Vanillin, a Potential Agent for the Treatment of Sickle Cell Anemia

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Vanillin, a food additive, has been evaluated as a potential agent to treat sickle cell anemia. Earlier studies indicated that vanillin had moderate antisickling activity when compared with other aldehydes. We have determined by high performance liquid chromatography that vanillin reacts covalently with sickle hemoglobin (HbS) both in solution and in intact red blood cells. Hemoscan equilibrium curves show a dose-dependent left shift, particularly at low oxygen tensions. Rheologic evaluation (pO2 scan, Ektacytometry) of vanillin-reacted HbS erythrocytes shows a dose-dependent inhibition of deoxygenation-induced cell sickling. Ektacytometry also suggests that vanillin may have a direct inhibitory effect on HbS polymer formation. Vanillin has no adverse effects on cell ion or water content. X-ray crystallographic studies with deoxyhemoglobin (HbA)-vanillin demonstrate that vanillin binds near His 103α, Cys 104α, and Gin 131β in the central water cavity. A secondary binding site is located between His 116β and Gin 117β. His 116β has been implicated as a polymer contact residue. Oxygen equilibrium, ektacytometry, and x-ray studies indicate that vanillin may be acting to decrease HbS polymerization by a dual mechanism of action; allosteric modulation to a high-affinity HbS molecule and by stereospecific inhibition of T state HbS polymerization. Because vanillin is a food additive on the GRAS (generally regarded as safe) list, and because it has little or no adverse effects at high doses in animals, vanillin is a candidate for further evaluation as an agent for the treatment of sickle cell disease.

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AT PRESENT THERE IS ONLY symptomatic treatment for sickle cell anemia (B6Glu in normal adult hemoglobin [HbA] → Val in sickle hemoglobin [HbS]). Over the last several years, we and others have discovered or identified a number of potent in vitro inhibitors of HbS polymerization. Unfortunately, the majority of these compounds (antigelling agents) have not proved suitable for human investigation. One reason for the lack of success in developing a clinically useful antisickling agent is the excessive amount of drug required to interact stereospecifically with the large quantity of Hb present in humans. The concentration of Hb within red blood cells (RBCs) is 5 mmol/L, a HbS homozygote with a blood volume of 4 L and a 25% hematocrit has approximately 322.5 g or 5 mmol of HbS. In theory, a two-to-one molar ratio (drug/HbS) is needed for 100% modification of HbS (because of the twofold symmetry axis that bisects the tetramer). A one-to-one molar ratio would occur if the agent binds on the twofold axis. However, it is conceivable that therapeutic efficacy could be obtained with 50% or less modification of HbS because 40% to 60% HbA heterozygotes are asymptomatic and 20% to 30% HbF Saudi homozygotes have very mild disease. Solubility measurements also indicate that these percentages of modified HbS could show clinical benefits.12 With a 25% hematocrit and a blood volume of 4 L, 1.5 g of drug (molecular weight = 300) would be needed to interact on a 1:1 molar ratio (50% modification). Considering that only a fraction of the drug is selectively transported into erythrocytes, while the remainder is metabolized or lost to other processes (excretion, fat depots, etc), daily dosages as high as 1 to 5 g might be needed for routine therapy. Lifetime treatment at these doses, even with very potent and selective HbS binding, would require an agent that is essentially nontoxic. For this reason, we began searching for a substance with little or no toxicity that might qualify as a template for chemical modification to increase antigelling activity.

Accordingly, our attention was drawn to a report by Zaug et al that evaluated the antisickling action of 29 aldehydes.13 In this study, vanillin, a food flavoring agent, was shown to have moderate in vitro antisickling activity. Beddell et al confirmed the modest activity of vanillin when compared to related aldehydes.14 Because vanillin is relatively nontoxic, we decided to first investigate in detail its antisickling and antigelling properties and then modify it in a rational manner to increase its activity profile. In the following battery of tests, vanillin exhibited a stronger activity profile than expected from the earlier two studies.13,14

Vanillin. Vanillin, 4-hydroxy-3-methoxybenzaldehyde (see structure below), is a flavorant present in foods, beverages, and tobacco. The highest average national level of use reported is 768 ppm, in confectioneries and frothings.13 The estimated daily intake is 11 mg/capita and possible average daily intake of vanillin from foods is 38.9 mg/capita.15 Acute oral LD50s are as follows: rat, 1.58 g/kg; guinea pigs, 1.40 g/kg; and rabbits, 3.0 g/kg.15 Several oral toxicity studies with rats were reported in which high levels of vanillin were consumed for extensive periods without adverse effects.15,16 For example, vanillin at 1.0% of the diet for 16 weeks, 0.1% for 27 to 28 weeks, 2.0% and 5.0% for 1 year, and 0.5%, 1.0%, and 2.0% for 2 years resulted in no significant differences between test and control rats with respect to body and organ weights, hematologic, and histopathology. The highest level consumed without adverse effects (5%) in rats19 is approximately equivalent to a daily intake of 2,500 mg/kg and is nearly 14,000 times the estimated per capita daily intake for a 60-kg person. A much earlier study17 reported growth depression and organ enlargement with rats on a 5% diet of vanillin for 91 days;
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VANILLIN

\[
\text{CHO} \quad \text{OCH}_3
\]

O\(\text{CH}_2\)\(_4\)COOH

VANILLIN

12C79

The structure of vanillin and a similar aldehyde, 12C79, that is currently in clinical trials for treatment of sickle cell anemia.

however, no adverse effects were observed when rats were administered vanillin orally either twice per week (300 mg/kg/dose) for 14 weeks or once a day (20 mg/kg/d) for 126 days. In man, vanillin is converted to vanillic acid in the liver and is excreted in the urine.\(^{26}\) Vanillic acid is also produced in the body as a result of the breakdown of epinephrine and norepinephrine.\(^{26}\) Because none of the above animal studies looked for hemoglobin interaction with vanillin, we are initiating such studies.

Vanillin was given GRAS (generally regarded as safe) status by the Flavor and Extract Manufacturers Association (FEMA) and recognized for food use by the Food and Drug Administration (FDA).

MATERIALS AND METHODS

Vanillin was purchased from Aldrich (Milwaukee, WI) and Sigma (St Louis, MO) Chemical Companies. Heparin or EDTA-anticoagulated blood samples were collected from normal donors (HbAA), from sickle cell homozygotes (HbSS), and from individuals with sickle cell trait (HbAS).

High performance liquid chromatography (HPLC) analyses were performed using a Perkin Elmer series 4 Liquid Chromatography Microcompressor-Controlled Delivery System, Perkin Elmer LC-95 UV/Visible spectrophotometer detector, Perkin Elmer LC-100 Laboratory Computing Integrator, a 4.6 x 250 mm SynChropak CM300 column, a weak cation exchanger, and 4.6 x 50 guard column SynChrom CCM103-5 (Linden, IN). The column was developed using developer A; 0.03 mol/L bis-Tris (Sigma), 0.0015 mol/L KCN at pH 6.40 with acetic acid; and developer B; 0.03 mol/L Bis-Tris, 0.0015 mol/L KCN at pH 6.40 with acetic acid.

Oxygen equilibria of HbSS and HbAA RBCs, preincubated with and without vanillin, were examined in a Hem-O-Scan (SLM Instruments Inc, Urbana, IL). For washed cell measurements, gas mixtures were 19% O\(_2\), 81% N\(_2\), or 100% N\(_2\).

For the ektacytometry studies, two different types of PO\(_2\) scans were used. Under a 100% nitrogen atmosphere; (2) during the subsequent scan, the ambient PO\(_2\) in the tonometer was increased from less than 3 to approximately 50 mm Hg; and (3) the osmolarity of the solution was increased to sickle HbAS cells (see the legend to Fig 4). The antigelling properties of vanillin were evaluated as previously described.\(^{2,3}\) The solubility ratio (HbS-vanillin)/(HbS) indicated no activity; however, it was discovered that the dithionite (used to deoxygenate HbS solutions completely) had reacted with vanillin. Moist nitrogen deoxygenation of HbS (without dithionite) resulted in nonreproducible ratios greater than one, indicating vanillin antigelling activity; however, complete deoxygenation of concentrated HbS solutions with moist nitrogen is difficult and is the likely source of the problem. The use of ferrous citrate as a replacement for dithionite in the gelation assay produced a brown precipitate and no increase in HbS solubility. It is known that ferric ions (produced from the deoxygenation of HbS) react with vanillin to produce dehydrodivanillin.\(^{27}\) This reaction, which is used as a color test for vanillin (brown at 20°C), is the likely reason that no antigelling activity was found.

For preparation of crystals, a 50 to one vanillin to human deoxy HbA incubation under nitrogen at room temperature was followed by reaction with cyanoborohydride according to the procedure of Manning and Manning.\(^{28}\) Because HbA crystals from high salt have the same crystal structure as HbS crystals grown from polyethylene glycol (PEG), we used the standard high salt HbA structure in our work. Crystals of borohydride-reduced and unreduced solutions of vanillin-Hb adduct were prepared according to Perutz.\(^{29}\) After several days, crystals were found in most tubes. X-ray data were collected with a Rigaku rotating anode and AFC-5R diffractometer (hk1l; -h - k - l) to 3.0-Å resolution. After the standard data processing and corrections, the R-factor between native and derivative data sets was 7.5% (8.5% weighted) on amplitude. The amplitudes and native phases\(^{29}\) were used to calculate a Fourier difference electron density map.

RESULTS AND DISCUSSION

Transport through RBC membranes and reaction with Hb.

To determine if transport and Hb reaction differences occur between HbAA and HbSS erythrocytes, we incubated under air whole blood from HbAA donors (1 hour with...
hematocrit 40%) and washed RBCs from HbSS donors (2 hours with hematocrit 10%) in the presence of varying concentrations of vanillin. At the end of the incubation period, the RBCs were washed and lysates were prepared for subsequent analysis. The results of cation-exchange HPLC Hb analysis are shown in Fig 1A and B. Note that vanillin adducts with HbA and HbS have shorter retention times than the control samples and the amount of Hb adduct increases with increasing concentration of vanillin. The shorter retention times for the adducts on a cation-exchange column indicate that vanillin-HbS and vanillin-HbA have less positive charge than the native protein. Both whole blood and washed cells sequestered vanillin and exhibited Hb reaction as indicated by HPLC.

At equivalent concentrations of vanillin, the ratio of Hb reacted/unreacted for washed HbS cells is greater than that observed for HbA RBCs in plasma (size of peaks in Fig 1). This difference can be attributed to a longer incubation time and higher drug to RBC ratio (10% hematocrit) used for the washed HbS cell study. The reaction of vanillin with HbA in whole blood demonstrates that vanillin can by pass plasma proteins, enter the RBC, and react with Hb. Therefore, we decided to evaluate and compare the action of vanillin with Hb SS whole blood.

Hb SS whole blood incubation study. A whole blood incubation study was performed under physiologic conditions at 37°C. Vanillin reacts with HbS at 2.5, 5, and 10 mmol/L vanillin, with a 2-hour incubation period giving the optimum yield (Fig 2 and Table 1). Longer incubation times did not show a decrease in the reacted Hb HPLC peak, indicating a long half-life for the Schiff base in RBC.

Therefore, it is possible that vanillin given in small doses over a period of time could steadily increase the amount of vanillin-HbS adduct.

Effect of vanillin-reacted Hb on oxygenation. Once it was established that vanillin bound to both HbA and HbS, its effect on the position of the oxy-Hb dissociation curves was studied. Vanillin reaction with HbA and HbS produced a dose-dependent left shift of the oxygen equilibrium curves in both Hb AA and SS samples (Fig 3A and B). In addition to an overall increase in Hb-oxygen affinity, vanillin has a major effect on the cooperativity that is normally observed at low oxygen tensions. For example, at a P0₂ of 10 mm Hg, the normal sigmoid-shaped curves for the controls have a more hyperbolic appearance for 5 and 10 mmol/L vanillin-reacted HbA and HbS (Fig 3). High concentrations of vanillin were used to ensure complete vanillin reaction with Hb. At a 50% hematocrit, 10 mmol/L vanillin would produce (if reacted completely and at a specific site on Hb) a 2 vanillin to 1 Hb adduct because Hb is 5 mmol/L in RBCs. With 10 mmol/L vanillin, the HbA and HbS oxygen equilibrium curves are superimposable from 0% to about 30% oxygen saturation. Comparison of the curves at 10% oxygen saturation (P0₂) demonstrates a larger left shift for HbS-10 mmol/L vanillin reaction (14 for control to 2.5 mm Hg for reacted) than for HbA-10 mmol/L vanillin reaction (10 for control to 2.5 mm Hg for reacted). The increased left shift at low P0₂ for the vanillin-HbS adduct that normalizes it to the oxygen equilibrium curve observed for the vanillin-HbA adduct indicates that a stereochemical inhibition of polymerization by vanillin-reacted T state HbS molecules is probably occurring. If this is the case, the reduction in polymerization is due to both the presence of R state vanillin-reacted HbS molecules (because R state...
molecules will not incorporate in the polymer) and T state vanillin-reacted HbS molecules (that are stereochemically inhibited from incorporation in the polymer).

Rheologic studies of vanillin-reacted HbSS and HbAS RBC. The rheologic effect of vanillin on HbS-containing RBCs was examined using PO₂ scan ektacytometry. Figure 4A depicts a sickling (deoxygenation) study in which this system was used to define the PO₂ at which HbS polymerization and cell sickling first became apparent. In the control cells, a marked change in cell rheology was observed at a PO₂ of approximately 40 mm Hg. In the cells preincubated with vanillin, the rheologic change did not occur until lower PO₂ levels were reached, a shift to the left that was dose dependent. Because we were looking for early changes in cell rheology, RBCs from HbSS homozygotes were used in the study shown here. However, similar data were also obtained when RBCs from HbAS heterozygotes were used. These results indicate that after exposure to vanillin, cell sickling does not begin until a lower PO₂ level is reached.

Figure 4B is an unsickling (reoxygenation) experiment, the purpose of which is to define the minimum EI value (EIₘᵦ) achievable after complete deoxygenation. Note that in the control cells, an EIₘᵦ of approximately 0.2 was observed. In the cells that had been preincubated in vanillin (5 mmol/L), an EIₘᵦ value of approximately 0.35 was noted. For agents that increase HbS solubility under completely deoxygenated conditions, the increase in EIₘᵦ is demonstrable using either HbSS or HbAS RBCs. This observation is best demonstrated using HbAS RBCs.

In previous studies, Johnson et al have shown that the mode of action of antisickling agents can be clarified by studying the relationship between EI and PO₂. Agents that increase oxygen affinity (eg, cyanate) reduce the PO₂ level at which a change in EI is first observed. Thus, the dose-dependent left shift of the EI versus PO₂ curves shown in Fig 4A is consistent with the increased oxygen affinity that we have shown to result from vanillin preincubation. On the other hand, agents that increase HbS solubility (eg, butyl urea), have been shown to increase EIₘᵦ, even under completely deoxygenated conditions. As shown in Fig 4B, vanillin increased EIₘᵦ from 0.2 to 0.35, a change highly suggestive of increased deoxy-HbS solubility.

These ektacytometry experiments suggest that the vanillin-HbS adduct inhibits polymerization via a dual mechanism of action: (1) by creating a high-affinity Hb variant, thereby increasing the number of R state HbS molecules; and (2) by causing mixed stereochemical inhibition of deoxy-HbS polymerization. A similar dual effect on HbS polymerization has been reported with pyridoxal, another aldehyde that is also thought to form a Schiff base adduct with HbS.

The effect of vanillin on RBC ion and water content. HbS polymerization and cell sickling are intimately linked to the intracellular concentration of HbS (mean corpuscular Hb concentration [MCHCs]). Therefore, any compound that increases cell ion content causing cell swelling will decrease MCHC and inhibit or retard cell sickling. On the other hand, a drug that causes the cells to lose ions and shrink will...
with Hb. The detection of a Schiff base adduct with a protein is difficult. Reduction of Schiff base double bonds with borohydride reagents is common to stabilize the adducts. For example, a number of aldehydes have been shown to form Schiff's base adducts with the terminal amino groups on Hb and the double bond reduced for analysis.3,27,28

However, we were successful in obtaining binding site information from crystals grown from solutions of both

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The results listed in Table 2 indicate that vanillin produces no significant changes in cell ion or water content in either AA or SS RBCs. The lack of major alteration of RBC ion content with vanillin is important, considering that other potent antigelling agents that we have developed dehydrate RBCs via activation of the KCl cotransport system (Orringer and Abraham, unpublished results).

X-ray crystallographic analysis. X-ray crystallographic studies of vanillin reacted deoxy-HbA were initiated to determine the binding site(s) of vanillin with the hope of explaining its allosteric and/or antigelling properties. It has been established that vanillin forms a Schiff base adduct

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vanillin-HbA adduct and HbA adduct reduced with cyanoborohydride. The difference electron density maps from crystals of the cyanoborohydride-reduced complex indicated major and minor binding sites for vanillin (Fig 5). The major site indicates that a pair of vanillin molecules has bound near a pair of a chain His 103 imidazole rings in the central water cavity. Positive and negative electron density contours indicate that residues Cys 104α and His 103α, and Gln 131β move to accommodate vanillin binding (Fig 6). A close contact of 2.74 Å in the native structure between atom OE1 of Gln 131β and atom CE1 of His 103α appears to have been dislodged (Fig 6, negative contour covering both residues). The minor site that could not be fit with the vanillin molecule because of low occupancy (Fig 7) indicates that vanillin has bound between His 116β and His 117β. His 117β has been shown to form a covalent adduct via a Michael addition reaction with the potent antigelling agent ethacrynic acid. Binding to His 116β or His 117β would suggest an antigelling mode of action for vanillin. It is

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**Fig 5.** A global view looking down the twofold axis of Hb. The major vanillin binding sites seen as heavy contours are in the central water cavity (upper right and lower left). The secondary sites are located on the outside of the molecule. The stronger secondary site is near His 317 (His 117β) and the weaker at His 717 (His 117β). Ethacrynic acid, another antigelling agent, also reacted with His 117β with very different occupancies between the two symmetry related His. The key for reading the amino acid residues are as follows: a three letter code for the amino acid plus a three digit number that signifies the residue and subunit location. The α subunit residues are numbered between 1 and 200; the β subunit residues from 201 to 400; the α subunit residues from 401 to 600; and the β subunit residues from 601 to 800. To find the standard residue notation simply subtract 200 from the values between 201 and 400; 400 from values between 401 and 600; and 600 from values between 601 and 800. For example, looking at four residue labels on the extreme corners of this figure, one sees at the upper right corner, SER452. This would be Ser 5h. Likewise, at the lower left corner, the symmetry-related SER452 is located. This would be Ser 5h. At the lower right corner, the symmetry-related SER452 is located. This would be Ser 5h. At the upper left corner, the symmetry-related residue, PRO258, is Pro 58β.
vanillin/Hb ratio, incubated under oxy conditions, and reaction at the terminal amino groups on the other group or low occupancy. Reaction at the terminal amino groups may be of interest in other protein systems.

Most likely point of reaction for an aldehyde would be at the C-terminal carboxyl group of Arg 141a and would be the vanillin molecule. Electron density maps because of disorder of the reacted group. Reaction at the terminal amino group on the a chains would disrupt a key salt bridge with the C-terminal carboxyl group of Arg 141a and would explain the left shift in the oxygen equilibrium curve.

Interestingly, deoxy crystals grown from a 4 to 1 mole vanillin/Hb ratio, incubated under oxy conditions, and without cyanoborohydride reduction, produced a difference electron density map that showed weak binding to the same sites observed in Fig 5. The binding of an aromatic aldehyde to or near a His residues appears to be novel and may be of interest in other protein systems.

Finally, HPLC spectra from crystals used in nine different x-ray analyses (that were grown from solutions of vanillin reacted and incubated with Hb under different conditions) indicates that the major HPLC peak for the reacted species may be caused by binding elsewhere (eg, at the terminal amino groups) and may not be related to the peaks observed in the electron density above.* We believe that the binding sites observed in our maps are probably secondary sites. Nevertheless, these results indicate that vanillin binds at a polymer contact site that should interfere stereochemically with the polymerization process as suggested from the oxygen equilibrium and the ektacytometry experiments.

**12C79 clinical study.** A similar aldehyde 12C79 (see structure) is currently undergoing clinical evaluation. It is not orally active and has a limited (4 hours) half-life when administered intravenously. However, results indicate that single 60-minute infusions of 12C79, up to 40 mg/kg, are well tolerated by sickle cell patients; and that greater than 30% modification of a high-affinity HbS is achievable without apparent adverse effect. Because the two molecules are chemically similar, the 12C79 study may provide a benchmark for further vanillin studies.

**CONCLUSIONS**

Vanillin has been shown to interact covalently with HbS, alter the rheologic properties of HbS containing erythrocytes in a positive fashion, and concentrate in erythrocytes. The reaction of vanillin with HbS produces a high-affinity Hb. Vanillin has been shown to transfer from plasma into erythrocytes, indicating that plasma protein binding, at these concentrations, does not hinder its availability to enter RBCs and react with HbS. X-ray crystallographic studies show that a secondary vanillin binding site is near or at a HbS polymer contact site. To our knowledge, this is the first time an x-ray crystallographic study has provided evidence for an aromatic aldehyde interaction or reaction with a histidine residue.

The above studies also indicate that vanillin may exhibit two separate mechanisms of action as an antisickling agent. First, it shifts the oxygen equilibrium curve to the left, thereby increasing the number of soluble R state HbS molecules. Second, ektacytometry, oxygen equilibrium studies, and x-ray crystallographic work indicate that the T state vanillin-HbS adduct may act directly to stereospecifically...
inhibit polymer formation. An antisickling agent such as vanillin that possesses a dual mechanism of action could exhibit synergistic behavior, meaning that a smaller dose would produce increased activity.

Vanillin has the following clinically attractive properties. It reacts covalently with HbS and, therefore, could be administered in smaller doses over a period of time to increase the percent modification of HbS. Also, the low molecular weight of vanillin (152) means that therapeutic doses near 1.0 to 4 gm/d or less might be practical. Finally, it is on the GRAS list, and has very low toxicity. Further studies are in progress to determine the pharmacokinetic properties of vanillin in animals. A single-dose study in humans is planned. Based upon these results, the potential clinical usefulness of vanillin in sickle cell disease will be evaluated.

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