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A Specific Inhibitor of Janus Kinase-3 Increases Survival in a Transgenic Mouse Model of Amyotrophic Lateral Sclerosis

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Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disorder involving the motor neurons of cortex, brain stem, and spinal cord. About 10% of all ALS patients are familial cases (FALS), of which 20% have mutations in the Cu,Zn-superoxide dismutase (SOD1) gene. The murine model for FALS, which overexpresses a FALS variant of the SOD1 gene, exhibits progressive limbic paralysis followed by death. Treatment of FALS mice with WHI-P131, a specific inhibitor of Janus kinase 3 (JAK3), increased survival by more than two months, suggesting that specific inhibitors of JAK3 may be useful in the treatment of human ALS. These results uniquely establish JAK3 as a novel molecular target for the treatment of FALS. © 2000 Academic Press

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive, fatal neurodegenerative disorder involving the motor neurons of the cortex, brain stem, and spinal cord (1). The onset of disease is usually in the fourth or fifth decades of life and affected individuals succumb within 2 to 5 years of the disease onset (2). ALS occurs in both sporadic and familial forms (3). About 10% of all ALS patients are familial cases (FALS), of which 20% have mutations in Cu,Zn-superoxide dismutase (SOD1) gene, suggesting that an abnormally functioning Cu,Zn-SOD enzyme may play a pivotal role in the pathogenesis and progression of FALS (4, 5).

Cu,Zn-SOD is a ubiquitous cytoplasmic enzyme that is constitutively expressed. The primary enzymatic function of Cu,Zn-SOD is to catalyze the dismutation of superoxide radicals to oxygen molecules and hydrogen peroxide, which can then be decomposed by other antioxidant enzymes, including catalase and glutathione peroxidase (6). However, Cu,Zn-SOD also has a peroxidative function and can generate $^{\bullet}OH$ radicals from its own dismutation product, hydrogen peroxide (7). It has been shown that mutations in Cu,Zn-SOD gene can result in a gain of peroxidative function that enhances motor neuron death in ALS (7, 8). More than 50 point mutations of the human Cu,ZnSOD gene have been found in FALS patients (9). Most of the mutations occur at the regions involved in the subunit folding which lead to the relative exposure of the active site to outside (10). Experimental data *in vitro* have demonstrated that hydroxyl radical generation by Cu,Zn-SOD is significantly elevated by one of these mutations, G93A (11). Yim *et al.* (8) expressed wild-type and mutant forms of Cu,Zn-SOD in Sf9 insect cells and reported that wild-type Cu,Zn-SOD and mutant G93A-Cu,Zn-SOD have identical dismutation activities but the $^{\bullet}OH$ radical generating peroxidative function of the G93A mutant is enhanced due to a small decrease in its K_m for hydrogen peroxide (8). The increased generation of oxygen free radicals, especially hydroxyl radicals, by mutant Cu,Zn-SOD is generally hypothesized to be the initiating factor that results in the sequence of events leading to the motor neuron death in FALS. Recently, we reported that transfection of PC12 neuron precursor cells with G93A-SOD gene results in increased production of hydroxyl ($^{\bullet}OH$) radicals and enhanced rate of apoptotic cell death (12). Notably, PC12 cells transfected with H63C/G93A-Cu,Zn-SOD gene with a dominant negative mutation in the catalytic site, that converts histidine at position 63 to cysteine, showed dramatically reduced $^{\bullet}OH$ radical production and apoptotic death rate (12). Thus, the gain of apoptosis-promoting function of G93A-Cu,Zn-SOD could be reduced by an active site mutation. These results provide

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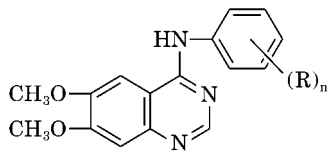
TABLE 1

6,7-Dimethoxyquinazoline Derivatives



No	R	Formula	mp(°C)	MW
WHI-P97	3-Br, 5-Br, 4-OH	$C_{16}H_{13}Br_2N_3O_3$	>300.0	455
WHI-P111	3-Br, 4-CH_3	$C_{17}H_{16}BrN_3O_2$	225.0–228.0	374
WHI-P131	4-OH	$C_{16}H_{15}N_3O_3$	245.0–248.0	297
WHI-P132	2-OH	$C_{16}H_{15}N_3O_3$	255.0–258.0	297
WHI-P154	3-Br, 4-OH	$C_{16}H_{14}BrN_3O_3$	233.0–233.5	376

additional genetic evidence for the hypothesis that the increased $^{\cdot}$OH radical production from the gain of peroxidative function by the Cu,Zn-SOD catalytic site induces cytotoxicity in neurons expressing the mutant FALS Cu,Zn-SOD gene.

Expression of high levels of human G93A-Cu,Zn-SOD causes an ALS-like motor neuron disease in transgenic mice (13). G93A-Cu,Zn-SOD-transgenic mice become paralyzed in one or more limbs due to motor neuron loss from the spinal cord and die within 6 months after birth (13). The purpose of the present study was to examine the effects of WHI-P131, a rationally designed Janus kinase 3 (JAK3)-specific tyrosine kinase inhibitor (14–16), on the onset of motor neuron disease in G93A-Cu,Zn-SOD-transgenic mice. WHI-P131 treatment significantly delayed the onset of paralysis, suggesting that further development of WHI-P131 may lead to an effective treatment program for ALS. Our study is the first to identify JAK3 as a molecular target for treatment of FALS.

MATERIALS AND METHODS

Chemicals. Genistein was synthesized as reported (17). The 6,7-dimethoxyquinazoline derivatives for this study were prepared by the condensation of 4-chloro-6,7-dimethoxyquinazoline and the substituted anilines as described recently in detail (14–16, 18).

In vitro determination of anti-oxidant activity. Anti-oxidant activity was measured using the total anti-oxidant status kit from Calbiochem (San Diego, CA). This spectrophotometric assay relies on the ability of anti-oxidants in the sample to inhibit the oxidation of ABST (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABST^{\cdot+}$ by metmyoglobin (a peroxidase) (19). The amount of $ABST^{\cdot+}$ produced was monitored at 620 nm using an ELISA plate reader set to read every 5 minutes for 90 minutes. The rate of $ABST^{\cdot+}$ production (ΔOD_{620}/min) was plotted against drug concentration (170 μM, 80 μM, 40 μM, and 20 μM) for determination of the EC_{50} values (i.e., the concentrations necessary for 50% inhibition).

In vivo determination of anti-oxidant activity. In vivo protection against oxygen singlet induced damage to the cranial vasculature was performed as described previously (20–22). Nine week old male Balb/c mice (Taconic, Germantown, NY) were kept in microisolator cages and fed regular rodent chow. Mice were injected with 300 μl of a 3 mg/mL solution of rose bengal in sterile phosphate buffered saline (PBS) via their tail vein. Mice were anesthetized with a ketamine/xylazine solution (200 mg ketamine/kg and 5 mg xylazine/kg) and illumination of the skull with cold green light (300 Watt Xenon arc lamp equipped with a 550 nm broadband interference filter having a 70 nm bandwidth which effectively blocks all heat generating infrared (Oriel Scientific, Stratford, CT) was performed on the shaved scalp by placing a 1.6 mm glass fiber optic light guide directly onto the scalp for 5 minutes. After 24 h, 300 μl of a 1 mg/mL solution of Evans Blue in PBS was administered intravenously. Thirty minutes later, the animals were anesthetized and perfused with PBS (for 5 minutes) followed by 4% phosphate buffered formalin. The lesion was well defined by the extravasated Evans Blue and lesion size in mm^2 was quantitated using the NIH Image 1.61 program in conjunction with a Pixera camera (Pixera Corp., CA). All numerical data are shown as mean ± SEM. Statistical differences among groups were performed by Student t-test using Instat, GraphPad Software (San Diego, CA). The drugs dissolved in DMSO were administered at a dosage of 16 mg/kg by IP administration every six h starting at 24 h pre-injury until 24 h post-injury in a volume of 50 μL per injection.

Murine FALS model. Heterozygous transgenic mice carrying the human SOD-1 (G93A) gene were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were treated either with vehicle (10% DMSO) or WHI-P131 at 12.5 mg/kg per day, everyday, except on weekends, starting at 60 days of age. These mice were monitored everyday for death. Life-table analysis was performed using Statview, Abacus Concepts, Inc. (Berkeley, CA).

RESULTS AND DISCUSSION

Previously, we have shown that the tyrosine kinase inhibitor genistein is protective against death and disease onset in the FALS model (21). The pleiotrophic activity profile of genistein, including its non-specific broad-spectrum tyrosine kinase inhibitory activity, anti-oxidant activity and hormone activity as a phytoestrogen, made it impossible to explain its protective activity. In the present study, we evaluated a specifc inhibitor of Janus kinase 3 (JAK3) as a potential FALS treatment agent. We first examined 3 rationally designed dimethoxyquinazoline compounds with JAK3

TABLE 2

Biological Activities of the Dimethoxyquinazoline Series

Compound	*In vitro* antioxidation: EC_{50} (μM)[b]	*In vivo* antioxidation: Lesion size (mm^2)	JAK3 inhibition[a]: IC_{50} (μM)
WHI-P97	392 ± 41	22.7 ± 5.6 (N = 5)	11.0
WHI-P111	2167 ± 197	16.7 ± 3.3 (N = 5)	>300
WHI-P131	24433 ± 3448	21.1 ± 2.9 (N = 5)	9.1
WHI-P132	132 ± 8	20.7 ± 2.6 (N = 5)	>300
WHI-P154	284 ± 12	Toxic (N = 5)	27.9
Genistein	32 ± 1	8.1 ± 1.0 (N = 20)	>300
Vehicle	N.A.[c]	17.7 ± 3.1 (N = 10)	N.A.

[a] Data from Ref. 14.

[b] Data are presented as mean ± SEM from four independent experiments.

[c] N.A., not applicable.

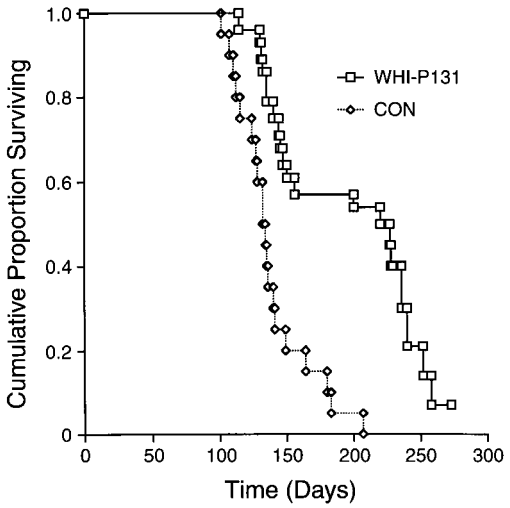


FIG. 1. Neuroprotection by WHI-P131 treatment in a mouse model for FALS. FALS mice were treated with vehicle (diamond, N = 24) or with WHI-P131 (square, N = 28). Increased survival was clearly observed in WHI-P131-treated FALS mice.

inhibitory activity, namely WHI-P97, WHI-P131, and WHI-P154 (14) as well as two control dimethoxyquinazoline compounds (viz., WHI-P111 and WHI-P132) and genistein for anti-oxidant activity *in vitro.* The chemical structures of these compounds are shown in Table 1. None of the dimethoxyquinazoline compounds exhibited anti-oxidant activity (Table 2). *In vitro,* WHI-P131, the most potent inhibitor of JAK3, had an anti-oxidant EC_{50} value of 24.4 mM, which is >26,000-fold higher than its IC_{50} value as a JAK3 inhibitor. By comparison, the anti-oxidant EC_{50} value of genistein was 32 μM. Similarily, none of the dimethoxyquinazoline compounds exhibited anti-oxidant activity *in vivo.* Unlike genistein (21), the JAK3 inhibitors were unable to protect the cranial vasculature against oxygen singlets generated by intracranial photoactivation of rose bengal (Table 2).

As shown in Table 2, WHI-P131, the most potent and specific inhibitor of JAK3, was singularly nontoxic *in vivo* and devoid of any anti-oxidant activity *in vitro* or *in vivo.* WHI-P131 also does not inhibit Janus kinases JAK1 and JAK2, the Src family kinase LYN, the ZAP/SYK family tyrosine kinase SYK, the TEC family tyrosine kinase BTK, and receptor family tyrosine kinase IRK (14–16). Furthermore, WHI-P131 showed very favorable pharmacokinetics in mice and monkeys and is nontoxic at dose level as high as 100 mg/kg (23). Therefore, WHI-P131 was chosen for further studies in FALS.

To determine whether JAK3 plays a role in FALS, we compared the survival of FALS mice treated with WHI-P131 (at a dose level of 12.5 mg/kg administered intraperitoneally on a once daily injection schedule) to the survival of vehicle-treated control FALS mice. Treatment was initiated 60 days following birth in order to closely mimic the clinical situation. Vehicle-treated control transgenic mice (N = 24) developed a rapidly progressive motor neuron disease affecting both upper and lower extremities and exhibited a median survival of 134 days (Fig. 1). WHI-P131 treatment significantly slowed the deterioriation of the neurologic status of treated transgenic mice (N = 28) yielding a median survival of 200 days (P = 0.0019) (Fig. 1). These results support the notion that neuronal salvage in FALS should be possible once diagnosis has been made, as fragmentation of the Golgi apparatus and vacuolar degeneration of the spinal cord motor neurons occur long before limbic paralysis and neuronal death (24, 25). By identifying JAK3 as a new molecular target in FALS therapy, our study indicates that further development of WHI-P131 may lead to an effective treatment program for FALS patients.

The exact molecular mechanism for the neuroprotective effects of WHI-P131 in FALS mice is unknown; however, c-jun expression is markedly increased in the spinal cord of the end-stage transgenic G93A-Cu,Zn-SOD-transgenic mice due to increased production of reactive oxygen intermediates (ROI) (13, 26–28) and WHI-P131 has been reported to inhibit ROI-induced pro-apoptotic biochemical signal transduction events, including c-jun expression, in irradiated cells (15). Therefore, we postulate that WHI-P131, when used as a single agent, improves the survival of G93A-Cu,Zn-SOD-transgenic mice by suppressing c-jun expression. In fact, overexpression of bcl-2, an antiapoptotic protein, has been shown to delay neuronal degeneration and simultaneously normalizes c-Jun expression in the motor neuron of FALS mice (29). c-Jun overexpression and phosphorylation have been shown to increase neuronal apoptosis (30–32) and inhibition of c-Jun phosphorylation by an indolocarbazole derivative has also been shown to prevent motor neuron apoptosis and injury-induced dedifferentiation *in vitro* as well as *in vivo* (33, 34).

REFERENCES

1. Mulder, D. W. (1982) *in* Human Motor Neuron Diseases (Rowland, L. P., Ed.), pp. 15–22, Raven, New York.
2. Williams, D. B., and Windebank, A. J. (1991) *Mayo Clin. Proc.* **66,** 54–82.
3. Ben Hamida, M., Hentati, F., and Ben Hamida, C. (1990) *Brain* **113,** 347–363.
4. Rosen, D. R., Siddique, T., Patternson, D., Figlewicz, D. A., Sapp, P., Hentati, A., Donalson, D., Goto, J., O'Regan, J. P., Deng, X-H., Rahmani, Z., Krizus, A., McKenna-Ysek, D., Cayabyab, A., Gaston, S. M., Berger, R., Tanzi, R. E., Haperin, J. J., Herzfeldt, B., Van den Bergh, R., Hung, W-Y., Bird, T., Deng, G., Mulder, D. W., Smyth, C., Laing, N. G., Soriano, E., Pericack-Vance, M. A., Haines, J., Rouleau, G. A., Gusella, J. S., Horvitz, H. R., and Brown, R. H., Jr. (1993) *Nature* **362,** 59–62.
5. Siddique, T., Figlewicz, D. A., Pericak-Vance, M. A, Haines, J. L., Roulean, G., Jeffers, A. J., Sapp, P., Hung, W-Y., Bebout, J., McKnna-Yasek, D., Deng, G., Horvitz, H. R., Gusella, J. F.,

Brown, R. H., Jr., Roses, A. D., *et al.* (1991) *N. Engl. J. Med.* **324,** 1381–1384.

6. Fridovich, I. (1986) *Adv. Enzymol.,* **58,** 61–97.
7. Yim, M. B., Kang, J-H., Yim, H-S., Kwak, H-S., Chock, P. B., and Stadtman, E. R. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5709–5714.
8. Wong, P. C., Pardo, C. A., Borchelt, D. R., Lee, M. K., Copeland, N. G., Jenkins, N. A., Sisodia, S. S., Cleveland, D. W., and Price, D. L. (1995) *Neuron* **14,** 1105–1116.
9. Deng, H-X., Hentati, A., Tainer, J. A., Iqbal, Z., Cayabyab, A., Hung, W-Y., Getzoff, E. D., Hu, P., Herzfeldt, B., Roos, R. P., Warner, C., Deng, G., Soriano, E., Smyth, C., Parge, H. E., Ahmed, A., Roses, A. D., and Siddique, T. (1993) *Science* **261,** 1047–1051.
10. Gurney, M. E. (1997) *J. Neurol.* **244,** S15–S20.
11. Wiedau-Pazos, M., Goto, J. J., Rabizadeh, S., Gralla, E. B., Roe, J. A., Lee, M. K., Valentine, J. S., and Bredesen, D. E. (1996) *Science* **271,** 515–518.
12. Liu, R., Narla, R. K., Kurinov, I., Li, B., and Uckun, F. M. (1999) *Radiation Res.* **151,** 133–141.
13. Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., Caliendo, J., Hentati, A., Kwon, Y. W., Deng, H.-X., Chen, W., Zhai, P., Sufit, R. L., and Siddique, T. (1994) *Science* **264,** 1772–1775.
14. Sudbeck, E. A., Liu, X. P., Narla, R. K., Mahajan, S., Ghosh, S., Mao, C., and Uckun, F. M. (1999) *Clin. Cancer Res.* **5,** 1569–1582.
15. Goodman, P. A., Niehoff, L. B., and Uckun, F. M. (1998) *J. Biol. Chem.* **273,** 17742–17748.
16. Malaviya, R., Zhu, D., Dibirdik, I, and Uckun, F. M. (1999) *J. Biol. Chem.* **274,** 27028–27038.
17. Uckun, F. M., Evans, W. E., Forsyth, C. J., Waddick, K. G., Tuel-Ahlgren, L., Chelstrom, L. M., Burkhardt, A., Bolen, J., Myers, D. E. (1995) *Science* **267,** 886–891.
18. Narla, R. K., Liu, X., Myers, D. E., and Uckun, F. M. (1998) *Clin. Cancer Res.* **4,** 1405–1414.
19. Miller, N. J., and Rice-Evans, C. A. (1996) *Redox Report* **2,** 161–171.
20. Trieu, V. N., and Uckun, F. M. (1998) *Biochem. Biophys. Res. Commun.* **247,** 277–279.
21. Trieu, V. N., and Uckun, F. M. (1999) *Biochem. Biophys. Res. Commun.* **258,** 685–688.
22. Trieu, V. N., Dong, Y., Zheng, Y., and Uckun, F. M. (1999) *Radiation Res.* **152,** 508–516.
23. Uckun, F. M., Ek, O., Liu, X.-P., and Chen, C.-L. (1999) *Clin. Cancer Res.* **5,** 2954–2962.
24. Mourelatos, Z., Gonatas, N. K., Stieber, A., Gurney, M. E., and Dal Canto, M. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5472–5477.
25. Kong, J., and Xu, Z. (1998) *J. Neuorosci.* **18,** 3241–3250.
26. Jaarsma, D., Holstege, J. C., Troost, D., Davis, M., Kennis, J., Haasdijk, E. D., and de Jong, V. J. M. B. (1996) *Neurosci. Lett.* **219,** 179–182.
27. Migheli, A., Piva, R., Atzori, C., Troost, D., and Schiffer, D. (1997) *J. Neuropath. Exp. Neurol.* **56,** 1314–1322.
28. Virgo, L., and de Belleroche, J. (1995) *Brain Res.* **676,** 196–204.
29. Kostic, V., Jackson-Lewis, V., de Bilbao, F., Dubois-Dauphin, M., and Przedborski, S. (1997) *Science* **277,** 559–562.
30. Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R., and Johnson, E. M. Jr. (1994) *J. Cell. Biol.* **127,** 1717–1727.
31. Eilers, A., Whitfield, J., Babij, C., Rubin, L. L., and Ham, J. (1998) *J. Neurosci.* **18,** 1713–1724.
32. Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L. L., and Ham, J. (1998) *J. Neurosci.* **18,** 751–762.
33. Glicksman, M. A., Chiu, A. Y., Dionne, C. A., Harty, M., Kaneko, M., Murakata, C., Oppenheim, R. W., Prevettte, D., Sengelaub, D. R., Vaught, J. L., and Neff, N. T. (1998) *J. Neurobiol.* **35,** 361–370.
34. Maroney, A. C., Glicksman, M. A., Basma, A. N., Walton, K. M., Knight, E. Jr., Murphy, C. A., Barlett, B. A., Finn, J. P., Angeles, T., Matsuda, Y., Neff, N. T., and Dionne, C. A. (1998) *J. Neurosci.* **18,** 104–111.