

COMPARATIVE STUDIES ON ANTISICKLING PROPERTIES OF THIOCYANATE, TELLURITE AND HYDROXYUREA

Oyewole, OI¹, Malomo, SO², Adebayo, JO³

ABSTRACT

Objective: Thiocyanate, hydroxyurea and tellurite are among chemical agents being used as antisickling drugs and currently receiving attention for research. The antisickling properties of these drugs was investigated and compared in this study.

Methodology: Human sickle blood was incubated with the drugs *in vitro* at concentrations related to the dose used by patients *in vivo*. Haemoglobin function and specific aspects of the sickling process were then measured by employing standard methods used in screening potential antisickling agents.

Results: All the drugs significantly inhibited ($P < 0.05$) sickling of deoxygenated sickle blood and formation of irreversibly sickled cell in a dose and time-dependent manner. Thiocyanate, hydroxyurea and tellurite inhibited sickling optimally at 20mM, 40mM and 50 μ M respectively. Thiocyanate and hydroxyurea prolonged sickle red blood cell life span as indicated in the significant decrease in haemolysis and osmotic fragility while tellurite increased these blood parameters. The three drugs also caused significant prolongation of delay time of haemoglobin S (HbS) polymerization while thiocyanate and hydroxyurea significantly increased ($P < 0.05$) both solubility ratio and oxygen affinity of HbS.

Conclusion: Results obtained in this study suggest that the three drugs have remarkable antisickling potential *in vitro* with thiocyanate being the most efficient followed by tellurite.

KEYWORDS: Antisickling properties, Hydroxyurea, Sodium thiocyanate, Potassium tellurite, Sickle blood.

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1. Oyewole OI, Ph.D, Department of Biochemistry, College of Medicine and Allied Health Sciences, University of Sierra Leone, Freetown.
 2. Malomo SO, Ph.D
 3. Adebayo JO, Ph.D
- 2-3: Department of Biochemistry, University, Ilorin, Ilorin, Nigeria.

Correspondence

Dr. Oyewole OI,
Department of Biochemistry,
College of Medicine and Allied Health Sciences,
University of Sierra Leone,
Freetown.
E-mail: ioluoye@yahoo.com

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INTRODUCTION

Sickle cell disease is a genetic blood disorder which occurs as a result of the presence of abnormal haemoglobin in the red blood cell of an individual. It is the most widespread and clinically significant disorder of haemoglobin, having a high frequency in Africa and the Middle East and among descendants of Africans in the Western world.¹ The abnormal haemoglobin S (HbS) differs from the normal haemoglobin A (HbA) in the sixth amino acid of the beta chain where valine is substituted for glutamic acid. This substitution place a non-polar residue on the outside of HbS and markedly reduce the solubility leading to sickling and polymer formation at low oxygen tension.² Some of the factors leading to clinical manifes-

tations of sickle cell disease have been attributed to functional abnormalities of sickle red blood cell which includes; reduced solubility and oxygen affinity, formation of irreversibly sickled cells (ISC), increased osmotic fragility, autohaemolysis and methaemoglobin formation.

Some promising antisickling agents earlier investigated caused haemolysis of sickle red blood cell at their effective dose levels and are therefore unsuitable for clinical use.³ Hydroxyurea, tellurite and thiocyanate are among the long list of chemical agents currently being used as antisickling drugs and undergoing intensive studies.⁴ The present study intend to compare the antisickling actions of these drugs and their effects on selected red blood cell parameters so as to further understand their mechanisms of action.

MATERIALS AND METHODS

Human sickle blood was obtained from patients who attended regular Clinic at the Haematology Department, University of Ilorin Teaching Hospital, Ilorin, Nigeria. In all cases, patients' consent was sought and approval for the study obtained from the Ethical Committee of the College of Medicine University of Ilorin, Nigeria. Sodium thiocyanate and potassium tellurite salts were products of Hopkins and Williams Ltd. Essex, England. Hydroxyurea is a product of British Drug House (Chemicals) Ltd., Poole England.

Sickling Inhibition Experiment: The procedure employed was based on the microscopic method described by Iyamu *et al.*⁵ 0.1ml blood sample was pipette into 1.0ml Hemox buffer (135mM NaCl, 5mM KCl and 30mM Tris, pH 7.4). Deoxygenation was achieved by adding 0.1ml 45mM sodium dithionite followed by the addition of 0.1ml drug sample. The mixture was incubated at 37°C for one hour after which samples were collected (with minimum exposure to air) and immediately fixed with buffered saline (130mM NaCl plus 20mM NaH₂PO₄ containing 2% glutaraldehyde). It was then mounted on a phase contrast microscope (x100 magnification) and viewed by two observers

who counted at least 100 cells to determine the number of sickle and normal cells. Cells were considered to be normal if they approximated the shape of a biconcave disc while those with elongated shape, star or wrinkle shape (with or without spike) are regarded as abnormal. For the ISC count, sample were exposed to air for 30 minutes before fixing in buffered saline.

Percent sickle cell =

$$\frac{\text{Number of sickle cells} \times 100}{\text{Total number of cells counted}}$$

Measurement of other Antisickling Properties:

Measurement of autohaemolysis and osmotic fragility of sickle red blood cell was performed following the procedure of Wolf.⁶ The method developed by Chang *et al.*⁷ was used for the measurement of HbS relative solubility ratio. Delay time of HbS polymerization was measured by the method described by Schechter *et al.*⁸ The method described by Benesch *et al.*⁹ was employed for the determination of percent oxyhaemoglobin and methaemoglobin.

Statistical Analysis: Data was analyzed using Duncan multiple range test following one-way analysis of variance (ANOVA) using SPSS 10.0 computer software package (SPSS Inc; Chicago, U.S.A). Differences at P<0.05 were considered significant.

RESULTS

The optimum sickling inhibitory concentration for thiocyanate, hydroxyurea and tellurite was found to be 20mM, 40mM and 50µM respectively. These are the minimum concentrations required to achieve maximum inhibition of sickling within one hour. Fig-1 show the time-dependent inhibition of sickling by the three antisickling agents. Blood incubated with thiocyanate had the least number of sickle cells (38%) compared to tellurite (50%) and hydroxyurea (63%) after two hours. Untreated sample had 93% sickle blood at the end of the incubation period. With respect to the inhibitory effect of the antisickling agents on ISC formation (Fig-2), the percentage ISC was reduced from 28% in the untreated blood to 14.1% in thiocyanate-treated blood, 16% in tellurite-

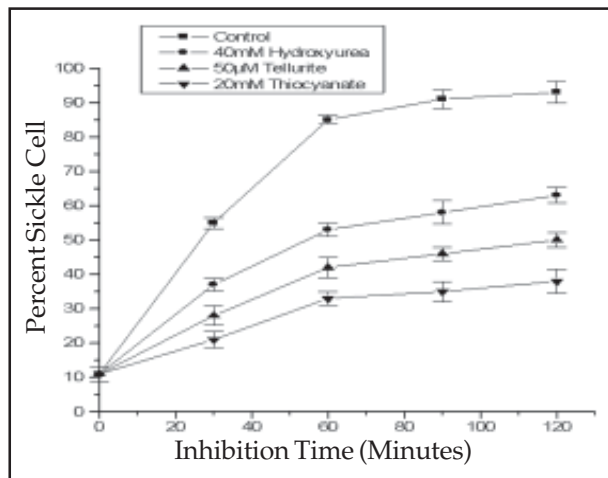


Fig-1: Time-Course inhibition of sickling of human sickle blood by Hydroxyurea, Tellurite and Thiocyanate

treated blood and 19.5% in hydroxyurea-treated blood after two hours 30 minutes of incubation.

Fig-3 shows that the osmotic fragility curve of sickle blood red cell was shifted to the left in the presence of tellurite while thiocyanate and hydroxyurea shifted it to the right. The effect of the drugs on some red blood cell indices is shown in Table-I. Tellurite significantly increased ($P<0.05$) red cell haemolysis by 18.6% compared to the control while thiocyanate and hydroxyurea significantly reduced it by 30.7% and 19.6% respectively. Thiocyanate and hydroxyurea significantly increased ($P<0.05$)

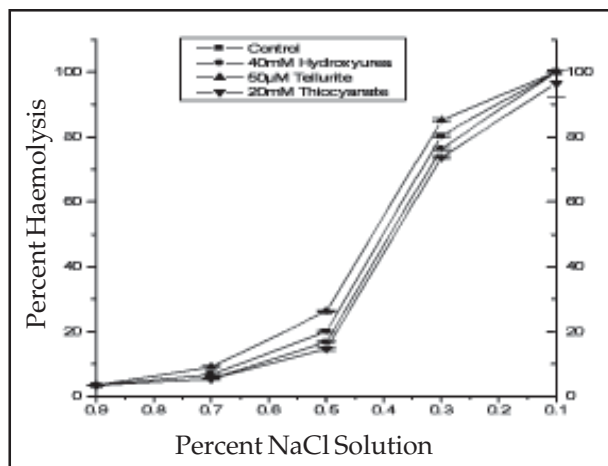


Fig-3: Effect of Hydroxyurea, Tellurite and Thiocyanate on Osmotic fragility of sickle red blood cell.

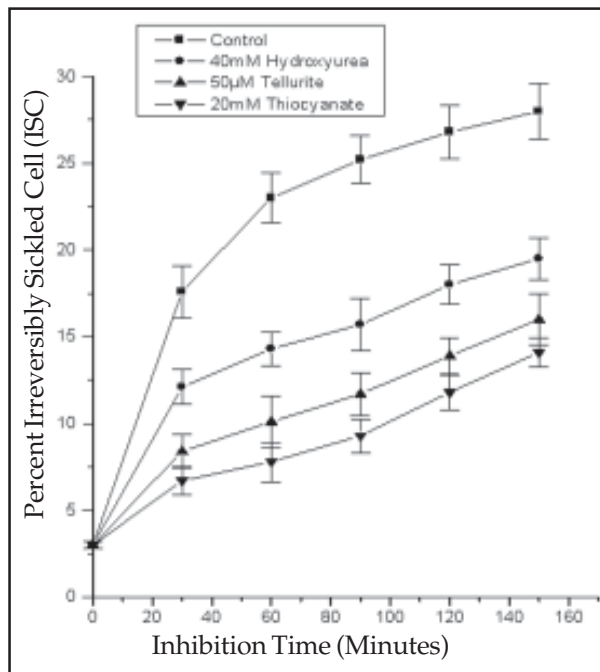


Fig-2: Inhibition of Irreversibly Sickled Cell (ISC) formation in sickle blood by Hydroxyurea, Tellurite and Thiocyanate.

percent oxyhaemoglobin by 111.0% and 39.0% respectively compared with the control while oxyhaemoglobin content was not affected by treatment with tellurite. In addition, tellurite and hydroxyurea significantly stimulated ($P<0.05$) methaemoglobin production by 61.5% and 45% respectively compared with the control. HbS solubility was significantly enhanced ($P<0.05$) in the presence of thiocyanate (130%) and hydroxyurea (54%) compared with the control while tellurite did not alter HbS solubility. All the drugs caused significant prolongation ($P<0.05$) of delay time of HbS polymerisation (tellurite 156%, thiocyanate 114% and hydroxyurea 71.7%) compared with the control as seen in Table-I.

DISCUSSION

In vitro Inhibition of Sickling: The abnormal shape of sickle erythrocyte in the deoxygenated state was markedly improved as shown by the significant reduction in percentage sickle cell upon incubation with the drugs. This sickling inhibition was found to be dose and time dependent. Thiocyanate appears to be the most

Table-I: Some Red Blood Cell Indices of Human Sickle Blood Incubated with Hydroxyurea, Tellurite and Thiocyanate

Indices	Control	Hydroxyurea	Tellurite	Thiocyanate
Oxyhaemoglobin concentration (%)	24.10±2.74 ^a	33.50±2.48 ^b	24.00±2.73 ^a	50.80±2.60 ^c
Methaemoglobin concentration (%)	1.95±0.12 ^a	2.83±0.10 ^b	3.15±0.13 ^c	1.97±0.09 ^a
Degree of haemolysis (%)	6.52± 0.10 ^a	5.24± 0.13 ^b	7.73± 0.18 ^c	4.52± 0.12 ^d
HbS solubility ratio	1.00±0.035 ^a	1.54±0.058 ^b	1.02±0.061 ^a	2.30±0.095 ^c
Delay time of HbS polymerization (minute)	11.30±1.50 ^a	19.40±2.20 ^b	29.00±1.80 ^c	24.20±2.10 ^d

Values are Mean ±SD, n=6. Values with different superscripts a, b, c and d along a row are significantly different at p<0.05.

efficient among the three antisickling drugs *in vitro* as the blood incubated with thiocyanate had the least number of sickle cells at the end of the incubation period. This might be because thiocyanate has a direct covalent modification effect on haemoglobin. Thiocyanate reacts with the amino terminal valine residue of haemoglobin and carbamylate it.¹⁰ When haemoglobin S is carbamylated, it is maintained in the R form which is not susceptible to sickling.

The mode of action of tellurite has been suggested to be by its ability to cause remarkable red blood cell swelling leading to cell hydration¹¹ while hydroxyurea has been suggested to act *in vitro* by reacting with haemoglobin to form nitrosyl haemoglobin, a modification which prevent red blood cell sickling.¹² The significant decrease (P<0.05) in percent ISC upon incubation with the drugs indicated that they might have a positive effect in maintaining membrane integrity and normal shape of red blood cells during sickling and thereby make them to regain their shape upon reoxygenation. Formation of irreversibly sickled cells has been attributed to conditions such as permanent membrane damage, calcium ion accumulation, repeated sickling-unsickling, ATP depletion and dehydration in some proportion of the red blood cell.¹³

Autohaemolysis and Osmotic Fragility: The degree of haemolysis and osmotic fragility of sickle red blood cell was significantly increased in the presence of tellurite while thiocyanate and hydroxyurea reduced these blood parameters. These results indicated that red blood cell becomes more fragile in the presence of tellurite and may induce haemolytic anaemia in

sickle cell patients while thiocyanate and hydroxyurea increased red cell life span. *In vitro* studies have demonstrated that tellurite (Te⁴⁺) ions can penetrate the erythrocyte membrane and, in the presence of reduced glutathione, form telluride (Te²⁺) which causes irreversible membrane damage and hence haemolysis.¹¹ Thiocyanate has the highest inhibitory effect on autohaemolysis and osmotic fragility probably because it carbamylates haemoglobin, a modification which increased red blood cell life span.¹⁴

Oxyhaemoglobin Content and HbS Solubility: The observed significant increase in percent oxyhaemoglobin and HbS solubility of sickle blood treated with thiocyanate and hydroxyurea is a proof that the two drugs have direct interaction with haemoglobin. Polymerisation and subsequent sickling of HbS is favoured when haemoglobin is deoxygenated. Rodgers¹⁵ suggested that one approach to solving the problem of sickle cell patients is to increase the oxygen affinity of their haemoglobin to such an extent that the proportion of deoxyHbS at tissue-oxygen tension is very low so that polymerisation and sickling does not occur. Chemical compounds that modify haemoglobin and increase its oxygen affinity and solubility will help to reduce the degree of sickling at any given partial pressure of oxygen.¹⁶

Thiocyanate modifies haemoglobin by irreversible carbamylation mainly of the N-terminal amino groups of all four chains with resulting increase in oxygen affinity.¹⁰ This may be as a result of stabilization of the oxy conformation of the haemoglobin molecule. Evidence has been presented that the amino terminal

valine residues form intramolecular salt bridges when haemoglobin is in the deoxy conformation.¹⁷ The presence of the uncharged carbamyl group on the amino terminal valine residue may interfere with this salt bridge and stabilize the oxy conformation.¹⁸ The formation of nitrosohaemoglobin by hydroxyurea might also account for the observed increase in oxygen affinity and solubility in hydroxyurea-treated blood.¹²

Methaemoglobin Formation: The inducement of methaemoglobin production in sickle blood caused by tellurite & hydroxyurea indicated a non-beneficial effect of the two drugs for sickle cell patients. This is because sickle blood is characterized by elevated production of MetHb, an abnormal brown pigment, which does not bind oxygen and has been reported to cause health risk and play important role in shortening the life span of sickle red blood cells.¹⁹

Delay Time of HbS Polymerization: The observed significant prolongation of delay time of HbS polymerization caused by the three drugs indicated their suitability as antisickling agents. Measurement of delay time has been suggested to be the most reliable tool in assessing the effectiveness of a potential antisickling agent. This is because it makes it possible to know how much inhibition of polymerization would be necessary to get a therapeutic effect in patients.²⁰

CONCLUSION

Results obtained from this study indicated that the three drugs are potent antisickling agents as evident from their remarkable ability to inhibit sickling of deoxygenated sickle red blood cells *in vitro*. Thiocyanate and hydroxyurea increased red cell life span *in vitro* while tellurite caused red blood cell osmotic fragility and may induce haemolytic anaemia in sickle cell patients. Thiocyanate and hydroxyurea interact directly with sickle red blood cell as indicated by increased solubility and oxygen affinity of sickle red blood cell in the presence of the two drugs. Thiocyanate appears to be the most efficient out of the three drugs tested followed by tellurite.

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