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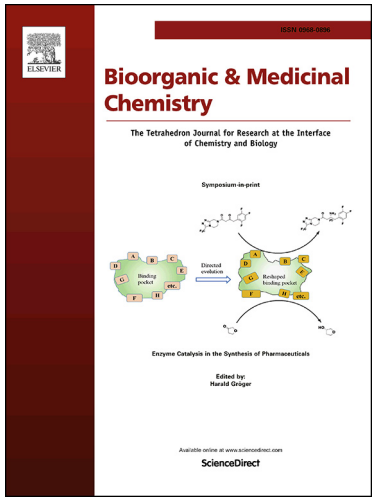

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Rational Design of Pyridyl Derivatives of Vanillin for the Treatment of Sickle Cell Disease.

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Abstract

Hypoxia-induced polymerization of sickle hemoglobin (Hb S) is the principal phenomenon that underlays the pathophysiology and morbidity associated with sickle cell disease (SCD). Opportunely, as an allosteric protein, hemoglobin (Hb) serves as a convenient and potentially critical druggable target. Consequently, molecules that prevent Hb S polymerization (Hb modifiers), and the associated erythrocyte sickling have been investigated--and retain significant interest--as a viable therapeutic strategy for SCD. This group of molecules, including aromatic aldehydes, form high oxygen affinity Schiff-base adducts with Hb S, which are resistant to polymerization. Here, we report the design and synthesis of novel potent antisickling agents (SAJ-009, SAJ-310 and SAJ-270) based on the pharmacophore of vanillin and INN-312, a previously reported pyridyl derivative of vanillin. These novel derivatives exhibited superior *in vitro* binding and pharmacokinetic properties compared to vanillin, which translated into significantly enhanced allosteric and antisickling properties. Crystal structure studies of liganded Hb in the R2 quaternary state in complex with SAJ-310 provided important insights into the allosteric and antisickling properties of this group of compounds. While these derivatives generally show similar *in vitro* biological potency, significant structure-dependent differences in their biochemical profiles would help predict the most promising candidates for successful *in vivo* pre-clinical translational studies and inform further structural modifications to improve on their pharmacologic properties.

Keywords:

Sickle cell disease, hemoglobin, relaxed state, aromatic aldehyde, antisickling, oxygen equilibrium, crystal structure, polymerization

1. Introduction

Hemoglobin (Hb) functions in equilibrium between the R-state (relaxed) possessing high oxygen (O_2) affinity, and T-state (tense) possessing low-O_2 affinity.[1,2,3] The R-state is an ensemble of high-affinity relaxed conformational states, including the R2, R3, RR2, RR3, and the classical R.[4–7] The equilibrium between the T and R states can be regulated by synthetic allosteric effectors that stabilize one state relative to the others.[6,7] Stabilization of the R-state, usually via the R2 or R3 or classical R conformation by allosteric effectors shifts the Hb oxygen equilibrium curve (OEC) to the left, producing a high-O_2 affinity Hb.[6–9] These types of allosteric effectors are potentially useful for treating sickle cell disease (SCD) since high-O_2-affinity sickle Hb (Hb S) does not undergo polymerization and subsequent RBC sickling.[6–19] The surface located αF-helix in Hb has been shown to enhance inter-strand polymer contact stabilization through a hydrogen-bond interaction between αAsn78 of the helix and a Hb molecule from an adjacent polymer strand. Consequently, the variant αAsn78Gly (Hb Stanleyville) is known to increase solubility of sickle Hb by abrogating the αAsn78 mediated hydrogen-bond interaction.[20,21] Hence, compounds that bind to Hb and stereospecifically destabilize polymer contacts are potential antisickling agents.[8,22]

Aromatic aldehydes, such as vanillin, Tucaresol, 5-HMF (and furan derivatives), and the most recent GBT-440 (Figure 1), are some of the most well-studied molecules that increase Hb oxygen affinity to prevent the hypoxia-induced sickle Hb polymerization and/or directly destabilize polymer contacts.[6–19] 5-HMF was investigated in phase I and II clinical trials for the treatment of SCD but was hampered by extensive oxidative metabolism.[10,23] GBT-440 is currently undergoing phase III clinical studies for the treatment of SCD (clinicaltrials.gov, NCT03036813). Early studies by Zaugg et al. reported the antisickling activities of vanillin and several of its analogs.[24] A subsequent study by Abraham and co-workers further studied vanillin for the treatment of SCD.[25] Vanillin undergoes extensive

metabolism resulting in low bioavailability, low potency, and overall reduces its pharmacologic effect. To understand the molecular basis of aromatic aldehyde antisickling activity, our group studied the co-crystal structures of aromatic aldehydes with Hb and showed that the compounds preferentially bind to the α-cleft of liganded R2-state Hb by forming Schiff-base interactions with the N-terminal αVal1 nitrogen atoms.[9] This mode of binding provides additional inter-subunit interactions across the subunit interfaces of liganded Hb that lead to stabilization of the R-state and increase the O_2 affinity of Hb. Based on these crystallographic findings that indicated a potential for appropriate modifications to vanillin (or its analogs), we designed and synthesized several pyridyl derivatives of vanillin, termed INN compounds, which demonstrated superior pharmacologic properties compared to vanillin.[8,26] The modification resulted in additional hydrophobic contacts with the binding site residues, explaining the much improved pharmacologic effect.[8] Some of the compounds, such as INN-312 appeared to exhibit dual antisickling effects by increasing the oxygen affinity of Hb, and by stereospecifically destabilizing polymer contacts via interactions with a surface located F-helix.[8] INN-312, the most potent from the early group of compounds, evolved as a unique and promising pharmacophore, suggesting not only the importance of a pyridinylmethoxy substitution but also the ortho placement of this moiety (relative to the aldehyde group); some properties also shared by GBT440.[17] In the present study we investigated INN-312 analogs (SAJ-310, SAJ-009 and SAJ-270) to further improve on the pharmacophore as a novel lead for development, and perhaps, provide newer knowledge on structure-activity relationships with mechanistic implications. We investigated these novel compounds for the general nature of their binding interactions with Hb, their effect on Hb O_2 affinity properties, inhibition of RBC sickling, as well as their *in vitro* pharmacokinetic/pharmacodynamic properties. We also conducted detailed crystallographic

studies on SAJ-310 complexed with Hb to gain insights into the compound's functional and biological activities.

2. Results and Discussion

2.1. Design and synthesis of SAJ compounds

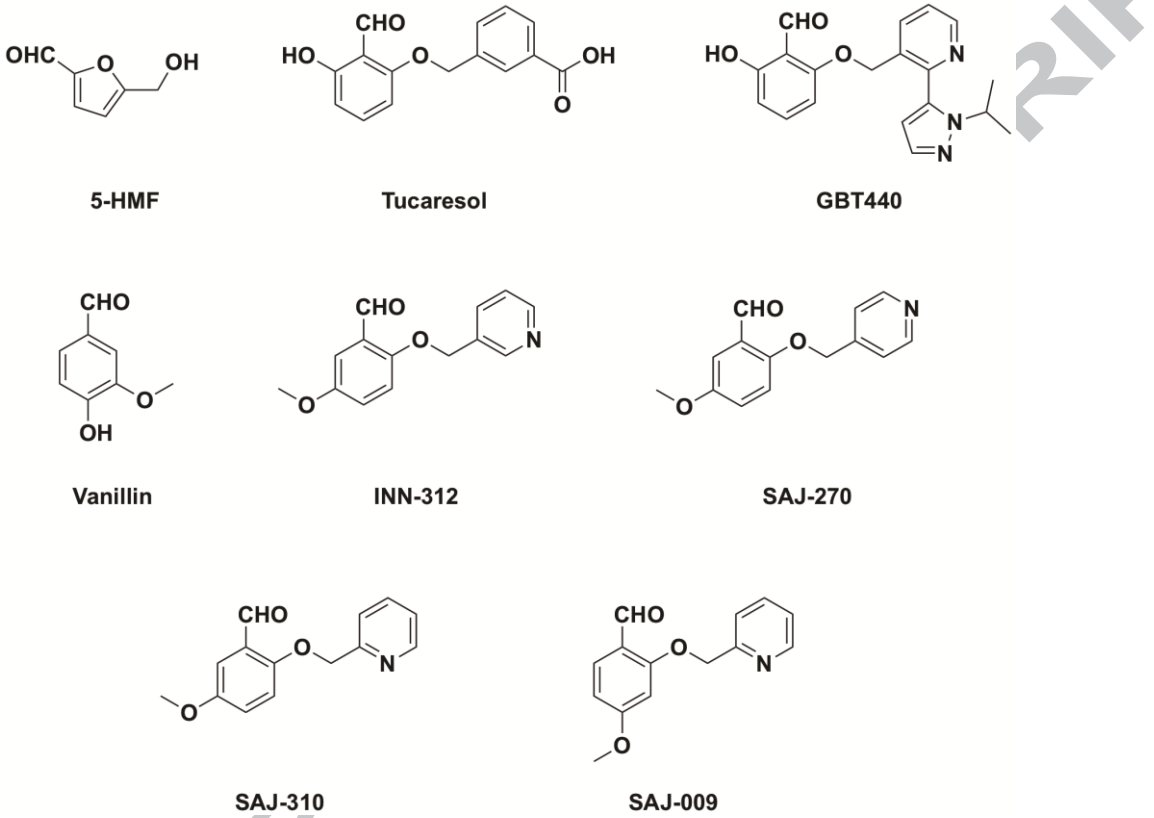
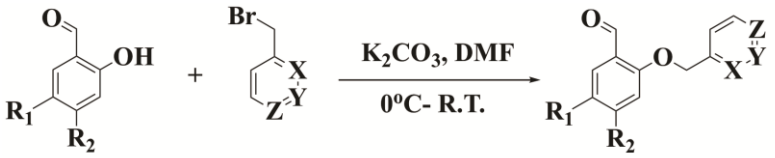


Fig. 1. Structures of 5-HMF, Vanillin, Tucaresol, GBT440, INN-312, SAJ-270, SAJ-310 and SAJ-009

We have previously studied several pyridyl derivatives of benzaldehyde-designated as INN compounds by incorporating a pyridinylmethoxy moiety on the vanillin pharmacophore (Figure 1).[8,26] The derivatives potently increased Hb affinity for oxygen and resulted in several-fold increase in antisickling effect when compared to the parent compound vanillin. Structural analysis demonstrated that like vanillin, two molecules of INN compounds form Schiff-base adducts with the N-terminal Val1 nitrogen atom of the α-subunits of the R2 conformer of Hb and stabilize the R-state.[8] As anticipated from our design, this class of

compounds made additional interactions with the protein, in part contributing to their enhanced functional and biological activities. Interestingly, the derivatives with the pyridinylmethoxy moiety at the ortho-position of the aldehyde group were generally more potent than the analogous para- or meta-positioned pyridinylmethoxy derivatives, with INN-312 being the most potent.[8] INN-312 binds and directs its ortho-positioned pyridinylmethoxy (pyridin-2-ylmethoxy) substituent (relative to the aldehyde moiety) towards the surface of Hb to make weak hydrophobic interaction with the surface located polymer stabilizing F-helix.[8] The enhanced antisickling potency of INN-312 is therefore likely due, in part, to the interactions with the F-helix. [8] Based on this promising lead, we further studied three ortho-substituted pyridinylmethoxy INN-312 analogs: SAJ-009, SAJ-270 and SAJ-310, for their functional and pharmacological properties (Figure 1). Our intent was to explore the position of the nitrogen atom on the pyridine ring, which influences the electronic property of the ring system and possibly interaction of the compound with the protein, and study its role in the pharmacological function of these types of compounds (SAJ-270 and SAJ-310). ~~We also surmised, based on the INN-312 crystal structure, that the ortho-located pyridine nitrogen atom of SAJ-310 could potentially allow hydrogen bond interaction between the pair of bound molecules through the pyridine nitrogen atom and pyridinylmethoxy oxygen atom to improve its binding interaction. Such an interaction would not be possible in INN-312 because of the meta-positioned pyridine nitrogen atom.~~ Also, to confirm that the change in the activity is due to the change of the position of the nitrogen atom on the pyridine ring alone, SAJ-009, carrying a para position methoxy group on the aromatic aldehyde was synthesized. Finally, it was anticipated that like INN-312, the pyridin-2-ylmethoxy of SAJ-270, SAJ-310 and SAJ-009 would direct towards the surface of the protein, resulting in not only improved binding interaction with the protein to increase Hb affinity for oxygen but also

make interactions with the F-helix to directly destabilize the polymerization process. The scheme for synthesizing the compounds is shown in Scheme 1.



INN-312: R_1= OCH_3, R_2= H, X= CH, Y= N, Z= CH
SAJ-270: R_1= OCH_3, R_2= H, X=CH, Y= CH, Z= N
SAJ-310: R_1= OCH_3, R_2= H, X= N, Y= CH, Z= CH
SAJ-009: R_1= H, R_2= OCH_3, X= N, Y= CH, Z= CH

Scheme 1

2.2. SAJ-310 binds to the α-subunit of liganded Hb in the R2 state conformation

We have previously shown that aromatic aldehydes that bind to Hb and increase the protein's affinity for oxygen with concomitant antisickling effect act by forming Schiff-base interactions with αVal1 of liganded R2 Hb.[8,9,11] In the present study, co-crystallization experiments were conducted with human carbonmonoxy Hb (COHb) complexed with SAJ-310, SAJ-009 and SAJ-270 using low salt precipitant to give needle crystals.[12] Unlike SAJ-310, which gave relatively larger needle crystals, crystals from SAJ-009 and SAJ-270 were very thin and poorly formed, making crystal manipulation difficult for X-ray diffraction data collection. The crystal structure of SAJ-310 was solved using molecular replacement with the native R2-state Hb structure (PDB code 1BBB) and refined to 1.95 Å. Structural statistics are summarized in Table 1 and the structure is deposited in the Protein Databank (PDB) with the ID code 6BNR.

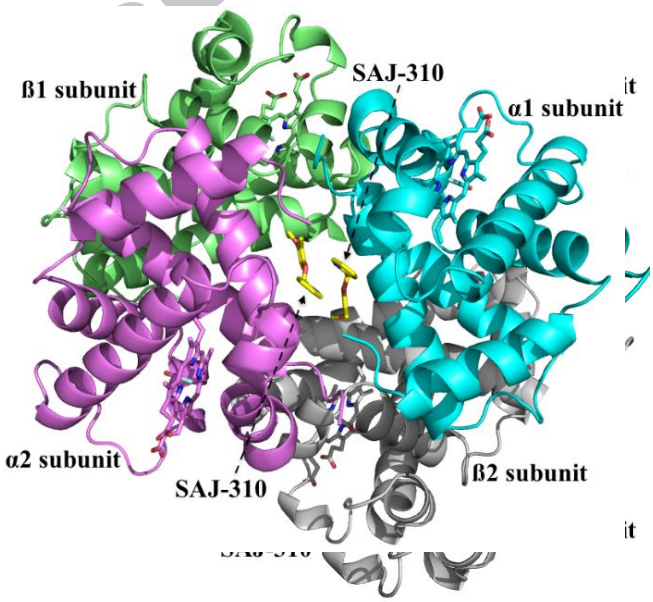
The overall tetrameric structure is indistinguishable (RMSD ≈ 0.3 Å) from 1BBB or the COHb complex structure with INN-312 (PDB code 3R5I).[8] As observed for the INN-312 complex structure (3R5I),[8] two molecules of SAJ-310 bind in a symmetry related fashion at the α-cleft of the Hb tetramer (Figure 2). The aldehyde groups of both molecules make Schiff-base interactions with the two N-terminal amines of the αVal1 residues, respectively (Figure 2).

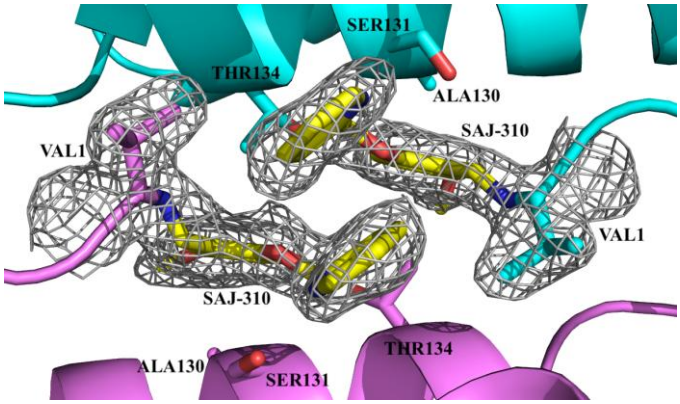
Table 1: Data collection and refinement statistics of carbonmonoxy Hb in complex with SAJ-310. Numbers in parentheses are for the highest resolution shell.

Data Collection Statistics	
Space group	$P2_12_12_1$
Unit-cell *a*, *b*, *c* (Å)	62.82, 83.55, 104.91
Resolution (Å)	29.40–1.95 (2.02 – 1.95)
Unique reflections	40595
Redundancy	3.97 (3.76)
Completeness (%)	99.1 (99.3)
Average **I**/σ(I)	11.8 (2.3)
R_{merge} (%)[a]	5.8 (46.7)
Refinement Statistics	
Resolution (Å)	29.40-1.95 (2.07-1.95)
No. of reflections	40456 (6308)
R_{work} (%)	20.6 (32.3)
R_{free} (%)[b]	26.4 (35.2)
R.m.s.d. bonds (Å)	0.007
R.m.s.d. angles (°)	1.6
Dihedral angles	
Most favored (%)	95.76
Allowed (%)	4.24
Average B (Å^2)/atoms	
All atoms	38.85
Protein	37.50
Hemes	33.61
SAJ-310	43.61
Water	48.55
PDB ID code	6BNR

[a]$R_{merge} = \Sigma_{hkl}\Sigma_i|I_i(hkl) - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i I_i(hkl)$. [b]$R_{free}$ was calculated from 5% randomly selected reflection for cross-validation. All other measured reflections were used during refinement.

A. **B.**





C.

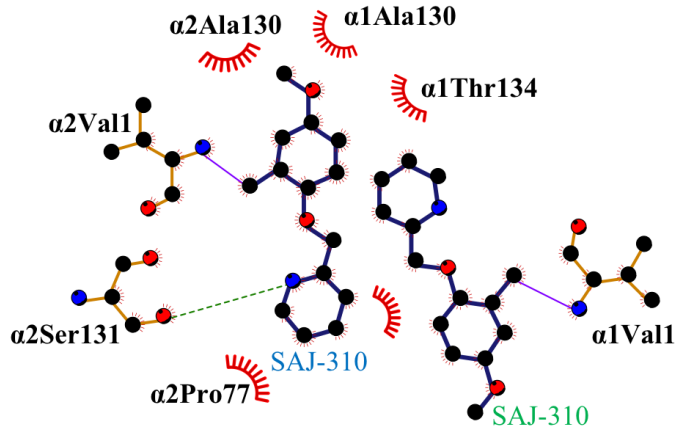


Figure 2: Crystal structure of Hb in the R2 conformation in complex with two molecules of SAJ-310 bound at the α-cleft. Hb subunits are shown as ribbons (α1 subunit in cyan, α2 subunit in magenta, ß1 subunit in green and ß2 subunit in grey) and hemes are shown as sticks. For clarity, not all binding site residues are shown but described in the text. (A) Overall structure of the complex showing bound SAJ-310 (yellow stick) in the central water cavity of the tetrameric protein. (B) Close-up view of SAJ-310 (yellow stick) binding with the final $2F_o$-F_c refined electron density map contoured at 1.0σ. (C) Two-dimensional contacts between one SAJ-310 molecule, the protein, and the second SAJ-310 molecule. The straight dashed lines indicate hydrogen-bond interactions and circular dashed lines indicate hydrophobic contacts.

Since both molecules bind in a symmetrical fashion, detailed SAJ-310 interactions with the protein will be focused on the α2Val1 binding compound. The Schiff-base interaction between SAJ-310 and Hb directed the pyridin-2-ylmethoxy group to make weak intrasubunit hydrophobic interactions (3.5-4.5 Å) with the surface-located F-helix residue of α2Pro77 (Figure 3). The F-helix has been noted to be involved in polymer stabilization through Asn78 mediated hydrogen-bonding interactions with other polymer strands.[22] We expect these hydrophobic interactions to perturb the polymer especially if the compound, as anticipated, binds to deoxygenated Hb, albeit weakly as previously noted for INN-312.[8] The benzaldehyde ring is sandwiched between, making intra- and inter-hydrophobic interactions with α2Ser131 and α1Thr134, respectively, while the pyridin-2-ylmethoxy oxygen atom appears to make intrasubunit hydrogen bonding interaction with the hydroxyl group of α2Ser131. Additionally, the pyridine rings from the two symmetry related bound compounds

make extensive 3.7-4.0 Å face-to-face π-π stacking interactions with each other (Figures 2 and 3). As previously noted,[8] the above SAJ-310-Hb interactions, which are also conserved in the INN-312 structure (Figure 3), not only should lead to stabilization of the R-state Hb and increase the protein affinity for oxygen, but may also directly destabilize polymer formation by perturbing the F helix.

There is one major significant difference between SAJ-310 and INN-312 protein interactions. In the SAJ-310 structure, the ortho-located pyridine nitrogen atom of each bound molecule makes direct intra-subunit hydrogen-bonding interaction with the hydroxyl group of α2Ser131(Figure 2C). This interaction is missing from the INN-312 structure because of its meta-located pyridine nitrogen atom, and could only interact with the protein via two water-mediated hydrogen bonding. We speculate that the direct interaction afforded by the meta-located pyridine nitrogen atom in SAJ-310 not only could lead to increase affinity with Hb but also result in slow dissociation of the compound from the binding site and could explain why SAJ-310 showed longer sustained functional effect when compared to INN-312 (vide infra).

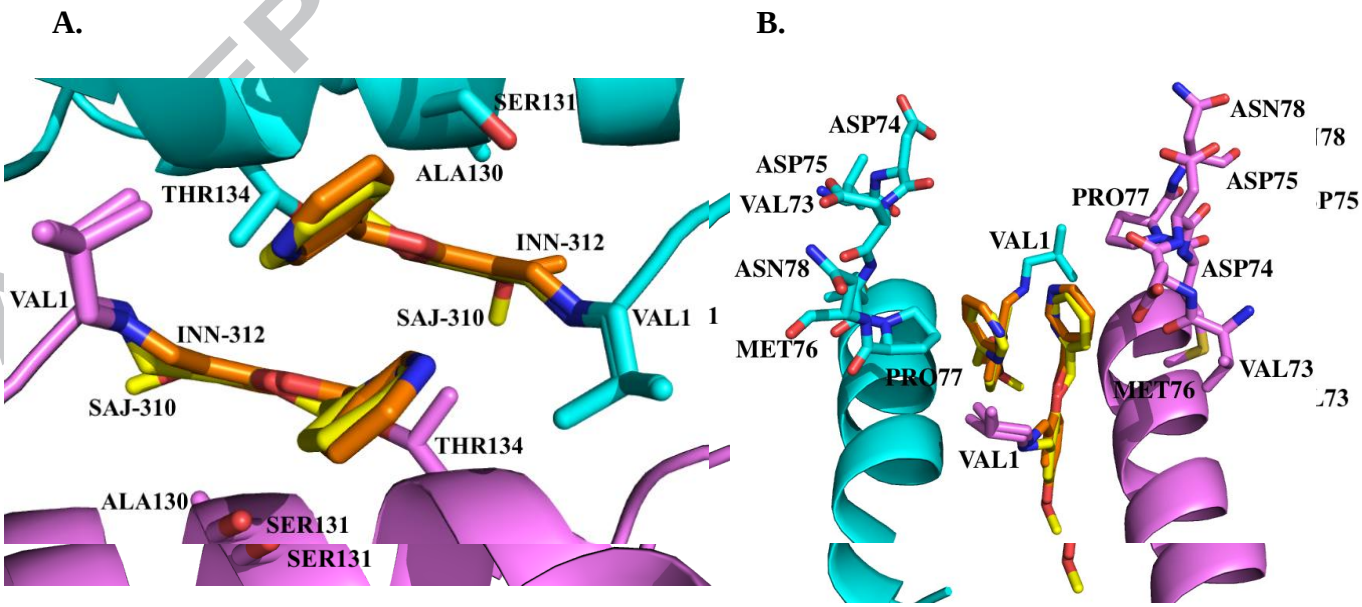


Figure 3: Structural comparison of molecules bound at the α-cleft of Hb. α1 subunit is shown in cyan and α2 subunit in magenta. **A.** Superposition of SAJ-310 (yellow) and INN-312

(brown) molecules showing interactions with residues from both A and F helices. B. Ninety-degree rotation view of **A**. The protein residues are from the SAJ-310 structure.

2.3. SAJ compounds bind and modify Hb

The pharmacologic properties of aromatic aldehydes, including allosteric, antisickling and pharmacokinetic are dependent on the compounds' ability to modify Hb as a result of the Schiff-base interaction between the aldehyde moiety and the N-terminal αVal1 nitrogen atom of Hb.[8,10,11] We conducted Hb modification studies of the compounds with cell free normal Hb (devoid of membrane and other red blood cell proteins), and normal whole blood by cation-exchange HPLC. When incubated with cell free Hb, INN-312, SAJ-310 and SAJ-270 showed similar reactivity of almost 100% adduct formation in 1 h, which was sustained for the full 90 minutes experiment (Figure 4). This compares to the <5% Hb modification observed for vanillin, clearly indicating vanillin's weak functional activity. Interestingly, SAJ-009 showed almost a linear relationship between time and % Hb modification, and at 90 minutes has modified only about 50% Hb, suggesting that SAJ-009 may achieve maximum effect ultimately beyond the 90-minute experimental time. In the absence of metabolizing enzymes, membranes and other blood proteins in the cell free Hb matrix, it is clear that INN-312, SAJ-270 and SAJ-310 exhibit quick onset or rapid rate of association with Hb, although the rate of adduct formation by SAJ-270 was slightly slower than that of INN-312 or SAJ-310. The three compounds, INN-312, SAJ-270 and SAJ-310 differ only in the positions of the nitrogen atom on the pyridine ring, suggesting that in the absence of other cell components, the slightly slower rate of SAJ-270 Hb association could be due to the para position of the pyridine nitrogen atom as compared to the meta and ortho positions in INN-312 and SAJ-310, respectively. As noted above, the rate of SAJ-009 association with Hb is much slower when compared to the other three compounds. SAJ-009 and SAJ-310 only differ in the position of their methoxy groups on the benzaldehyde ring (para and meta to the

aldehyde, respectively), therefore the unusually slow rate of SAJ-009 Hb association is likely due to the para methoxy group vs the meta position in the other three compounds.

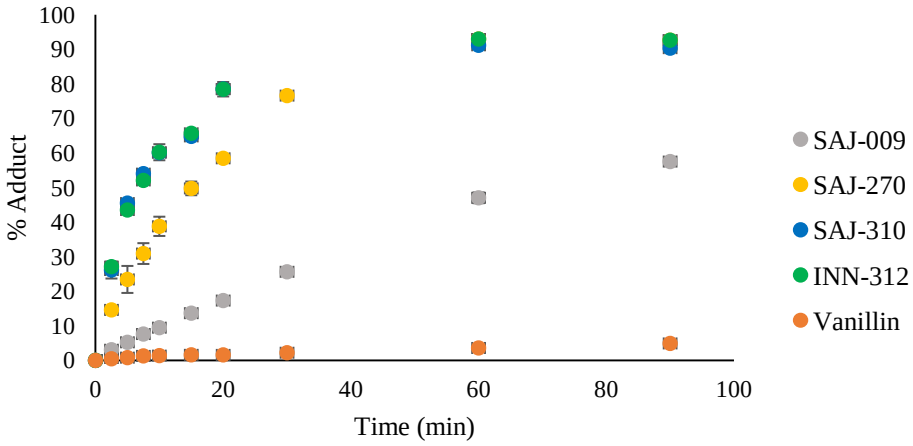
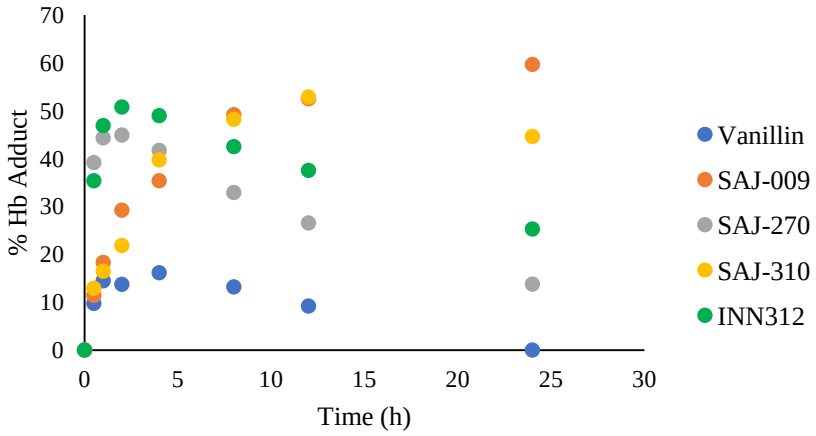


Figure 4: Time dependent modification of HbA in cell free hemoglobin incubated with test compounds

We next conducted similar cation-exchange HPLC experiments using freshly drawn normal whole blood from volunteers with informed consent. When incubated with the hematocrit-adjusted whole blood suspensions (30% HCT), all compounds at 2 mM concentrations modified intracellular Hb in a time-dependent manner, and importantly, demonstrated significant enhanced levels of Hb modification, in comparison to vanillin (Figure 5). SAJ-270 and INN-312 reached maximum Hb modifications (~45% and 50%) in ~2 hrs, followed by decrease to ~15% and 25%, respectively in 24 h. Thus, membrane and/or other blood proteins appear to slow down the transport of SAJ-270 and INN-312 to bind with Hb, as their maximum effect occurred faster at 1 hr in cell free Hb (Figures 4 and 5). As observed in the cell free Hb study, the rate of Hb modification by SAJ-009 was very slow but continuous even up to 24 h where it showed Hb modification of ~60% (Figure 5). These observations suggest SAJ-009 to be the most potent and have the most prolonged effect of the tested compounds. On the other hand, it is the slowest to reach maximum effect. Also, the fact that SAJ-009 showed similar slow rate of Hb modification both in cell free Hb and whole blood suggests an intrinsic slow rate of association with Hb. What is most interesting and

quite unexpected is that in contrast to the cell free Hb study, SAJ-310 in whole blood behaved similarly as SAJ-009 (Figures 4 and 5), i.e. showing a much slower rate of Hb modification in whole blood. Thus, cell membrane and/or other proteins appear to slow SAJ-310 transport to bind with Hb, even more so than INN-312 and SAJ-270. The ortho-pyridine nitrogen atom in SAJ-310, the only structural difference between INN-312 and SAJ-270 may explain this observation. In addition, the para-methoxy group in SAJ-009 may also be having effect in the compound transport through the cellular content and/or rate of association with Hb.

A.



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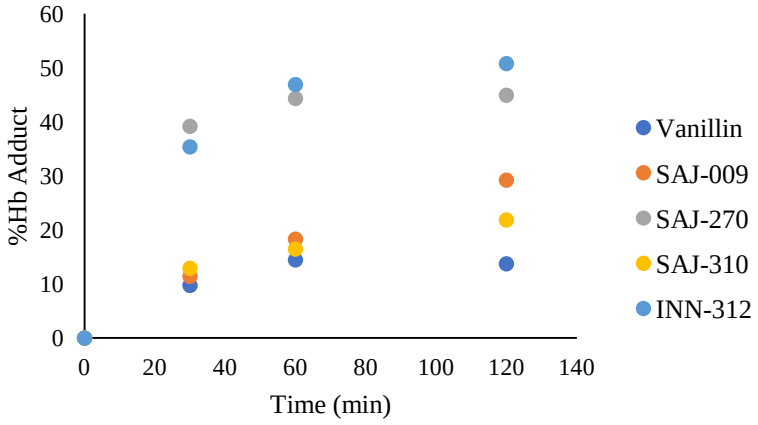

Figure 5: Time dependent modification of HbA in normal blood incubated with test compounds. A. The full 24 hr experimental result. B. Result truncated to 2 hr time point.

Unlike SAJ-009 that did not show any decline in Hb modification even after 24 hours, SAJ-310, INN-312 and SAJ-270 after attaining maximum level of ~50% Hb modification at 12 h (SAJ-310) or ~2 hr (INN-312 and SAJ-270), begun to decline to 45%, 25% and 15%, respectively (Figure 5). Antisickling aromatic aldehydes are known to undergo metabolism to the corresponding pharmacologically inactive acid analogs in liver, plasma, and RBC.[12,28] The metabolism in the RBC may occur prior to compound binding to and/or after dissociation from Hb. The fact that INN-312 and SAJ-270 (with similar potency as SAJ-310) associate faster with and also decline faster from Hb than SAJ-310 or SAJ-009 suggest that INN-312 and SAJ-270 are more susceptible to enzymatic metabolism likely due to faster unbinding or dissociation rate from Hb. It is worth pointing out that SAJ-310 and SAJ-009, especially the latter, associate very slowly with Hb in whole blood but still exhibit potent effect, suggesting the compounds to be more resistant to metabolism outside or inside the RBC. As observed in the crystal structure of SAJ-310, not only are the two molecules involved in strong π-π interactions with each other, but also make direct hydrogen-bond interactions between their ortho-positioned pyridine nitrogen atoms and the hydroxyl group of Ser131. The hydrogen-bond interaction is absent in INN-312, and expected to be absent in SAJ-270 also, because the pyridine nitrogen atoms of these two compounds are located in the meta- and para-positions, respectively. This unique hydrogen-bond interaction in SAJ-310 may increase its affinity to Hb, and importantly slow its dissociation from Hb when compared to INN-312 and SAJ-270, in part explaining the compound's resistant to blood metabolism. We expect SAJ-009, also with an ortho-positioned nitrogen atom to exhibit similar hydrogen-bond interaction, and in addition to the para-positioned methoxy group on the benzaldehyde ring may also explain this compound's apparent resistant to blood metabolism.

2.4. SAJ compounds prevent SS RBC sickling by modifying intracellular Hb S and altering oxygen affinity

The antisickling properties of aromatic aldehydes are primarily due to their interaction with sickle Hb and consequent increase in its affinity for oxygen. We thus investigated the compounds abilities to modify sickle Hb, increase sickle Hb oxygen affinity, and prevent RBC sickling. The studies were conducted by incubating sickle erythrocytes (hematocrit: 20%) in the absence or presence of 0.5 mM, 1 mM, and 2 mM concentration of test compound in air at 37°C for 1 h, followed by further incubation under hypoxic condition (4% oxygen/96% nitrogen) for 2 hr. Aliquot samples were then used for the three different studies as described in the experimental section.

Table 2: Hemoglobin adduct formation, oxygen equilibrium, and antisickling studies using homozygous sickle red blood cells with test compounds[a]

	Modified Hb (%)[b]			ΔP_{50} (%)[c]			Sickling Inhibition (%)[d]		
	0.5mM	**1mM**	**2mM**	**0.5mM**	**1mM**	**2mM**	**0.5mM**	**1mM**	**2mM**
Vanillin	3.3±3.3	5.6±3.2	12.0±4.6	1.2±1.2	10.6±2.8	21.0±10.3	4.2±2.1	7.3±2.8	7.3±2.6
SAJ-009	12.0±6.6	24.8±6.8	51.6±6.4	8.1±2.5	20.9±8.1	34.2±10.3	7.6±3.8	13.5±3.0	51.3±11.9
SAJ-270	32.8±9.8	58.7±13.2	82.5±4.7	7.9±3.5	31.5±12.8	55.6±12.6	21.7±9.3	60.4±10.2	85.9±5.2
SAJ-310	26.3±9.5	55.3±14.3	79.5±5.9	22.0±12.1	38.1±10.6	61.4±12.1	24.3±10.4	64.8±7.4	90.3±3.4
INN-	34±5.2	79±5.3	100±7.9	24.8±2.2	49.2±2.7	75.8±4.9	14.9±7.8	59.5±2.4	90.5±4.3

312			

[a]All studies were conducted with SS cells suspensions (20% hematocrit) incubated with 0.5 mM, 1 mM and 2 mM of each test compound. The results are the mean values ± SD for three separate experiments (biological replicates). The final concentration of DMSO was <2% in all samples, including in control samples. [b]Hb S adduct values obtained from HPLC elution patterns of individual hemolysates after incubation of compounds with SS cells. [c]P_{50} is the oxygen pressure at which the hemolysates are 50 % saturated with oxygen. ΔP_{50} (%) was determined as:

$$\Delta P_{50}\ (\%) = \frac{P_{50} \text{ of lysates from untreated cells} - P_{50} \text{ of lysates from treated cells}}{P_{50} \text{ of lysates from untreated cells}} \times 100$$

[d]Antisickling studies with SS cells were conducted under hypoxia (4% Oxygen/96% Nitrogen).

When subjected to the *in vitro* sickling assay under hypoxic conditions, the compounds reduced the sickling of SS cells in a concentration-dependent manner that is significantly superior to vanillin (Table 2; Figure 6). While the effects were comparable at higher concentrations for SAJ-270, SAJ-310, and SAJ-312, we observed a slightly higher potency at 0.5 mM concentrations for SAJ-310 and SAJ-270 (~ 22 and 24% inhibitions, respectively), compared to INN-312 (~ 15% inhibition). This may suggest a secondary, additional mechanism of antisickling action, considering that the levels of modified hemoglobin are comparable between the three candidate molecules at the given concentration (Table 2 and vide infra). Furthermore, the degree of shift in oxygen equilibria is comparable between SAJ-310 and INN-312, but is remarkably lower in SAJ-270, supporting the hypothesis of a secondary mechanism of action (Table 2 and vide infra).

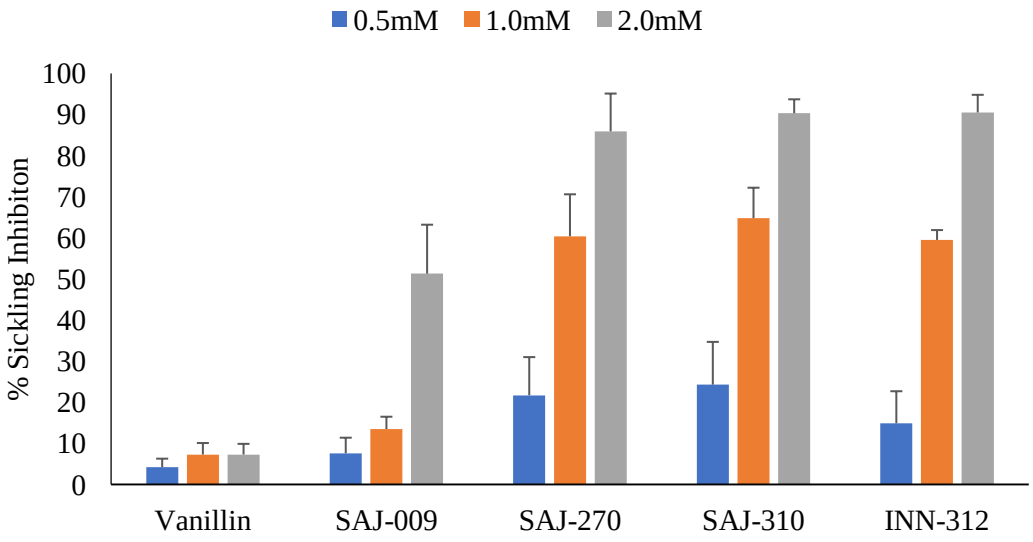

Figure 6: Concentration-dependent inhibition of SS cell sickling under hypoxia for 2 h. All SAJ compounds and INN-312 demonstrated strong concentration-dependent inhibition of sickling, with SAJ-009 showing the weakest effects under these experimental conditions.

Aliquots from the antisickling study were hemolyzed and utilized for Hb modification study by cation-exchange HPLC analyses. All compounds modified Hb S in a concentration dependent manner, concordant with the antisickling results (Table 2; Figure 7). Also, expectedly vanillin showed the lowest Hb modification levels (3.3, 5.6 and 12% at 0.5, 1 and 2 mM, respectively), with increasing effects by SAJ-009 (12, 25, 52%), SAJ-270 (26, 55, 80%), SAJ-310 (33, 59, 83%), and INN-312 (34, 80, 100%). These findings confirm previous observations that the slow association rate of SAJ-009 with Hb, and perhaps, impediment by other cellular components are likely contributing to its relatively low Hb modification levels during the 3-hr sickling assay period. Nonetheless, we expect both SAJ-009 and SAJ-310 to exhibit higher and sustained potency beyond 3 hours, as observed in the study with normal whole blood (Figure 5).

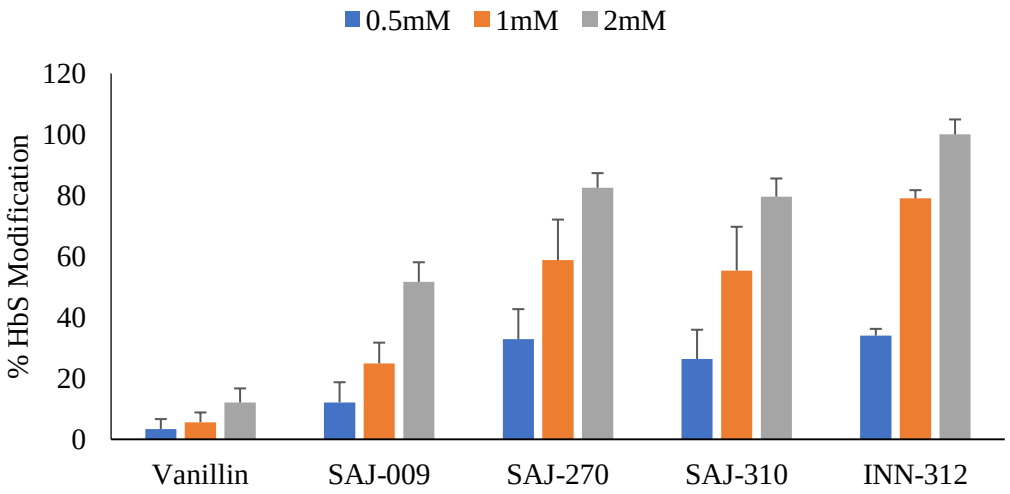

Figure 7: Concentration dependent modification of Hb S incubated with test compounds. Hemolysates from the antisickling assay were subjected to cation-exchange HPLC analyses, and levels of modified Hb were assessed in triplicates. Near complete modification of Hb S is observed at 2 mM concentrations of SAJ-270, SAJ-310 and INN-312.

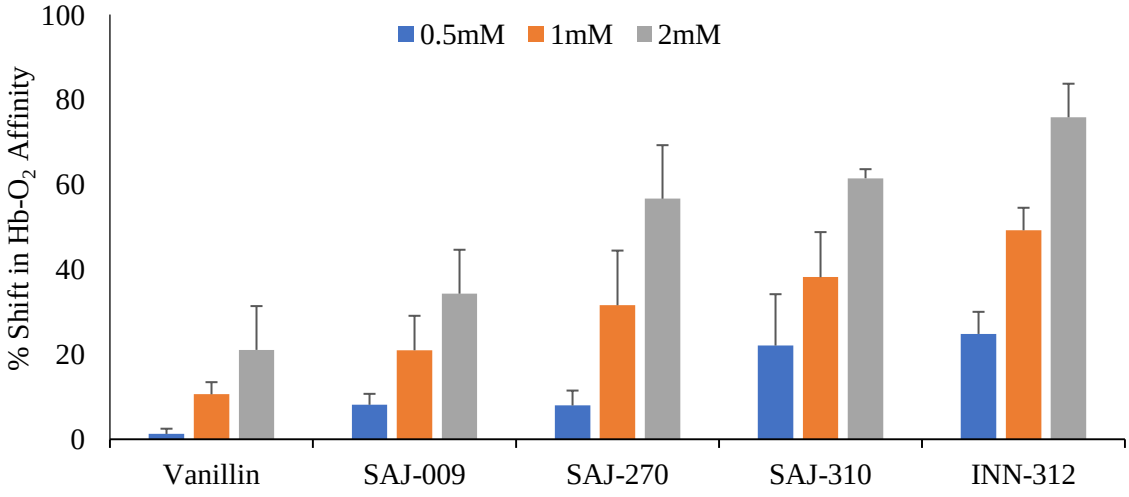

Figure 8: Tests compounds shifted in oxygen equilibrium significantly (ΔP_{50}) in a concentration-dependent manner.

When aliquots of Hb S-complex lysates from the antisickling studies were investigated in the OEC assay, we observed a similar concentration dependent effect on increasing Hb S affinity for oxygen. As expected, vanillin showed the lowest allosteric potency (1, 11, 21%; Figure 8). Among the novel test compounds, SAJ-009 exhibited the least potency (8, 21, 34%), while higher levels were observed for SAJ-270 (8, 32, 56%), SAJ-310 (22, 38, 61%) and INN-312 (24, 49, 76%) (Table 2; Figure 8). These findings are consistent with our

expectations based on the hemoglobin-binding assays. The greater allosteric potency of SAJ-310 over SAJ-270 even though the latter modifies Hb at a higher rate is probably due to the additional interaction(s) afforded by the direct hydrogen bond interaction between the ortho-positioned nitrogen atom in the pyridine ring of SAJ-310 and the hydroxyl group of αSer131, which leads to better stabilization of the R-state Hb complex.

3. Summary and Conclusion

Aromatic aldehydes have been studied extensively as potential antisickling agents, with vanillin being one of the earliest such compounds to undergo clinical trials for the treatment of SCD. Vanillin required high doses to elicit pharmacologic effect due to low potency, and importantly, poor bioavailability due to high susceptibility to oxidative metabolism. We have rationally and systematically modified vanillin along with several of its analogs to yield derivatives that overcome these pharmacologic disadvantages. Part of our earlier modifications led to the synthesis of INN-312, which showed significant improvement over vanillin,[8] thus establishing--for the first time--the ability of pyridyl derivatives of benzaldehyde scaffold to be exploited as potential SCD therapeutics, and laying the foundation for follow-up extensive SAR studies. In the current study, we synthesized and studied novel analogs of INN-312, including SAJ-009, SAJ-270 and SAJ-310, by investigating the effects of 1) the nitrogen atom position in the pyridine ring, and 2) the methoxy position on the benzaldehyde ring, separately, or in combination (Figure 1). As anticipated, the SAJ compounds are significantly more potent than vanillin: modified Hb S and increased its affinity for oxygen, and importantly inhibit hypoxia-induced SS cell sickling. SAJ-009 appears to be least susceptible to oxidative metabolism and also modify Hb the most but at a delayed time of about 10 hours. At shorter experimental times, this compound exhibits very weak allosteric and antisickling effect due to its slow onset. We speculate, based on our

findings that perhaps the position of the nitrogen atom plays an important role in dissociation from Hb, a phenomenon we also noticed in SAJ-310. However, the methoxy moiety at the para- position relative to the aldehyde in INN-009 appears to be responsible for the slow association rate. Conversely, INN-312 and SAJ-270 both demonstrated a more rapid onset of action and exhibited potent activities in increasing Hb affinity for oxygen and preventing RBC sickling. However, their activity began to decline in whole blood after 4 hrs, likely due to high rate of dissociation from Hb and/or increase susceptibility to oxidative metabolism. This could be due to the position(s) of the nitrogen atoms (para in SAJ-270 and meta in INN-312). Even though SAJ-310 showed slower association with intracellular, it demonstrated comparably high potency, while the activity is sustained in blood significantly longer than INN-312 and SAJ-270, suggesting a better metabolic stability. Importantly, SAJ-310 showed the most potent antisickling activity at lower compound concentration. These findings suggest that SAJ-310 is potentially a more promising lead candidate than other members of its class, and warrants further investigations, including *in vivo* PK/PD studies, as well as additional structural optimization.

4. Materials and Methods

Normal whole blood was collected from adult donors at the Virginia Commonwealth University after informed consent, in accordance with regulations of the IRB for Protection of Human Subjects. Hb was purified from discarded normal blood samples using standard procedures.[29] Leftover blood samples from patients with homozygous SS were obtained and utilized, based on an approved IRB protocol at the Children's Hospital of Philadelphia, with informed consent.

All reagents used in the synthesis and functional assays were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA) and utilized without additional purification. Melting points were determined on a Fisher-Scientific melting point

apparatus (Serial# 410N0117), and were uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker 400MHz spectrometer and tetramethylsilane (TMS) was used as an internal standard. Peak positions are given in parts per million (δ). Column chromatography was performed on silica gel (grade 60 mesh; Bodman Industries, Aston, PA). Routine thin-layer chromatography (TLC) was performed on silica gel GHIF plates (250 μm, 2.5 x 10 cm; Analtech Inc., Newark, DE). MS spectra were obtained from a Perkin Elmer Flexar UHPLC with AxION 2 Time of Flight (TOF) Mass Spectrometer, and the molecular weight of the compounds was within 0.005% of calculated values. Infrared spectra were obtained on Thermo Nicolet iS10 FT-IR. Purity of the compounds was determined by HPLC using Varian Microsorb 100-5 C18 column (250 x 4.6 mm), using Prostar 325 UV-Vis (254 nm) as the detector.

4.1. Synthesis:

The SAJ compounds were synthesized according to standard procedures for similar compounds [8,26] and the detailed procedures are given below.

Synthesis of INN-312 was carried out as previously reported.[8] ^{1}H-NMR (400 MHz, DMSO-d6): δ 10.36 (s, 1H), 8.71 (d, *J* = 1.6 Hz, 1H), 8.56 (dd, *J* =1.64 Hz, *J* = 4.8 Hz, 1H), 7.92 (m, 1H), 7.44 (m, 1H), 7.32 (d, *J* = 9.08 Hz, 1H), 7.27 (dd, *J* = 3.16 Hz, *J* = 9.04 Hz, 1H), 7.19 (d, *J* = 3.08 Hz, 1H), 5.28 (s, 2H), 3.76 (s, 3H).

Synthesis of 4-methoxy-2-(pyridine-2-ylmethoxy)benzaldehyde, *SAJ-009*:

2-hydroxy-4-methoxybenzaldehyde (152 mg, 1.0 mmol), 2-bromomethylpyridine hydrobromide (253 mg, 1.0 mmol) were dissolved in dimethylformamide (DMF), and the mixture was cooled at 0 °C. Potassium carbonate (340 mg, 2.5 mmol) was then added portion wise. The reaction mixture was stirred at 0 °C to room temperature for 16 hours. Following

that, the mixture was diluted with ethyl acetate (100 mL), washed with water (3 x 30 mL), brine (30 mL), dried over Na_2SO_4, and filtered. The ethyl acetate was evaporated under reduced pressure and the crude product was purified by column chromatography (hexanes/EtOAc; 2:1) to give a white solid, 140 mg, 58%. Mp: 70-71°C; IR (Diamond, cm^{-1}): 2852, 1677; ^{1}H-NMR (400 MHz, DMSO-d6): δ 10.30 (s, 1H), 8.60 (m, 1H), 7.87 (m, 1H), 7.71 (d, *J* = 8.7 Hz, 1H), 7.64 (d, *J* = 7.8 Hz, 1H), 7.37 (m, 1H), 6.81 (d, *J* = 2.2 Hz, 1H), 6.68 (dd, *J* = 8.7, 1.8 Hz, 1H), 5.36 (s, 2H), 3.85 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d6): δ 187.3, 165.9, 156.0, 149.1, 137.1, 130.8, 123.1, 121.7, 118.5, 107.2, 99.6, 70.9, 55.8. MS (ESI) *m/z* found 274.15 $(M + H)^+$, 296.14 $(M + Na)^+$. The purity of the compound was checked by HPLC and was found to be 99.8% pure.

Synthesis of 5-methoxy-2-(pyridine-4-ylmethoxy)benzaldehyde, *SAJ-270*:

Target compound was prepared as previously described with only slight modification[26] as a white solid. Mp: 56-57 °C; IR (Diamond, cm^{-1}): 2861, 1672; ^{1}H-NMR (400 MHz, DMSO-d6): δ 10.45 (s, 1H), 8.58 (dd, *J* = 4.6, 1.4 Hz, 1H), 7.50 (d, *J* = 5.8 Hz, 2H), 7.24 (m, 3H), 5.31 (s, 2H), 3.76 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d6): δ 188.9, 154.6, 153.5, 149.8, 145.7, 125.0,122.8, 121.7, 115.8, 110.8, 68.7, 55.6. MS (ESI) *m/z* found 266.0789 $(M + Na)^+$, 298.1054 $(M + MeOH + Na)^+$. The purity of the compound was checked by HPLC and was found to be 99.2% pure.

Synthesis of 5-methoxy-2-(pyridin-2-ylmethoxy)benzaldehyde, *SAJ-310*:

Target compound was prepared as previously described with only slight modification[26] as an off-white crystal. Mp: 111-112 °C; IR (Diamond, cm^{-1}): 2869, 1671; ^{1}H-NMR (400 MHz, DMSO-d6): δ 10.44 (s, 1H), 8.58 (d, *J* = 4.6 Hz, 1H), 7.87 (m, 1H), 7.60 (d, *J* = 7.8 Hz), 7.35 (m, 1H), 7.25 (m, 3H), 5.31 (s, 2H), 3.76 (s, 3H); ^{13}C-NMR (100 MHz, DMSO-d6): δ 188.9,

156.3, 154.9, 149.1, 137.0, 125.0, 123.0, 122.9, 121.6, 116.0, 110.5, 71.5, 55.5. MS (ESI) *m/z* found 266.0790 $(M + Na)^+$, 298.1054 $(M + MeOH + Na)^+$. The purity of the compound was checked by HPLC and was found to be 99.5% pure.

4.2. Crystallization, Data collection and Structure determination of liganded Hb in complex with SAJ-310:

Freshly made solution of SAJ-009, SAJ-270 or SAJ-310 in DMSO was added to deoxygenated (deoxy) Hb (30mg/ml protein) at Hb tetramer-compound ratio of 1:10. Following, the respective mixture was saturated with carbon monoxide and allowed to incubate for 2h to form the COHb-compound complex. Sodium cyanoborohydride ($NaBH_3CN$) was then added to this mixture to reduce the Schiff-base interaction formed between the aromatic aldehyde of the compound and the protein to the corresponding irreversible alkylamine covalent bond. The resulting solution was crystalized using 10-20% PEG6000, 100mM HEPES buffer (pH 7.4) using the batch method. Cherry red needle crystals were obtained for all complexes; however, those of SAJ-009 and SAJ-270 were very thin and unusable for X-ray data collection, while crystals of SAJ-310 were relatively bigger that resulted in useful X-ray diffraction data. Full X-ray diffraction data for the SAJ-310 crystal was collected at 100° K using a Molecular Structure Corporation (MSC) X-Stream Cryogenic Cooler System (The Woodlands, TX) and an R-Axis IV image plate detector. The crystals were first cryoprotected with 80 μL mother liquor mixed with 62 μL 50% PEG6000. The dataset was processed with the d*trek software (Rigaku) and the CCP4 suite of programs.[30] The crystals are in the space group $P2_12_12_1$ with the typical cell constant of a=62.82, b=83.55, c=104.91. The crystal structure of the SAJ-310 complex was determined by a molecular replacement method with the Phenix program,[31,32] using the native R2-state crystal structure (PDB ID code 1BBB) as a search model.[4] The initial refinement using

Phenix identified two molecules of SAJ-310 bound at the α-cleft of the central water cavity, oxygen molecules bound at all four distal heme sites, and several water molecules. These molecules were added to the model, followed by several cycles of refinements with the CNS program[33] to a final Rfactor/Rfree of 20.76%/26.86% at 1.95 Å. Model building and correction were carried out using COOT.[34] The atomic coordinates and structure factors of SAJ-310 have been deposited in the RCSB Protein Data Bank with the accession code 6BNR. Detailed crystallographic parameters are reported in Table 1.

4.3. Time dependent and Concentration Dependent Adduct Formation using Normal Whole Blood and Hemoglobin:

The compounds SAJ-009, SAJ-270, SAJ-310, and the controls INN-312 and vanillin were used to conduct concentration-dependent and time-dependent studies on Hb adduct formation using normal whole blood. The study was performed in a 96-well deep well plate (Thermo Scientific), where each compound at 0.5 mM, 1.0 mM and 2.0 mM concentrations was added to 600 μL of whole blood (30% hematocrit) or Hb (1.56 mM) and incubated at 37°C for 24 h with shaking (at 140 rpm). At 0.5, 1, 2, 4, 8, 12 and 24 h time intervals, a 50 μL aliquot of blood/Hb was removed from each well using a multichannel pipet and added to respective tubes containing 50 μL of Na-cyanoborohydride and borohydride mixture (1:1 v/v 50mM stock) to terminate the Schiff-base reaction, fix the Schiff-base adducts, and reduce the free reactive aldehyde.[35] We have previously optimized these conditions. After mixing, the tubes were stored immediately at -80°C until ready for analysis using cation-exchange HPLC (Hitachi D-7000 Series, Hitachi Instruments, Inc., San Jose, CA) as previously described.[9,11] The observed Hb adduct in %Hb modification were plotted as a function of time (h); peak adduct concentration was obtained by visual inspection, and area under the curve, AUC (0-24), was obtained by linear trapezoidal rule across the entire 24 h measurement period.

4.4. Hemoglobin Modification, Oxygen Equilibrium and Antisickling Studies Using Human Sickle Blood

Blood suspensions from subjects with homozygous SCD (hematocrit 20%) were incubated under air in the absence or presence of 0.5 mM, 1.0 mM and 2.0 mM concentration of SAJ-009, SAJ-270, SAJ-310and the controls INN-312 and vanillin at 37°C for 1 h to ensure that binding has attained equilibrium. Following, the suspensions were incubated under hypoxic condition (4% oxygen/96% nitrogen) at 37°C for 2 h. Aliquot samples were fixed with 2% glutaraldehyde solution without exposure to air, and then subjected to microscopic morphological analysis of bright field images (at 40x magnification) of single layer cells on an Olympus BX40 microscope fitted with an Infinity Lite B camera (Olympus), and the coupled Image Capture software. The residual samples were washed in phosphate-buffered saline, and hemolyzed in hypotonic lysis buffer for subsequent analyses.

For the OEC study, approximately 100 μL aliquot samples from clarified lysate obtained from the antisickling study were added to 4 ml of 0.1 M potassium phosphate buffer, pH 7.0, in cuvettes and subjected to hemoximetry analysis using Hemox^{TM} Analyzer (TCS Scientific Corp.) to assess P_{50} shifts. Degree of P_{50} shift (ΔP_{50}) was expressed as percentage fractions of control DMSO-treated samples. Finally, for the Hb adduct formation study, clarified lysates, also from the above antisickling study, were subjected to cation-exchange HPLC (Hitachi D-7000 Series, Hitachi Instruments, Inc., San Jose, CA), using a weak cation-exchange column (Poly CAT A: 30 mm x 4.6 mm, Poly LC, Inc., Columbia, MD). A commercial standard consisting of approximately equal amounts of composite Hb F, A, S and C (Helena Laboratories, Beaumont, TX), was utilized as reference isotypes. The areas of new peaks, representing Hb S adducts were obtained, calculated as percentage

fractions of total Hb area, and reported as levels of modified Hb. All assays were conducted in three biological replicates on different days.

Statistical Analyses

All functional and biological assays evaluating antisickling properties, Hb modification and oxygen affinity changes were conducted in three biological replicates. Results are reported as mean values with standard deviations, from triplicate analyses.

Accession Codes

The atomic coordinate and structure factor files have been submitted to the Protein Data Bank under an accession code 6BNR.

Acknowledgements

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