogenate coincubated with IDX. In IDX-treated hamsters, clinical signs of disease were delayed and survival time was prolonged. Neuropathological examination showed a parallel delay in the appearance of brain changes and in the accumulation of PrPres and PrP amyloid.

Prion diseases, such as scrapie of sheep, spongiform encephalopathy of cattle, and Creutzfeldt-Jakob disease of humans, are transmissible neurodegenerative conditions characterized by the accumulation of protease-resistant forms of the prion protein (PrP), termed PrPres, in the brain (*1*). Unlike the normal PrP, PrPres has a large amount of β-sheet secondary structure and a strong tendency to aggregate into amyloid fibrils (*2*). Deposition of PrPres and PrP amyloid is accompanied by nerve cell degeneration and glial cell proliferation, leading to the clinical signs of disease (*3*). In scrapie-infected hamsters, survival time is prolonged by the amyloid-binding dye Congo red (*4*). Congo red inhibits the accumulation of PrPres in scrapie-infected neuroblastoma cells without affecting the metabolism of normal PrP (*5*).

The anthracycline 4′-iodo-4′-deoxy-doxorubicin (IDX) (Fig. 1A) is a recently developed derivative of doxorubicin, a drug of proven efficacy in a large number of malignancies (*6*). The administration of IDX to eight patients with plasma cell dyscrasias complicated by immunoglobulin light-chain amyloidosis resulted in the partial resorption of amyloid deposits in three cases. In addition, four of the eight patients exhibited discernible clinical improvement, two patients showed reduced symptoms of the disease, and the other two patients were rendered stable (*7*). This unexpected activity of the compound was confirmed by further studies showing that IDX binds strongly to amyloid fibrils of different chemical composition, inhibits in vitro assembly of insulin into amyloid fibrils, and reduces amyloid deposits in a murine model of reactive amyloidosis (*8*). Thus, IDX can be regarded as a prototype of a class of drugs that are able to inhibit amyloidogenesis, reverse tissue deposition of amyloid fibrils, or both.

To examine this activity of IDX, we first investigated the ability of the compound to bind to PrP amyloid. Serial sections of the cerebral cortex and cerebellum from scrapie-infected hamsters and patients with Creutzfeldt-Jakob disease were incubated with 10^{-7} M aqueous solution of IDX or with the amyloid-binding fluorochrome thioflavine S and were analyzed by fluorescence microscopy (*9*). The comparison of adjacent sections showed that all amyloid deposits revealed by thioflavine S exhibited the characteristic fluorescence of IDX (Fig. 1, B and C).

We then investigated the effects of IDX on experimental scrapie, which is regarded as a model for prion diseases (*1*). Three groups of female Syrian hamsters (10 animals per group) were used for the study. The infected controls were injected intracerebrally with 30 μl of 1% (w/v) brain homogenate obtained from hamsters infected with the 263K strain of scrapie that were in the terminal stage of the disease. The IDX-treated hamsters were injected with the same inoculum, coincubated for 1 hour at room temperature with 2.9 mM IDX before intracerebral inoculation. The uninfected controls were injected with 30 μl of 1% suspension of normal hamster brain. Hamsters infected with scrapie agent coincubated with IDX lived significantly longer than those infected with scrapie alone (Fig. 2A). Thus, although all scrapie-infected hamsters had died by 94 days after infection (mean ± SEM = 88.5 ± 1.9 days), IDX-treated hamsters lived up to 128 days [mean ± SEM = 116 ± 5.6 days; $P < 0.01$, one-way analysis of variance (ANOVA)].

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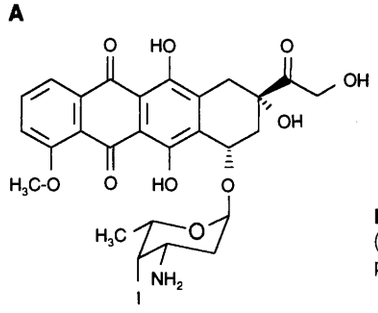

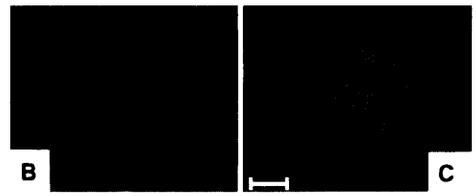

Fig. 1. (**A**) Chemical structure of IDX. (**B**) Binding of IDX and (**C**) thioflavine S to PrP amyloid in brain sections from a patient with Creutzfeldt-Jakob disease. Scale bar, 50 μm.