Structure-Function Correlations in the Reaction of Bis(thiosemicarbazonato) Copper(II) Complexes with Ehrlich Ascites Tumor Cells

Daniel T. Minkel, Leon A. Saryan, and David H. Petering

ABSTRACT

Examination of a series of substituted bis(thiosemicarbazonato) copper(II) complexes shows that their reaction with Ehrlich ascites tumor cells can be understood in terms of the relative reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes correlates directly with their respective terms of the relative reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups. In general, the order of reactivity of the complexes with sulphydryl groups.

INTRODUCTION

During the investigation of the antitumor properties of bis(thiosemicarbazones), a variety of derivatives were made and an interesting structure-function correlation was observed (11). For a given group, ethoxyethyl or methyl, the compounds with R2, R3 = H or R2 = CH3 and R3 = H were activated by copper ion. However, the N-dimethylthiosemicarbazones with R2 = CH3 could not be converted into cytotoxic species by complexation with copper.

In the preceding article (8) a mechanism of reaction of CuKTS and Ehrlich cells was set forth. In essence the evidence supports an oxidation-reduction reaction between CuKTS and cellular thiols (Equation A).

Cu(II)KTS + 2RSH → Cu(II)SR + R2RSSR + H2KTS (A)

in which copper becomes distributed throughout the cell, inhibiting a variety of processes such as DNA synthesis and mitochondrial respiration and in which 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) is released to partition out of the cell (2, 3, 13). The validity of the mechanism is tested in the current work with the use of a set of bis(thiosemicarbazonato) copper(II) complexes containing both active and noncytotoxic metal chelates.
Comparative Reactions of Copper Complexes with Ehrlich Cells

Radioactivity in the acid-insoluble fraction was taken as a measure of DNA synthesis; counts remaining in the incubation medium were used to evaluate uptake of labeled deoxynucleotide into the cell.

Effects of Complexes upon Cell Respiration. As outlined earlier, sample and reference suspensions of Ehrlich cells in MEM supplemented with 10% ascites fluid were examined in tandem with a Yellow Springs Instruments Model 53 oxygen analyzer for their ability to carry out respiration (3). Complexes were added after stable O2 consumption rates were observed for both sample and reference suspensions and rates of respiration were monitored for 3 to 4 hr with periodic reoxygenation of samples.

Partition Coefficients. The UV-Vis spectra (200 to 800 nm) of equimolar concentrations of complexes in 10.0 ml H2O (or buffer) and 10.0 ml 1-octanol (Eastman Organics, Oak Brook, Ill.) were obtained. Then solutions were combined and equilibrated. Spectra were obtained on 3 successive days to assure that equilibrium was attained, and mass balance was calculated to ensure that no dissociation or decomposition had occurred. Concentrations and partition coefficients were calculated from published extinction coefficients (12, 13).

Cytotoxicity of CuKTS and CuKTSM2. The procedure is similar to one previously used (1). Ehrlich ascites cells are isolated from female Swiss mice and suspended in MEM. Complexes are incubated with cells for 20 min at 37°C. After centrifugation, removal of medium, and resuspension in fresh medium, 4 x 10^6 treated cells are injected into mice, 5 mice/group, and the weight changes of the mice are followed over time as a measure of tumor growth.

RESULTS

In the preceding paper (8) a rapid reaction of Cu(II)KTS with Ehrlich cells was described which was taken to be the primary interaction of the complex with cells. The reaction was followed conveniently by observing the rate of decrease of the visible absorbance band of CuKTS. This was translated into an observed rate constant, kobs, for the reaction. Table 2 summarizes the behavior of a series of bis(thiosemicarbazonato) copper(II) complexes in the presence of cells. Although some complexes, CuKTS, 3-ethoxy-2-oxobutyrildehyde bis(N4-dimethylthiosemicarbazone) copper(II), and pyruvaldehyde bis(thiosemicarbazone) copper(II), and pyruvaldehyde bis(N4-methylthiosemicarbazone) copper(II), were found in a representative experiment in which samples were counted on a Beckman LS-150 liquid scintillation instrument with automated quench determination.

Table 2

<table>
<thead>
<tr>
<th>Complex</th>
<th>kobs^a (sec^-1)</th>
<th>k/k0</th>
<th>Eui^b (mV)</th>
<th>k/k0^c</th>
<th>Relative in vitro cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CuKTS</td>
<td>8.29 x 10^-3</td>
<td>1.0</td>
<td>-178</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>2. CuKTSM</td>
<td>3.59 x 10^-3</td>
<td>0.43</td>
<td>-188</td>
<td>0.84</td>
<td>0.5</td>
</tr>
<tr>
<td>3. CuKTSM2</td>
<td>8.97 x 10^-3</td>
<td>0.0036</td>
<td>-283</td>
<td>0.007</td>
<td>c &lt;0.008 0 0.2</td>
</tr>
<tr>
<td>4. CuPTSM</td>
<td>3.94 x 10^-4</td>
<td>0.475</td>
<td>-188</td>
<td>3.7</td>
<td>4.63</td>
</tr>
<tr>
<td>5. CuPTSM2</td>
<td>4.32 x 10^-4</td>
<td>0.521</td>
<td>-208</td>
<td>3.7</td>
<td>2.44</td>
</tr>
<tr>
<td>6. CuPTSM2</td>
<td>1.26 x 10^-4</td>
<td>0.0132</td>
<td>-287</td>
<td>0.015</td>
<td>c &lt;0.008 0.25</td>
</tr>
<tr>
<td>7. CuGTS</td>
<td>1.26 x 10^-4</td>
<td>0.0132</td>
<td>-98</td>
<td>165</td>
<td>c &lt;0.008 0.88</td>
</tr>
</tbody>
</table>

^a Reaction with cell suspension, 19.7 mg cell protein per ml; initial concentration of complexes ~0.1 mm.
^b Inhibition of Walker 256-nitrogen mustard resistant respiration (Table 3; Ref. 11).
^c Footnote 13.
^d Footnote 3.
^e Inhibition of ascites cell respiration (Table 4).
^f For definitions, see Table 1.
ato) copper(II), react rapidly with cells, CuKTS, pyruvaldehyde bis(\(N^4\)-dimethylthiosemicarbazonato) copper(II), and CuGTS react much more slowly. This is the same pattern of reactivity of complexes seen in the modified Arai-Suzuki cytotoxicity test in which Walker cells are used (Table 2; Ref. 11). In Chart 1 \(\log k/k_0\) is plotted versus \(E_{1/2}\), the half-wave reduction potentials of the complexes. On the same graph and plotted similarly are the data for the rates of reactions of the copper chelates with dithiothreitol. A comparison is thus provided between substituent effects upon a cellular reaction thought to involve thiol oxidation and effects seen in a model system for examining thiol oxidation by bis(thiosemicarbazone) copper(II) complexes.

That the trend in the rates of the cellular reactions is not well correlated with the partition coefficient is illustrated in Table 3. Clearly, the similarities and marked differences in reactivity seen in Table 2 cannot be due to simple differences in membrane uptake and transport of the complexes.

Turning from the delineation of the initial reaction of this complex with Ehrlich cells to a consideration of the biochemical effects of these agents, we carried out studies of the inhibition of DNA synthesis in these cells with the reactive and unreactive complexes, CuKTS and CuKTS.

Experiments measuring the dose-response relationship of thymidine incorporation into acid-insoluble material and thymidine uptake by Ehrlich tumor cells revealed basic differences in the response of the cell to CuKTS and CuKTS complexes. The percentage of total radioactivity in each compartment was compared to control at 5, 10, and 20 min after the addition of radioactive precursors, and the results were pooled to obtain a percentage of control average value.

In Chart 2, the closed circles represent the percentage of DNA synthesis compared to control at various concentrations of CuKTS expressed in relation to the quantity of cell protein present. DNA synthesis is almost totally repressed at a concentration of 2 nmoles CuKTS per mg protein. Inhibition of thymidine transport is much more gradual. At 2 nmoles CuKTS per mg, approximately 75% of control rate of uptake is occurring. Transport is still taking place at 15% of control at CuKTS concentrations of 200 nmoles/mg protein.

Chart 3 shows that the effects of CuKTS upon DNA synthesis and thymidine uptake follow nearly the same concentration dependence in contrast to the response of the cells to CuKTS. At 2 nmoles CuKTS per mg protein, both processes are suppressed to 65% of control. At 45 nmoles/mg, transport of thymidine is 20% of control and DNA synthesis is 5% of control. Higher concentrations were not achieved because of the low solubility of this complex in aqueous media.

Superimposition of the data from Charts 2 and 3 shows that the patterns of thymidine transport repression are similar for both drugs. For CuKTS, however, DNA synthesis is repressed at much lower concentrations of drug than for CuKTS, reflecting a basic difference in the interaction of these 2 complexes with the cell.

Examination of the degree of inhibition of respiration of Ehrlich cells by several copper complexes shows that CuKTS and 3-ethoxy-2-oxobutyraldehyde bis(\(N^4\)-methylthiosemicarbazone) copper(II) are substantially more inhibitory than are CuKTS or CuGTS (Table 4). The concentration of CuKTS used here is substantially higher and the percentage of inhibition after long incubation is lower than needed for the perturbation of DNA synthesis shown in Chart 2.

It is necessary to show that the biochemical effects seen here and elsewhere occur at concentrations of complex that decrease cell viability. Hence, using the same incubation medium and temperature and working at levels of drugs known to inhibit DNA synthesis or cell respiration, we incubated cells with CuKTS or CuKTS, isolated them.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Partition coefficient (1-octanol-H_2O)</th>
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</thead>
<tbody>
<tr>
<td>H_2KTS</td>
<td>12</td>
</tr>
<tr>
<td>CuKTS</td>
<td>30.5</td>
</tr>
<tr>
<td>CuKTS</td>
<td>37</td>
</tr>
<tr>
<td>CuKTS</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CuKTS</td>
<td>3.5</td>
</tr>
<tr>
<td>CuKTS</td>
<td>25</td>
</tr>
<tr>
<td>CuKTS</td>
<td>40</td>
</tr>
<tr>
<td>CuGTS</td>
<td>-3</td>
</tr>
</tbody>
</table>

a Partition coefficients for 1-octanol-0.1 M KH_2PO_4 buffer at pH 7.2 and 5.5, respectively, were 20.8 and 18.5 for H_2KTS and 42.5 and 40.4 for CuKTS.

b For definitions, see Table 1.

c Precipitate forms between layers. CuGTS is highly insoluble in both octanol and water. A small fraction (~3%) of the total remains in each layer to give value calculated.

Table 3
Partition coefficients for distribution of thiosemicarbazones between 1-octanol and aqueous phase

<table>
<thead>
<tr>
<th>Compound</th>
<th>Partition coefficient (1-octanol-H_2O)</th>
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<tr>
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</tr>
<tr>
<td>CuKTS</td>
<td>25</td>
</tr>
<tr>
<td>CuKTS</td>
<td>40</td>
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<tr>
<td>CuGTS</td>
<td>-3</td>
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</tbody>
</table>

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Table 4

<table>
<thead>
<tr>
<th>Complex</th>
<th>nmoles/mg</th>
<th>No. of trials</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuKTS</td>
<td>50</td>
<td>4</td>
<td>65</td>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>CuKTSM(^a)</td>
<td>70</td>
<td>1</td>
<td>75</td>
<td>33</td>
<td>16</td>
</tr>
<tr>
<td>CuKTSM(_2)</td>
<td>150</td>
<td>3</td>
<td>93</td>
<td>88</td>
<td>67</td>
</tr>
<tr>
<td>CuGTS</td>
<td>125</td>
<td>1</td>
<td>125</td>
<td>114</td>
<td>108</td>
</tr>
<tr>
<td>H(_2)KTS(^a)</td>
<td>100</td>
<td>2</td>
<td>102</td>
<td>108</td>
<td>100</td>
</tr>
<tr>
<td>H(_3)KTSM(^a)</td>
<td>60</td>
<td>1</td>
<td>98</td>
<td>76</td>
<td>73</td>
</tr>
</tbody>
</table>

\(^a\) For definitions, see Table 1.

Comparative Reactions of Copper Complexes with Ehrlich Cells

DISCUSSION

In the previous paper a mechanism of reaction of CuKTS, with tumor cells was set forth summarized by Reaction A.

\[
\text{ComplexCuKTS} \rightarrow \text{CuKTSM} \\
\text{CuKTSM} \rightarrow \text{CuGTSH}_{2}KTS \\
\text{HzKTSM} \rightarrow n_{\text{moles/mg}}
\]

from the medium and then injected the cells into mice. Chart 4 shows the growth pattern of several groups of mice. The ascites tumor grows rapidly in mice given untreated cells and in animals given cells pretreated at a level of CuKTSM\(_2\) that completely inhibits DNA synthesis. In contrast, no growth is evident in mice given injections of cells that had been incubated with CuKTS, 5 nmoles/mg, enough to prevent DNA synthesis but not to halt respiration over several hr, and CuKTS, 50 nmoles/mg, which affects both DNA synthesis and respiration. These animals were kept for observation. No tumors developed after treatment with the higher concentration of drug, but 1 tumor did begin to develop 40 days after injection with cells treated with the low CuKTS concentration.

The availability of a series of substituted thiosemicarbazones with differing cytotoxicity toward Walker cells according to a modification of the Arai-Suzuki method has permitted a test of the generality of this mechanism within the bis(thiosemicarbazonato) copper(II) class of complexes.

Although the Arai-Suzuki method has been used as a general method to measure drug cytotoxicity, in fact it utilizes reduction of méthylène blue by mitochondria for its indicator of cellular effect and therefore is not directly monitoring cell viability. In order to obtain some assurance that there is a definite correlation between the pattern of cytotoxicity for the bis(thiosemicarbazonato) copper(II) species in this system with directly measured effects on cell viability, the 2 extremes of activity CuKTS (+) and CuKTSM\(_2\) (−) were tested for their effects on the growth of Ehrlich cells in mice. As shown in Chart 4, CuKTS is effective at 5 nmoles/mg while CuKTSM\(_2\) is completely ineffective at 50 nmoles/mg, thus confirming the biological difference suggested by the modified Arai-Suzuki method.

A series of complexes, both active and inactive in the modified Arai-Suzuki test, was reacted with Ehrlich cells, and the rates of reaction were spectrophotometrically determined. The pseudo-first-order rate constants have been graphed in a linear free-energy correlation plot as log \(k/k_{\text{ref}}\) versus \(E_{1/2}\), in which the half-wave reduction potential
serves effectively as a substituent constant for these complexes (13). One can see a linear relationship with the single exception of CuGTS, with the active complexes being dissociated by cells and the ineffective complexes being very stable. As in the cytotoxicity test, CuGTS is ineffective, presumably because of its insolubility in both aqueous and lipid organic media (Table 3). Unreactive complexes such as CuKTSM₂ turn the cells dark pink, indicative of substantial uptake into the lipophilic region of the cells. The finding is similar to the observation with CuKTS in heat-treated cells in which no thiols are available for reaction (8).

The slope of this graph can be considered a measure of the sensitivity of the reaction between bis(thiosemicarbazone) copper(II) complexes with cells to changes in peripheral substituents [R₁ to R₄ (Chart 1 of preceding paper (8)]. It is quite similar to the slope of the chemical reaction of this series of complexes with the model thiol, dithiothreitol. In that reaction, as well as the one with cells, as alkylation increases in R₁-R₄ the complexes become progressively slower in their reaction. The agreement adds further credence to the proposed mechanism of reaction in which the rate-determining step is the reaction of the copper chelate with thiols. The trend in the rate constants does not correlate well with the 1-octanol-water partition coefficient. Hence, as with studies of the temperature dependence of the rate of reaction of CuKTS with cells, it does not appear that the discriminatory factor is membrane uptake of these compounds (8).

In other studies of phenyl-substituted phenylglyoxal bis(thiosemicarbazone) copper(II) complexes, a trend of increasing inhibition of Ehrlich cell respiration with increasing electron donor character of the substituent has also been observed (5). Therefore, in terms of the known chemistry of these complexes and the postulated mechanism of reaction of CuKTS with cells, the variation in reactivity among these compounds is directly due to differences in structure which, thermodynamically and kinetically, make them more or less able to react with thiol groups.

Several indicators of cellular integrity have been analyzed as a function of treatment with these previously defined cytotoxic and noncytotoxic copper bis(thiosemicarbazones). Degree of inhibition of mitochondrial respiration is directly correlated with extent of initial reaction of complexes with cells (3). Interestingly, since the initial reaction of active complexes with cells is much faster than is the decline in oxygen consumption of Ehrlich cells, it is now clear that copper ion in some other form than the bis(thiosemicarbazone) copper(II) complex reacts with mitochondria. It had been shown previously that reactive bis(thiosemicarbazone) copper complexes are potential inhibitors of oxidative phosphorylation in isolated mitochondria (3). In whole cells, however, the reactive species is cell-bound copper. Nevertheless, in agreement with those studies, CuKTSM₂ and pyruvaldehyde bis(N⁺,N'-dimethylthiosemicarbazone) copper(II) are unreactive toward mitochondria in whole cells as well as in isolated form.

As had been shown in earlier work by Booth and Sartorelli (2), CuKTS is an effective inhibitor of DNA synthesis. In concentration dependence studies the current work shows that a 20-min incubation of cells with CuKTS at the 5-nmole/mg level leads to almost complete inhibition of DNA synthesis. At higher concentration thymidine uptake is also inhibited. DNA synthesis is more sensitive to CuKTS than is uptake of thymidine, or oxidative phosphorylation. Likewise, the kinetics of the inhibition of uptake and DNA synthesis are much faster than those for the inhibition of respiration. Therefore, contrary to an earlier hypothesis, it appears that these effects of CuKTS are biochemically unrelated (10). The fact that CuKTS-treated cells eventually show widespread inhibition of cellular processes involving sites in the nucleus and mitochondria, with DNA synthesis being particularly sensitive, suggests a general poisoning of the cell by copper. This rather broad pattern of reactions is consistent with the evidence for widespread distribution of copper among many structural components of the cell (8).

This interpretation also agrees with the earlier report that CuKTS inhibits a broad spectrum of reactions leading to the synthesis of DNA, including thymidine kinase (2, 10). However, CuKTS had no effect on the isolated enzyme (2). The difference in behavior of thymidine kinase in vivo and in vitro may mean that CuKTS reacts elsewhere to produce Cu(l)SR (Equation A). Once formed this cell-bound species may then have a distribution pattern of its own including binding to thymidine kinase. Thymidine kinase could be unreactive with CuKTS in vitro simply because of lack of available thiols.

In light of the profound inhibition of DNA synthesis by CuKTS, it was at first surprising to find that CuKTSM₂, as a noncytotoxic complex, also inhibited DNA synthesis. The primary mechanism seems to be the inhibition of thymidine uptake, which coincides in concentration dependence of inhibition by CuKTSM₂ with that for DNA synthesis. In this circumstance labeled thymidine could not reach the nucleus for incorporation into DNA although the replication apparatus was still intact. A physical interaction between CuKTSM₂ and the plasma membrane is proposed in which the complex alters in some way the structure of the membrane related to the mediated transport of thymidine. That there has been no irreversible damage to the cell is indicated by the rapid growth of CuKTSM₂-treated cells injected into mice (Chart 4). Presumably, CuKTSM₂ gradually partitions itself into the i.p. cavity allowing for reactivation of thymidine transport. In contrast, a net chemical reaction has occurred with CuKTS, leaving toxic copper ions in the cell to inhibit irreversibly tumor growth.

REFERENCES

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