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Niprisan (Nix-0699) improves the survival rates of transgenic sickle cell mice under acute severe hypoxic conditions

EFEMWONKIEKIE W. IYAMU,[1] ERNEST A. TURNER[2] AND TOSHIO ASAKURA[1,3] [1]*Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA,* [2]*ProHealth Medical Center, Franklin, TN, and* [3]*Department of Pediatrics and Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA*

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Summary. The substitution of glutamic acid by valine at the sixth position of the beta-globins of haemoglobin S (Hb S) causes a drastic reduction in the solubility of the deoxy form of Hb S. Under hypoxic conditions, deoxy-Hb S molecules polymerize inside the cells, forming rigid, sickled cells. We studied the effect of Niprisan (Nix-0699), a naturally occurring antisickling agent, on the survival of transgenic (Tg) sickle mice under severe acute hypoxic conditions (60 min). Before hypoxia exposure, the mice were treated by gavage once daily for 7 d with 0 mg/kg ($n = 10$), 10 mg/kg ($n = 5$), 50 mg/kg ($n = 5$), 300 mg/kg ($n = 4$) or 500 mg/kg ($n = 5$) of Nix-0699. The mean survival times of the untreated and treated mice were 10, 25, 39, 55 or 60 min respectively. The percentage of sickled cells in the venous blood of the treated mice was lower than that in control mice and was dose dependent. Histological examination of the lungs of the control mice showed entrapment of massive numbers of sickled cells in the alveolar capillaries, although the degree of such entrapment decreased with the increased dose of Nix-0699. Nix-0699 may be a promising option for the treatment and management of patients with sickle cell disease.

Keywords: sickle cell disease, antisickling agent, hypoxia, transgenic sickle mice, P_{50}.

Sickle cell disease (SCD) is a hereditary blood disorder caused by a single amino acid substitution (Glu → Val) at the sixth position of the β-globin chains of haemoglobin. This single amino acid substitution causes a significant reduction in the solubility of the deoxy form of sickle haemoglobin (deoxy-Hb S), causing polymer formation inside the red blood cells (RBCs). Rigid sickled cells occlude capillaries, causing tissue and organ damage. In an attempt to find agents that inhibit the polymerization of deoxy-Hb S, oxygen, carbon monoxide and sodium nitrite were used to reduce the amount of deoxy-Hb S (Beutler & Mikus, 1961; Laszlo *et al*, 1969; Beutler, 1975). However, these compounds did not give the much-needed beneficial effects to patients based on a reduction in the number and severity of painful events as the criteria for successful treatment (Ballas, 1994). A great number of other agents were also tested to find drugs that shift the oxygen dissociation equilibrium curve towards the left, increase the solubility of deoxy-Hb S, inhibit or delay the polymerization of deoxy-Hb S, induce the production of Hb F and inhibit the adhesion of RBCs to endothelial cells (Abraham & Perutz, 1986; Al-khatti *et al*, 1988; Ley, 1991; Perrine *et al*, 1993). Among the various potential antisickling agents that have been tested to date, only hydroxyurea (HU) is used for the treatment of SCD patients (Charache *et al*, 1992; Fibach *et al*, 1993). Although HU is effective in many patients, not all patients respond to it. In addition, some patients suffer adverse effects including myelosuppression. In a multicentre clinical trial, only about 44% of patients on HU therapy had an annual reduction in the rate of painful episodes, and some patients did not respond to HU therapy (Charache *et al*, 1995).

Niprisan (Nix-0699) is a plant extract product (Wambebe *et al*, 2001a). The herbal medicine has been used locally in Nigeria to prevent the painful crises that are associated with SCD. Studies *in vitro* showed that Nix-0699 possesses a very potent antisickling effect without causing either haemolysis or methaemoglobin formation (Iyamu *et al*, 2002). As reported previously (Iyamu *et al*, 2002), the results of the *in vitro* studies on the mechanism of action of Nix-0699 suggested that this herbal extract revealed multiple effects that are beneficial for the treatment of SCD. It contains a

Correspondence: Dr Efemwonkiekie W. Iyamu, Division of Hematology, The Children's Hospital of Philadelphia, Abramson Pediatric Research Center, 3516 Civic Center Blvd, Room 314 I, Philadelphia, PA 19104-4399, USA. E-mail: iyamu@email.chop.edu

compound or compounds that increase the solubility of deoxy-Hb S, prolong the delay time before deoxy-Hb S polymerization and shift the oxygen equilibrium curve of Hb S towards the left, thereby increasing the oxygen affinity of Hb S. Clinical trials of Nix-0699 have been conducted at two different institutions in Nigeria, and the results showed that the drug significantly reduced the number of vaso-occlusive events in SCD patients. This observation was accompanied by an increase in body weight in 60% of the total number of patients enrolled in the clinical trials (Wambebe *et al*, 2001a,b).

To our knowledge, there has been no documented study using a transgenic (Tg) animal model to establish the therapeutic efficacy of Nix-0699 for SCD. Tg mice models have been used to investigate the pathogenesis of, and assess therapeutic interventions in, SCD (Trudel *et al*, 1994; De Franceschi *et al*, 1999). In our present study, we investigated the tolerance of Tg sickle mice to acute, severe hypoxic stress after the administration of various doses of Nix-0699. We also examined *in vivo* erythrocyte sickling, P_{50} of erythrocytes, met-haemoglobin/membrane-associated denatured Hb (MADH) levels and tissue histology before and after hypoxic stress with or without pretreatment with Nix-0699.

MATERIALS AND METHODS

Transgenic sickle mice. The Tg sickle mice used in this study produced 100% human β^s-globin and approximately equal amounts of mouse and human α-globins. They were derived from the original founder animals that had been produced by co-injection of DNA constructs of hα- and hβ^s-globin genes ligated to the human β-globin locus control region into the pronuclei of fertilized mouse eggs (Ryan et al, 1990). A colony of 42 Tg sickle mice was propagated from a breeding pair by crossing transgenic animals with C57B1/6 (Hb β^{single} haplotype) mice or with mice heterozygous for mouse β-thalassaemia (Hbβ^{dt}) on a C57B1/6 background (Jackson Laboratory, Bar Harbor, ME, USA). The α and β chain components were assessed by high-performance liquid chromatography (HPLC) or electrophoresis. All mice (body weight < 34 g) were housed in a viral-free environment in standard approved chambers (≤ five mice/cage). Food and water were provided ad libitum, and no significant changes in body weight were observed during the treatment period.

Although these mice are relatively healthy under normoxic conditions, they develop acute, sickling-dependent pulmonary sequestration upon exposure to hypoxic conditions. In this study, we used 5% oxygen (5% O_2/95% N_2) because, upon exposure of these mice to 5% O_2, they exclusively develop pulmonary sequestration. All procedures involving the use of mice were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia.

Nix-0699 and other reagents. The formulated extract (Nix-0699) was obtained from the National Institutes for Pharmaceutical Research and Development, Abuja, Nigeria. A stock solution of the drug (500 mg/ml) was prepared by dissolving the dry powder (w/v) of Nix-0699 in water. Appropriate doses of the drug were prepared by diluting the stock solution in the appropriate amount of water, and were administered to Tg sickle mice by gavage. We periodically measured the absorption spectrum and the antisickling effect of the stock solution after appropriate dilution, and confirmed that both the absorption spectrum and the *in vitro* antisickling effect of the stock solution were maintained over the period of this study. All other chemicals were purchased from Sigma Chemical (St Louis, MO, USA) and used without further purification.

Hypoxia studies. Nineteen Tg sickle mice were divided into four groups, and treated with various doses of Nix-0699 once daily for 7 d. The animals were then exposed to hypoxia after 7 d of treatment. The survival time of Tg sickle or wild-type mice under the hypoxic conditions was recorded up to 60 min or until death. A gas tank containing a mixture of 5% O_2/95% N_2 was used with a continuous controlled flow of the gas. Four groups of Tg sickle mice were administered by gavage 10 ($n = 5$), 50 ($n = 5$), 300 ($n = 4$) or 500 mg/kg/d ($n = 5$) Nix-0699 for 7 d before the hypoxia experiment. An equivalent volume of water was administered by gavage to the control mice (Tg sickle mice, $n = 10$; and wild-type mice, $n = 6$) for 7 d. During the hypoxia exposure, the gas flow was carefully monitored. In the hypoxia experiment, the Tg sickle mice were exposed to hypoxia for up to 60 min. Any surviving mice at 60 min were euthanized by cervical dislocation under anaesthesia. In all cases, after the mouse died, an aliquot of whole blood cells was collected by cardiac puncture for the determination of haematological parameters and the mouse was immediately dissected. The heart, lungs, brain, liver, spleen and kidneys were fixed in 10% phosphate-buffered formaldehyde. Tissue samples were embedded in paraffin according to standard methods. Sections were cut and stained by a haematoxylin–eosin (HE) solution for light microscopy. The alveolar surface area of the lungs was calculated using the following equation: $Y = 1{\cdot}87M\hat{}0{\cdot}888$, where Y is the alveolar surface area (m^2) and M is the mouse body weight in kg (Lechner, 1978; Peters, 1986).

Erythrocyte sickling. To determine the percentage of sickled cells, blood samples were obtained from the tail vein before, during and after hypoxia exposure. Aliquots (5–10 μl) of blood were collected at specific time intervals during the hypoxia exposure and fixed with 2% glutaraldehyde solution without exposure to air. In order to determine the time- and dose-dependent antisickling effects of Nix-0699, an aliquot of blood was collected from each group of Tg sickle mice ($n \geq 2$) that had been treated with Nix-0699 for 7 d, followed by incubation in Hemox buffer for 5 h under low oxygen pressure (4% O_2). The percentage of sickle or discoidal erythrocytes was compared between pre- and post-hypoxic samples with and without Nix-0699 treatment. Morphological analysis of erythrocytes (at least 200 cells on four or five optical fields) was performed for all samples using a computer-assisted image analysis system (Horiuchi *et al*, 1990) as described further below. The percentage of elongated sickled cells and percentage of total sickled cells (elongated + star-shaped sickled cells) were each plotted against time.

Image analysis. RBC morphology of control and drug-treated mice was evaluated by a computer-assisted image analysis system (Horiuchi *et al*, 1990). The RBCs were analysed by measuring various parameters including the area, perimeter and the lengths of the long (a) and short (b) axes of each red blood cell. Circular shape factor (CSF) represents the degree of deviation from the circular shape (elongated + star-shaped cells), whereas elliptical shape factor (ESF) represents the degree of elongation (elongated cells). CSF and ESF were calculated by the following equations:

$$\mathrm{CSF} = 4\pi \times \mathrm{area}/(\mathrm{perimeter})^2$$

$$\mathrm{ESF} = \mathrm{b/a}$$

The CSF and ESF are useful for distinguishing deformed from normal cells and for quantifying the degree of deformation. The use of a computer-assisted image analysis system enabled an objective, accurate and rapid evaluation of the morphology of a large number of cells.

Oxygen dissociation curve. In order to determine whether the prolongation of the survival time of Tg sickle mice by Nix-0699 under hypoxia is attributed to its effect on the oxygen-binding property of Hb, the oxygen dissociation curve of the erythrocyte suspensions was determined with a Hemox analyser (TCS Scientific, New Hope, PA, USA) (Asakura, 1979; Festa & Asakura, 1979). Briefly, blood collected from mice was suspended in Hemox buffer, pH 7·4, followed by measurement of the oxygen dissociation curve with a Hemox analyser by flushing with nitrogen. The P_{50} was determined as the partial pressure of oxygen at which 50% of the haemoglobin molecules were bound with oxygen.

Formation of met-haemoglobin (met-Hb) and membrane-associated denatured Hb (MADH). Blood samples collected in a microcapillary tube were used to determine the met-haemoglobin and MADH levels before and after drug treatment. Aliquots of cells (20 μl) were collected, washed three times with physiological saline and lysed with haemolysing solution, and the absorption spectrum of the supernatant was determined with a Hitachi U-3410 recording spectrophotometer (Tokyo, Japan). The concentration of met-Hb in the supernatant was determined by measuring the absorbance at 630 nm. The formation of MADH was determined as described previously (Asakura *et al*, 1977).

Effects of Nix-0699 on haematological parameters and erythrocyte haemolysis. As hydration of red blood cells reduces deoxy-Hb S polymerization, we investigated whether Nix-0699 reduced RBC size. Aliquots (10 μl) of blood were collected, and the complete blood cell count (CBC) was determined with a Hemavet counter (CDC Technologies, Oxford, CT, USA). To determine the extent of haemolysis, the absorbance of the supernatant was measured at 415 nm. The degree of haemolysis was expressed as a percentage of the total Hb level.

Statistical analyses. Data are expressed as mean ± standard error of the mean (SEM). Except where indicated, the Kaplan–Meier method with log-rank test was used to compare the 60-min survival rate between groups. The Student's *t*-test was used for comparison of the P_{50} and percentage of sickling between the drug-treated groups and the control group. The level of significance was set at $P < 0{\cdot}05$.

RESULTS

Effect of Nix-0699 on the percentage of sickled cells in the peripheral blood of Tg sickle mice under hypoxia

As shown in Fig 1, pretreatment of Tg sickle mice with Nix-0699 reduced the percentage of sickled cells in a dose-dependent manner. Upon acute hypoxic (5% O_2) exposure, the percentage of sickled cells in the blood of the control mice increased sharply. However, in mice that had been pretreated with 500 mg/kg Nix-0699, the original discoidal shape was retained in nearly all cells during hypoxia exposure. In order to determine whether the antisickling effect of Nix-0699 is maintained after blood collection, blood samples were collected from control and drug-treated mice that had been kept under normoxic conditions and then incubated in Hemox buffer for 5 h under a more severe hypoxic condition (4% O_2). Figure 2 shows the time course of the degree of sickling of SS cells *in vitro* in blood samples from Tg sickle mice that had been treated with different doses of Nix-0699. In the absence of Nix-0699 administration, ≈70% of the cells underwent sickling at 5 h. In contrast, upon incubation of blood samples obtained from mice treated with 10, 50, 300 or 500 mg/kg Nix-0699 for 5 h, the percentage of sickled cells was 35%, 20%, 10% or 8·5% respectively. Furthermore, the exposure of wild-type mice to hypoxia did not show significantly improved survival compared with that of the control Tg sickle mice, as the average survival time of wild-type mice was 12 min (results not shown).

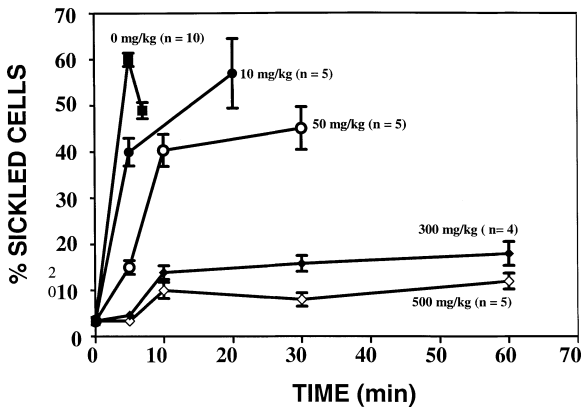

Fig 1. Degree of SS cell sickling in Tg sickle mice over time, upon hypoxic exposure. Five groups of Tg sickle mice were treated with 0 ($n = 10$), 10 ($n = 5$), 50 ($n = 5$), 300 ($n = 4$) or 500 ($n = 5$) mg/kg/d Nix-0699 for 7 d. Thereafter, the mice were exposed to hypoxia for 60 min. During hypoxic exposure, aliquots of blood were collected from the tail vein under venous oxygen pressure, and the cells were fixed in 2% glutaraldehyde solution without exposure to air. The percentage of sickled cells was determined by the computer-assisted image analysis system as described in Materials and Methods.

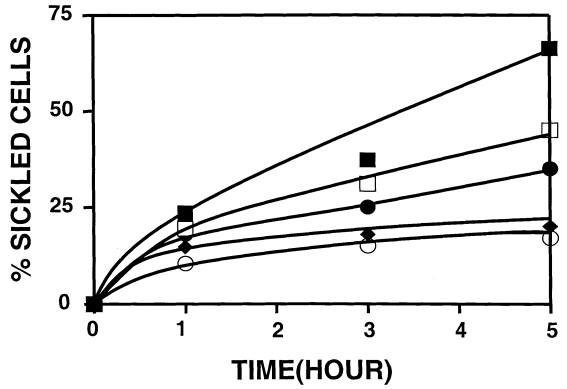

Fig 2. Time course of the effect of Nix-0699 on sickling of SS cells. Four groups of Tg sickle mice were administered 10 (open squares), 50 (closed circles), 300 (closed diamonds) or 500 (open circles) mg/kg/d Nix-0699 [or an equivalent volume of water for the control (closed squares)] by gavage for 7 d. At the end of the treatment period (when the mice were under normoxic conditions), a blood sample was collected and incubated under more hypoxic conditions (4% O_2) for 5 h. At the indicated time intervals, an aliquot of the blood sample was collected without exposure to air. The percentage of sickled cells was determined by the computer-assisted image analysis system by counting at least 200 cells from five optical fields. Data obtained from a typical experiment performed in duplicate using blood samples from at least two Tg mice per dose are presented.

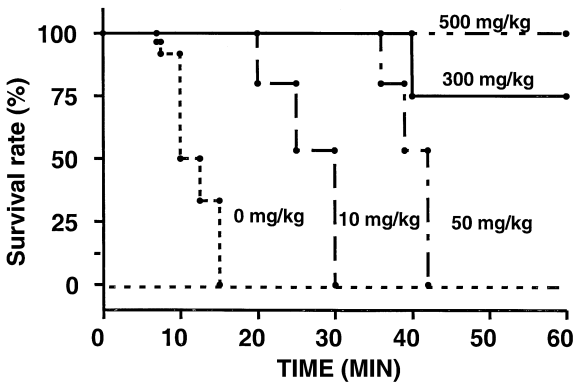

Fig 3. Kaplan–Meier survival curves during 60 min of hypoxic exposure of transgenic sickle mice. Five groups of Tg sickle mice were treated with 0 (group 1, $n = 10$), 10 (group 2, $n = 5$), 50 (group 3, $n = 5$), 300 (group 4, $n = 4$) or 500 (group 5, $n = 5$) mg/kg/d of Nix-0699 for 7 d. Thereafter, the mice were exposed to hypoxia for 60 min, and the survival time of these mice was then determined as described in Materials and Methods. The survival time of the drug-treated groups was significantly longer than that of the Tg sickle control mice: group 5 ($P < 0{\cdot}0001$), group 4 ($P < 0{\cdot}0001$), group 3 ($P < 0{\cdot}001$), group 2 ($P < 0{\cdot}01$).

Effect of Nix-0699 on the survival time of Tg sickle mice

Figure 3 shows the Kaplan–Meier survival curves of Tg sickle mice upon exposure to hypoxia after treatment with various amounts of Nix-0699. Upon treatment of the animals with 300 or 500 mg/kg Nix-0699, almost all of the Tg sickle mice (8/9) survived for the entire 60-min study period under severe acute hypoxia (5% O_2). However, one of the mice in the 300 mg/kg Nix-0699-treated group died at 45 min. In the 50 mg/kg and 10 mg/kg Nix-0699-treated groups, the mean survival time was 39 and 25 min, respectively, whereas all of the control mice (10/10) died at a mean survival time of 10 min.

Histopathological examination of the organs

Histopathological staining of various organs obtained after euthanasia of the control and drug-treated mice was performed. Histological examination showed entrapment of massive numbers of sickled cells in the lungs of untreated mice and in the lungs of mice treated with lower doses (≤ 50 mg/kg) of Nix-0699 that died before the full experimental period of 60 min. However, there were no significant pathological alterations in the other organs of both treated and untreated mice (results not shown), suggesting that the cause of death of these mice was pulmonary sequestration by rigid sickled cells. As shown in Fig 4A, the pulmonary capillaries of the control Tg sickle mice were packed with RBCs and contained seemingly sickled cells, thereby reducing the alveolar surface area from $5{\cdot}9 \times 10^{-2} \pm 0{\cdot}002$ m^2 (value under normoxic conditions; $n = 4$) to $1{\cdot}99 \times 10^{-2} \pm 0{\cdot}001$ m^2 ($n = 10$). In contrast, the alveolar surface area of 500 mg/kg Nix-0699-treated mice was within the normal range ($5{\cdot}7 \times 10^{-2} \pm 0{\cdot}003$ m^2), and retained RBCs were rarely seen in the alveolar capillaries of the lungs of this group of treated mice (Fig 4B). In the wild-type mice, no apparent engorgement of RBCs was observed in the lungs or in the other organs studied, indicating that the cause of death of these mice may not be related to entrapment of RBCs in the alveolar capillaries. Further, the difference in values of the alveolar surface area was not statistically significant between the group of mice treated with lower doses of Nix-0699 that died before the full experimental period of 60 min and the control group (results not shown).

Effect of Nix-0699 on haematological parameters

In order to study the potential adverse effects of Nix-0699, aliquots of whole blood cells were collected after death, and the degree of haemolysis, formation of met-Hb, change in the size of RBCs, the level of MADH and the oxygen-binding affinity of Hb were determined. The results indicated that Nix-0699 did not cause any of the above-mentioned adverse effects. Further analysis of the mean cell volume (MCV), mean cell Hb concentration (MCHC) and red cell distribution width (RDW) before and after hypoxia exposure indicated no significant differences in these haematological parameters between the drug-treated and control mice. The results of the effect of Nix-0699 on the haematological parameters in Tg sickle mice after 7-d treatment with Nix-0699 are shown in Table I. There was also no apparent difference in the oxygen-binding affinity between the drug-treated mice and the control group, when the blood samples obtained from mice were subjected to oxygen equilibrium curve (OEC) analysis. The fact that we did not observe any difference in the P_{50} values may be attributed to the dilution of the drug because

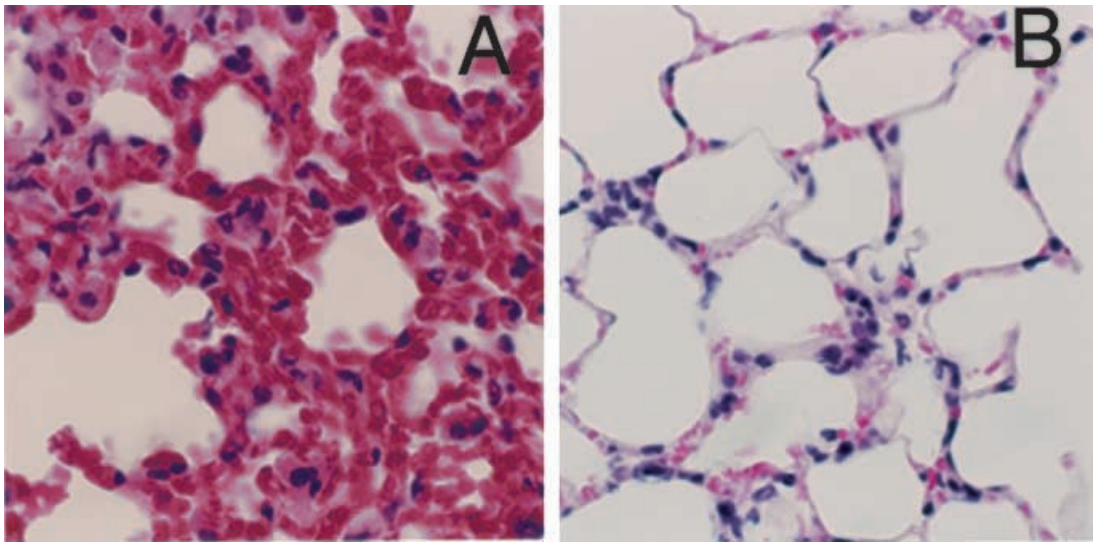

Fig 4. Histopathological analysis of the lungs of control and Nix-0699-treated Tg sickle mice. Control and Nix-0699-treated Tg sickle mice were exposed to hypoxia. After death, or after the full experimental period of 60 min, mice were sacrificed, and the organs were immediately collected as described in Materials and Methods. The alveolocapillary walls of control mice (A) were heavily engorged with sickled cells, resulting in greatly reduced alveolar space. In contrast, the lungs of Tg sickle mice that had been pretreated with 500 mg/kg Nix-0699 (B) were remarkably normal compared with the control (alveolar surface area; $P < 0{\cdot}001$) (haematoxylin and eosin, original magnification $\times 100$).

Table I. Effect of Nix-0699 on haematological parameters in Tg sickle mice*.

	Dose of Nix-0699 (mg/kg)				
Parameter	0 ($n = 10$)	10 ($n = 5$)	50 ($n = 5$)	300 ($n = 4$)	500 ($n = 5$)
HCT (%)	53·1 ± 1·6	54·3 ± 0·7	54·6 ± 1·1	52·3 ± 0·9	53·4 ± 1·6
MCV (fl)	45·4 ± 0·8	45·6 ± 1·3	44·6 ± 0·7	43·9 ± 1·2	44·9 ± 0·9
MCH (pg)	26·4 ± 0·8	25·4 ± 1·0	25·2 ± 0·9	26·0 ± 1·2	26·2 ± 0·7
MCHC (g/dl)	36·1 ± 1·0	35·3 ± 1·3	34·9 ± 1·2	35·6 ± 1·8	37·0 ± 0·7
Hb (g/dl)	15·3 ± 1·1	14·8 ± 0·6	15·2 ± 0·8	15·0 ± 1·1	14·7 ± 1·2
RDW (%)	35·3 ± 0·9	35·7 ± 0·1	34·8 ± 1·4	34·7 ± 1·3	34·7 ± 1·7

Tg sickle mice were treated with different doses of Nix-0699 for 7 d. At the end of the treatment period, and after hypoxia and death, a blood sample was collected, and the haematological parameters were determined as described in Materials and Methods.

HCT, haematocrit; MCH, mean cell Hb.

*Data are presented as the mean ± standard deviation.

very small samples are suspended in a large volume of Hemox buffer before analysis with the Hemox analyser.

DISCUSSION

Our present study demonstrated the ability of Nix-0699 to improve the survival of Tg sickle mice that were subjected to severe hypoxic stress. The cause of this survival advantage seems to be the direct interaction of Nix-0699 with Hb S. Our *in vitro* studies showed that Nix-0699 not only prolonged the delay time before deoxy-Hb S polymerization, but also inhibited sickling of SS cells (Iyamu *et al*, 2002). Further, our *in vitro* results also showed that Nix-0699 slightly reduced the P_{50} value. However, we did not observe any significant differences in the P_{50} values between the drug-treated and control mice in our *in vivo* study. Small animals such as mice have a higher metabolic rate than humans, and their haemoglobin has a low affinity for oxygen (40–44 mmHg) to facilitate its unloading to tissues (Schmidt-Nielsen, 1984; Uchida *et al*, 1998). This right-shifted OEC in wild-type mice in comparison with the OEC of humans decreases the efficiency of oxygen transport under severe hypoxic conditions (5% O_2). This problem was lessened in our Tg sickle mice because the P_{50} value of the blood of 100% $h\beta^s$-globin Tg mice is 27 ± 2 mmHg, owing to the presence of mouse/human hybrid haemoglobin (unpublished

observations; Reilly *et al*, 1994). Because of the similarity in the P_{50} value of these Tg mice to that of human patients (Hb SS = 32 ± 2 mmHg), one would expect to observe similar oxygen-binding characteristics of the haemoglobin of Tg sickle mice to those of human Hb S and Hb A (P_{50}, 26·5 ± 0·8 mmHg). The lack of a difference in the oxygen affinity of the haemoglobin of Nix-0699-treated mice in comparison with that in the control mice may result from as yet unknown binding kinetics of Nix-0699 and Hb S. As has already been documented in the binding of some ligands with Hb (Parkhurst, 1979), it is feasible that the oxygen dissociation curve may not reveal certain final details (stepwise binding) of the overall reaction between Hb S and Nix-0699, as it is also possible that the reaction of Hb S with Nix-0699 could be much faster during transit in the microvasculature than would be observed *in vitro*. Another possible explanation for the observed similarity in the P_{50} values between the treated and untreated Tg sickle mice may be attributed to the significant dilution of the drug concentration when the OEC was determined by the Hemox analyser. A marked decrease in the percentage of sickled cells in the blood of treated mice supports our assumption that the affinity of Hb S was increased in the blood of Nix-0699-treated mice. Additional studies are therefore needed to understand better these apparent differences.

Sickling of SS cells

In this study, we demonstrated that, upon exposure of untreated Tg sickle mice to hypoxia, the percentage of sickled cells in the venous blood increased to as much as 60% and the mice died within 10 min. The characteristic morphology of SS cells in these mice under hypoxia has been well documented (Reilly *et al*, 1994). When these Tg mice were exposed to hypoxia (5–12% O_2), sickled cells with blunt edges appeared. These sickled cells are partially oxygenated sickled cells (POSCs) and contain a small amount of fragmented deoxy-Hb S fibres (Asakura *et al*, 1994). Immediately before death, however, sickled cells with pointed edges appear in the venous blood. These sickled cells contain dense and well-aligned deoxy-Hb S fibres and are rigid. Histopathological studies of various organs showed that sickled cells were trapped in the alveolar capillaries of the lungs. As shown in this study, oral administration of Nix-0699 prevented the hypoxia-induced pulmonary sequestration.

Mechanism of the antisickling effect of Nix-0699

In view of our recent *in vitro* findings (Iyamu *et al*, 2002), coupled with the current study, we believe that Nix-0699 specifically interacts with Hb S, as even a low dose of the drug (10 mg/kg) significantly reduced the percentage of sickled cells in Tg sickle mice. Because there was no increase in the RBC size, the *in vivo* antisickling effect of Nix-0699 cannot be attributed to a reduction in MCHC. It is therefore plausible to conclude that the interaction of Nix-0699 with Hb S prevents the formation of Hb S polymers within the erythrocytes, prevents cell sickling and improves the survival of Tg sickle mice under hypoxia. However, the precise binding site of Nix-0699 with the Hb S molecule remains uncertain. In this regard, X-ray crystallography may help to elucidate the interaction site of the active agent with the Hb S molecule. Furthermore, although work on the structural elucidation of the active compound(s) in Nix-0699 has not been performed, phytochemical screening for possible chemical components of Nix-0699 by Wambebe and co-workers revealed that this natural product contains alkaloids, saponins, flavonoids, glycosides and trace amounts of anthraquinones (Dr C. Wambebe, personal communications). Interestingly, chalcone and nicotine (an alkaloid) have been observed directly to inhibit fibril formation in amyloid disease (Lin *et al*, 2001; Ono *et al*, 2002). It was suggested that these fibril inhibitors could find increased application in other molecular polymerization diseases such as sickle cell anaemia. Also, a well-known local anaesthetic, procain (an alkaloid derivative), has long been implicated as a potential antisickling agent (Palek *et al*, 1977). The identification of the presence of flavonoids in Nix-0699 could well explain the low toxicity of this agent in laboratory animals and humans, as flavonoids have been shown to have potent antioxidant properties (Silva *et al*, 2002). Taken together, the glycosides, alkaloids, flavonoids and anthraquinones (in trace amounts) in Nix-0699 could act as adjuvant substances that enhance the activity of the components actually responsible for the antisickling effect. This synergy may involve protection of the active substance from degradation by enzymes, or it may facilitate transport across barriers such as the cell membrane or provide signals (as some known signalling molecules are glycoside derivatives) to the host's cells that result in higher efficacy of Nix-0699. It is important to study the structure of the active compound(s) in order to establish the structure–activity properties of Nix-0699.

Although the entrapment of sickled erythrocytes in the microvasculature may have been the cause of death in the control mice and those mice treated with lower doses of the drug (≤ 50 mg/kg), it is also likely that, in the survivors, reductions in platelet and erythrocyte adhesion to the endothelium played an important role (Hebbel & Mohandas, 1994; Kaul *et al*, 1995). In this regard, we propose that direct interaction of Nix-0699 with Hb S (based on our *in vitro* and *in vivo* studies) may not be the sole mechanism of action of this agent. Multiple mechanisms and sites may be involved in the beneficial effects of orally administered Nix-0699. Further studies are therefore needed to clarify the mechanism(s) of action of Nix-0699 through which the sickling of SS cells under hypoxia is inhibited, leading to the improved survival of hypoxia-exposed Tg sickle mice.

It should be noted that many antisickling agents that inhibited sickling by interacting directly with Hb S *in vitro* have been abandoned because of the unacceptable dose that was required to inhibit the sickling of SS cells *in vivo* (Orringer *et al*, 1994). A recent clinical study by Wambebe *et al* (2001a) indicated that Nix-0699 is tolerable, and daily oral administration of 12 mg/kg Nix-0699 to SCD patients for 6 months reduced the frequency of painful episodes and hospital admissions in comparison with those in SCD patients who received a placebo ($P < 0·05$). As to the

toxicity of Nix-0699, oral administration of Nix-0699 to SCD patients for 12 months showed no acute toxicity to the liver and kidneys as assessed by the activities of the liver enzymes and serum creatinine level respectively (Wambebe *et al*, 2001b). However, a more extensive multicentre clinical study is required to determine the interindividual variability and efficacy of Nix-0699 in a large group of patients. It would also be important to study the beneficial effects of Nix-0699 in Tg sickle mice that are kept under normoxic and/or chronic hypoxic conditions. Further, a long-term toxicity profile of Nix-0699 in a large group of Tg sickle mice would be worth investigating.

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