

MEDICAMENT FOR AUGMENTING FETAL HEMOGLOBIN

The present invention is directed to inhibiting or reversing the switching in in vivo or in vitro from production of γ to β -globin, thus
5 augmenting the production of fetal hemoglobin. In particular, the present invention envisages controlling the fetal hemoglobin switch by introducing into the bloodstream of the fetus or child or into erythroid cultures a compound of the
10 formula I, described hereinbelow.

Normal adult hemoglobin comprises four globin proteins, two of which are α proteins and two of which are β proteins. Diseases known as sickle cell
15 syndromes are associated with disorders in the β chain of the hemoglobin. However, in mammals, and particularly in humans, during fetal development, the fetus produces a fetal hemoglobin which comprises, instead of β -globin proteins, two γ -globin proteins.
20 At some point during fetal development or infancy, depending on the particular species and individual, there is a so-called globin switch wherein the erythrocytes in the fetus switch from making

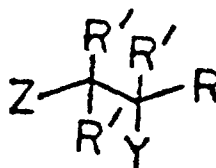
predominantly γ -globin to making predominantly β -globin. It has been observed, however, that increased levels of fetal hemoglobin (derived from γ -globin) ameliorate the severity of sickling disorders. It has also been observed that subjects heterozygous for hereditary persistence of fetal hemoglobin syndromes (HPFH) and sickling hemoglobin (HbS) are clinically asymptomatic of sickle cell anemia. Also, infants with sickle cell anemia do not usually develop the symptoms of the disease until approximately four months of age when their fetal hemoglobin levels decrease. These observations suggest that a method for increasing the levels of fetal hemoglobin would be beneficial to patients with sickle cell syndromes.

It is thus an object of the present invention to enable inhibiting or reversing the γ to β -globin switch in a fetus or infant to maintain increased fetal hemoglobin levels in those individuals with sickle cell syndromes.

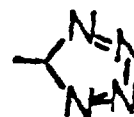
Since inhibition of the γ to β -globin gene switch has been observed in infants of diabetic mothers (IDM), it appears that disturbances in normal metabolites in the bloodstream might disrupt a normally timed and fixed gene-switch. Two metabolites, insulin and butyric acid, have been observed as being elevated in infants of diabetic mothers. Some investigators have shown that factors in serum can effect the γ to β -globin switching, particularly around the time the switch occurs. There has been, however, heretofore no development of a non-toxic therapeutic method or natural agent to increase fetal hemoglobin in subjects with sickle cell disease.

It is thus another object of the present invention to provide an agent for maintaining a high level of γ chain synthesis (thereby maintaining high fetal hemoglobin levels), without toxicity and long term side effects, by using a physiologic factor rather than a chemotherapeutic agent.

The present invention enables, by the manufacture of of an appropriate medicament, a method for ameliorating β -globin disorders in mammals comprising the step of periodically introducing into the bloodstream of said mammal during its gestation period and/or early infancy and thereafter, an amount of a compound of the formula I:



wherein R is $-\text{CO}_2\text{R}_1$, $-\text{SO}_2\text{R}_1$, $-\text{SO}_3\text{R}_1$, or



and R_1 is H, M, or alkyl 1-4 carbons and M is a cation; Z is $-\text{CH}_3$, $-\text{X}$, or $-\text{CX}_3$; and X is Cl, F, Br, or I; Y is H, $-\text{NH}_2$, $-\text{NH}_3^+$, or F; and R' is H or F; or mixtures of these, sufficient to inhibit or reverse fetal γ to β -globin switching. The medicament according to the present invention is particularly useful for ameliorating in humans the clinical effects of sickle cell anemia.

Other aspects of the invention are:-

- 5 (1) A method of monitoring the clinical use of a medicament manufactured in the use according to the invention , in which method γ -globin to β -globin switching is monitored by adding to an in vitro cell culture from the mammal an effective amount of a compound as defined above, or mixture thereof, to determine if additional treatment is needed.
- 10 (2) A method for diagnosing disease in a mammal comprising the steps of culturing a biological sample from the mammal with an effective amount of a compound as defined above, or mixture thereof, and determining
- 15 the change in β -globin synthesis of the sample as compared to a control sample cultured in the absence of such compound.

20 The present invention permits a method for ameliorating the clinical effects of β -globin disorders, particularly the disorder of sickle cell anemia and β -thalassemias. The present invention is advantageous in that the compounds utilized are physiologic factors, i.e., natural metabolites found

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in the bloodstream of mammals and are thus, when introduced into the bloodstream, not likely to have toxic or undesirable long term side effects.

5 In accordance with the present invention, a compound of the formula I or a mixture of two or more thereof, are introduced into the bloodstream of the subject shown to or suspected of having a β -globin disorder, such as sickle cell anemia or β -thalassemias. The foregoing may be administered as their non-toxic
10 salts, such as their sodium salts, ammonium salts, potassium salts, and the like. Preferred compounds are L- β -amino-n-butyric acid, L- α -amino-n-butyric acid, DL- α -amino-n-butyric acid, sodium butyrate, β -chloro-D-alanine, 3-chloro-propionic acid, 5-(2-chloroethyl)tetrazole, heptafluoro-butyric acid,
15 α -amino propane sulfonic acid, and sodium propanesulfinate.

Methods used to introduce the compound will be any convenient method normally used to introduce
20 pharmaceuticals into the bloodstream, such as by injection or infusion, catheter, syringe, transcutaneous patch, in-dwelling depot delivery systems, and the like. Oral or parenteral administration may also be utilized.

25 The exact size of an effective dose of a compound according to the method of the present invention will depend on a number of factors including the particular recipient and the severity of condition, thus the route of administration will be ultimately
30 at the discretion of the attendant physician.

While it is possible to utilize the compounds in vivo as a raw chemical, it is preferable to present them as a pharmaceutical formulation. The formulation of

the present invention comprises a compound as previously described together with one or more acceptable carriers therefor and, optionally other therapeutic ingredients. The carriers must be
5 acceptable in the sense of being compatible with other ingredients of the formulation and not dilatory as to the recipient.

For oral administration, the formulations may be presented as discrete units such as capsules, or
10 tablets each containing a predetermined amount of the active ingredient, as a powder or granules, as a solution or suspension in an aqueous or nonaqueous liquid, as an oil in water liquid emulsion or as a water in oil liquid emulsion.

15 Formulations for parenteral administration include aqueous or nonaqueous sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended
20 recipient. The formulation may be presented in dose unit or multi-dose containers such as ampules or vials and may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier, for example water, for injections.

25 Preferred unit dosage formulations are those containing a daily dose or a unit daily subdose, or an appropriate fraction thereof.

As further application of the compounds according to the present method, they may be utilized in vitro to
30 cell cultures taken from patients to determine the potential efficacy of further treatment for the β -globin disorders, such as sickle cell anemia. The compounds may be thus used in vitro in cell cultures

from patients to determine whether further addition of one of the compounds would result in continued inhibition or reversal of the globin switch.

The frequency and dosages of administration of the
5 above compounds will depend upon when the compound is introduced, whether the subject is a fetus or an infant, the size and weight of the subject, the condition of the pregnant mother, and the like. Generally, injections beginning at a dosage as low as
10 about 12 mg/kg body weight per day during gestation, particularly prior to the thirty-second week of gestation in humans, will delay the γ to β switching. Typically, larger dosages in the range of 500-1000 mg/kg of estimated fetal weight will be useful.
15 Higher dosages may be utilized at the discretion of the physician. Since apparently the switching process is not complete in humans until approximately four months after birth, treatment may be preferentially initiated after birth up until
20 about the fourth month of infancy and continued as long as necessary to maintain enhanced fetal hemoglobin levels in the patient. However, initiation of treatment even subsequent to the fourth month may also be effective.

25 All treatment should preferably occur prior to the fourth month of infancy since the γ to β switching process is difficult to reverse. Although treatment with one of the above compounds prior to the fourth month of infancy will inhibit the γ to β -switching
30 process, treatment subsequent to that period may also achieve the desired clinical results, i.e., the amelioration of the effects of the β -globin disorder. Therefore, if the switching process is inhibited even to the extent of 10 to 20% (that is, the subject
35 makes 10 to 20% more γ -globin than would be expected

if the switch were allowed to occur), this may be sufficient to ameliorate the symptoms of the disease.

The method according to the present invention may be utilized in vivo, or in vitro as a diagnostic test.

5 For the in vitro test erythroid cultures, such as that obtained from cord blood mononuclear cells in Iscove's Modified Dulbecco's Medium with 0.9% methylcellulose, may be used as described by Stamatoyannopoulos et al., Blood, 54, 440-450 (1979)
10 and Friedman et al., J. Clin. Invest., 75, 1359-1368 (1985). The cultures may be formed with and without the addition of various metabolites whose concentrations are observed to increase in infants of
15 diabetic mothers (IDM). To show the effect of such factors, insulin (0.1-100 ng/ml), Insulin Growth Factor I (4-10 ng/ml), Isoleucine (4-10 mcg/ml), and butyric acid (0.01-10 mM) were added to the cultures. Some cultures were performed in 95% nitrogen to
20 simulate a hypoxic intrauterine environment, and IDM plasma was used instead of fetal calf serum in some cultures. Colonies were harvested and incubated with ³H-leucine under the same culture conditions for the last 24 hours. Globin production was analyzed by
25 electrophoresis on Triton-urea gels, autoradiography, and densitometry and by radioligand immunoassay of the total and fetal hemoglobin in pg per Bfu-e-derived cell was performed as described in Stamatoyannopoulos et al. and Friedman et al., ibid.
30 In 10 Cfu-e (colony-forming unit-erythroid) cultures from five normal infants and five term IDM mean β -globin synthesis (expressed as percent of total non- α synthesis), was 42.3% β -globin and 17.3% respectively. This is equivalent to the β -globin levels produced in infants' reticulocytes. This
35 difference in percent β -globin synthesis between the IDM and normal infant Cfu-e was ($p < .001$, Student's

t-test) and indicates that these relatively late erythroid progenitors are committed to a pattern of globin production in the fetal environment. The percentage of β -globin synthesized by Bfu-e (burst-forming unit-erythroid) from normal term infants did not differ statistically from the percentage produced by their Cfue. However, Bfu-e from IDM produced a significantly higher percentage of the β -globin than did the infants Cfue and reticulocytes ($p < .05$).

This difference indicates that switching to increased β -globin production occurred once the more primitive progenitors were removed from the abnormal intrauterine environment or that the process began in vivo. Thus, the apparent in vitro switching by Bfu-e cultures from premature infants of less than 33 weeks gestation and from term IDM (pre-switch infants) is an assay system for testing the effect on globin switching of physiological parameters peculiar to the diabetic intrauterine environment, in which the globin switch is delayed. The addition of insulin, IGF-I, isoleucine, or low oxygen conditions cause the Bfu-e to synthesize the equivalent or more β -globin than control cultures.

Sodium butyrate (at 0.1 mM concentrations), caused Bfu-e from pre-switch infants to synthesize significantly less β -globin than did Bfu-e in control cultures. This effect of sodium butyrate was not found consistently in infants in whom the switch had occurred before birth, however, increased γ -globin synthesis was found consistently with α -amino-n-butyric acid. A significant increase in accumulation of hemoglobin F in the cord blood erythroid colonies also occurred in nine out of eleven cultures from seven infants, grown in the presence of sodium-butyrate compared to control cultures ($p < .05$). This effect on increasing hemoglobin F in culture was not

due to toxicity since the pH of the culture media was not effected, and similar numbers of Bfu-e and Cfu-e were found in cultures grown in the presence or absence of the butyrates. Radioimmunoassay of Bfu-e
5 hemoglobin indicates complete hemoglobinization (>15 pg/cell) in the presence of butyrate, demonstrating that the observed increase in hemoglobin F synthesis and accumulation were not an artifact of retardation of globin production.

10 The following examples are provided by way of illustration, however, the invention is not intended to be limited in any manner thereby.

EXAMPLE 1

Cultures of erythroid progenitors from blood obtained
15 during the first three years of life of Hb SS (sickle cell syndrome) infants are tested. It is found that DL- α , L- α or L- β butyric acid or their sodium salts, or β -Cl-D-alanine, 3-CL-propionic acid, 5-(2-chloroethyl)tetrazole, heptafluoro-butyric acid,
20 α -aminopropane sulfonic acid, or sodium propane sulfinate, increase γ -globin synthesis in two-thirds of infant cord blood erythroid colonies, SS samples from infants, even when the infants' reticulocytes and control colonies (Bfu-e) synthesize nearly all
25 β -globin.

EXAMPLE 2

In peripheral blood erythroid cultures established from infant sickle cell anemia patients it was found that butyrate enhances γ globin synthesis by
30 approximately 14% compared to control cultures from the same patients. This would achieve corresponding

Hb F synthetic levels of greater than 20% in most patients.

EXAMPLE 3

5 A Dexter culture is established from cord blood from
an infant with sickle cell anemia whose mother has
diabetes. This infant was prenatally diagnosed and
has almost no β^S globin on electrophoresis.
Progenitors assayed weekly for globin synthesis from
this infant's erythroid progenitors cultured in the
10 presence and absence of α -amino-n-butyric acid and
insulin showed that the combination of these two
agents prevented switching at two weeks. Her control
cells switched from 9% β^S globin to 20%. The
treated cells still synthesize only 9% β^S globin.

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EXAMPLE 4

Four fetal sheep were treated with continuous
infusions of butyrate in utero and the fetal to adult
(β) hemoglobin switching process was delayed in three
of the four. In one, the "switch" was completely
20 inhibited. This lamb had 100% fetal globin at birth,
a time when controls produce 80-100% adult globin.
When the infusion was begun in this lamb, β (adult)
globin was only 4%. In two of the lambs, the switch
was delayed; these two lambs already produced
25 approximately 15% adult globin when the infusion was
started; they produced half of that found in controls
at birth. In the fourth fetal lamb in whom adult
 β -globin production was already 35% when the infusion
started, a response was not found, implying that
30 early treatment may be of primary importance in
inhibiting the process.

EXAMPLE 5

Erythroid cultures (progenitors) from twelve sickle cell anemia patients of various ages were cultured for 14 days in presence of 0.1 mM sodium butyrate, and the total hemoglobin and fetal hemoglobin were assayed. The results are shown below in Table 1. In all cases the percent fetal hemoglobin produced was greater in the treated cultures than in untreated controls.

TABLE 1

Radio-Ligand Assay of Hemoglobin Produced
by Erythroid Progenitors

	Patient	Total Hb, pg per cell	HbF, pg per cell	Percent HbF
1.	Control	32.6	4.0	12.3
	Butyrate	32.5	7.1	21.8
2.	Control	16.2	1.8	11.1
	Butyrate	15.9	3.7	23.3
3.	Control	29.3	1.8	6.3
	Butyrate	24.5	3.05	12.4
4.	Control	29.4	5.9	20.0
	Butyrate	30.0	7.8	26.0
5.	Control	24.6	2.7	11.0
	Butyrate	28.2	7.2	25.5
6.	Control	25.6	4.18	16.3
	Butyrate	18.8	6.65	35.4
7.	Control	25.6	7.92	30.9
	Butyrate	19.7	7.35	37.3
8.	Control	24.7	1.63	6.6
	Butyrate	21.4	3.61	16.9
9.	Control	12.9	2.27	17.6
	Butyrate	18.0	4.2	23.2
10.	Control	44.5	32.1	72.1
	Butyrate	41.5	43.2	100.0
11.	Control	12.5	5.2	41.6
	Butyrate	19.7	10.9	55.3
12.	Control	11.1	4.5	40.5
	Butyrate	12.6	5.8	46.0

EXAMPLE 6

Erythroid cultures from five β -thalassemia patients were cultured in the presence of sodium butyrate as described in Example 6 and the α /non- α globin ratios were assayed. The results are shown in Table 2. In all cases the butyrate lowered the ratio compared to control cultures, indicating a lowering of excess α -globin levels (which cause the damage by an increase in γ -globin). The lowering of the excess α -globin levels ameliorates, and in some instances may eliminate, the need for chronic transfusions.

TABLE 2

Alpha to Non-Alpha Globin Ratios

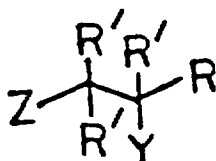
<u>Patient Phenotype</u>	<u>Control</u>	<u>Butyrate</u>
1. β^+ Thalassemia	2.5	1.6
2. β^+ Thalassemia	1.75	0.91
3. B^+ Thalassemia	1.76	1.33
4. HbE- β^+ Thalassemia	4.65	4.11
5. HbE- β^+ Thalassemia	5.7	2.8

EXAMPLE 7

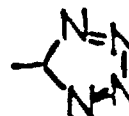
Three fetal sheep are treated with continuous infusions in utero as in Example 4, using heptafluorobutyric acid. In all three sheep, decrease in β -globin is observed within three days, thus evidencing a reversal of the switch. This effect persists for 5 days off treatment.

CLAIMS:

1. The use of a compound of the formula I:



wherein R is $-\text{CO}_2\text{R}_1$, $-\text{SO}_2\text{R}_1$, $-\text{SO}_3\text{R}_1$, or



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and R_1 is H, M, or alkyl 1-4 carbons and M is a cation; Z is $-\text{CH}_3$, $-\text{X}$, or $-\text{CX}_3$; and X is Cl, F, Br, or I; Y is H, $-\text{NH}_2$, $-\text{NH}_3^+$, or F; and R' is H or F, or a mixture thereof, in the manufacture of a medicament for ameliorating β -globin disorders in a mammal by introducing said compound into the bloodstream of said mammal periodically during its gestation period and/or infancy in an amount and frequency sufficient to inhibit or reverse fetal γ to β -globin switching.

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2. The use according to claim 1 wherein said mammal is human and said disorder is sickle cell anemia or β -thalassemia.

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3. The use according to claim 1 or claim 2 wherein said compound comprises α -amino-n-butyric acid, sodium butyrate, β -chloro-D-alanine, 3-chloro-propionic acid, 5-(2-chloroethyl)tetrazole, heptafluorobutyric acid, α -aminopropanesulfonic acid, and/or sodium propanesulfinate.
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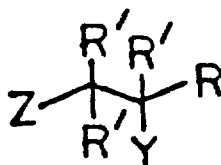
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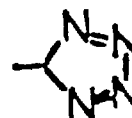
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4. The use of according to any one of claims 1 to 3 wherein said γ to β -globin switching is inhibited to the extent that γ -globin synthesis in said mammal remains at 10 to 20% above normal levels after birth.

5. A compound of the formula I:



wherein R is $-\text{CO}_2\text{R}_1$, $-\text{SO}_2\text{R}_1$, $-\text{SO}_3\text{R}_1$, or



and R_1 is H, M, or alkyl 1-4 carbons and M is a cation; Z is $-\text{CH}_3$, $-\text{X}$, or $-\text{CX}_3$; and X is Cl, F, Br, or I; Y is H, $-\text{NH}_2$, $-\text{NH}_3^+$, or F; and R' is H or F, or a mixture thereof, for use in the manufacture of a medicament for ameliorating β -globin disorders in a mammal by introducing said compound into the bloodstream of said mammal periodically during its gestation period and/or infancy in an amount and frequency sufficient to inhibit or reverse fetal γ to β -globin switching.

6. A method of monitoring the clinical use of a medicament manufactured in the use according to any one of claims 1 to 4, in which method γ -globin to β -globin switching is monitored by adding to an in vitro cell culture from the mammal an effective amount

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of a compound as defined in claim 1, or mixture thereof, to determine if additional treatment is needed.

- 5 7. A method for diagnosing disease in a mammal comprising the steps of culturing a biological sample from the mammal with an effective amount of a compound as defined in claim 1, or mixture thereof, and determining the change in β -globin synthesis of the
10 sample as compared to a control sample cultured in the absence of such compound.

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