DEVELOPMENT OF A NEW STRATEGY FOR ANTIMALARIAL DRUGS

Principal Investigators: Prof. Shalom Sarel and Prof. Dan T. Spira
Grantee Institution: The Hebrew University of Jerusalem

Collaborators:
Dr. Shelly Avramovici-Grisaru, The Hebrew University of Jerusalem
Dr. Nipon Wongvisetsirikul, King Mongut’s Institute of Science and Technology, Bangkok, Thailand

Project Number: CDR Program C-7160
Grant Number: DPE-5544-G-SS-7021-00

A. I. D. Grant Project Officer: Joyce E. Frame

Project Duration: 8/12/87 - 2/11/92
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Content</td>
<td>I</td>
</tr>
<tr>
<td>Summary</td>
<td>II</td>
</tr>
<tr>
<td>Research Objectives</td>
<td>1-3</td>
</tr>
<tr>
<td>Methods and Results:</td>
<td></td>
</tr>
<tr>
<td>Synthetical Methodologies</td>
<td>4-17</td>
</tr>
<tr>
<td>Inhibition of Ribonucleotide Diphosphatase Reductase</td>
<td>18-24</td>
</tr>
<tr>
<td>Inhibition of Growth of <em>Plasmodium falciparum</em></td>
<td>25-35</td>
</tr>
<tr>
<td><em>ab initio</em> Calculations of Proton Binding</td>
<td></td>
</tr>
<tr>
<td>Energies, and Distribution of Electronic Charges</td>
<td>36-38</td>
</tr>
<tr>
<td>Proton Binding Constants and Species Distribution</td>
<td>38-46</td>
</tr>
<tr>
<td>over pH</td>
<td></td>
</tr>
<tr>
<td>Complex Formation Constants with Fe(II) and Fe(III)</td>
<td>47-56</td>
</tr>
<tr>
<td>Redox Potentials of Some Antimalarials</td>
<td>57-58</td>
</tr>
<tr>
<td>Determined by Cyclovoltametry</td>
<td></td>
</tr>
<tr>
<td>Project Activities</td>
<td>59-60</td>
</tr>
<tr>
<td>Impact, Relveance and Technology Transfer</td>
<td>60</td>
</tr>
<tr>
<td>Project Productivity</td>
<td>61</td>
</tr>
<tr>
<td>Future Work</td>
<td>61</td>
</tr>
<tr>
<td>Bibliography</td>
<td>62-63</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
</tr>
<tr>
<td>Reprints of Four Published Articles</td>
<td></td>
</tr>
</tbody>
</table>
Executive Summary

Some 70 chelating agents based on pyridoxal and salicylaldehyde were prepared according to standard methods, and their structures characterized and identified by elementary analysis, and chemical spectroscopy (IR, UV, NMR, MS).

About 30 chelators were tested against ribonucleotide diphosphate reductase. Depending on structure, the compounds ranged from essentially non-active, to significant inhibitors, relative to hydroxyurea as reference compound. Their relevance to biological properties is herein discussed.

Some 35 compounds were tested against Plasmodium falciparum (FCR-3) maintained in human A+ erythrocytes. Compounds (13) and (29) were outstanding in their ability to kill the drug-resistant malaria parasites at concentrations as low as 5-10 μM within 24 hrs., with IC$_{50}$ values of 1.2 μM and 1.9 μM, respectively. This interesting discovery prompted a study of the mechanism of action. Towards this end we secured pertinent data as follows: i) Fe$^{2+}$ and Fe$^{3+}$ ions blocked the inhibitory effect of the chelators; ii) early removal of cheletable iron from the erythrocyte-parasite complex renders protection to the parasite, indicating that iron is essential for the parasiticidal action, in other words, the antimalarial activity is iron-dependent.

Ligand (29) exhibit moderate affinity for Fe(II), and much stronger affinity for Fe(III). Study has shown that the Fe(II)-, and Fe(III)-complexes differ considerably in their sensitivity towards pH. Whereas the 1:1-ligand-Fe(II) is stable in the pH range 4-9, 1:1-Fe(III)-ligand is stable only at pH < 6. At pH > 6, it transforms into corresponding 1:2-Fe(III)-complex.

ESR measurements indicated that 1:1-Fe(II)-chelate of (29), produces C-centered free radicals, both in presence and in absence of living cells. The Fe(III)-complex produces free radicals only in the presence of actively metabolizing cells.

Most significantly, Fe(II)-(29) complex induces breaks both in the molecular λ phage DNA, and in a lower molecular pBR322 supercoiled plasmid DNA. This was reasonably extended to explain the antimalarial action in vitro. This offers an interesting venue to antimalarial drug design implicating intracellular generation of harmful free radicals by iron-dependent inducers. The rapid spread of multi-drug resistant strains of the malarial parasites prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms. It is highly likely that (13) and/or (29), or another analogue could demonstrate other antiparasitic and/or antitumor activity of potential therapeutic utility.
Research Objectives

This project aimed at developing a new chemotherapeutic strategy against the drug-resistant strains of Plasmodium falciparum, the main causative parasite for human malaria. Already in the mid-'80 World Health Organization (WHO) has alarmed the international scientific community over the rapid spreading of a new species of Falciparum malaria into areas such as South America and South East Asia, which hitherto were considered not to be endemic for Falciparum malaria. It is unfortunate that to this date the world medical arsenal does not possess effective weapons to meet this health challenge.

Iron is an essential element to virtually all living organisms. An invading pathogen must compete with its host for iron. If the parasite cannot obtain sufficient iron, its proliferation may be retarded and the infection attenuated. Host defences may involve iron withholding. A decrease in serum iron has been observed in malarial infections in man. This drop in serum iron levels during parasitemia can be regarded as a mechanism of non-specific resistance. Iron chelators have, therefore, been shown to affect the growth and development of parasites both \textit{in vitro} and \textit{in vivo}.

It was not long ago that the antimalarial drug - chloroquine - has been one of the most successful antimicrobial agent ever produced by mankind. Its beneficial effects on public health have been enormous. Now, when chloroquine is not effective any more against the resistant parasite, novel chemical weapons are promptly needed to counter the menace of the dreadful disease.

The guiding idea was to devise a novel antimalarial strategy based on a new mechanism of action, targeted at protozoal processes implicating iron. For good reasons we selected two keynote cell-targets of the parasite, controlling cell-proliferation and cell-survival. One, relates to the metalloenzyme which controls the synthesis of DNA, and the second, to the stability of the DNA as a bio-macromolecule.

The group of ribonucleotide diphosphate reductases (RdR) play a central role in the DNA bio-syntheses, catalyzing the conversion of nucleotide diphosphates, NDPs, to deoxynucleotide diphosphates, dNDPs. The metalloenzymes posses two subunit structures, B$_1$ and B$_2$, in which the B$_2$ subunit posses an unusual bi-nuclear iron center, and an organic tyrosyl radical as cofactors. Inhibition of RdR could be effected by targeting either at the iron-center, via metal-chelation (mode a, see Chart 1), or, at the tyrosyl-radical co-factor, by interaction with an electron-transfer (ET) reductant (mode b, Chart 1).
POSSIBLE MODES FOR ANTIMALARIAL ACTION IMPLICATING Fe-CHEMISTRY

INHIBITION OF DNA SYNTHESIS via Inactivation of Ribonucleotide Diphosphate Reductase (RDPR)

Mode \( a \)

RDPR

\[ \text{B}_2 \text{ subunit possess Fe(III)-O-Fe(III) as cofactor (active)} \]

removal of Fe-O-Fe chelator → APO-RDPR (inactive)

Mode \( b \)

RDPR

possessing as cofactor + e⁻

Unstable-RDPR

O-centered radical

\[ \text{electron-transfer} \]

Mode \( c \)

\[ R^* + DNA \quad \text{Fe-mediated electron-transfer} \quad [R]^* + [DNA]^- \]

[DNA]⁻ → Fragmentation
Another possible mode for antimalarial action implicating iron chemistry is illustrated by mode c. In this mode, the parasite is killed due to a damage to its DNA, caused by a free radical. Through electron-transfer, the DNA macromolecule becomes an highly unstable anionic radical, which fragments as formed.

In the framework of this project, the israeli and the thai partners have produced some 70 structurally related candidates for antimalarial evaluation. They were related to vitamin B₆ (pyridoxal), a co-factor that Nature has invented for playing pivotal role as an electron-sink in numerous critical biochemical pathways (amino-acid metabolism).¹⁸

The drug design aimed at producing new pyridoxal-based chelators, comprised of four distinctly different domains (see, Chart 2), capable of penetrating into the parasite iron-pool, depriving it from its iron, and, in turn, inhibiting the synthesis of DNA in the parasite cell. Moreover, these molecules are capable of internal electron-trasferring, rendering damage to the parasite DNA. The four domains comprise: (a) an electrophoric domain, conferring electron-sink properties; (b) a transition-metal binding domain; (c) a hydrogen binding domain; and (d) a lipophilic domain, endowing facile trans-membrane transport capabilities to the molecule.

Chart II

![Diagram of drug design concept](chart_2.png)
Methods and Results
Synthetical Methodologies

Structurally, the synthetical ligands comprise ten classes of hydrazones of 2-hydroxy aromatic-aldehydes, acyl hydrazones, thioacyl-hydrazones, guanidino-hydrazones, and aryl-hydrazones, outlined in Charts II - VIII. They comprise of two different aromatic rings which are connected by bridges made of hydrazonic moieties. Six of these contain bridges which are capable of transmitting electronic effects, as follows: (i) CH=N-NH (an hydrazone group), (ii) CH=N-NH-CO (an acyl hydrazone group), (iii) CH=N-NH-CO-NH-N=CH, (iv) CH=N-NH-CS-NH-N=CH,
(v) CH=N-NH-[aromatic ring]-NH-N=CH. The remaining four, contain chains of carbon and nitrogen which are incapable of transmitting electronic effects. These are: (vi) CH=N-NH-CO-[CH2]x-CO-NH-N=CH, (vii) CH=N-[CH2]x-N=CH, (viii) CH=N-NHCO-CH2, (ix) CH=N-NHCOO-CH2, and (x) CH=N--[CH2]x-N=CH.

Most of the chelators presented in Charts III-VII were prepared in very good yields following the standard method outlined by equation (3)\textsuperscript{19}. The two-stage pathway, implying sequences (equ. 1) and (equ. 2) were employed for the special cases of the symmetrical bis-hydrazones, compds. 8 (Chart IV) and 67 (Chart VII), and for the unsymmetrical azines, compds. 9 and 19 (Chart IV). The redox active pyridoxylidenium salts, ligands 22 - 31 (Chart V), were produced conveniently by N-alkylation of the respective aryl- and acyl hydrazones following equ. 4\textsuperscript{20-22}. Depending on reaction conditions, the respective O\textsuperscript{2}-acetate (mono), 17 (Chart IV), and O\textsuperscript{1},O\textsuperscript{2}-diacetates, 16 and 18, could be produced according to equ. 5 & 6. Finally, the corresponding 1:1-, and 1:2-transition metal complexes were easily produced according to equ. 7.

Depending on properties (mainly solubility in H\textsubscript{2}O) the chelators were selectively screened for activities against the metalloenzyme - ribonucleotide diphosphate reductase (RdR) - and against the growth of the drug-resistant species of Plasmodium falciparum. The methodologies and results are given in the sequence.

To shed some light on structure-activity-relationship, four representative chelators {PIH, (10), (25), and (29)} were selected for quantum mechanical and thermodynamical studies. These included, ab initio calculations, ionization constants - pKa values, species distribution in aqueous solutions, pH dependence of carbon-13 magnetic resonance spectra, formation constants of the Fe(II) and Fe(III) complexes, and redox potentials by cyclic voltametry.
Methods of Synthesis

(Formation of hydrazones by condensation reaction. Alkylation at pyridinic ring-nitrogen. Acetylations at alcoholic and phenolic moieties of pyridoxal. Complex formation with metals.)

\[
\begin{align*}
\text{OH} & \quad \text{CHO} & + & H_2NNH_2 & \rightarrow & \text{OH} & \quad \text{CH}=\text{NNH}_2 & + & H_2O & \text{(equ. 1)} \\
\text{OH} & \quad \text{CH}=\text{NNH}_2 & + & RCHO & \rightarrow & \text{OH} & \quad \text{CH}=\text{NNH}-R & + & H_2O & \text{(equ. 2)} \\
\text{OH} & \quad \text{CHO} & + & R\cdot\text{NH-NH}_2 & \rightarrow & \text{OH} & \quad \text{CH}=\text{NNH}-R & + & H_2O & \text{(equ. 3)} \\
\text{R} & = & \text{aryl, alkyl, CO-aryl, CO-alkyl}
\end{align*}
\]

\[
\begin{align*}
\text{OH} & \quad \text{CH}=\text{NNH}-R & + & R^1\cdot X & \rightarrow & \text{OH} & \quad \text{R}^1\cdot \text{N} & \quad \text{CH}=\text{NNH}-R & \text{(equ. 4)} \\
R^1 & = & \text{CH}_3, \text{CH}_2\text{CH}_3 & ; & X & = \text{Br, I}
\end{align*}
\]

\[
\begin{align*}
\text{OH} & \quad \text{CH}=\text{NNH}-R & + & (\text{CH}_3\text{CO})_2O & \rightarrow & \text{OH} & \quad \text{CH}=\text{NNH}-R & \text{(equ. 5)} \\
\text{OH} & \quad \text{CH}=\text{NNH}-R & + & (\text{CH}_3\text{CO})_2O & \rightarrow & \text{OH} & \quad \text{CH}=\text{NNH}-R & \text{(equ. 6)} \\
\text{OH} & \quad \text{CH}=\text{NNH}-R & + & M^{2+} & \longrightarrow & \text{OH} & \quad \text{CH}=\text{NNH}-R & \text{(equ. 7)}
\end{align*}
\]
COMPOUNDS PREPARED ACCORDING TO METHODS OUTLINED IN CHARTS III - VII

CHART III
Halogenated Salicyl Aldehyde 2-Pyridyl Hydrazones

1-[3,5-Dichlorosalicylidene]-2-[2'-pyridyl]hydrazine (1)
1-[5'-Bromosalicylidene]-2-[2''-pyridyl]hydrazine (2)
1-[3,5-Dichlorosalicylidene]-2-[2'-N-methylpyridinium]hydrazine Iodide (3)

CHART IV
Pyridoxal Aryl Hydrazones

1-[Pyridoxylidene]-2-[phenyl]hydrazine (4).
1-[Pyridoxylidene]-2-[4'-methoxyphenyl]hydrazine Hydrochloride (5)
1-[Pyridoxylidene]-2-[4'-nitrophenyl]hydrazine (6).
1-[Pyridoxylidene]-2-[4'-carboxyphenyl]hydrazine Hydrochloride (7).
1,1'-[Di-pyridoxylidene]-2,2'-[ortho-xylidene]di-hydrazin (8).
1-[Pyridoxylidene]-2-[5'-nitrofururylidene]hydrazine (9).
1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine (10).
1-[Pyridoxylidene]-2-[2'-pyrimidyl]hydrazine (11).
1-[Pyridoxylidene]-2-[2'-pyrazyl]hydrazine (12).
1-[Pyridoxylidene]-2-[1'-phthalazyl]hydrazine Dihydrochloride (13).
1-[Pyridoxylidene]-2-[6'-puriny]hydrazine Hydrochloride (14).
1-[Pyridoxylidene]-2-[5'-1'H-tetraazolyll]hydrazine: (1) dihydrate (15-I); (2) sulphate (15-II).
1-[Pyridoxylidene-0\textsubscript{1},0\textsubscript{2}-diacetate]-2-[2'-pyridyl]hydrazine (16).
1-[Pyridoxylidene-O\textsubscript{2}-acetate]-2-[2'-pyrimidyl]hydrazine (17).
1-[Pyridoxylidene-O\textsubscript{1},0\textsubscript{2}-diacetate]-2-[2'-pyrimidyl]hydrazine (18).
1-[Pyridoxylidene]-2-[2'-oxo-3'-sulfonic acid naphthylidy]hydrazine (19).
Copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine sulphate (20a)
Di-copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyridyl]hydrazine (20b).
Copper(II)-chelate of 1-[Pyridoxylidene]-2-[2'-pyrimidyl]hydrazine sulphate.
Iron(II)-chelates of 1-[Pyridoxylidene-O\textsubscript{2}-acetate]-2-[2'-pyrimidyl]hydrazine.
CHART V
Pyridoxylidenium Salts of Aryl Hydrazones

1-[N-Methylpyridoxylidenium]-2-[phenyl]hydrazine iodide (22).
1-[N-Methylpyridoxylidenium]-2-[4'-methoxyphenyl]hydrazine iodide (23).
1-[N-Methylpyridoxylidenium]-2-[4'-nitrophenyl]hydrazine iodide (24).
1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25).
The MeOH-Compound of 1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide
1-[N-Methylpyridoxylidenium]-2-[2'-(1'-methylpyridylidene)hydrazine iodide (26).
1-[N-Methylpyridoxylidenium]-2-[2'-pyrimidyl]hydrazine iodide (27).
1-[N-Methylpyridoxylidenium]-2-[4'-methyl-2'-pyrazyl]hydrazine iodide (28).
1-[N-Ethoxycarbonylmethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine Bromide (29).
1-[N-Ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide (30).
Di-[1-[N-Ethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine]-amine Di-chloride (31).
1-[Pyridoxylidene]-2-[3'-chloro-2-pyridyl]hydrazine Hydrochloride (32).
1-[Pyridoxylidene]-2-[5'-chloro-2-pyridyl]hydrazine Hydrochloride (33).
1-[Pyridoxylidene]-2-[3',5'-dichloro-2-pyridyl]hydrazine Hydrochloride (34).
1-[Pyridoxylidene]-2-[3',4',5',6'-tetrachloro-2-pyridyl]hydrazine HCl (35).
1-[Pyridoxylidene]-2-[6'-chloro-4'-pyrimidyl]hydrazine Hydrochloride (36).
1-[Pyridoxylidene]-2-[4'-hydroxy-6'-methyl-2'-pyrimidyl]hydrazine (37).
Iron(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyridyl]hydrazine Bromide (38).
Iron(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide (39).
Copper(II)-chelate of 1-[N-ethoxycarbonylmethyl pyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide (40).

CHART VI
Hydrazidic Derivatives of Pyridoxal

Pyridoxal Semicarbazone Hydrochloride (41).
Pyridoxal Thiosemicarbazone Hydrochloride (42).
Pyridoxal Guanidino-hydrazone (43).
Pyridoxal Carbohydrazone Hydrochloride (45).
Pyridoxal Thiocarbohydrazone Hydrochloride (46).
Pyridoxal Oxamic Acid Hydrazone Hydrochloride (47).
Pyridoxal Oxalic Acid Hydrazone Hydrochloride (48).
Bis-Pyridoxal Carbohydrazone Hydrochloride (49).
Bis-Pyridoxal Thiocarbohydrazone Hydrochloride (50).
Bis-[N-Methylpyridoxylenium] Carbohydrazone Di-iodide (51).
Pyridoxal Adipic Acid Hydrazone Hydrochloride (52).
Bis-Pyridoxal Adipic Acid Hydrazone Di-Hydrochloride (53).

CHART VII
Pyridoxal Acyl Hydrazone

1-[Pyridoxylidene]-2-[2'-furoyl]hydrazine hydrochloride (54)
1-[Pyridoxylidene]-2-[isonicotinoyl]hydrazine. Pyridoxal Isonicotinoyl Hydrazone, (55)
1-[Pyridoxylidene]-2-[4'-quinolino]hydrazine (56).
1-[Pyridoxylidene]-2-[N1'-methylpyridiniumcarbonyllhydrazine Iodide. Pyridoxal N-Methylisonicotinoyl Hydrazone Iodide (57).
1-[N-Methylpyridoxylidenum]-2-[N1'-methylpyridiniumcarbonyllhydrazine Diiodide. Pyridoxal Isonicotinoyl Hydrazone Dimethiodide (58).
5-Bromosalicyl Isonicotinoyl Hydrazone Methiodide (59).
3,5-Dichlorosalicyl Isonicotinoyl Hydrazone Methiodide (60).
1-[5'-Chloro-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyllhydrazine Bromide (61).
1-[5'-Bromo-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyllhydrazine Bromide (62).

2:1-Ligand:copper(II) of 1-[5'-Bromo-salicylidene]-2-[N-ethoxycarbonylmethyl-nicotinoyllhydrazine Sulphate (63).
2:1-Ligand:copper(II) of 1-[5'-Bromo-salicylidene]-2-[isonicotinoyl]hydrazine Formate (64).

The respective 1:1-ligand:copper(II) complex of 1-[pyridoxylidene]-2-[5'-chloro-2'-pyridyl]hydrazine sulphate (65)
1:1-Ligand:copper(II) complexes of 1-[pyridoxylidene]-2-[3',5'-dichloro-2'-pyridyl]hydrazine (66a) and (66b).
1,1'-Bis-[pyridoxylidene]-2-2'-[1,4-phthalazyldi]hydrazine Trisulphate (67).
Bis-pyridoxal Ethylene-Diazone (68).
Pyridoxal 4-Methoxybenzyloxycarbonyl Hydrazone (69).
1-[Pyridoxylidene]-2-[N-pyridiniumacetyl]hydrazine Chloride (70).
**PYRIDOXAL-BASED CHELATORS**

**CHART III**

*Halogenated Salicyl Aldehyde 2-Pyridyl Hydrazones*

1-[3,5-Dichlorosalicylidene]-2-[2'-pyridyl]hydrazine

1-[5-Bromosalicylidene]-2-[2'-pyridyl]hydrazine

1-[3,5-Dichlorosalicylidene]-2-[2'-N-methylpyridylene]hydrazine

**CHART IV**

*Pyridoxal Aryl Hydrazones*

1-[Pyridoxylidene]-2-[phenyl]hydrazine

1-[Pyridoxylidene]-2-[4'-methoxyphenyl]hydrazine

1-[Pyridoxylidene]-2-[4'-nitrophenyl]hydrazine

1-[Pyridoxylidene]-2-[4'-benzene-carboxylic acid]hydrazine

1,1'-[Dipyridoxylidene]-2,2'-[ortho-xylidene]di-hydrazine
CHART IV

Pyridoxal Aryl Hydrazones

1-[Pyridoxylidene]-2-[5'-nitrofururylidene]hydrazine

1-[Pyridoxylidene]-2-[2''-pyridyl]hydrazine

1-[Pyridoxylidene]-2-[2''-pyrimidyl]hydrazine

[Pyridoxylidene]-2-[2'-pyrazyl]hydrazine

1-[Pyridoxylidene]-2-[1'-phthalayl]hydrazine

1-[Pyridoxylidene]-2-[6'-purinyl]hydrazine

1-[Pyridoxylidene]-2-[2''-tetrazolyl]hydrazine

1-[Pyridoxylidene-O^1,O^2-diacetate]-2-[2' pyridyl]hydrazine

1-[Pyridoxylidene-O^2-acetate]-2-[2' pyrimidyl]hydrazine
II-3. **Copper(II) and Iron(II)-chelates of 1-[Pyridoxylidene]-2-[2'-pyridyl]
and [2'-pyrimidyl]hydrazine**

- Copper(II)-chelate of 1-[pyridoxylidene] 2-[2'-pyridyl]hydrazine Sulphate
- Dicopper(II)-chelate of 1-[pyridoxylidene] 2-[2'-pyridyl]hydrazine Dihydroxide
- Iron(II)-chelate of 1-[pyridoxylidene] 2-[2'-pyrimidyl]hydrazine Sulphate
CHART V

Pyridoxal-Betaine Salts of Aryl Hydrazones

1-[N1-Alkylpyridoxylidenium]-2-[Aryl]hydrazine Halides

1-[N-Methylpyridoxylidenium]-2-[phenyl]hydrazine

1-[N-Methylpyridoxylidenium]-2-[4'-methoxy-phenyl]hydrazine

1-[N-Methylpyridoxylidenium]-2-[4'-nitrophenyl]hydrazine

1-[N1-Alkylpyridoxylidenium]-2-[Heteroaryl]hydrazine Halides

1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine Iodide

1-[N-Methylpyridoxylidenium]-2-[2''-N-methyl pyridinium]hydrazine Iodide

1-[N-Methylpyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Iodide

1-[N-Methylpyridoxylidenium]-2-[4'-methylpyrazyl]hydrazine Diiodide

1-[N-Methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine Bromide

1-[N-Methylpyridoxylidenium]-2-[2'-pyrimidyl]hydrazine Bromide
CHART V

1-\([N^1-\text{Alkylpyridoxylidenium}]\)-2-[\text{Heteroaryl}]\text{hydrazine}\) Halides

![Chemical Structures](image)

Di-\([1-[N-\text{Ethylpyridoxylidenium}]\)-2-[2'-pyridyl]\text{hydrazine}\)-amine Dichloride

![Chemical Structures](image)

Iron(II)=chelates of 1-\([\text{Pyridoxylidene}]\)-2-[2'-pyridyl] and [2'-pyrimidyl]\text{hydrazine}\) Sulphates

![Chemical Structures](image)
CHART VI
Hydrazidic Derivatives of Pyridoxal

Pyridoxal Semicarbazone and Thio-semicarbazone

(41) Pyridoxal Semicarbazone
(42) Pyridoxal Thiosemicarbazone
(43) Pyridoxal Guanidinoxyazone

Pyridoxal Carboxydrazone and Thiocarboxydrazone

(44) 11-Sodiumcarboxylpyridoxyl-
denium Semicarbazone Chloride
(45) Pyridoxal Carboxydrazone
(46) Pyridoxal Thiocarboxydrazone

Pyridoxal Semioxamazines

(47) Pyridoxal Oxamic Acid Hydrazone
(48) Pyridoxal Oxalic Acid Hydrazone

Bispyridoxal Carboxydrazone and Thiocarboxydrazone

(49)
(50)
**Bis-[N-Methylpyridoxylenium] Carbohydrazone Di-iodide**

![Chemical Structure](image1)

(51)

**Mono- and Bis-Pyridoxal Adipic Acid Hydrazone**

![Chemical Structure](image2)

(52)

Pyridoxal Adipic Acid Hydrazone

![Chemical Structure](image3)

(53)

Bis-Pyridoxal Adipic Acid Hydrazone

**CHART VII**

**Pyridoxal Acyl Hydrazones**

![Chemical Structure](image4)

(54)

Pyridoxal 2-Furoyl Hydrazone (PFH)

![Chemical Structure](image5)

(55)

Pyridoxal Isonicotinoyl Hydrazone (PIH)
Pyridoxal 4-Quinoline Acyl Hydrazone

Pyridoxal N-Methylisonicotinoyl Hydrazone Iodide

N-Methylpyridoxylenedium N-Methylisonicotinoyl Hydrazone Diodide

5-Bromosalicyl Isonicotinoyl Hydrazine

3,5-Dichloresalicyl N-Methylisonicotinoyl Hydrazine Iodide

-Chlorosalicyl N-Ethoxycarbonylmethyl -isonicotinoyl Hydrazine Bromide

5-Bromosalicyl N-Ethoxycarbonylmethyl -isonicotinoyl Hydrazine Bromide

2:1-Ligand:Cu Complex of 5-Bromosalicyl N-Ethoxycarbonyl methylisonicotinoyl Hydrazine Sulphate
2:1-Ligand:Cu Complex of 5-Bromosalicyl Isonicotinoyl Hydrazine Formate

Bis-Pyridoxal 1,4-Phthalazinyl Dihydrazone Dihydrochloride

Copper(II)-chelate of (34)

Bis-Pyridoxal Ethylene Diazone

Pyridoxal 4-Methoxy-benzyloxy carbonyl Hydrazone

1-[Pyridoxylidene]-2-[N-pyridinium-acetyl]hydrazone Chloride
Inhibition of Ribonucleotide Diphosphate Reductase

Some half of the chelators produced in this project underwent testings of activity against the metalloenzyme ribonucleotide diphosphate reductase (RdR). The enzyme was isolated from a subcutaneously growing murine tumor (sarcoma 180 implanted in $B_6D_2F_3$ male mice).

Tests of activity of the metalloenzyme, RdR, was followed by the method of Takeda and Weber, published in 1981\textsuperscript{24}. Cytidine was separated from deoxycytidine by use of thin-layer-chromatography over a period of 4 hours on cellulose foil. Distribution of radioactivity was evaluated on an automatic thin-layer-chromatography linear analyzer. Hydroxyurea was used as a reference compound through all experiments. The results obtained are assembled in Tables 1-4, and illustrated by Fig. 1.

Biological Relevance of Results

From Tables 1-4, and Fig. 1, it can be seen that on the basis of their ability to inhibit the activity of RdR the chelating ligands could be divided into two distinctly different classes: 1) a group of very weak inhibitors of RdR, related to pyridoxal aryl hydrazones, of which PIH is a representative; 2) a group of strong RdR inhibitors related to pyridoxal pyridyl hydrazone, represented by pyridoxal 2-pyridyl hydrazone (PPH, 10). PIH is essentially non-toxic to the enzyme RdR. However, removal of the carbonyl group from the acyl-hydrazone bridge connecting the two pyridinic rings in PIH (PIH $\rightarrow$ PPH) confers anti-RdR activity to the molecule. Moreover, replacing the pyridyl ring in PPH by 2-pyrimidinic ring enhances activity against RdR. Likewise, chlorination of the pyridinic moiety in PPH (10 $\rightarrow$ 34) decreases effectively the activity of RdR. N-Alkylation of the pyridoxal ring-nitrogen manifests an opposite effect on the activity of the metalloenzyme.

Paradoxically, these trends do not parallel with those on growth of drug-resistant species of \textit{Plasmodium falciparum} (CDR-3). Thus, whereas the non-active PPH becomes highly active against \textit{P. falciparum} as result of N$^1$-alkylation (10 $\rightarrow$ 29), the inhibitory effect of 29 drops below the level of hydroxyurea. Similarly, going from 29 to 30, the antimalarial activity decreases whereas the anti-RdR activity increases. The same occurs on going from PPH to 34.

\textit{The data produced here indicates that there is no direct correlation between the ability of the chelating ligands to inhibit the growth of \textit{Plasmodium falciparum} (CDR-3) in vitro, and their ability to inhibit the action of \textit{RdR}. The anti-RdR activity in the killing process of the parasite appears to be one factor among others.}
<table>
<thead>
<tr>
<th>Chelator No.</th>
<th>Chelator Formula</th>
<th>Molecular Weight</th>
<th>Concentration (mM)</th>
<th>Inhibition of RdR (%)</th>
<th>Chelator Hydroxyurea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$C_{12}H_{10}N_3OCl_2$</td>
<td>282</td>
<td>1.0</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.033</td>
<td>65</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0033</td>
<td>34</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00033</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>$C_{12}H_{10}N_3OBr$</td>
<td>292</td>
<td>0.01</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>$C_{13}H_{12}N_3O_{1.5}Cl_2$</td>
<td>432</td>
<td>0.033</td>
<td>58</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0033</td>
<td>34</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00033</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>$C_{13}H_{15}N_4O_2Cl$</td>
<td>294.5</td>
<td>0.077</td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>$C_{12}H_{13}N_5O_2$</td>
<td>259</td>
<td>1.0</td>
<td>78</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>40</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>30</td>
<td>15</td>
</tr>
<tr>
<td>14</td>
<td>$C_{13}H_{13}N_7O_2$</td>
<td>299</td>
<td>1.0</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.033</td>
<td>72</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0033</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00033</td>
<td>18</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>$C_{17}H_{18}N_4O_4$</td>
<td>342</td>
<td>1.0</td>
<td>65</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>68</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>31</td>
<td>15</td>
</tr>
</tbody>
</table>
Table 2
INHIBITION OF RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE BY CHELATORS
INHIBITION TEST

<table>
<thead>
<tr>
<th>Chelator No.</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>Concentration (mM)</th>
<th>Inhibition of RdR (%)</th>
<th>Chelator Hydroxyurea</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>C_{14}H_{15}N_{5}O_{3}</td>
<td>301</td>
<td>1.0</td>
<td>84</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>45</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>27</td>
<td>15</td>
</tr>
<tr>
<td>18</td>
<td>C_{16}H_{17}N_{5}O_{4}</td>
<td>343</td>
<td>1.0</td>
<td>79</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>43</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>24</td>
<td>15</td>
</tr>
<tr>
<td>20</td>
<td>C_{13}H_{14}N_{4}O_{6}SCu</td>
<td>417.5</td>
<td>1.0</td>
<td>90</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>90</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>21</td>
<td>C_{12}H_{17}N_{5}O_{8}SCu</td>
<td>454.5</td>
<td>1.0</td>
<td>86</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>78</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>38</td>
<td>15</td>
</tr>
<tr>
<td>26</td>
<td>C_{15}H_{21}N_{4}O_{3}l</td>
<td>432</td>
<td>1.0</td>
<td>-</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>27</td>
<td>C_{15}H_{19}N_{4}O_{2}l</td>
<td>414</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>29</td>
<td>C_{17}H_{20}N_{4}O_{4}Br</td>
<td>423</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>30</td>
<td>C_{16}H_{20}N_{5}O_{4}Br</td>
<td>425</td>
<td>1.0</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>76</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>79</td>
<td>15</td>
</tr>
<tr>
<td>Chelator No.</td>
<td>Formula</td>
<td>Molecular Weight</td>
<td>Concentration (mM)</td>
<td>Inhibition of RdR (%)</td>
<td>Chelator</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>-----------------------</td>
<td>----------</td>
</tr>
<tr>
<td>30</td>
<td>C$<em>{16}$H$</em>{20}$N$<em>{5}$O$</em>{4}$Br</td>
<td>425</td>
<td>1.0</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>76</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>79</td>
<td>15</td>
</tr>
<tr>
<td>34</td>
<td>C$<em>{13}$H$</em>{13}$N$<em>{4}$O$</em>{2}$Cl$_{3}$</td>
<td>362.5</td>
<td>1.0</td>
<td>83</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>78</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>79</td>
<td>15</td>
</tr>
<tr>
<td>35</td>
<td>C$<em>{13}$H$</em>{11}$N$<em>{4}$O$</em>{2}$Cl$_{5}$</td>
<td>432.5</td>
<td>1.0</td>
<td>43</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>18</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>37</td>
<td>C$<em>{13}$H$</em>{15}$N$<em>{5}$O$</em>{3}$</td>
<td>289</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>56</td>
<td>C$<em>{18}$H$</em>{17}$N$<em>{4}$O$</em>{3}$Cl</td>
<td>372.5</td>
<td>1.0</td>
<td>--</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.027</td>
<td>63</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0027</td>
<td>32</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00027</td>
<td>11</td>
<td>--</td>
</tr>
<tr>
<td>57</td>
<td>C$<em>{15}$H$</em>{17}$N$<em>{4}$O$</em>{3}$I</td>
<td>428</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>58</td>
<td>C$<em>{16}$H$</em>{20}$N$<em>{4}$O$</em>{3}$I</td>
<td>570</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>60</td>
<td>C$<em>{14}$H$</em>{12}$N$<em>{3}$O$</em>{2}$Cl$_{2}$</td>
<td>452</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>Chelator No.</td>
<td>Chelator Formula</td>
<td>Molecular Weight (g/mol)</td>
<td>Concentration (mM)</td>
<td>Inhibition of RdR (%)</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------</td>
<td>-------------------------</td>
<td>-------------------</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>$\text{C}<em>{17}\text{H}</em>{17}\text{N}<em>{3}\text{O}</em>{4}\text{BrCl}$</td>
<td>441.5</td>
<td>1.0</td>
<td>--</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
<td>77</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.002</td>
<td>10</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0002</td>
<td>18</td>
<td>--</td>
</tr>
<tr>
<td>62</td>
<td>$\text{C}<em>{17}\text{H}</em>{17}\text{N}<em>{3}\text{O}</em>{4}\text{Br}_2$</td>
<td>505</td>
<td>1.0</td>
<td>--</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>63</td>
<td>$\text{C}<em>{34}\text{H}</em>{134}\text{N}<em>{6}\text{O}</em>{11}\text{Br}<em>4\text{S}</em>{0.5}\text{Cu}$</td>
<td>1103.5</td>
<td>1.0</td>
<td>71</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>64</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>35</td>
<td>15</td>
</tr>
<tr>
<td>64</td>
<td>$\text{C}<em>{27}\text{H}</em>{20}\text{N}<em>{6}\text{O}</em>{6}\text{Br}_2\text{Cu}$</td>
<td>749.5</td>
<td>1.0</td>
<td>59</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>28</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>65</td>
<td>$\text{C}<em>{13}\text{H}</em>{13}\text{N}<em>{4}\text{O}</em>{6}\text{Cl}\text{SCu}$</td>
<td>452</td>
<td>1.0</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>65</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>27</td>
<td>15</td>
</tr>
</tbody>
</table>
ACTIVITY OF PYRIDOXAL-BASED CHELATORS IN THE RIBONUCLEOSIDE DIPHOSPHATE REDUCTASE (RdR) INHIBITION TEST

<table>
<thead>
<tr>
<th>Chelator No.</th>
<th>Formula</th>
<th>Molecular Weight</th>
<th>Concentration (mM)</th>
<th>Inhibition of RdR (%)</th>
<th>Chelator Hydroxy urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>C&lt;sub&gt;13&lt;/sub&gt;H&lt;sub&gt;12&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;SCu</td>
<td>486.5</td>
<td>1.0</td>
<td>85</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td>83</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>67</td>
<td>C&lt;sub&gt;24&lt;/sub&gt;H&lt;sub&gt;26&lt;/sub&gt;N&lt;sub&gt;8&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;Cl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>561</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>68</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;H&lt;sub&gt;22&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>358</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td>69</td>
<td>C&lt;sub&gt;17&lt;/sub&gt;H&lt;sub&gt;18&lt;/sub&gt;N&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
<td>344</td>
<td>0.01</td>
<td>0</td>
<td>15</td>
</tr>
</tbody>
</table>
Inhibition of RdR by 10 mM Chelator Relative to 10mM Hydroxyurea (71)
Inhibition of Growth of *Plasmodium falciparum*

**EFFECT OF AQUEOUS SOLUTIONS OF CHELATORS OF VARIOUS CONCENTRATIONS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF THE POTENTIAL DRUGS**

The new series of pyridoxal-based chelators (2) - (70) were tested for their inhibitory effect on the growth of drug-resistant species of Plasmodium falciparum during a period of 2 hours of incubation.

Parasites were synchronized by the gelatin sedimentation method and diluted with fresh red cells to 0.3% parasitemia and 4% haematocrit. They were dispensed into various six-wells macro plates and left to stand for about an hour. The medium was then replaced with 40 μM of the various chelators. The parasites were then incubated in the chelator medium for 2 hours at 37°C. After the 2 hrs. incubation period, the chelator medium was washed out and the parasites washed thrice with RPMI washing solution. They were then cultured in normal growth medium. The concentrations were maintained throughout the three days culture period. Parasite growth was determined by both microscopic slide counts and incorporation of 3H-hypoxanthine.

The chelator solutions were prepared by dissolving the appropriate amount in a minimal volume of double distilled water to give a stock solution of 1 mM. Further serial dilutions were done in RPMI growth medium to give the required working concentrations of 2, 5, 10, and 20 μM.

**EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS IN DIMETHYL SULFOXIDE (DMSO)**

Parasites were synchronized by the gelatin sedimentation method and diluted with fresh red cells to 0.3% parasitemia and 4% haematocrit. They were dispensed into various six-wells macro plates and left to stand for about an hour. The medium was then replaced with 40 μM of the various chelators. The parasites were then incubated in the chelator medium for 2 hours at 37°C. After the 2 hrs. incubation period, the chelator medium was washed out and the parasites washed thrice with RPMI washing solution. They were then cultured in normal growth medium. The concentrations were maintained throughout the three days culture period. Parasite growth was determined by both microscopic slide counts and incorporation of 3H-hypoxanthine.

Because of low solubility in water, chelators no. 4, 5, 9, 11, 17, 18, 24, 29, 38, 55, and 57, were first dissolved in 1 molar aqu. DMSO. They were thereafter diluted with RPMI washing solution to the required working concentrations. The same concentration of DMSO was added to the RPMI washing solution used as the control. First concentration of DMSO in the test solutions was 0.01 M. A positive control was normal RPMI washing solution, namely, without DMSO.
Biological Importance of Results

Structure-Activity-Relationship study (see, Chart VIII) indicated strong electronic influence on antimalarial activity. Thus, the antimalarial activity decreases dramatically on $23 \rightarrow 22 \rightarrow 7$, and $25 \rightarrow 27 \rightarrow 22$ transformations. But, it increases on $24 \rightarrow 9$, and $22 \rightarrow 23$ conversions. Electron-releasing substituents (methoxy group, OCH$_3$) on the aromatic ring enhances the antimalarial activity, whereas electron-withdrawing substituents (NO$_2$, COOH) exerts an opposite effect. Moreover, the $\pi$-electron densities, and the pKa values of the heterocyclic ring of the ligands profoundly affects the antimalarial activity.

From Tables 7-11, and Fig. 2, it can be seen that 1-[pyridoxylidene]-2-[1'-phthalazyl] hydrazine (13), 1-[N-ethoxycarbonylmethylpyridoxylidienium]-2-[2'-pyridyl]hydrazine (29), pyridoxal semicarbazone (41), and pyridoxal oxalic acid hydrazone (48) are outstanding in their ability to inhibit in vitro the growth of the chloroquine-resistant *Plasmodium falciparum* (FCR-3). The corresponding IC$_{50}$ values (concentration causing 50% inhibition of growth) found were:

$$\text{IC}_{50}: \quad (13) = 1.2 \, \mu\text{M}; \quad (29) = 1.9 \, \mu\text{M}; \quad (41) = 14.2 \, \mu\text{M};$$

It is worthy of note that chloroquine, which is effective only against the sensitive species of *Plasmodium falciparum*, has the value IC$_{50} = 0.12 \, \mu\text{M}$, and quinine has the value of IC$_{50} = 0.30 \, \mu\text{M}$.

The results produced here clearly indicate that pyridoxal hydrazones when properly substituted provide novel class of antimalarials operating on distinctly different mode of action as reported earlier in 1990 in a series of three articles.$^{14,25,26}$
Substituent Effects on Growth Inhibition of Dientate and Tridentate Chelators Based on Pyridoxal

<table>
<thead>
<tr>
<th>Inhibition (%) of [(^{3}H)] hypoxanthine Incorporation by Chelators at 40(\mu)M Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dientate</strong></td>
</tr>
</tbody>
</table>
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
88% |
| **Tridentate**                                    |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
55% |
| **Dientate**                                      |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
33% |

<table>
<thead>
<tr>
<th>Dientate &gt; Tridentate &gt; Dientate</th>
</tr>
</thead>
</table>
| dientate \ 61\% \  \\
| dientate \ 28\% \  \\
| inactive, \ 0\% |

Electron-Release Ordering: \(\text{OCH}_3 > H\)
Electron-Withdrawn Ordering: \(H < NO_2 \geq \text{COOH}\)

\(\pi\)-Electron Density Ordering:
\[
\begin{align*}
\uparrow & \quad < \quad \uparrow \\
\text{pentagon} & \quad \text{cyclopentene} & \quad \text{pyridine}
\end{align*}
\]
\(\pi\)-excessive \  \(\pi\)-deficient

<table>
<thead>
<tr>
<th>Basicity</th>
<th>pK(_a), 20°C</th>
</tr>
</thead>
</table>
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
5.23 |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
3.5 |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
1.3 |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
0.6 |
| \[
\begin{align*}
&\text{CH}_3\text{N}-\text{OH} \\
&\text{H} \quad \text{I} \\
&\text{H} \quad \text{I}
\end{align*}
\]  \\
4.93 |
Table 5

INHIBITION OF GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS

<table>
<thead>
<tr>
<th>Chelato No.</th>
<th>FORMULA</th>
<th>MW</th>
<th>SLIDE COUNTS</th>
<th>% PARTISEMIA</th>
<th>(^3)H-HYPOXANTHINE INCORPORATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAY 1</td>
<td>DAY 2</td>
<td>DAY 3</td>
</tr>
<tr>
<td>control (norm. Med)</td>
<td>0.91</td>
<td>1.691</td>
<td>4.86</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C(<em>{12})H(</em>{10})N(_3)OBr</td>
<td>292</td>
<td>0.83</td>
<td>1.91</td>
<td>4.08</td>
</tr>
<tr>
<td>3</td>
<td>C(<em>{13})H(</em>{12})N(_3)O(_1.5)Cl(_2)I</td>
<td>432</td>
<td>0.58</td>
<td>1.41</td>
<td>3.25</td>
</tr>
<tr>
<td>7</td>
<td>C(<em>{15})H(</em>{16})N(_3)O(_1)Cl</td>
<td>337.5</td>
<td>2.16</td>
<td>1.91</td>
<td>14.70</td>
</tr>
<tr>
<td>10</td>
<td>C(<em>{13})H(</em>{15})N(_4)O(_2)Cl</td>
<td>294.5</td>
<td>1.08</td>
<td>1.75</td>
<td>3.25</td>
</tr>
<tr>
<td>13</td>
<td>C(<em>{16})H(</em>{18})N(_5)O(_2.5)Cl(_2)</td>
<td>391</td>
<td>0.58</td>
<td>0.41</td>
<td>0.66</td>
</tr>
<tr>
<td>19</td>
<td>C(<em>{18})H(</em>{17})N(_3)O(_7.5)Na</td>
<td>418</td>
<td>0.66</td>
<td>1.83</td>
<td>3.83</td>
</tr>
<tr>
<td>25</td>
<td>C(<em>{14})H(</em>{17})N(_4)O(_2)I</td>
<td>400</td>
<td>0.41</td>
<td>0.58</td>
<td>1.41</td>
</tr>
<tr>
<td>26</td>
<td>C(<em>{15})H(</em>{21})N(_4)O(_3)I</td>
<td>432</td>
<td>0.75</td>
<td>0.66</td>
<td>2.25</td>
</tr>
<tr>
<td>27</td>
<td>C(<em>{15})H(</em>{19})N(_4)O(_2)I</td>
<td>414</td>
<td>0.83</td>
<td>1.58</td>
<td>2.91</td>
</tr>
<tr>
<td>28</td>
<td>C(<em>{14})H(</em>{20})N(_5)O(_2.5)I(_2)</td>
<td>552</td>
<td>0.66</td>
<td>1.16</td>
<td>2.41</td>
</tr>
<tr>
<td>29</td>
<td>C(<em>{17})H(</em>{20})N(_4)O(_2)Br</td>
<td>423</td>
<td>0.33</td>
<td>0.25</td>
<td>0.58</td>
</tr>
<tr>
<td>30</td>
<td>C(<em>{15})H(</em>{20})N(_5)O(_4)Br</td>
<td>425</td>
<td>0.83</td>
<td>1.33</td>
<td>4.5</td>
</tr>
<tr>
<td>41</td>
<td>C(<em>{9})H(</em>{13})N(_4)O(_3)Cl</td>
<td>260.5</td>
<td>0.91</td>
<td>0.41</td>
<td>0.58</td>
</tr>
<tr>
<td>42</td>
<td>C(<em>{9})H(</em>{13})N(_4)O(_2)SCl</td>
<td>276.5</td>
<td>1.58</td>
<td>2.08</td>
<td>15.70</td>
</tr>
<tr>
<td>44</td>
<td>C(<em>{11})H(</em>{14})N(_4)O(_5)ClNa</td>
<td>330.5</td>
<td>1.91</td>
<td>2.08</td>
<td>16.1</td>
</tr>
</tbody>
</table>
Table 6

EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF *Plasmodium falciparum* AFTER 2 HRS. EXPOSURE TO 40 μM OF CHELATORS

<table>
<thead>
<tr>
<th>Chelato No.</th>
<th>FORMULA</th>
<th>MW</th>
<th>SLIDE COUNTS % PARTISEMIA</th>
<th>³H-HYPOXANTHINE INCORPORATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAY 1</td>
<td>DAY 2</td>
</tr>
<tr>
<td>CONTROL (norm. Med)</td>
<td></td>
<td></td>
<td>0.91</td>
<td>1.83</td>
</tr>
<tr>
<td>45</td>
<td>C₉H₁₄N₅O₃Cl</td>
<td>275.5</td>
<td>1.66</td>
<td>1.66</td>
</tr>
<tr>
<td>46</td>
<td>C₉H₁₄N₅O₂SCl</td>
<td>291.5</td>
<td>1.50</td>
<td>1.25</td>
</tr>
<tr>
<td>47</td>
<td>C₁₀H₁₃N₄O₄Cl</td>
<td>288.5</td>
<td>1.58</td>
<td>1.58</td>
</tr>
<tr>
<td>48</td>
<td>C₁₀H₁₄N₅O₄Cl</td>
<td>303.5</td>
<td>0.33</td>
<td>0.25</td>
</tr>
<tr>
<td>49</td>
<td>C₁₇H₂₂N₆O₅Cl₂</td>
<td>461</td>
<td>1.58</td>
<td>2.16</td>
</tr>
<tr>
<td>50</td>
<td>C₁₇H₂₂N₆O₄SCl</td>
<td>441.5</td>
<td>1.83</td>
<td>1.50</td>
</tr>
<tr>
<td>51</td>
<td>C₁₉H₂₆N₆O₅I₂</td>
<td>672</td>
<td>1.50</td>
<td>1.91</td>
</tr>
<tr>
<td>53</td>
<td>C₁₇H₂₂N₆O₄SCl₂</td>
<td>477</td>
<td>1.25</td>
<td>1.91</td>
</tr>
<tr>
<td>54</td>
<td>C₁₃H₁₃N₃O₄</td>
<td>275</td>
<td>1.58</td>
<td>2.16</td>
</tr>
<tr>
<td>55</td>
<td>C₁₄H₁₅N₄O₃I</td>
<td>322.5</td>
<td>2.08</td>
<td>1.58</td>
</tr>
<tr>
<td>57</td>
<td>C₁₅H₁₇N₄O₃I</td>
<td>428</td>
<td>1.91</td>
<td>2.16</td>
</tr>
<tr>
<td>61</td>
<td>C₁₇H₁₇N₃O₄BrCl</td>
<td>441.5</td>
<td>0.91</td>
<td>1.25</td>
</tr>
<tr>
<td>70</td>
<td>C₁₅H₁₇N₄O₃Cl</td>
<td>336.5</td>
<td>2.16</td>
<td>2.08</td>
</tr>
</tbody>
</table>
Figure 2

Growth inhibition of P. falciparum after 2 hrs. Exposure to 40 mM of Chelator
Table 7

EFFECT OF CHELATORS ON GROWTH OF DRUG-RESISTANT SPECIES OF Plasmodium falciparum AFTER 2 HRS. EXPOSURE TO 40 \( \mu \text{M} \) OF CHELATORS IN DIMETHYL SULFOXIDE (DMSO)

<table>
<thead>
<tr>
<th>Chelato No.</th>
<th>FORMULA</th>
<th>MW</th>
<th>SLIDE COUNTS</th>
<th>(^{3}\text{H}-\text{HYPOXANTHINE INCORPORATION}</th>
<th>% PARTISEMIA</th>
<th>COUNT</th>
<th>% GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>DAY 1</td>
<td>DAY 2</td>
<td>DAY 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>( \text{C}<em>{14}\text{H}</em>{15}\text{N}<em>{3}\text{O}</em>{2} )</td>
<td>257</td>
<td>2.53</td>
<td>3.75</td>
<td>17.25</td>
<td>115726</td>
<td>67</td>
</tr>
<tr>
<td>5</td>
<td>( \text{C}<em>{15}\text{H}</em>{18}\text{N}<em>{3}\text{O}</em>{3}\text{Cl} )</td>
<td>323.5</td>
<td>1.25</td>
<td>2.30</td>
<td>8.25</td>
<td>68214</td>
<td>39</td>
</tr>
<tr>
<td>9</td>
<td>( \text{C}<em>{13}\text{H}</em>{12}\text{N}<em>{4}\text{O}</em>{5} )</td>
<td>304</td>
<td>1.83</td>
<td>3.75</td>
<td>13.5</td>
<td>115715</td>
<td>67</td>
</tr>
<tr>
<td>11</td>
<td>( \text{C}<em>{12}\text{H}</em>{13}\text{N}<em>{5}\text{O}</em>{2} )</td>
<td>259</td>
<td>1.0</td>
<td>1.41</td>
<td>3.58</td>
<td>48939</td>
<td>28</td>
</tr>
<tr>
<td>17</td>
<td>( \text{C}<em>{14}\text{H}</em>{15}\text{N}<em>{5}\text{O}</em>{3} )</td>
<td>301</td>
<td>2.58</td>
<td>3.25</td>
<td>17.75</td>
<td>131861</td>
<td>76</td>
</tr>
<tr>
<td>18</td>
<td>( \text{C}<em>{16}\text{H}</em>{17}\text{N}<em>{5}\text{O}</em>{4} )</td>
<td>343</td>
<td>1.92</td>
<td>3.30</td>
<td>15.50</td>
<td>125781</td>
<td>72</td>
</tr>
<tr>
<td>24</td>
<td>( \text{C}<em>{15}\text{H}</em>{19}\text{N}<em>{4}\text{O}</em>{5}\text{I} )</td>
<td>462</td>
<td>2.41</td>
<td>3.58</td>
<td>16.50</td>
<td>124105</td>
<td>72</td>
</tr>
<tr>
<td>29</td>
<td>( \text{C}<em>{18}\text{H}</em>{24}\text{N}<em>{4}\text{O}</em>{4}\text{Br} )</td>
<td>440</td>
<td>0.40</td>
<td>0.58</td>
<td>1.16</td>
<td>15744</td>
<td>9</td>
</tr>
<tr>
<td>38</td>
<td>( \text{C}<em>{17}\text{H}</em>{22}\text{N}<em>{4}\text{O}</em>{9}\text{Br}\text{SFe} )</td>
<td>594</td>
<td>1.75</td>
<td>3.58</td>
<td>18.25</td>
<td>132132</td>
<td>76</td>
</tr>
<tr>
<td>55</td>
<td>( \text{C}<em>{14}\text{H}</em>{15}\text{N}<em>{4}\text{O}</em>{3}\text{Cl} )</td>
<td>322.5</td>
<td>2.83</td>
<td>3.75</td>
<td>18.25</td>
<td>137085</td>
<td>79</td>
</tr>
<tr>
<td>57</td>
<td>( \text{C}<em>{15}\text{H}</em>{17}\text{N}<em>{4}\text{O}</em>{3}\text{I} )</td>
<td>428</td>
<td>1.58</td>
<td>3.25</td>
<td>18.25</td>
<td>160562</td>
<td>93</td>
</tr>
<tr>
<td>Norm. Medium without DMSO</td>
<td></td>
<td></td>
<td>2.92</td>
<td>3.50</td>
<td>18.50</td>
<td>184826</td>
<td>100</td>
</tr>
<tr>
<td>Norm. Medium with 0.01 M DMSO</td>
<td></td>
<td></td>
<td>2.75</td>
<td>3.83</td>
<td>19.50</td>
<td>172951</td>
<td>100</td>
</tr>
<tr>
<td>COMPOUND</td>
<td>FORMULA</td>
<td>CONCENTRATION</td>
<td>% INHIBITION</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>---------------</td>
<td>--------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(μM)</td>
<td>DAY 1</td>
<td>DAY 2</td>
<td>DAY 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C_{14}H_{15}N_{3}O_{2}</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>--</td>
<td>28</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>41</td>
<td>31</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C_{15}H_{18}N_{3}O_{3}Cl</td>
<td>5</td>
<td>14</td>
<td>3</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>28</td>
<td>9</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>50</td>
<td>44</td>
<td>45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>C_{18}H_{17}N_{3}O_{7.5}Na</td>
<td>5</td>
<td>23</td>
<td>22</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>41</td>
<td>45</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>50</td>
<td>56</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25a</td>
<td>C_{14}H_{17}N_{4}O_{2}I</td>
<td>2</td>
<td>63</td>
<td>83</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>74</td>
<td>83</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>71</td>
<td>91</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>74</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25b</td>
<td>C_{14}H_{21}N_{4}O_{3}I</td>
<td>2</td>
<td>64</td>
<td>79</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>64</td>
<td>83</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>71</td>
<td>87</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>76</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COMPOUND</td>
<td>FORMULA</td>
<td>CONCENTRATION (µM)</td>
<td>% INHIBITION</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-------------</td>
<td>--------------------</td>
<td>--------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>C_{15}H_{19}N_{4}O_{2}I</td>
<td>2 3 5 10 20</td>
<td>33 45 68 87 99</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>C_{14}H_{20}N_{5}O_{2.5}I_{2}</td>
<td>2 5 10 20</td>
<td>0 21 48 57 76 91 89 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>C_{18}H_{24}N_{4}O_{4}Br</td>
<td>2 5 10 20</td>
<td>40 34 58 68 76 40 75 87 100 100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 10
EFFECT OF CONTINUOUS EXPOSURE OF Plasmodium falciparum TO VARIOUS CONCENTRATIONS OF CHELATORS

Inhibition Test

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (mM)</th>
<th>% PARASITEMIA DAY 1</th>
<th>% PARASITEMIA DAY 2</th>
<th>% PARASITEMIA DAY 3</th>
<th>% INHIBITION OF GROWTH ON DAY 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>5</td>
<td>57</td>
<td>50</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>35</td>
<td>31</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>29</td>
<td>5</td>
<td>93</td>
<td>68</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>71</td>
<td>43</td>
<td>33</td>
<td>67</td>
</tr>
<tr>
<td>41</td>
<td>5</td>
<td>93</td>
<td>93</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>93</td>
<td>100</td>
<td>71</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>93</td>
<td>68</td>
<td>67</td>
<td>33</td>
</tr>
<tr>
<td>55</td>
<td>5</td>
<td>78</td>
<td>68</td>
<td>58</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65</td>
<td>44</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>Artemisin</td>
<td>5</td>
<td>21</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>21</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
### Table 11

**EFFECT OF 3 DAYS EXPOSURE OF Plasmodium falciparum TO VARIOUS CONCENTRATIONS OF CHELATORS**

Inhibition Test

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>CONCENTRATION (mM)</th>
<th>3H-HYPOXANTHINE INCORPORATION</th>
<th>INHIBITION %</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>5</td>
<td>4199</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2697</td>
<td>82</td>
</tr>
<tr>
<td>29</td>
<td>5</td>
<td>9390</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6425</td>
<td>55</td>
</tr>
<tr>
<td>41</td>
<td>5</td>
<td>16162</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>17168</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>5</td>
<td>15877</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10621</td>
<td>26</td>
</tr>
<tr>
<td>55</td>
<td>5</td>
<td>9779</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7828</td>
<td>45</td>
</tr>
<tr>
<td>artemisin</td>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Structure-activity-relationship study indicated that the antimalarial activity of ligands included in this investigation appears to be controlled by electronic effects. In view of this we deemed it important to calculate distribution of electronic charges in (29), most promising new antimalarial candidate against chloroquine-resistant species, and in related active compounds. In addition, we undertook the determination of proton binding energies at four sites of ligand (10) for assignment of pKa values derived by potentiometric titrations.

Proton Binding Energies at Ionizable Sites of 1-[Pyridoxylidene]-2-[Pyridyl]hydrazine (10)

SCF Calculations

Ab initio calculations were based on sto-3g set\(^ {28} \). Full optimization was performed for the unprotonated species, L\(^ - \), of (10). For each computation of proton-binding, the optimization was confined to site position of binding. The results of Muliken population analysis\(^ {29} \) and its protonated species were calculated by CNDO/\(^ 2 \)\(^ {30} \), were attested by comparison with data produced for (10) by use of ab initio calculations. The results summerized in Table 12 were obtained from two different modes of calculation. Qualitatively, they are identical. The distribution of electronic charges around the 19-21 atoms in (10), (25), (29), and PIH are presented in Table 13. All calculations were performed by use of IBM RISC/6000 computer of The Hebrew University of Jerusalem.

<table>
<thead>
<tr>
<th>Binding Sites</th>
<th>Total Energy(^ a ) (in atomic units)</th>
<th>Binding Energy(^ b ) (in kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L(^ - )</td>
<td>- 854.33597</td>
<td>---</td>
</tr>
<tr>
<td>O(^ 2 )</td>
<td>- 855.47231</td>
<td>713.05</td>
</tr>
<tr>
<td>N(^ 3 )</td>
<td>- 855.35986</td>
<td>642.49</td>
</tr>
<tr>
<td>O(^ 1 )</td>
<td>- 855.24823</td>
<td>572.44</td>
</tr>
<tr>
<td>N(^ 1 )</td>
<td>- 855.16845</td>
<td>522.38</td>
</tr>
<tr>
<td>N(^ 4 )</td>
<td>- 855.11898</td>
<td>491.34</td>
</tr>
</tbody>
</table>

a) ab initio STO-\( \# \)G basis set calculations; b) The binding energy relative to the deprotonated species L\(^ - \)
Table 13

Distribution of Electronic Charges in (10), (25), (29), and PIH

<table>
<thead>
<tr>
<th>Atom</th>
<th>(10)</th>
<th>(25)</th>
<th>$\Delta^1$</th>
<th>(29)</th>
<th>$\Delta^2$</th>
<th>PIH</th>
<th>$\Delta^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(1)</td>
<td>-0.146</td>
<td>0.034</td>
<td>-0.180</td>
<td>0.032</td>
<td>-0.177</td>
<td>-0.142</td>
<td>-0.003</td>
</tr>
<tr>
<td>C(1)</td>
<td>0.047</td>
<td>0.098</td>
<td>-0.051</td>
<td>0.095</td>
<td>-0.048</td>
<td>0.046</td>
<td>0.001</td>
</tr>
<tr>
<td>C(2)</td>
<td>0.008</td>
<td>0.012</td>
<td>-0.004</td>
<td>0.014</td>
<td>-0.006</td>
<td>0.010</td>
<td>-0.002</td>
</tr>
<tr>
<td>C(3)</td>
<td>-0.026</td>
<td>0.052</td>
<td>-0.078</td>
<td>0.050</td>
<td>-0.076</td>
<td>-0.028</td>
<td>0.002</td>
</tr>
<tr>
<td>C(3)</td>
<td>-0.026</td>
<td>0.052</td>
<td>-0.078</td>
<td>0.050</td>
<td>-0.076</td>
<td>-0.028</td>
<td>0.002</td>
</tr>
<tr>
<td>C(4)</td>
<td>0.167</td>
<td>0.165</td>
<td>0.001</td>
<td>0.167</td>
<td>0.000</td>
<td>0.171</td>
<td>-0.004</td>
</tr>
<tr>
<td>C(5)</td>
<td>0.052</td>
<td>0.113</td>
<td>-0.060</td>
<td>0.111</td>
<td>-0.059</td>
<td>0.051</td>
<td>0.004</td>
</tr>
<tr>
<td>O(1)</td>
<td>-0.250</td>
<td>-0.231</td>
<td>-0.019</td>
<td>0.233</td>
<td>-0.017</td>
<td>-0.249</td>
<td>-0.001</td>
</tr>
<tr>
<td>C(6)</td>
<td>-0.19</td>
<td>-0.037</td>
<td>0.019</td>
<td>-0.039</td>
<td>0.021</td>
<td>-0.019</td>
<td>0.000</td>
</tr>
<tr>
<td>C(7)</td>
<td>0.142</td>
<td>0.140</td>
<td>0.002</td>
<td>0.140</td>
<td>0.002</td>
<td>0.142</td>
<td>0.001</td>
</tr>
<tr>
<td>O(2)</td>
<td>-0.267</td>
<td>-0.264</td>
<td>-0.003</td>
<td>0.265</td>
<td>-0.003</td>
<td>0.266</td>
<td>0.001</td>
</tr>
<tr>
<td>N(3)</td>
<td>-0.161</td>
<td>-0.143</td>
<td>-0.018</td>
<td>0.143</td>
<td>-0.018</td>
<td>-0.157</td>
<td>0.004</td>
</tr>
<tr>
<td>C(9)</td>
<td>0.217</td>
<td>0.208</td>
<td>0.009</td>
<td>0.209</td>
<td>0.008</td>
<td>-0.004</td>
<td>--</td>
</tr>
<tr>
<td>C(10)</td>
<td>-0.083</td>
<td>-0.070</td>
<td>-0.012</td>
<td>-0.071</td>
<td>-0.012</td>
<td>-0.010</td>
<td>--</td>
</tr>
<tr>
<td>C(11)</td>
<td>0.064</td>
<td>0.069</td>
<td>-0.005</td>
<td>0.069</td>
<td>-0.005</td>
<td>0.073</td>
<td>--</td>
</tr>
<tr>
<td>C(12)</td>
<td>-0.055</td>
<td>-0.042</td>
<td>-0.013</td>
<td>-0.043</td>
<td>-0.012</td>
<td>0.072</td>
<td>--</td>
</tr>
<tr>
<td>C(13)</td>
<td>0.102</td>
<td>0.106</td>
<td>-0.004</td>
<td>0.106</td>
<td>-0.004</td>
<td>0.001</td>
<td>--</td>
</tr>
<tr>
<td>N(4)</td>
<td>-0.209</td>
<td>-0.213</td>
<td>0.004</td>
<td>-0.215</td>
<td>0.006</td>
<td>-0.136</td>
<td>0.073</td>
</tr>
<tr>
<td>C(8)</td>
<td>0.062</td>
<td>0.024</td>
<td>-0.037</td>
<td>0.027</td>
<td>-0.034</td>
<td>0.071</td>
<td>0.009</td>
</tr>
<tr>
<td>N(2)</td>
<td>-0.051</td>
<td>0.002</td>
<td>-0.053</td>
<td>0.000</td>
<td>-0.052</td>
<td>-0.047</td>
<td>0.005</td>
</tr>
<tr>
<td>CC(1)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>0.333</td>
<td>--</td>
</tr>
<tr>
<td>O(3)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>-0.336</td>
<td>--</td>
</tr>
</tbody>
</table>

$\Delta^1$ - is the difference in charge between respective atoms of (10) and (25); $\Delta^2$ - is the charge difference between respective atoms of (10) and (29), and $\Delta^3$ relates to similar differences in charge between PIH and (10).
Biological Relevance of Results

From ab initio calculation data assembled in Table 12 it can be seen that the ease of proton removal from the possible binding sites decreases in the ordering:

\[ \text{PYR ring-}N^4 > \text{Pyridoxal-ring } N^1 > O^1H > N^3H > O^2H \]

Thus, the highest binding energy rests between H and alcoholic oxygen (O^2), whereas the binding energy between H and phenolic oxygen (O^1) is 140.61 kcal/mol smaller. The least binding energy is between H and pyridinic ring-N^4, which is 31.04 kcal/mol smaller than the pyridoxyl-N^1, which in turn, is 50.06 kcal/mol smaller than that of the phenolic bond.

Proton Binding Constants and Species Distribution Over pH Range 1.5 - 12.0

Two of the most active antimalarials produced in this study, (13) and (29), consist of two different rings, each of which exists in the aqueous media as several prototropic species. The behaviour of the pyridoxal moiety has been thoroughly analyzed, and this species was shown to be present in aqueous media under at least four pH-dependent structures. ab initio Calculations of the parent compound, (10), have shown that it possesses 4 proton ionizable sites, and, depending on pH, it could exist in aqueous solution in 4 different structural species. We deemed it essential to identify the pH range in which the antimalarial compounds could largely exist in the neutral form adequate for iron chelation. Towards this end we undertook a study aimed at determining the pKa's, and species distribution of (29), and of three related compounds, (10), (25), and (55).

Protonation Constants

Stock solutions.

Nitric acid and sodium hydroxide solutions were obtained from "Merck" (Titrisol). The concentrations of these solutions were checked regularly by acid-base titrations in the course of electrode calibration\(^{31}\). Stock solutions of 0.2 mM of (10), (25), (29), and PIH were freshly prepared before each titration by dissolving the respective ligand in in 0.15 M KNO\(_3\) (Merck Analytical grade). Double-distilled water was used throughout the experiments.
Potentiometric titrations

Potentiometric (pH) titrations were used to determine the protonation constants for ligands (10), (25), (29), and (55). Potentiometric titration data were obtained using a purpose-built automatic titrator of PHM62 Radiometer pH meter, with a 5 ml piston burette, a digital electrometer and stirrer, which all were under the control of a programmable calculator. Potentiometric measurements were carried out in a capped titration vessel, fitted with a GK 2322C combined electrode and a fibre-tipped saturated calomel electrode. The titrations were performed under inert atmosphere by flowing pure N₂ to the titration vessel. The entire apparatus was kept in a thermostat at 37 ± 0.5°C. The electrode calibrations were made in a 0.15 M KNO₃ at 37°C by acid-base titration under identical above conditions.

Analysis of the titration data for (10), (25), (29), and PIH was generally performed using the SUPERQUARD computer program under IBM RISC/6000 computer machine of The Hebrew University of Jerusalem. The data were between 300 and 500 for the calculations.

Species Distribution Plot as a Function of pH

Analysis of the titration curves for (10), (25), (29), and PIH yielded four pKa values for the two unsubstituted ligands, (10) and PIH, and three each for the other two N₁-alkylated ligands, (25) and (29). Plotting of species distribution in solution of PIH, (10), (25), and (29) against pH, in the range pH 1.5-12.5 (Figures 3-4a-c, and Table 14), clearly indicates that at pH < 3, three to four sites on the molecules are protonated. From plots in Fig. 3-4a-c, it can be seen that at pH 5.2, the molecules of PIH and (10) exist in solution as 4:1-mixtures of the cationic species of \( \text{H}_3\text{L}^+ \), and of the neutral form of \( \text{H}_2\text{L} \), respectively, and with the absence of the anionic species, \( \text{HL}^- \). The molecules of (25) and (29), by contrast, do not contain at all the cationic \( \text{H}_2\text{L}^+ \) species, but rather contain to some extent the anionic \( \text{HL}^- \) species, in a 10:1 ratio of \( \text{H}_2\text{L} \) : \( \text{HL}^- \). Most interestingly, at physiological pH (7.2), molecules (10) and PIH in solution exist respectively, as 2:1 and 1:1-mixtures of the neutral \( \text{H}_2\text{L} \) and of the singly charged anionic \( \text{HL}^- \) species, whereas the corresponding ratio for N₁-alkylated molecules, (25) and (29), at the same pH (7.2), is 1:2 (see, Table 14). Evidently, lowering the physiological pH by 2 pH-units, confers predominancy to the neutral forms of the N₁-alkylated molecules, (25) and (29), causing loss of this dominancy to parent molecules, in (10), and in PIH. These results would indicate that the chelating agents would be lipophilic, and furthermore, that (25) and (29), would be able to diffuse through cell membrane, allowing absorption from the stomach and access to intracellular iron pools. In PIH and (10), the presence of the neutral species would be maximal at pH 6 and hence maximal absorption would probably occur in the small intestine where the pH is in this range. The
CHART A1

Pyridoxal Pyridyl Hydrazone
Protonated-Neutral-Deprotonated Species

Preferred Conformation: trans-transoid

Pyridoxal Isonicotinoyl Hydrazone

PIH
enhancement of percentage of H$_2$L species on pH decrease from 7.2 to 5.2 as result of N$^1$-alkylation (10 $\rightarrow$ 29 conversion) could explain, at least partly, the observed remarkable enhancement in the in vivo antimalarial activity on 25 $\leftrightarrow$ 10 $\rightarrow$ 29 conversions. All ligands are virtually fully deprotonated at pH > 11. The species distribution of (10), (25), (29), and PIH as a function of pH are shown in Figures 3 - 4a-c. They agree well with the data of related structures in the literature$^{34,35,36}$.

### Table 14

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pH = 5.2</th>
<th>pH = 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(H$_3$L$^+$) : (H$_2$L$^-$)</td>
<td>[H$_2$L$^-$] : [HL$^-$]</td>
</tr>
<tr>
<td>(10)</td>
<td>20 : 80</td>
<td>-----</td>
</tr>
<tr>
<td>(25)</td>
<td>-----</td>
<td>87 : 13</td>
</tr>
<tr>
<td>(29)</td>
<td>-----</td>
<td>93 : 7</td>
</tr>
<tr>
<td>PIH</td>
<td>17 : 83</td>
<td>-----</td>
</tr>
</tbody>
</table>

**The Assignment of pKa Values to Ionisable Sites O$^1$, N$^1$", N$^3$", and N$^4$**

The assignment of pKa values to ionisable sites (O$^1$, N$^1$", N$^3$", and N$^4$") on the ligands could not be made with confidence by comparison with its components: pyridoxal, the acid hydrizde, and related compounds$^{34}$, nor with PIH$^{35,36}$. Caution is necessary because theoretical calculations indicate that pyridoxal pyridyl hydrazone (10) is likely to lose its pyridoxal ring N$^1$- proton first, prior to its phenolic proton. To avoid possible ambiguities, a $^{13}$C NMR study as a function of pH for (10), (25), (29), and PIH, was undertaken, in spite of difficulties due to low solubilities of the ligands at pH > 9.

**Carbon-13 Nuclear Magnetic Resonance Study**

The sensitivity of carbon-13 nuclear magnetic resonance ($^{13}$C NMR) to electronic structure is well known$^{37}$. Luckily, the complete studies of the $^{13}$C NMR spectroscopy of the pyridine...
Figure 3. Species distribution plot over pH 1.5-12 for Pyridoxal Isonicotinoyl Hydrazone. 

$H_4L^{2+}$ is the second protonated species at pyridinic $N^4$; $H_3L^+$ is the first protonated species at pyridoxal ring $N^1$; $H_2L$ is neutral $\text{PIH}$; $HL^{-}$ is the first deprotonated species; $HL^{-}$ is the second deprotonated species of $\text{PIH}$.
Figure 4a. Species distribution plot over 1.5 - 2 pH for Pyridoxal Piridyld Hydrazone (10); $H_4L^2+$ is the second protonated species at pyridinic ring N$^4$; $H_3L^+$ is the first protonated species of pyridoxal ring N$^1$; $H_2L$ is neutral species of (10); $HL^-$ is the first deprotonated species of (10); $L^2-$ is the second de-protonated species of Pyridoxal Pyridyl Hydrazone.
Figure 4b. Species distribution plot over pH 1.5 - 12.0 for 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25). $H_3L^{2+}$ is the first protonated species of (25); $H_2L^+$ is the non-protonated species of (25); $HL$ is the first deprotonated species of (25); $HL^-$ is the second deprotonated species of (25).
Figure 4c. Species distribution plot over pH 1.5 - 12.0 for 1-[N-ethoxycarbonylmethyl-pyridoxylidenium]-2'-pyridyl]hydrazine bromide (29); H$_3$L$^{2+}$ is the first protonation species; H$_2$L$^+$ is the non-protonated species; HL is the first deprotonated species; HL is the second deprotonated species.

species dist 4 2/1/94 16:16
ring, the vitamin B-6 Schiff bases, and their metal complexes, are well recorded in the literature\textsuperscript{37-41}, enabling their use for assignment resonances of pyridoxal pyridyl hydrazone and its analogues. Indeed, the assignment of carbon chemical shifts was done by comparison with pyridoxal and pyridine. We have measured the $^{13}$C NMR spectral assignments for (10), (25), (29), and PIH, and their pH dependence.

Analysis of $^{13}$C NMR data permits to assign most positively that the respective pK\textsubscript{a} values of $2.39 \pm 0.31$ and $2.31 \pm 0.15$, $7.30 \pm 0.42$ and $7.43 \pm 0.35$, obtained for ligands (25) and (29) (see Table 15), belong correspondingly to protons bound to pyridinic ring N$^4$ and phenolic O$^1$. Thus, it can be established that the ease of proton removal from the four binding sites in ligands included in this study decrease in the ordering:

$$\text{PYR ring-N}^4 > \text{Pyridoxal-ring N}^1 > \text{O}^1\text{H} > \text{N}^3\text{H} > \text{O}^2\text{H}$$

in excellent agreement with the theoretical calculations put forward previously, and consistent with the findings of Dubois and co-workers\textsuperscript{35}.

**BIOLOGICAL RELEVANCE OF RESULTS**

Compound (10) is inactive in vitro against the malaria parasite. However, putting a methyl substituent at the electrophoric center (at the pyridoxal ring-nitrogen) (10 $\rightarrow$ 25) confer notable antimalarial activity to the molecule. Substituting methyl for ethoxycarbonylmethyl group (25 $\rightarrow$ 29) dramatically enhances the antimalarial activity of the molecule. This parallels the trends in species distribution with pH. At pH 5.2, the $\{H_2L\}:\{HL^-\}$ ratio in (29) is 13:1, but in (10) the $\{LH^+\}$ species is missing. Instead, a positively charged species is present, with $\{H_3L^+\}:\{H_2L\}$ ratio of 1:4. Significantly, at pH 7.2, the $\{H_2L\}:\{HL^-\}$ ratio is 1:2 in (29), and 2:1 in (10). This correlation between antimalarial activity with pH-dependent species distribution merits further investigations, since it could provide a clue for the mode of action of the new antimalarials.
Table 15

Prototautomomerism pKa of PIH, (10), (25), and (29)

<table>
<thead>
<tr>
<th>pK</th>
<th>PIH</th>
<th>(10)</th>
<th>(25)</th>
<th>(29)</th>
<th>PBH&lt;sup&gt;9&lt;/sup&gt;</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt; 1</td>
<td>2.45 ± 0.10</td>
<td>2.62 ± 0.09</td>
<td>2.39 ± 0.31</td>
<td>2.31 ± 0.15</td>
<td>---</td>
<td>PYR ring N</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt; 2</td>
<td>4.54 ± 0.07</td>
<td>4.63 ± 0.05</td>
<td>---</td>
<td>---</td>
<td>4.59 ± 0.04</td>
<td>PDX ring N</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt; 3</td>
<td>7.44 ± 0.14</td>
<td>7.96 ± 0.07</td>
<td>7.30 ± 0.42</td>
<td>7.43 ± 0.35</td>
<td>8.47 ± 0.04</td>
<td>PDX OH</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt; 4</td>
<td>9.94 ± 0.50</td>
<td>9.84 ± 0.07</td>
<td>9.76 ± 0.76</td>
<td>9.70 ± 0.51</td>
<td>11.40 ± 0.04</td>
<td>NH-N=</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt; 5</td>
<td>13.35 ± 0.02</td>
<td>13.35 ± 0.01</td>
<td>13.33 ± 0.04</td>
<td>13.34 ± 0.04</td>
<td>---</td>
<td></td>
</tr>
</tbody>
</table>
Complex Formation Constants With Fe(II) and Fe(III)

In the framework of this project we reported\textsuperscript{25,26} that the remarkable \textit{in vitro} antimalarial activities of (13), (25), (29) and related structures are iron-dependent\textsuperscript{14}. The active antimalarial species was shown to be the Fe(II)-chelate which acts presumably as a carbon-centered radical capable of damaging the parasite DNA\textsuperscript{26}. This was verified by electron spin resonance (ESR)\textsuperscript{25} studies. These prompted a study of the coordination chemistry underlying interactions between the redox-active ligands and iron(II), and/or Fe(III). Since such information is important for understanding the \textit{in vivo} behaviour of these drugs, we have undertaken a study of \textit{complex formation} between ions of Fe\textsuperscript{2+} and of Fe\textsuperscript{3+} and the active agents: (10), (25), (29), and PIH.

\textit{Iron Complex Formation}

\textit{Stock Solutions.}—The acid, HNO\textsubscript{3}, and the base, NaOH, were purchased from Merck (Tritrisol). Double distilled water was used through all experiments. Solutions of Fe(NO\textsubscript{3})\textsubscript{2} were prepared by mixing solutions of FeSO\textsubscript{4} and Ba(NO\textsubscript{3})\textsubscript{2} in freshly distilled water. The supernatant was decanted and filtered under oxygen-free nitrogen, HNO\textsubscript{3} added to give a final acid concentration of 0.05 M and a final [Fe(II)] of approx. 0.1 M. The exact [Fe(II)] was determined by titration with standard KMnO\textsubscript{4}.

Formation constants of the Fe(III) complexes were estimated by a combination of spectrophotometry and potentiometry. Spectrophotometry was utilized to measure initial complexation at pH 1.5-3.2. Potentiometric titration (H\textsuperscript{+}-titrant) were then employed to determine the acid dissociation constants of the Fe(III) complexes at higher pH. Dilute solutions with [Fe(III)] = 1-5 \times 10\textsuperscript{-5} M were used to avoid precipitation. The more soluble complexes of Fe(II) were studied at [Fe(II)] = 2 \times 10\textsuperscript{-4} M over pH range 2-10 by potentiometry under oxygen-free nitrogen. The titration apparatus and procedures for pH measurements are described above. The measurements were made at 37\textdegree C at an ionic strength of 0.15 M KNO\textsubscript{3}.

As elaborated above, the pyridoxal-based series of synthetic ligands exist in aqueous solution as several pH-dependent species. It was assumed that H\textsubscript{2}L was the most protonated species of the ligand that can bind significantly to the metal ions. Hence, data fitting began with 8 parameters: the formation constant (β) and molar absorptivities (ε), of the four mono- and bis-complexes of H\textsubscript{2}L: i) M[H\textsubscript{2}L], ii) M[H\textsubscript{2}L]\textsubscript{2}, and of HL\textsuperscript{−}: iii) M[HL\textsuperscript{−}], iv) M[HL\textsuperscript{−}]\textsubscript{2}, and their standard deviations providing a measure of goodness-of-fit using the non-linear algorithm. Various combinations of metal complexes were evaluated in this fashion to give the best standard deviation. Speciation plots were calculated from the mass balance for metal and ligand at each pH value. Variables were the measured pH, [L]T and [M]T derived from the initial totals and the titration volumes, and the parameters were the acid dissociation constants of the complexes.
The pyridoxal-based series of synthetic ligands exist in aqueous media as several pH-dependent species. For the analysis of the experimental data, each experimental point was considered to represent four variables: i) initial total concentration of the ligand \([L]_T\); ii) initial total concentration of the metal \([M]_T\); iii) pH; iv) molar absorptivities \((e)\), and/or potentiometric values. We assume that the neutral species \(H_2L\) is the most protonated species of the ligand that can bind significantly to the metal ions. Hence data fitting begins with 8 parameters: the formation constants and molar absorptivity of the four mono-, and bis-complexes of \(H_2L\), namely, \(M\{H_2L\}\) and \(M\{H_2L\}_2\), and of \(HL^-\), i.e., \(M\{HL^-\}\) and \(M\{HL^-\}_2\). Various combinations of metal complexes were evaluated in this fashion to give the best standard deviation. Specification plots were calculated from the mass balance for metal and ligand at each pH value. Variables were the measured \(pH\), \([L]_T\) and \([M]_T\) derived from the initial totals and the titration volumes, whilst the parameters were the acid dissociation constants of the complexes.

The association of the ligand with the metal can be represented generally as follows:

\[
\begin{align*}
H_2L + M &\rightarrow M\{HL\} + H^+ & \beta_1 &= \frac{[M\{HL\]}{[H^+]} / [M] \{H_2L\} \\
2H_2L + M &\rightarrow M\{HL\}_2 + 2H^+ & \beta_2 &= \frac{[M\{HL\}_2]}{[H^+]^2} / [M] \{H_2L\}^2 \\
H_2L + M + H_2O &\rightarrow M\{HL\}(OH) + 2H^+ & \beta_3 &= \frac{[M\{HL\}(OH)]}{[H^+]^2} / [M] \{H_2L\} \\
H_2O &\rightarrow OH^- + H^+ & \beta_4 &= \frac{[H^+] \{OH^-\}}{[H_2O]}
\end{align*}
\]

where \(H_2L\) is free ligand, M is either Fe(II) or Fe(III), \(M\{HL\}\) is 1:1-iron complex, \(M\{HL\}_2\) is 1:2-iron:ligand complex, and \(M\{HL\}(OH)\) is a 1:1:1-iron:ligand:hydroxyl complex. Table 16 summarizes the formation constants \(\beta_2\) and \(\beta_3\) for \((10), (25), (29), \) and \(PIH\).

**Table 16**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>(\log\beta_2^*)</th>
<th>(\log\beta_3^{**})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10)</td>
<td>11.01 ± 0.32</td>
<td>9.56 ± 0.52</td>
</tr>
<tr>
<td>(25)</td>
<td>11.86 ± 0.22</td>
<td>8.74 ± 0.22</td>
</tr>
<tr>
<td>(29)</td>
<td>11.80 ± 0.75</td>
<td>8.40 ± 0.44</td>
</tr>
<tr>
<td>PIH</td>
<td>---</td>
<td>8.67 ± 0.22</td>
</tr>
</tbody>
</table>

\(*\beta_2 = \{L(OH)Fe^{II}\}{H^+}^2/[Fe^{2+}]{H^+}; \quad **\beta_3 = \{L_2Fe^{III}\}{H^+}^2/[Fe^{3+}]{HL}^2\)
Complexation of Pyridoxal-Based Chelators with Fe(II) and Fe(III) pH Dependence

Fe$^{2+}$ $\rightarrow$ Fe(II) stable in the range pH 2-8

(L$^+$, X$^-$)-Fe(II)

metal ion self-reduction

Fe$^{3+}$ $\rightarrow$ Fe(III) stable below pH 4.0

(L$^+$, X$^-$)-Fe(III)-（L$^+$, X$^-$)

metal ion self-oxidation
Ultraviolet Spectra of Ligands, LH: (10), (25), (29), and their Respective 1:1-Fe(III)-Complexes, MLH, at pH 3.5

Fig. 3a. 1-[Pyridoxylidene]-2-[2'-pyridyl] hydrazine (10)

Fig. 3b. 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25)

Fig. 3c. 1-[N-Ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide (29)
Figure 6a. Visible Spectra of 1:1-Fe(II)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (10) at pH 4.0, 5.0, and 7.0

Figure 7a. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (10) at 37°C in 0.15 M KNO₃.
**Figure 6b.** Visible Spectra of 1:1-Fe(II)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25) at pH 4.0, 4.3, and 7.7.

**Figure 7b.** Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-methyl-pyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25) at 37°C in 0.15 M KNO₃.
Figure 6c. Visible Spectra of 1:1-Fe(II)-chelate of 1-[N-ethoxycarbonylmethylpyrdoxyl idenium]-2-[2'-pyridyl]hydrazine bromide (29) at pH 4.0, and 7.3

Figure 7c. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-ethoxycarbonylmethylpyrdoxylidenium]-2-[2'-pyridyl]hydrazine bromide (29) at 37°C in 0.15 M KNO₃
Figure 8a. Visible Spectra of 1:1-Fe(III)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (10) at pH 4.0, 5.3, and 7.1

Figure 9a. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[pyridoxylidene]-2-[2'-pyridyl]hydrazine (10) at 37°C in 0.15 M KNO₃
Figure 8b. Visible Spectra of 1:1-Fe(III)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25) at pH 4.0, 4.3, and 7.7.

Figure 9b. Species distribution plot over pH 3.5 - 8.5 for 1:1-, and 1:2-Fe(III)-chelate of 1-[N-methylpyridoxylidenium]-2-[2'-pyridyl]hydrazine iodide (25) at 37°C in 0.15 M KNO₃.
Figure 8c. Visible Spectra of 1:1-Fe(III)-chelate of 1-[N-Ethoxycarbonylmethylpyridoxylidinium]-2-[2'-pyridyl]hydrazine bromide (29) at pH 4.0, 5.0, and 6.8.

Figure 9c. Species distribution plot over pH 3.5 - 8.5 for 1:1-Fe(II)-chelate of 1-[N-ethoxycarbonylmethylpyridoxylidinium]-2-[2'-pyridyl]hydrazine bromide (29) at 37°C in 0.15 M KNO₃.
Redox Potentials of Antimalarials

Determined by Cyclovoltametry
CYCLIC VOLTAMETRY OF AN ANTIMALARIAL CHELATOR AND OF ITS Fe(II)-CHELATE

in water containing 0.5 M KCl at ambient temp.

concentration: 5 \mu M; pH = 4.41
peak A, Red Potential: +0.30 volt; 85 \mu A
peak B, Oxid Potential: +0.63 volt; 132 \mu A

Conc.: ligand = 5\mu M; Fe^{2+} = 2\mu M; pH = 2.07
peak C, Red Potential: +0.38 volt; 95 \mu A
peak D, Red Potential: +0.23 volt; 25 \mu A
peak E, Oxid Potential: +0.62 volt; 125 \mu A
peak F, Oxid Potential: +0.36 volt; 37 \mu A
CYCLIC VOLTAMETRY OF AN ANTIMALARIAL Fe(II)-CHELATE OF 1-[N-METHYL-PYRIDOXYLIDENE]-2-[2'-PYRIDYL]HYDRAZINE IODIDE

Solvent: Water; in KCl 0.5μM; Temp.: Ambient

Conc.: ligand = 5μM; Fe$^{2+}$ = 2μM; pH = 5.87
peak G, Red Potential: +0.46 volt; 47 μA
peak H, Red Potential: +0.38 volt; 60 μA
peak I, Red Potential: +0.20 volt; 37 μA
peak J, Oxid Potential: +0.63 volt; 130 μA
peak K, Oxid Potential: +0.35 volt; 42 μA

Conc.: ligand = 5μM; Fe$^{2+}$ = 2μM; pH = 7.44
peak L, Red Potential: +0.48 volt; 20 μA
peak M, Red Potential: +0.44 volt; 20 μA
peak N, Red Potential: +0.10 volt; 40 μA
peak O, Oxid Potential: +0.68 volt; 132 μA
peak P, Oxid Potential: +0.32 volt; 37 μA

Conc.: ligand = 5μM; Fe$^{2+}$ = 2μM; pH = 10.83
peak Q, Red Potential: +0.06 volt; 50 μA
peak R, Red Potential: -0.34 volt; 45 μA
peak S, Oxid Potential: +0.81 volt; 190 μA
peak T, Oxid Potential: +0.72 volt; 225 μA
Project Activities

Over the entire project period the activities included: i) participation in national and international symposia dedicated to subjects related to the project, and ii) publication of research accounts in international scientific journals. They list as following:

List of meetings:


Symposium Lecture: "From Pyridoxal To Antimalarials Active Against Drug-Resistant Plasmodium falciparum", held in Jomien, Thailand, 11-14 December 1990.


Publications:


Impact, Relevance and Technology Transfer

In retrospect, the rationale that initiated this project proved to have a sound basis in reality. Indeed, it opened new venues in the combat against the drug-resistant strains (FCR-3) of the malaria parasites which are responsible for the rapid spread of falciparum malaria in South East Asia and South America.

The greatest impact of results produced in this project is likely to be on the minds of the researchers dedicated to malaria chemotherapy, since the new discoveries are based on hitherto unknown mechanism of action. The new antimalarial agents were shown to be parasiticidal, killing the parasites within minutes or hours, depending on structure and concentration.

The results fully warrant a new project aimed at enhancing the scale and deepening the depth of testings in model animals.

The collaborators from Thailand learned many lessons during the joint endeavor. They have acquired new skills in modern experimental chemistry (ESR, cyclovoltametry etc.), molecular biology, and computational chemistry. In estimation of parasitemia they have learned to use the more accurate $^3$H-hypoxanthine incorporation method which is highly reliable. The training acquired via involvement in the process of data production, analysis of data, and discussion of results immensely improved the capabilities of the collaborating country scientists.
Project Productivity

The project accomplished almost all of the proposed goals, except the in vivo testings.

Future Work

The new conceptual findings in the project opened interesting venues to antimalarial drug design implicating intracellular generation of harmful free radicals by iron-dependent inducers. This prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms. Towards this end more work should be done on five relevant topics conducive to therapeutically safe agents active against drug-resistant falciparum malaria. The ordering of topics below is tentative, requiring more thought for preferences in designing a working research plan.

(i) in vivo testings of chelating agents which exhibited remarkable in vitro antimalarial activity.

(ii) Structure-activity-relationship study to uncover factors determining the antimalarial activity of pyridoxal-based chelators.

(iii) Synthesis and assessment of antimalarial activity of a new series of metal-chelates where the metal equals: Fe(II), Fe(III), Cu(II), Co(III), and Pd(II), and the respective ligands are: pyridoxal 1-phthalazyl hydrazone, and/or di-pyridoxal 1,4-phthalazyl di-hydrazone and other related analogues.

(iv) Inhibition tests of chelating agents against haem polymerase which blocks the detoxification of ferri-protoporphyrin IX, and poisons the parasite food vacuole.

(v) To uncover factors affecting transmembrane transport of chelating agents and their metal-chelates through blood red-cells.

(vi) The importance of affinity of chelating agents to Fe(II) and/or Fe(III) to effect antimalarial activity.
Bibliography

2. Policy Decisions and Statements by WHO, PAHO, etc.
   (b) S.J. Oppenheimer, Parasitology Today, 1989, 3, 77-79.


(b) A. Albert, *Nature*, 1956, 177, 525.


MECHANISM-BASED APPROACHES TOWARDS CHEMOTHERAPIES OF THALASSEMAIA AND MALARIA

Shalom Sarel, Schelly Crisaru, Chaim Hershko, Gabriela Link, Dan Spira and Eugene Iheanacho
Hebrew University of Jerusalem, Jerusalem, Israel

SUMMARY

The synthesis, coordination chemistry and biological activities of a new class of iron chelators based on pyridoxal are described herein. These comprise two types of aryl hydrazones of pyridoxal represented by the general formulae (I) and (II). Both function in vitro and in vivo as tridentate chelators. They are essentially non-toxic and compare with desferrioxamine (desferal, DF) with regard to affinity and specificity for iron. Unlike desferrioxamine, they are orally effective and considerably less expensive. They represent excellent candidates for remedy of iron overloads in thalassemia and blood transfusional patients.

Depending on structure, some of the new chelators can kill cultures of chloroquine-resistant strains of P. falciparum at \(10^{-6}\) concentration. Their mode of action is believed to involve an intracellular iron-catalyzed Fenton-type reaction which places increased "oxidative stress" both on the real cells and on the more sensitive parasitic invaders.

INTRODUCTION

The thalassemias are among the commonest inherited blood disorders in the world, of which beta-thalassemia major (known also as Cooley's anaemia) alone is responsible for 100,000 child deaths per year.1,2,3 Most significantly, it is also among the five major haemoglobin abnormalities that confer resistance to Plasmodium falciparum, the main causative agent of human malaria which affects one quarter of the world's population and is responsible for 1,000,000 deaths per year in Africa alone.4 The five genetically controlled traits are:

1. Sickle-cell anaemia, the normal haemoglobin A (\(\alpha_2\beta_2\)) in the red cell being replaced by a chemical variant Hbs.
2. Thalassemia, in its \(\alpha\)-form, the red cells contain the totally ineffective haemoglobin variant Hbr (\(\alpha_1\)).
3. Hereditary persistence of foetal haemoglobin (HPFH), part of the thalassemia syndrome in which the red cells contain an unstable haemoglobin variant Hbf (\(\alpha_2\gamma_2\)).
4. Haemoglobin E disease, part of the thalassemia syndrome, containing HbE variant, an unstable haemoglobin.
5. Favism, common among Mediterranean and Middle Eastern populations, in which the red cells are deficient of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD), controlling the redox transitions: GSSG + 2NADPH \(\rightarrow\) 2GSH + 2NADP
The growth of *P. falciparum* in culture has been shown to be inhibited on exposure to the above haemoglobin variants. The normal person has no obvious defence mechanism against invasion by the malaria parasite. Evolutionwise, the genetic alteration of normal haemoglobin appears to be the only survival means for the body to combat the malaria parasites.

Immunity to malaria could also be promoted by induction state of iron-deficiency. The case in point is the resistance to malaria acquired by anaemic women during pregnancy which fades away upon administration of iron.

This communication deals with chemotherapeutic strategies aimed at (i) designing orally effective and inexpensive chelators capable of removal of toxic accumulation of iron in thalassemic and iron overloaded patients, and (ii) the molecular engineering of orally effective, safe, and inexpensive antimalarials, mechanistically implicating iron.

**THALASSEMA**

Thalassemia is a syndrome of inherited abnormalities of haemoglobin which arises from defects in the genes controlling the manufacture of either alpha- or beta-haemoglobin chains. It occurs not only in people of Mediterranean extraction but also in the Middle East, the Indian subcontinent, South-East Asia and Africa and among immigrant populations from these areas. If the gene for production of $\beta$ chains is abnormal, the blood disorder is called $\beta$-thalassemia, and when the $\alpha$ chains are affected, it is known as $\alpha$-thalassemia. Reduced production of the affected globin chain leads to an excess of the other which tends to precipitate particularly in the nucleated red cells in the bone marrow resulting in distorting the vault of the skull and facial bones.

The anaemia in $\beta$-thalassemia major becomes apparent soon after birth. The child fails to thrive, is prone to infection, developing bony distortions and giving mongoloid appearance. These children die young.

**IRON OVERLOAD**

The picture can be dramatically modified by regular blood transfusions to suppress the abnormal bone marrow activity and to maintain normal levels of haemoglobin. About 200 mg of iron is estimated to accumulate as a result of each unit of blood transfused since it cannot be excreted from the body when the transfused cells are ultimately destroyed. Most patients die in their teens from liver or heart failure. As the body has no means of significantly increasing iron excretion, about 1 or 2 individuals in a population of 10,000 require treatment with chelating agents for safe removal of toxic amounts of iron from the body. The iron overload can successfully be treated by applying the naturally occurring chelator desferrioxamine (DF) which safely removes surpluses of iron from the body. Although DF is satisfactory with regard to
affinity and specificity for iron and it is essentially non-toxic, it is unsatisfactory in other respects, such as its poor absorption from the gastrointestinal tract and it is expensive. It has a very short half-life and has no effect on plasma ferrokinetics. This prompted studies which brought about the uncovering of some novel candidates, representing a new class of chelators based on pyridoxal of the general structures (I) and (II).

**CHEMICAL SYNTHESIS**

The syntheses of (I) and (II) were straightforward, as delineated in Chart 1. The pyridoxal is condensed with the corresponding aryl or acyl-hydrazine to form (I), and when required the latter was exposed to the action of alkyl halide or an α-bromoester to yield (II).

![Chemical Structures]

---

**Chart 1**
BIOLOGICAL TESTING

The biological testings involved hypertransfused rats which were labelled by intravenous injection of $^{59}$Fe-ferritin. The ability of the new chelators to mobilize radio-iron from hepatic parenchymal stores followed from a single subcutaneous injection of 40mg of the compound into the labelled rat. The radio-iron was excreted totally, mainly in the stool. The oral effectiveness was assessed from administration of 40mg of the chelator by the gastric tube followed by estimation of total radio-iron excretion. The results of some selected compounds, (1) to (5), are given in Table 1.

<table>
<thead>
<tr>
<th>Code Number</th>
<th>Blood</th>
<th>Liver</th>
<th>Spleen</th>
<th>Urine</th>
<th>Faeces</th>
<th>Total Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.4±1.6</td>
<td>12.1±0.6</td>
<td>0.47±0.03</td>
<td>0.1±0</td>
<td>3.1±0</td>
<td>3.2±0</td>
</tr>
<tr>
<td>Desferral</td>
<td>8.7±1.6</td>
<td>2.7±0.3</td>
<td>0.14±0.03</td>
<td>71.9±3.6</td>
<td>13.5±0.7</td>
<td>85.4±4.3</td>
</tr>
<tr>
<td>1</td>
<td>33.3±2.1</td>
<td>12.8±0.2</td>
<td>0.56±0.02</td>
<td>3.7±0.2</td>
<td>22.7±1.1</td>
<td>26.4±1.3</td>
</tr>
<tr>
<td>2</td>
<td>13.3±2.3</td>
<td>6.8±0.4</td>
<td>0.51±0.02</td>
<td>39.0±3.1</td>
<td>27.0±2.2</td>
<td>66.0±3.3</td>
</tr>
<tr>
<td>3</td>
<td>6.4±0.4</td>
<td>3.3±0.4</td>
<td>0.16±0.04</td>
<td>29.5±0.6</td>
<td>57.2±1.1</td>
<td>86.7±1.7</td>
</tr>
<tr>
<td>4</td>
<td>4.2±0.2</td>
<td>2.9±0.2</td>
<td>0.06±0.03</td>
<td>28.9±1.0</td>
<td>67.3±2.4</td>
<td>96.2±3.4</td>
</tr>
<tr>
<td>Comp.5*</td>
<td>29.1±2.2</td>
<td>8.2±0.8</td>
<td>0.36±0.03</td>
<td>30.6±1.2</td>
<td>8.6±0.3</td>
<td>39.2±1.6</td>
</tr>
</tbody>
</table>

*pyridoxol isonicotinoyl hydrazone dimethiodide

From Table 1 it can be seen that when the tested compounds were given orally to the animals enhanced excretion of radio-iron took place, considerably above controls. The observed in vivo activities of two compounds were comparable to and even exceeded that of desferrioxamine (which were given by injection). These results represent a very significant progress in providing new chelators of potential clinical usefulness in the management of patients with transfusional iron overload.
Study of the coordination chemistry of these compounds was undertaken with a view to uncovering factors underlying the biological activities. Chemical and spectroscopic evidences indicate that the new chelators function as tridentate ligands, forming two sets of metal complexes with metal:ligand ratios of 1:1 and 1:2, depending on the pH. Thus, 1:1-complexes are stable at pH values below 4, whereas the 1:2 complexes are highly stable at pH values above 5.5 (Chart 2). The apparent binding constants with metals were measured (see Table 2), following spectroscopic methods. From Table 2 it can be seen that the affinity for iron is quite high at neutral pH. Looking at Mössbauer spectroscopic data presented in Table 3 reveals that iron(II) in the complexes tends to lose one electron to form high-spin Fe(III)-complexes.

![Diagram](chart2.png)
<table>
<thead>
<tr>
<th>LIGAND</th>
<th>COPPER CHELATION</th>
<th>IRON CHELATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu:L ratio</td>
<td>Fe:L ratio</td>
</tr>
<tr>
<td></td>
<td>Cu:L ratio</td>
<td>Cu:L ratio</td>
</tr>
<tr>
<td>DESFERRIOXAMINE</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PII</td>
<td>1:1 405 14</td>
<td>1:1 402 18</td>
</tr>
<tr>
<td>PPH (I)</td>
<td>1:2 443 12.8</td>
<td>1:2 420 2.15</td>
</tr>
<tr>
<td>PPH*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPHE (II)</td>
<td>1:1 460 70</td>
<td>1:2 439 42</td>
</tr>
</tbody>
</table>

(* with ferric ions at comparable conditions)
Table 3. MOSSBAUER RESULTS

![Chemical structure diagram]

<table>
<thead>
<tr>
<th>Form</th>
<th>%</th>
<th>I.S.</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH insoluble</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe$^{2+}$: 30</td>
<td>0.93(1)</td>
<td>2.38(1)</td>
<td></td>
</tr>
<tr>
<td>EtOH soluble</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe$^{3+}$: 70</td>
<td>0.370(6)</td>
<td>0.810(6)</td>
<td></td>
</tr>
<tr>
<td>Fe$^{2+}$: 50</td>
<td>0.94(1)</td>
<td>2.26(2)</td>
<td></td>
</tr>
</tbody>
</table>

Malaria

Malaria is caused by protozoan parasites belonging to the genus Plasmodium, of which the four species: P. falciparum (often fatal), P. vivax, P. malariae and P. ovale are essentially exclusive to human beings. They have a complex life cycle in both the insect and the vertebrate host. Our practical attention was directed at the three-stage development sequence: ring forms, trophozoite and schizont.

Malaria has undergone a resurgence over the past decade, and, despite its high incidence, no revolutionary new drugs have been introduced over the past three decades. The widespread appearance of chloroquine-resistant strains of P. falciparum prompts the development of new antimalarial drugs, operating most desirably by entirely new mechanisms, to replace those to which P. falciparum has become resistant. In conformity with this line the most logical approach would be of making use of parasite metabolism for targeting the
drug action at vulnerable sites of the organism. Towards this end two different approaches have been adopted. One, making use of the susceptibility of the malaria parasites to free oxygen radical-reduced oxidative stress. The second, to affect the iron pool of the parasite controlling the biosynthesis both of metallo-protein oxidases and of the iron-dependent ribonucleotide diphosphate reductase (RdR) by applying chelators inhibitory to these enzymes. This might exert selective inhibition on the growth of *P. falciparum*. Cultures of the chloroquine-resistant strain of *P. falciparum* (FCR-3) were suspended in solutions containing chelators and then cultured for 3 days. Growth of parasites was determined by either slide counts or by incorporation of $^3$H-hypoxanthine. The parasite growth was monitored by microscopic slide counts. The effects of one representative of the new chelators on the growth of *P. falciparum* chloroquine-resistant strains are demonstrated in Figures 1 and 2. The effect of added Cu$^{2+}$ ions is shown in Figure 3. Table 4 summarizes the data on inhibitory effects of some of the new chelators on the growth of *P. falciparum* and on the enzyme activity of RdR with a view to their capability of removal of iron from the body.

![Fig. 1. Growth curves of *P. falciparum* in human red cells as a function of Lig-2 concentration. A synchronous of 3% schizonts was used as the starting parasitemia.](image1)

![Fig. 2. Growth curves of parasites pre-treated with 40 μl Lig-3 and normal medium respectively for 2 hours before culturing.](image2)

![Fig. 3. Pre-complexing chelator with copper salt: the chelator was pre-complexed with copper. This was then used in medium for growing the parasite as in previous experiments. 1% mu culture was used for initial parasitemia.](image3)
<table>
<thead>
<tr>
<th>CHELATOR Code No.</th>
<th>% EXCRETION OF RADIO-IRON IN RATS</th>
<th>% CYTOXICITY ON P388 CELLS OF 10(\mu)g/m(l)</th>
<th>% INHIBITION OF P. falciparum at 2.10(^{-5}) M</th>
<th>% INHIBITION OF RdR (10^{-3}) M HYDOXYUREA = 81%</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG-6</td>
<td>36.4</td>
<td>-</td>
<td>16.6 at 2.10(^{-5}) M</td>
<td>1.10(^{-5})</td>
</tr>
<tr>
<td>SG-9</td>
<td>86.7</td>
<td>-</td>
<td>90.9</td>
<td>1.10(^{-5})</td>
</tr>
<tr>
<td>SG-11</td>
<td>34.2</td>
<td>100</td>
<td>93.4</td>
<td>none</td>
</tr>
<tr>
<td>SG-14</td>
<td>-</td>
<td>100</td>
<td>86.6 at 2.10(^{-5}) M</td>
<td>1.10(^{-5})</td>
</tr>
<tr>
<td>SG-15</td>
<td>96</td>
<td>13 (non-toxic)</td>
<td>90.3 at 2.10(^{-5}) M</td>
<td>none</td>
</tr>
<tr>
<td>SG-18</td>
<td>-</td>
<td>none</td>
<td>88.6 at 2.10(^{-5}) M</td>
<td>none</td>
</tr>
<tr>
<td>SG-32</td>
<td>-</td>
<td>none</td>
<td>100</td>
<td>none</td>
</tr>
</tbody>
</table>

\(^{21}\)
ANTIMALARIAL MODE OF ACTION

The data presented in Table 4 provides no clue for understanding the impressive antimalarial activity displayed by the chelators. The clue, however, emerged when the effect of antioxidants on antimalarial activity was searched. Towards this end desferrioxamine was selected since it effectively blocks the iron-catalysed Fenton-type reaction, generating highly toxic oxygen species such as \( \text{H}_2\text{O}_2 \), superoxide (\( \text{O}_2^- \)), and hydroxyl (\( \cdot\text{OH} \)) radicals. As suspected, the presence of desferrioxamine entirely blocked the antimalarial activity of the pyridoxal-based chelators. This suggested that the latter functions most likely as electron-transfer oxidants, mediating transfer of an electron from iron(II) to an appropriate "acceptor". When the latter is molecular oxygen, a uni-valent reduction process ensues, generating the toxic oxygen species (see Chart 3).

\[
\begin{align*}
\text{H}^+ + \text{CH}_3 \quad + \text{Fe}^{3+} \quad \rightarrow \quad \text{H}^+ + \text{CH}_3 \\
\text{H}^+ + \text{CH}_3 \quad + \text{Fe}^{3+} \quad \rightarrow \quad \text{H}^+ + \text{CH}_3 \\
\end{align*}
\]

\[\text{R = NiCOAr, NHAr}\]

**Chart 3.** Suggested mechanism for pyridoxal induced electron transfer process.

The superoxide radical is efficiently and specifically scavanged by superoxide dismutase (SOD), catalyzing the disproportionation of \( \text{O}_2^- \) into \( \text{H}_2\text{O}_2 \):

\[
\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

In the presence of ferrous ions the Haber-Weiss reaction takes place:

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \quad \rightarrow \quad \text{O}_2 + \text{OH}^- + \cdot\text{OH}
\]
The toxic oxygen species are formed intraerythrocytically in large quantities when the red blood cells are abnormal as in the case of sickle cell anaemia and thalassemia. This implies enhanced resistance to malaria parasites by haemolytic anaemia patients, as is the case.

Corroboration of this mechanistic view was forthcoming from Electron Spin Resonance studies of cultures of *P. falciparum* exposed to the simultaneous action of both the chelator and iron ions. The appearance of strong ESR signals was attributed to carbon-centered free radicals as portrayed by Figures 4 and 5.

**CONCLUSION**

Among the metals playing vital roles in health and diseased organisms, iron is the most important one. This communication amply underlines the importance of implicating iron in designing successful chemotherapeutical strategies against parasites capable of developing resistance to drugs. In Rudyard Kipling’s words: "Cold Iron": "Cold is for the mistress - silver for the maid - copper for the craftsman, cunning at his trade. Good! said the Baron, sitting in his hall, but Iron - Cold Iron - is the master of them all".
A - Ligand in absence of iron ions
but in presence of P. falciparum and PBN
Hepes/NaHCO$_3$

B - Lig-[Fe(III)] complex
in presence of P. falciparum
and PBN, Hepes/NaHCO$_3$

C - Lig-[Fe(III)] complex in presence of PBN, but
in the absence of living cells

Fig. 5. ESR spectra of Ligand-Fe(III) complexes in the presence
(A) and (B) and in the absence of P. falciparum (C),
PBN as a spin trap.
REFERENCES

22. Fizames, C., Sarel, S., and Avromovici-Grisaru, S., to be published.
Inhibition of *Plasmodium falciparum* growth by a synthetic iron chelator

**Eugene N. Iheuacho**, Amram Samuni, Schelly Avramovic-Grisaru, Shalom Sarel and Dan T. Spira

1 The Kuvim Centre for the Study of Infectious and Tropical Diseases; 2 Department of Molecular Biology; 3 Department of Pharmaceutical Chemistry, Hebrew University-Hadassah Medical School, Jerusalem, Israel

### Abstract

The susceptibility of the chloroquine-resistant malaria parasite *Plasmodium falciparum* (FCR-3) to a pyridoxal-based iron chelator was tested. 10 μM of the chelator 1-(N-ethoxycarbonylmethyl-pyridoxylidenium)-2(2'-pyridyl)hydrazine bromide (code name L2-9) effectively inhibited growth *in vitro* of the parasites. Presaturation of the chelator with either ferric or ferrous iron partially blocked the inhibitory effect. Two hours' exposure of parasites to 20 μM L2-9 was sufficient to inhibit their growth irreversibly. Desferrioxamine blocked the inhibitory effect of L2-9. It is suggested that the chelator may be acting by generating free radicals in complexing intracellular iron.

### Introduction

Drug-resistant malaria has now become a major health problem. World-wide malaria eradication appears to be less likely, and millions of lives are in jeopardy as a result of this threat. The fast growing prevalence of drug-resistant malaria therefore calls for rational biochemical approaches for the development of new antimalarial chemotherapy. The exquisite sensitivity of malaria parasites to oxidant stress and radical-inducing drugs makes this approach a logical candidate for antimalarial activity.

With the possible exception of lactobacilli, iron is an essential growth element to virtually all living organisms (NÉILANDS, 1981), and its importance and influence on infections is well documented (WEINBERG, 1984). An invading pathogen must compete with its host for iron. If the pathogen cannot obtain sufficient iron, its proliferation may be retarded and the infection attenuated (PAYNE & FINKELSTEIN, 1978). Host defences may involve withholding iron: serum iron levels fall with inanition, and phagocytic cells, which accumulate sites of inflammation, bring with them lactoferrin, a protein which sequesters iron (ROESES, 1980). The drop in serum iron levels of mammalian hosts during bacterial infections (KLUGER & ROTHENBERG, 1979) can be regarded as a mechanism of non-specific resistance. A similar decrease in serum iron has been observed in malarial infections in man (USANGA, 1983). Many microorganisms show enhanced virulence *in vivo* or faster growth *in vitro* when the concentration of available iron is increased (MCFARLANE et al., 1970; MURRAY et al., 1980; LOO & LALONDE, 1984). Iron chelators have, therefore, been shown to affect the growth and development of parasites both *in vivo* and *in vitro* (RAVENTOS-SUAREZ et al., 1982; FRITSCH et al., 1985, 1987).

Desferrioxamine (DFO), an iron-specific chelating agent, inhibits growth of the malarial parasite, *Plasmodium falciparum*, in cultures (RAVENTOS-SUAREZ et al., 1982; FRITSCH et al., 1985; PETO & THOMPSON, 1986). However, the poor oral absorption and short half-life of DFO in the plasma limit its usefulness as an antimalarial agent. There is therefore a need continually to explore the antimalarial properties of other iron chelators for possible clinical usefulness. In the present work, the effect of a new pyridoxal-based iron chelator on the growth and development of a chloroquine-resistant strain of *P. falciparum* was studied. The results showed that parasite growth could be effectively inhibited by this iron chelator, which has no effect on normal red blood cells; it seemed to be acting not by merely depriving the parasite of iron but primarily by initiating an adverse biochemical reaction which led to parasite death.

### Materials and Methods

*P. falciparum*, chloroquine resistant strain FCR-3, was obtained from the stock of a continuous line maintained in our laboratory. Normal red blood cells, group A* or O*, were obtained from healthy donors who had no prior contact with malaria. Serum and plasma from healthy donors were obtained from the Hadassah Hospital blood bank. The pyridoxal-based iron chelator 1-(N-ethoxycarbonylmethyl-pyridoxylidenium)-2(2'-pyridyl)hydrazine bromide, code-named L2-9, was synthesized by us. Iron chelates were prepared by mixing L2-9 anaerobically with either FeSO₄ or FeCO₃, at 1:1 molar ratios. Radiolabelled [³H]hypoxanthine (specific activity 100 Ci/mmol) was purchased from NEN Corporation (Boston, Massachusetts, USA).

Parasites were grown in 9 cm Petri dishes using the candle jar method (JENSEN & TRAGER, 1977). They were cultured in group A* or O* red blood cells in RPMI 1640 medium containing 25 mm HEPES buffer, 2 g/litre NaHCO₃, 100 μg/ml gentamicin (Sigma) and 10% human serum or plasma type A. Parasites were synchronized by a combination of sorbitol treatment (LAMBROS & VANDERBERG, 1979) and gelatine sedimentation (JENSEN, 1978). Parasitaemia was determined by both microscopic counts of the number of parasites per 1000 cells and by [³H]hypoxanthine incorporation (GOLENSKY et al., 1981). The cells were harvested with a MASIL II* automatic cell harvester on glass fibre filters and their radioactivity determined in a Beckman LS 7500 liquid scintillation counter.

All experiments were begun with highly synchronized parasite cultures of either schizonts or ring stages. Since the inoculum varied from experiment to experiment, parasite growth depended on the inocu-
Illum and \( ^{3}H \) hypoxanthine incorporation and growth curves were comparable only within a given experimental set and not between different experiments. All experiments were repeated at least thrice. All microscopic and radioactive counts presented here are averages of at least triplicate determinations.

**Results**

In the first series of experiments parasites were cultured for 3 d in the presence of varying concentrations of L2-9. The results (Fig. 1) show that 20 μM L2-9 completely inhibited parasite growth in less than 24 h, while 10 μM L2-9 achieved nearly total inhibition on day 2. To study the effect of iron, the experiment was repeated with 20 μM preformed iron chelates of L2-9-Fe(II) and L2-9-Fe(III). As seen in the Table, Fe(III) and, to a lesser extent, Fe(II) blocked the inhibitory effect of L2-9. To investigate the possibility that L2-9 affects the red blood cells rather than the parasites, normal erythrocytes were pre-incubated with 40 μM L2-9 for 24 h and subsequently washed with RPMI 1640 before being infected with the parasites. Chelator-treated red cells sustained parasite growth as effectively as untreated normal cells, indicating that L2-9 does not affect parasite-free erythrocytes.

To measure the minimal exposure time required for drug-induced killing of parasites, synchronized schizonts were suspended in 20 μM L2-9 and incubated at 37°C for 1 h or 2 h. Thereafter, the cells were washed twice and cultured in the normal way. The results showed that L2-9 had no effect on the parasite in the first hour but an inhibition of more than 75% occurred within the second hour of incubation (Fig. 2). To test which growth stage of the parasite is susceptible to the drug, 2 h pulse treatment was given at various times throughout the growth cycle. Cultures were synchronized and adjusted with fresh erythrocytes to 0.1% parasitemia and 4% hematocrit. At times corresponding to specific growth stages of the parasite, the cells were exposed to 20 μM L2-9 for 2 h, washed thrice and re-cultivated. The results (Fig. 3) showed that, while there was marked growth inhibition at the period corresponding to the late trophozoites and schizonts, there was no inhibition at the ring stage, suggesting that the inhibitory effect of L2-9 is stage-dependent. A differential count of the various parasite stages after 2 h pulsing showed no deviation from the normal growth pattern. This means that parasites surviving the 2 h treatment continued their normal life cycle, implying that L2-9 does not modify the parasite's growth.

Because desferrioxamine (DFO) is known to sequester iron ions, its effect on the inhibitory activity of L2-9 was tested. The parasite-infected erythrocytes were exposed to DFO and L2-9 (40 μM each) for 2 h. DFO alone had a minimal inhibitory effect on the parasites, but in combination with L2-9, DFO fully blocked the inhibitory effect of L2-9. A 15 min pre-incubation of parasites with DFO was necessary for the effect of DFO on L2-9 to be manifested (Fig. 4).

**Table. Effect of pre-complexing iron with L2-9 on its antiplasmodial activity**

<table>
<thead>
<tr>
<th>Culture media</th>
<th>[^{3}H]hypoxanthine (cpm)</th>
<th>Percentage incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal medium</td>
<td>8298</td>
<td>100</td>
</tr>
<tr>
<td>10 μM L2-9</td>
<td>1331</td>
<td>16</td>
</tr>
<tr>
<td>20 μM L2-9</td>
<td>302</td>
<td>3</td>
</tr>
<tr>
<td>20 μM L2-9 + Fe(II)</td>
<td>6682</td>
<td>80</td>
</tr>
<tr>
<td>20 μM L2-9 + Fe(III)</td>
<td>7679</td>
<td>92</td>
</tr>
</tbody>
</table>

*Gelatin-synchronized parasites were grown in RPMI growth medium containing either only L2-9 or its complexes with iron. L2-9 was chemically pre-complexed with either Fe(II) or Fe(III) and these were used in the growth medium. \[^{3}H\]hypoxanthine was added 24 h after the start of the experiment, and the cells were harvested 24 h later. The results shown are the means of triplicate counts.
Discussion

This study has shown that L2-9, a pyridoxal-based iron chelator, at a concentration of 20 μM inhibited P. falciparum growth in vitro. If the drug exerts its antiparasitic effect by sequestering the iron essential for parasite growth, pre-saturation of the chelator with exogenous iron should diminish or abolish its antiparasitic action, as shown for DFO (RAVENTOS-SUAREZ et al., 1982; LATTSH et al., 1985; PETO & THOMPSON, 1986; HERSIKO & PETO, 1988). The present results indeed showed that pre-saturating the chelator with iron greatly reduced its antiparasitic action. This finding apparently supports the assumption that L2-9 acts by depriving the parasite of necessary iron. This conclusion, however, does not account for the finding that Fe(III), despite its lower solubility, was more effective than Fe(II) in abolishing the inhibitory action of L2-9. Moreover, this conclusion does not agree with the effect of DFO on the chelator. If L2-9 inhibits the growth of parasites by simply withdrawing iron, its combined effect with DFO would be expected to be either additive or synergistic. On the contrary, a combination of DFO and L2-9 was antagonistic. This antagonistic effect depended on the sequence of addition of DFO and L2-9 to the culture, and was manifested only when DFO, which has a higher affinity for iron, was added at least 15 min before the addition of L2-9. Early removal of chelatable iron from the erythrocyte-parasite complex appeared to protect the parasite, suggesting that iron ions were instrumental in the antiparasitic activity of L2-9. Parasite killing could involve free radical intermediates generated by L2-9 upon its binding with iron. Considerable controversy exists as to whether natural sequestered forms of iron or possibly other transition metal ions are effective in catalyzing the Fenton and Haber-Weiss reaction (AMARAGOS & JOHNSTON, 1981; GUTTENRIDGE et al., 1982; HALLIWELL, 1982; IPO et al., 1983; WINTEBOURN, 1983; BALDWIN et al., 1984; VERCELLOTTI et al., 1985; PRATISCH et al., 1987). However, iron activity strongly depends upon the nature of its coordinating ligand. Different ligands alter the redox potential, the activity and binding mode of a complexed transition metal (RICHARD, 1984). Since iron ions readily undergo redox reactions which could lead to free radical formation (GUTTENRIDGE et al., 1979; BUTCHER et al., 1983), it is tempting to speculate that a similar mechanism is operative also in the antiparasitic action of L2-9. L2-9 is a redox-active compound. In cyclic voltametry in water, it exhibits a redox transition at approximately +1.0 V versus calomel. Indeed L2-9 induces carbon-centered radicals as proven by electron spin resonance spectroscopy. The radical is initiated by the internal redox reaction of the L2-9 iron complex during an Fe(II)→Fe(III) transition (unpublished observations).

It is, therefore, very likely that L2-9 exerts its antiparasitic action in a dual manner, withholding iron from the parasite and generating free radical intermediates, both mechanisms leading to the death of the parasites.

Acknowledgement

This study was supported by CDR Program grant C7-16U No. 5544-G-SS-7021-00, and by the US NIH-NIAID (Research Contract NO1-AI-22668).

References


GROWTH INHIBITION OF PLASMODIUM FALCIPARUM INVOLVING CARBON CENTERED IRON-CHELATE RADICAL (L', X')-Fe(III) BASED ON PYRIDOXAL-BETAINE. A NOVEL TYPE OF ANTIMALARIALS ACTIVE AGAINST CHLOROQUINE-RESISTANT PARASITES

EUGENE N. IHEANACHO,† SHALOM SAREL,‡ AMRAM SAMUNI,§ SCHELLY AVRAMOVICI-GRISARU‖ and DAN T. SPIRAT†

†The Kuvin Centre for the Study of Infectious and Tropical Diseases, §Department of Molecular Biology, Hebrew University — Hadassah Medical School, ‖Department of Pharmaceutical Chemistry, Hebrew University School of Pharmacy, Jerusalem, Israel

(Received March 27th, 1990; in revised form April 18th, 1990)

Malaria parasites have been shown to be more susceptible to oxidative stress than their host erythrocytes. In the present work, a chloroquine resistant malaria parasite, Plasmodium falciparum (FCR-3) was found to be susceptible in vitro to a pyridoxal based iron chelator — (1-[N-ethoxycarbonylmethylpyridoxylidenium]-2-[2'-pyridyl]hydrazine bromide — (code named L2-9). 2h exposure to 20 μM L2-9 was sufficient to irreversibly inhibit parasite growth. Desferrioxamine blocked the drug effect, indicating the requirement for iron. Oxygen however, was not essential. Spectrophotometric analysis showed that under anoxic conditions, L2-9-Fe(II) chelate undergoes an intramolecular redox reaction which presumably involves a one electron transfer and is expected to result in the formation of free radical. Spin trapping coupled to electron spin resonance (ESR) studies of L2-9-iron chelate showed that L2-9-Fe(II) produced free radicals both in the presence and absence of cells, while L2-9-Fe(III) produced free radicals only in the presence of actively metabolising cells.

KEY WORDS: red blood cells, Plasmodium falciparum, iron chelates, free radicals, electron spin resonance.

ABBREVIATIONS: RBC, red blood cells; DFO, desferrioxamine; ESR, electron spin resonance.

INTRODUCTION

The widespread of multidrug resistant strains of the malarial parasite — P. falciparum that are now resistant to the quinine-related antimalarials calls for fundamental change in the design of novel chemotherapeutical agents. Clinical observations have shown that iron metabolism and malarial infection are closely interrelated, and several studies have been designed to explore the effect of iron chelators on the growth of P. falciparum.3-6 The binding of iron to redox-active sequestering agents could give...
rise to opposite biological effects, namely, either to enhancement of an oxidative (free radical) damage to the cell,\(^1\) or inversely to an inhibition of such an effect. Mechanistically, chelators can operate by several distinctly different modes. One mode may involve mediation in transferring of the metal-ion to a cell receptor, invoking a damaging process to the latter. Another mode may invoke modification of the electrochemical gradient of the metal-ion. Alternatively, the chelator may act as a catalyst for converting essential cell metabolites into harmful free radicals by promoting a single-electron-transfer (SET) process.

*In vivo* and *in vitro* observations suggest that oxidative stress plays a dominant role in the defense against parasitic infections. Malarial parasites have been shown to be more susceptible to oxidative stress than their host erythrocytes.\(^8\)-\(^\text{11}\) Injection of malaria infected mice with alloxan which is known to increase in vivo the production of highly reactive oxygen species such as \(O_2^\cdot\), \(H_2O_2\), and \(^1\)OH markedly reduced the parasitemia.\(^\text{12}\)-\(^\text{13}\) There is also evidence that activated macrophages contribute through reactive oxygen intermediates (ROI) and other secretory products to rodent immunity against malaria.\(^\text{14}\) The concept of free radical induced damage and the greater susceptibility of parasitized erythrocytes to oxidant damage provides a number of possibilities for malaria control.

In the present study, the chloroquine-resistant strain of *P. falciparum* is shown to be susceptible to 20 \(\mu\)M of an iron chelator (L2-9 = \(L^+\), \(X^-\)) based on pyridoxal-betaine. Most significantly, the latter was observed to inhibit the growth of parasites after 2 hours of incubation.\(^\text{17}\) This inhibition could be reversed by equimolar concentration of desferrioxamine (DFO). This result could be rationalized by viewing the chelator as functioning not only as a metal sequestrant capable of depriving the parasite of essential iron, but also as an inducer of free radical intermediates which lead to the death of the parasite. To substantiate this rational, we undertook an electro spin resonance (ESR) study (using spin traps such as PBN and DMPO) of L2-9. Most significantly, we observed that whereas the Fe(II)-chelate \([L^+, \ X^-\cdot\text{Fe(II)}]\) induces the production of carbon-centered free radicals both in the presence and in the absence of cells (parasites), the corresponding Fe(III)-chelate \([L^+, \ X^-\cdot\text{Fe(III)}]\) could do so only in the presence of living cells.

**MATERIALS AND METHODS**

Chemicals: The ligand, 1-[\(N\)-ethoxycarbonylmethyl-pyridoxylidenium]-2-[2'-pyridyl] hydrazine bromide (code named L2-9) was synthesized by exposing pyridoxal pyridyl hydrazone to the action of ethyl bromoacetate. Desferrioxamine B methanesulphonate (DFO) was obtained from CIBA. Horsham, Sussex-UK. \(\alpha\)-phenyl-\(N\)-tert-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline-\(N\)-oxide (DMPO) were purchased from Aldrich. DMPO was purified before use by distillation or by activated charcoal. The DMPO concentration in aqueous solutions was determined spectroscopically. \(\text{Fe}_2(\text{SO}_4)\)\(_3\) and FeSO\(_4\) were obtained from Fisher Scientific Co. (Silver Spring, MD); RPMI 1640 from GIBCO (Grand Island, NY USA); HEPEs from Sigma Chem. Co. (USA). All other chemicals were of analytical grade.

**Parasites:** *P. falciparum* (FCR-3) was obtained from a stock of a continuous line maintained in human A\(^+\) erythrocytes in RPMI 1640 medium containing 10% plasma.\(^\text{15}\) Parasites were synchronized by either the sorbitol treatment,\(^\text{16}\) gelatine sedimentation\(^\text{17}\)
or a combination of both. Before use, parasites were washed twice with RPMI 1640 without plasma and once with HEPES/NaHCO₃ buffer (pH 7.2), and resuspended in the HEPES/NaHCO₃ buffer to a concentration of 10⁵ cells/ml.

*Influence of DFO on the inhibitory potential of L2-9.* Parasites were synchronized by gelatin sedimentation and adjusted to 0.4% parasitemia and 4% hematocrit. This was dispensed equally into 15 ml centrifuge tubes. To these were added either 4 ml of RPMI 1640 normal growth medium, L2-9, DFO or 2 ml each of DFO and L2-9. For the L2-9/DFO mixture, parasites were first suspended in 2 ml of 80 μM DFO for 15 min, and then 2 ml of 80 μM L2-9 was added to give a final L2-9/DFO concentration of 40 μM. All four tubes were incubated at 37°C for 2 h. After the 2 h incubation, the cells were washed thrice with RPMI washing solution and cultured in normal growth medium. Parasitemia was determined daily by microscopical counts of number of parasites per 1000 cells.

**ESR measurements:** Spectra were recorded with a Varian E4 X-band spectrometer (9.5 GHz) with a field modulation frequency of 100 kHz, using non-saturating microwave power and modulation amplitude of 1 G. Measurements were made using a gas permeable teflon capillary which was inserted inside quartz tubing and placed in spectrometer.

**Spectroscopic measurements:** Measurements were made with a dual beam Kontron model Uvikon 860 spectrophotometer at room temperature in quartz cuvettes with optical path length of 1 cm. In order to prevent hydrolysis, iron was initially dissolved in sulphuric acid at pH 2.0 and complexed to the chelator anaerobically at the acid pH, [L2-9]/[iron] = 1:1. The concentration was adjusted to 0.5 mM L2-9 with triple distilled water. Just before use, the pH of the complex was adjusted to 7.2 using HEPES buffer to give a final L2-9-Fe concentration of 50-100 μM and 40 mM buffer concentration.

**RESULTS**

The exposure of the parasites to 20 μM L2-9 for 2 h resulted in inhibition of the *P. falciparum* growth. This inhibitory effect could be reversed by adding an equimolar concentration of DFO. This reversal action was observed only when the parasites were suspended first in DFO medium for 15 min followed by addition of the chelator (L2-9). If the parasites were added to a mixture containing DFO and the pyridoxal-based chelator, no effect of DFO was observed (Figure 1).

Spectrophotometric studies showed that under anoxic conditions and at pH 5.0-5.6, the reaction between the ligand and Fe(II) gives rise to a green coloured complex characterised by an absorption band \(\varepsilon_{L2-9-Fe(II)}^{525\text{nm}} = 5.25 \text{mM}^{-1}\text{cm}^{-1}\). Within 30-60 min and even in the absence of oxygen, the green L2-9-Fe(II) chelate gradually changes to a red L2-9-Fe(III) chelate having an extinction coefficient \(\varepsilon_{L2-9-Fe(III)}^{408\text{nm}} = 19.05 \text{mM}^{-1}\text{cm}^{-1}\) (Figure 2). The L2-9-Fe(III) generated from L2-9-Fe(II) under anoxic conditions was spectrophotometrically similar, though not equal to that formed by the direct chelating of L2-9 to Fe(III) ion, having \(\varepsilon_{L2-9-Fe(III)}^{412\text{nm}} = 60.8 \text{mM}^{-1}\text{cm}^{-1}\). The anoxic conversion of L2-9-Fe(II) to L2-9-Fe(III) indicates some intramolecular redox process which presumably involves a one electron transfer and is therefore expected to yield some
FIGURE 1 Effect of Desferrioxamine (DFO) on the antimalarial activity of L2-9. Gelatin synchronized parasites \textit{P.falciparum} culture was dispensed in aliquots of 5 ml of either normal medium (control), L2-9, DFO or L2-9 + DFO. All concentrations were 40 \mu M at final hematocrit of 4\% and a parasitemia of 0.2\%. They were incubated at 37\^\circ C for 2 h. In the case of L2-9 + DFO, DFO was first added and left to stand for 15 min before addition of L2-9. After incubating for 2 h, parasites were washed thrice with RPMI 1640 washing solution and cultured in RPMI complete medium. Parasitemia was determined daily by microscope counts of number of parasites per \(5 \times 10^3\) erythrocytes. Each value represents an average of 3 counts.

FIGURE 2 Absorption spectra of the anaerobic oxidation of Fe(II) to Fe(III) by L2-9. 1 mM Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\) in 10 mM sulphuric acid at pH 2.0, was complexed to L2-9 anaerobically in a ratio of [L2-9]/[iron] = 1:1. The concentration was adjusted to give 50 \mu M (L2-9)-Fe(II) in 40 mM HEPES buffer pH 7.4. Change in spectra with time after complexing were (a) 20 min; (b) 1 h; (c) 2 h; (d) 3 h; and (e) 20 h. The assay was performed anaerobically at room temperature using a dual beam Kontron model Uvikon 860 spectrophotometer.
FIGURE 3  Electron spin resonance (ESR) spectra of L2-9-Iron complexes. L2-9-Fe(II) was prepared anoxically and immediately scanned for ESR signal in the presence and absence of cells, (a) 15 mM PBN; (b) 135 mM DMPO; (c) in the presence of 15 mM PBN and 10^8 P. falciparum schizonts/ml; (d) (L2-9)-Fe(III), 15 mM PBN and 10^8 P. falciparum schizonts/ml.
reduced intermediate of the ligand L2-9. A tentative explanation for this observation is that Fe(III)-(L', X-) tends to enter into a demonstration reaction, as follows: 2Fe(III)-(L', X-) → Fe(II)-(L', X-) + Fe(III)-(L', X-). To investigate this possibility, the reaction was studied using electron spin resonance (ESR) spectroscopy. When the ligand-iron II complex was anaerobically prepared and scanned for the presence of free radicals, no ESR signal was detected. Because the lack of any observable ESR signal could be due to instability of the active intermediates, the spin trapping technique was utilized. L2-9-Fe(II) was anaerobically incubated in the presence of spin traps such as PBN and DMPO. In the presence of either PBN or DMPO, six lines ESR signals were accumulated and persisted. Both ESR spectra were characteristic of a carbon centered spin adduct having $a_N = 15.75G$ and $a_H = 4.25G$ of PBN-R' (Figure 3a) and $a_N = 15.75G$ and $a_H = 23.5G$ of DMPO-R' (Figure 3b) respectively. The same ESR signals were observed when the experiments were repeated under aerobic conditions but no spin adducts of \('OH or O_2^{' were detected.

L2-9-Fe(III), unlike L2-9-Fe(II), did not elicit any detectable spin adduct. It was therefore anticipated that in the presence of metabolising cells, which are capable of reducing Fe(III) to Fe(II), the generation of free radicals by both chelates would be potentiated. When the experiments were repeated using Plasmodium falciparum (10'schizonts/ml), the chelates of both Fe(II) and Fe(III) gave rise to ESR signals identical to those observed in cell-free systems (Fig. 3c and 3d). However, the spin adduct signal generated by L2-9-Fe(II) was bigger than that formed by L2-9-Fe(II).

DISCUSSION

In this study we have shown that the chelator L2-9 (L', X- in chart) induces the production of free radicals in the presence of Fe(II). In the presence of metabolising cells or reducing agents, free radicals were produced by both Fe(II) and Fe(III). It thus appears that L2-9 inhibits the growth of parasites not just by the simple chelating process of iron withholding but by the generation of free radicals. The death of parasites after two hours of incubation with L2-9 is attributable to some active intermediates induced by the chelator in the presence of Fe(II) rather than a simple nutritional deprivation of iron.

The production of oxygen-derived free radicals via the iron catalyzed Haber-Weiss reaction is well documented. However, considerable controversy exists as to whether the natural sequestered forms of iron or possibly other transition metal ions are effective in catalyzing this reaction.\textsuperscript{18-21} The activity of iron is dependent upon the ligand to which the metal is complexed, since various chelators alter the redox potential and the reactivity of the bound metal ion to varying extents.\textsuperscript{24,25} Iron chelators such as EDTA and DTPA inhibit or increase lipid peroxidation stimulated by chelatable iron depending on the ratio of chelator to iron, whereas DFO inhibits at all concentrations tested.\textsuperscript{25,26} Clark et al.\textsuperscript{27} have reported the in vivo and in vitro killing of Plasmodium vinсkei vinсkei and Plasmodium falciparum by \textit{t}-butyl hydroperoxide (\textit{t}-BHP) which generates oxygen derived free radicals. It was also shown that alloxan, a generator of reactive oxygen intermediates, cleared mice of lethal infection with Plasmodium vinсkei.\textsuperscript{13} In both experiments, pre-treatment with DFO for 15 min reportedly abolished the action of \textit{t}-butyl hydroperoxide and alloxan. The present results suggest that free radical intermediates are instrumental in L2-9 toxicity.
towards the parasites. The failure, however, to detect DMPO-OH spin adduct in the present study and the anoxic toxicity of L2-9 do not support the hypothesis that oxygen-derived radicals such as 'OH or superoxide, play any role in the drug's effect.

Oxidant generating drugs have been previously shown to have anti-malarial properties, but the precise mechanism by which the human malarial parasite is killed by a variety of radical generating systems still remain unclear. The data produced here permits to suggest that the death of the parasite is likely caused by a sort of damaging process arising from the break down of an essential component of the cell, such as the DNA macromolecule. Evidences indicating damage to DNA by L2-9-Fe(II) is forthcoming. This is substantiated by the appearance of ESR signals only when living cells are present in a mixture of the chelate L2-9-Fe(III) with spin traps such as PBN or DMPO, indicating that living cells are involved in a type of single electron-transfer [SET] as expressed by Eq. 3. The tendency of the DNA macromolecule to function as an electron-transfer oxidant (an electron acceptor) in redox reactions, is well documented. It is plausible therefore to suggest that the iron chelate assumes most likely the role of an electron-transfer-reductant (an electron donor) in the latter process. Accordingly we attribute reductive properties to the carbon-centered free radical of structure (L, X')-Fe(III) (see chart). The latter most likely arises from a reversible intramolecular SET shift (Eq. (2)), involving an initially formed (L', X')-Fe(II) intermediate (Eq. (1)). The (DNA) anion radical (eq. (3)) is known to be highly labile, tending to fragment and thus leading to the death of

**CHART**

![Chart](image-url)
the cell.

\[ [H_2L^+, X^-] + Fe^{2+} \rightarrow 2H^+ + [L^+, X^-]-Fe(II) \]  
(1)

\[ [L^+X^-]-Fe(II) \xrightarrow{\text{intramolecular electron transfer}} [L^+, X^-]-Fe(III) \]  
(2)

\[ [L^-, X^-]-Fe(III) + DNA (parasite) \xrightarrow{\text{SET}} [L^+, X^-]-Fe(III) + [DNA^-] \text{ parasite} \]  
(3)

Parasite-[DNA]^- \rightarrow \text{Decomposition products}  
(4)

Because of the low concentration of the ligand (20 \( \mu \text{M} \)) for effective antimalarial activity, its mode of action could not be explained in terms of mass-action alone. It could however be understood in terms of steady-state theory, by which the truly active species, namely, the carbon-centered free radical \([L^-, X^-]-Fe(III)\) is regenerated in the presence of living cells as soon as consumed. The free radical regeneration could be envisioned to result from a cascade of redox events involving ubiquitous cell reductants by \((\text{red-(I)}-H_2), (\text{red-(II)}-H_2), \text{etc.},\) with matching electrochemical gradient as expressed by Eqs. (5) and (6).

\[ 2[L^-, X^-]-Fe(III) + [\text{red-(I)}-H_2] \rightarrow 2H^+ + [L^+, X^-]-Fe(III) \]  
(5)

\[ [\text{ox-(I)}] + [\text{red-(II)}-H_2] \rightarrow [\text{red-(I)}-H_2] + [\text{ox-(II)}] \]  
(6)

Essentially it suggests that the antimalarial free radical species \([L^-, X^-]-Fe(III)\) plays a pivotal role in catalyzing the generation of electrons from ubiquitous cell reductants, and in mediating the transfer of the electrons to the parasite-DNA, by a multi-stage ET process, as outlined below:

\[ \text{[red-(I)}-H_2] + 2\text{DNA-parasite} \xrightarrow{\text{catalyst}} [\text{ox-(I)}] + 2H^+ + 2[\text{DNA}^-] \text{- parasite.} \]  
(7)

The novel type of lipophilic-hydrophilic chelator based on pyridoxal-betaine (L2-9) described here is a representative of a family of new antimalarials operating by a hitherto unknown mechanism involving the generation and transfer of electrons from cell reductants to the parasite DNA (Eqs. (3-7)). The death of the parasite is believed to follow from the breakdown of the resulting DNA anion radical ([DNA]^-) (eqn. 4).14,38

\[ \text{[DNA]^-} \xrightarrow{\text{fragmentation}} [L^+, X^-]-Fe(III) \]  

\[ 2H^+ + [\text{ox-(I)}] \]

The greater susceptibility of parasitized erythrocytes to oxidant damage14,38 and the relative abundance of iron in parasitized cells provide a number of possibilities for the design of new anti-malarial drugs based on intracellular iron dependent radical inducers.

References

GROWTH INHIBITION OF PLASMODIUM FALCIPARUM


38. A.O. Wozencraft (1986) Damage to malaria infected erythrocytes following exposure to oxidant generating systems. Parasitology, 92, 559-567.

Accepted by Prof. G. Czapski
Fe (II)-CHELATES BASED ON REDOX-ACTIVE PYRIDOXAL-BETAINES AS C-CENTERED RADICALS CAUSING SINGLE- AND DOUBLE-STRAND SCISSIONS TO DNA

EUGENE N. IHEANACHO†, SHALOM SAREL‡, AMRAM SAMUNI*, SHELLY AVRAMOVICI-GRISARU‡, and DAN T. SPIRA†

†The Kavli Centre for the Study of Infectious and Tropical Diseases; ‡Dept. of Molecular Biology; Hebrew University — Hadassah Medical School; *Dept. of Pharmaceutical Chemistry; Hebrew University School of Pharmacy, Jerusalem — Israel

The ability of 1-[N-Ethoxycarbonylmethyl]pyridoxalhemiacetal [L2-9 = L',X ]-[Fe(II)] chelate [L2-9-Fe(II)] to induce breaks both in the 43kb linear double-strand phage DNA, and in the 4383 base pair supercoiled pBR322 plasmid DNA is here described. Neither the free ligand nor Fe(III) alone demonstrated any effect on the DNA. The cleaving ability is shown to occur instantaneously under strictly anaerobic conditions, either in the presence or absence of the enzyme catalase. It is also shown to be dose dependent. Thus, at a DNA:L2-9-Fe(II) molar ratio of 3.7:1, the linear DNA is randomly cleaved into fragments ranging from 23.4kb to 4.3kb, whereas at approximately 1:1 molar ratio, the range extends down to 2.5kb fragments. By contrast, at 1:2.7 [plasmid DNA]: chelate-Fe(II) molar ratio, a single-strand nick was observed, and a double strand break was noted at a 1.50 ratio [plasmid DNA]: chelate-Fe(II). A multi-stage redox cycling involving a carbon-centered (L,X )-Fe(III) radical capable of transferring an electron to the DNA to form high unstable [DNA]•-amino-radical is invoked to explain the degradation of the macromolecule. Possible modes for regeneration of the chelate-Fe(III) radical both at the cell-free and at the cell levels are proposed.

KEY WORDS: Iron chelates, free radicals, DNA breakage.

INTRODUCTION

The increase in drug resistant strains of the malarial parasite — P. falciparum underscores an urgent need for the development of new chemotherapeutic strategies against malaria. Though immunopreventive and immunocurative strategies are still being experimented upon, the prospect for the development of an effective vaccine looks bleak.† Chemotherapy therefore remains the major weapon for reducing malaria morbidity and mortality. Elucidation of the mechanism of resistance and the mode of action of chemotherapeutic agents are important in the development of new chemotherapeutic strategies.

Iron chelation may form the basis of a new class of antimalariais. Laboratory and field observations lend support to this possibility.‡ The binding of iron to redox
active sequestering agents may give rise to opposite biological effects, namely, either to the enhancement of an oxidative (free radical) damage, or inversely, to an inhibition of such effect. Mechanistically, chelators can operate by several distinctly different modes. One mode may involve mediation in transferring of the transition metal-ion to a cell receptor, invoking a damaging process to the latter. Another mode may involve modification of the electrochemical potential of the metal-ion and as a consequence to affect essential redox processes in the cell. Alternatively, the chelator may act as a catalyst for converting essential cell metabolites into harmful free radicals by promoting single electron transfer (SET) processes.

Free radical attack on DNA has been shown to have a serious effect on living organisms by causing strand breaks in the DNA. The tendency of the DNA macromolecule to function as an electron-transfer oxidant is well documented. Cochrane et al. have shown in studies with isolated DNA that hydrogen peroxide (H2O2) generates hydroxyl radicals (·OH), following Fenton reaction at the vicinity of the DNA and that the amount of the ·OH formed correlates with the percentage of DNA strand breaks.

By use of a representative of a new class of iron chelators based on pyridoxal-betaine – [(1-[N-ethoxycarbonylmethyl]-pyridoxylidenium)-2-[2'-pyridyl]hydrazine bromide] – (code named L2-9), we have developed a model for a new chemotherapeutic strategy against the chloroquine resistant malaria parasite – Plasmodium falciparum (FCR116). Previously, we have shown that the antimalarial action of the new chelator involves a carbon-centered free radical chelate (L2-9Fe(II)–[L2-9]2Fe(III)). In the present study, we show that the free radicals generated by the chelator and Fe2+ affects the DNA. Nick translation and electrophoresis of L2-9-Fe(II) complex treated DNA showed that the latter suffers both single and double strand fissions only by the chelator-iron complex and not by either the chelator, or the iron (Fe2+) alone.

MATERIALS AND METHODS

Chemicals

The iron chelator – L2-9 was synthesized by exposing pyridoxal pyridyl hydrazine to the action of ethyl bromoacetate. Ferric and ferrous sulphates were obtained from Fisher Scientific Co. (Silver Spring, MD, USA); RPMI 1640 from Gibco (Grand Island, NY, USA); HEPES from Sigma Chemical Co (St Louis, MO, USA); Nick translation System Kit from NEN research Products (Boston, MA, USA); Calf thymus (CT), Lambda (λ) DNA, Plasmid (pBR322), catalase and mineral oil from Sigma Chemical Co. All other chemicals were of analytical grades.

Chelator-iron (II) complex [L2-9-Fe(II)] was prepared immediately before use by mixing 1mM L2-9 with 1mM FeSO4 (v/v) to give a 500µM complex. This was diluted to the required working concentrations (0.5-40µM) with RPMI 1640 medium without plasma (washing solution).

Nick translation. The method used as per the instructions in the manufacturer’s manual for Nick translation system [32P]. Briefly, the following components were added as follows: [2-32P]dCTP – 5µl; Nick translation buffer – 5µl; cold deoxynucleoside triphosphate mixture (dCTP) 4µl; either L2-9-Fe(II) treated or untreated CT DNA – 2µl (the final concentration in the reaction mixture was 0.5µg);
INDUCED DAMAGE TO DNA BY A PYRIDOXAL BASED IRON CHELATOR

309

translation grade water – 7 µl; DNA polymerase I (Pol 1, 0.6 units/µl -- 2 µl. These gave a total reaction mixture of 25 µl. This was incubated at 10°C for 2h. The reaction was terminated by the addition of 200 µl Nick translation stop buffer. The mixture was then passed through a sephadex G-50 column using a 1ml syringe. The effluent was collected and 10 µl sample placed on a Whatman GFC filter paper and radioactivity counted.

\begin{equation}
\text{Gel electrophoresis (Neutral gel).} \quad 2 \mu l \text{ of } \lambda \text{ DNA (0.5 µg/µl) was incubated with 8µl of various concentrations (0.5 µM–40 µM) of freshly prepared L2-9-Fe(II) complex for 2h. After the 2h incubation period, 0.5 µg of the treated DNA was run in an agarose gel (0.8%) using 50 mM Tris-Acetate-EDTA (TAE) pH 7.5 as the running buffer. The plasmid (pBR322) was treated likewise with the L2-9-Fe(II) complex and run in 1% agarose gel in TAE buffer pH 7.5. The gel was run at 5 V/cm for 3h.}
\end{equation}

To measure the time course of DNA cleavage by the chelate, 1 µl of plasmid pBR322 (0.5 µg/µl) was incubated with 5 µl of 40 µM freshly prepared L2-9-Fe(II) complex for various lengths of time (0–60 minutes). The reaction was stopped by the addition of 2 µl loading buffer containing 1 mM EDTA. To mimic the effect of an anoxic condition, a thin layer of mineral oil (heavy white oil) was placed over one of the reaction mixtures and incubated for 60 minutes. To test if hydroxyl radicals (-OH) was involved in L2-9 induced radical damage, catalase (60µg) was added to one of the reaction mixtures and incubated for 60 minutes. The mixtures were run in 0.8% agarose gel in TAE buffer pH 7.5 at 1.5 V/cm for 14 hours (overnight) at room temperature.

\section*{RESULTS}

Using the nick translation method of Kelly et al. (1970),17 the ability of L2-9-Fe(II) complex to induce nicks in DNA was tested. The result (Table) showed that L2-9-Fe(II) complex induced nicks in DNA. CT DNA treated with L2-9-Fe(II) complex incorporated [32P]dCTP 3 times as much as the untreated one. CT DNA treated with DNase I incorporated [32P]dCTP only twice as much as the L2-9-Fe(II) treated one. L2-9-Fe(II) could therefore be said to have introduced nicks or breaks in the DNA half as effectively as the DNase I.

\begin{table}[h]
\centering
\caption{ Incorporation of [32P]dCTP into L2-9-Fe(II) treated CT DNA}
\begin{tabular}{ll}
\hline
CT DNA treated with: & Counts \\
\hline
RPMI medium (without chelate) & 136492 ± 15121 \\
L2-9-Fe(II) in RPMI medium & 322504 ± 1754 \\
DNase I & 619230 ± 10332 \\
\hline
\end{tabular}
\end{table}

Counts are expressed as mean ± SD of triplicate determinations. CT DNA was suspended in either 40µM L2-9-Fe(II) in RPMI medium or RPMI medium alone for 3h and thereafter used for the nick translation reaction.

Standard Deviation.
FIGURE 1 Gel electrophoretic pattern of λ phage DNA treated with L2-9-Fe(II) complex. λ phage DNA was suspended for 2h in RPMI medium (without plasma) containing various concentrations of L2-9-Fe(II). The treated DNA was then separated on a 0.8% agarose gel, stained with ethidium bromide and visualized under UV illumination. Lane a, DNA digested with HindIII; lane b, DNA in medium without L2-9-Fe(II); lanes c-h, DNA in medium containing 40, 20, 10, 5, 1, and 0.5μM L2-9-Fe(II) complex respectively. Lanes i, and j, DNA in medium containing 40μM L2-9, and Fe(II) alone, respectively. The final amount of the DNA applied to each well was 0.5μg.

The ability of the chelator-iron complex to induce nicks in DNA was also tested by gel electrophoretic analysis of both λ phage and pBR322 plasmid DNA. Figure 1 shows the gel electrophoretic pattern of λ phage DNA treated with the chelator-Fe(II) complex. The cutting ability of the complex is dose dependent. 40μM of the complex formed fragments ranging from 2.5kb to 23.1kb. The 20 and 10μM complexes formed less number of fragments, while the 5 and 1μM complexes formed only fragments of 9.4 and 23.1kb (using λ DNA cut with restriction endonuclease HindIII as the marker). Introducing nicks in a supercoiled plasmid would result in either its relaxation (for single strand nicks) or its linearization (for double strand nicks). When pBR322 plasmid was incubated with the complex for 2h, both single and double strand breakers were induced by 40, 20, and 10μM complexes; while the 5, 1, and 0.5μM complexes induced only single strand breaks (Figure 2). Neither L2-9 nor Fe(II) alone had any effect on the DNA.

The time course of DNA cleavage by the chelate was followed by incubating 0.5μg pBR322 with 40μM complex for times 0-60 minutes. The result (Figure 1) showed that the DNA cleavage was rapid, occurring in less than 5 minutes. Oxygen was not necessary for the cleavage to occur, and catalase had no effect on it, indicating the non-participation of hydroxyl radicals in the L2-9 induced radical damage.
DISCUSSION

From Figures 1 and 2, it is evident that L2-9-Fe(II) [(L',X)-Fe(II)] is able to induce breaks both in the high molecular λ phage DNA (26.57 × 10^6 mol. wt., from E.coli strain C600), a 43000 base pair linear double strand DNA, and in a lower molecular pBR322 plasmid DNA (2.7 × 10^6 mol. wt., from E.coli strain RPI), a 4363 base pair supercoiled DNA. Neither the free ligand (L-9) nor Fe(II) alone exhibited any effect on the DNA. The cleaving ability of (L',X)-Fe(II) is shown to be both instantaneous and dose-dependent. At molar ratio 3.7:1.0 of Lλ DNA : (L',X)-Fe(II), the macromolecule is randomly cut into fragments ranging from 23100 to 4360 base pairs, and at approximately 1:1 - molar ratio, the range extends down to 2500 base pair fragments.

In contrast to the observed chain breaking yield (DNA/Fe-chelate × 100) of 370% in λ DNA, the yield of macromolecular scission in the supercoiled DNA plasmid is considerably lower (37-4%). Thus, at [pBR322 plasmid DNA] : [(L',X)-Fe(II)] molar ratio of 1:2.7, the supercoiled DNA undergoes a single strand nick leading to its relaxation, whereas at 1:50 molar ratio, a double-strand scission takes place giving rise to its linearization. From Figure 3 it can be seen that these DNA chain cuts occur...
FIGURE 3  Gel electrophoretic pattern of plasmid pBR322 treated with 40μM 12,9-Fe(III) complex b times ranging from 0-60 minutes. 5μg of plasmid pBR322 was incubated with 5μl of 40μM fresh prepared 12,9-Fe(III) complex for various lengths of time (0-160 minutes). The reaction was stopped by addition of 2μl loading buffer containing 1M EDTA. It was separated on a 0.8% agarose gel, stained with ethidium bromide and visualized under UV illumination. Lane a, untreated plasmid. Lane b, untreated plasmid under anaerobic condition, lane c plasmid treated with 100μM 12,9 alone. Lane d, treated with 40μM chelate for 60 minutes, lane e, treated with 40μM chelate + 0.5mg catalase for 60 minutes, lane f treated with 40μM chelate under anaerobic condition for 60 minutes, lanes g-k, treated with 40μM chelate and incubated for 30, 20, 10, 5, and 0 minutes respectively. The amount of DNA in each well was 0.5μg. The gel was run at 1 V/cm at 4°C for 14 hours (overnight) at room temperature.

instantaneously under anaerobic conditions, and that they are unaffected by the presence of the enzyme catalase, implying the uninvovlement of reactive oxygen species (ROS) in the chain breakings.

ESR study12 has shown that (L',X )-Fe(II) exist as a transient carbon-centered free radical presumably of L',X -Fe(III) structure, and its X-ray crystal analysis14 indicated that the ligand (L',X ) utilizes all available hydrogen acceptors and donors for intra- and inter-molecular hydrogen contacts. On the basis of data now available, it is permissible to invoke a multi-stage process for the macromolecular chain degradation initiated by (i) binding (L',X )-Fe(II) to DNA chain, followed by (ii) single-electron-transfers (SET): (L',X )-Fe(II) → (L',X )-Fe(II) + DNA → (L',X )-Fe(III) + [DNA] , and (iii) degradation of the anion − radical [DNA] into [base pair fragments] , then finally by (iv) redox-cycling: [base pair fragment] + (L',X )-Fe(III) → DNA-fragments + (L',X )-Fe(II), to start all over again. The discrepancy in the chain breaking yields underlined above could be rationalized in terms of redox gradients, being higher for the linear [DNA] than for the circular/relaxed [DNA] species.
INDUCED DAMAGE TO DNA BY A PYRIDOXAL BASED IRON CHELATOR

\[(L'\cdot X) + Fe(II) \rightarrow (L'\cdot X) - Fe(II) \rightarrow (L'\cdot X) - Fe(III)\]

Oxidized metabolites + (L'\cdot X) - Fe(II) \rightarrow (L'\cdot X) - Fe(III) + \{DNA\}

The mechanistic scheme portrayed above could reasonably be invoked to explain the *in vitro* cytocidal effect of L2-9 on the drug resistant malarial parasite *Plasmodium falciparum*. It implies that the ligand sequesters first the endogenous cellular Fe's to form the respective Fe(II)-complex, since pre-treatment of the parasite culture with a stronger iron chelator – desferrioxamine – obliterates the antimalarial properties of L2-9.

The \((L'\cdot X)\)-Fe(II) complex is prone to an internal electron transfer to form the respective radical species capable of delivering an electron to the DNA after its binding to the macromolecule to yield an unstable \[[\text{DNA}]^+\] which decomposes as soon as formed (see chart). It is of interest to compare the mode of action of \((L'\cdot X)\)-Fe(III) with that of the iron chelate radical-cation Fe(II)-Dox' \(^{21}\) originating from sequestration of the anti-tumour antibiotic doxorubicin (DOX) (adriamycin) with Fe's, following the sequence: Fe(III)-Dox \rightarrow Fe(II)-Dox'. Whereas the former does not require molecular oxygen to damage DNA, the latter by contrast, does require \(O_2\) to produce ROS via the reaction: Fe(II)-Dox' + \(O_2\) \rightarrow Fe(III)-Dox + O\(_2\)\(^{\cdot}\) (ROS). The ligand in \((L'\cdot X)\)-Fe(II) functions apparently as an internal electron transfer oxidant, whereas DOX in Fe(III)-Dox functions as an internal electron transfer reductant. \(^{21}\) The antimalarial action of ditaluric/alloxan, \(^{22}\) divicine, \(^{22}\) and butyl hydroperoxide, \(^{22}\) seems also to involve ROS \(^{22}\) via redox cycling. \(^{22}\) In the latter, electrons appear to emerge from endogenous cellular reductants, \(AH_2\), which in combination with a redox metal (Cu/Fe) complex and oxygen can generate ROS via \(3AH_2 + 20_2 \rightarrow 3A + 2H_2O + 2\cdot OH\) (net reaction).

Antimalarial chelators based on redox-active pyridoxal-betaine seems to operate quite differently, following a hitherto unknown redox cycle:

\[AH_2 + 2Fe(III) - (L'\cdot X) \rightarrow A + 2Fe(III) - (L'\cdot X)\]

\[\{\text{DNA}\}^+ + Fe(III) - (L'\cdot X) \rightarrow DNA + Fe(III) - (L'\cdot X)\]

Conceptually, the ability of L2-9 to interact with and cut DNAs (\(\lambda\) phage and plasmid) was reasonably extended to explain its antimalarial action *in vitro*. This offers an interesting venue to antimalarial drug design implicating intracellular generation of harmful free radicals by iron-dependent inducers. The rapid spread of multi-drug resistant strains of the malarial parasites prompts the use of new models for drug design to provide more effective antimalarials operating by hitherto unknown mechanisms. It is highly likely that L2-9, or its analogues could demonstrate other antiparasitic and/or antitumor activity of potential therapeutic utility.
Acknowledgement

This study was supported by CDR Program grant C7 - 160, No.5544-G-SS-7021-00.

References


Accepted by Prof. G. Czapski