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Gold binding sites in red blood cells ☆

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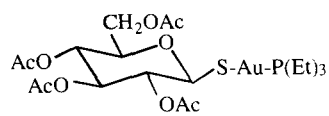
Abstract

Gold distribution and binding sites in blood and red blood cells (RBCs) have been determined. RBCs were separated from plasma and lysed. The cytosol was separated from membranes which were then solubilized via detergents. Total gold in each fraction was measured via flow injection analysis (FIA) with inductively coupled plasma mass spectroscopy (ICP-MS) detection. Various high-performance liquid chromatography (HPLC) techniques such as ion-pairing, reversed-phase and size-exclusion chromatography have been applied to RBC samples prepared by incubation with specific compounds and to RBCs from rheumatoid arthritis (RA) patients. Preliminary studies of RA patients' samples indicate very different gold uptake into RBCs depending on the particular patient. Size-exclusion chromatography indicates that gold in the lysate is not bound principally to hemoglobin but rather to a significantly higher molecular weight species (about 330 000 Da). Low molecular weight species in the ultrafiltered RBC lysate include the dicyanogold(I) anion and possibly the bis(glutathione)gold(I) complex. Incubation experiments have been designed to measure dicyanogold(I) and gold drug uptake by RBCs. Experiments with 4,4'-diisothiocyanatostilbene-2,2'-D,L-sulfonic acid (DIDS), an anion channel blocker, indicate that dicyanogold(I) enters the cell by some path other than the anion channel. The inhibition of gold uptake on addition of free cyanide suggests that the loss of cyanide from dicyanogold(I) is important in dicyanogold(I) uptake by RBCs. Given the rapid uptake of dicyanogold(I) and its apparently high tolerance in humans, this material is suggested as a possible therapy in the treatment of AIDS.

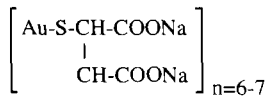
Keywords: Gold metabolism; Rheumatoid arthritis; Gold drugs; Gold complexes; Cyano complexes; HPLC; ICP-MS

1. Introduction

Gold compounds are among the few therapeutic agents that can cause remission of aggressive rheumatoid arthritis (RA). Since these agents are quite toxic and are effective for only about 50% of RA patients treated [1], there is a need for a more specific and effective gold-based therapy. Consequently, the mechanism of action of the compounds is a question of practical and theoretical interest [2]. Despite a considerable research effort stimulated by the development of the oral compound auranofin (Fig. 1), no definite mechanism of action has been established [3,4]. No enzyme system has appeared as a key participant although many have altered activity [5]. Previous efforts have been concerned with the determination of plasma gold levels either to provide a marker of therapeutic action or to aid in studies of transport and in vivo chemistry [6,7]. It was found that the main gold binding site in plasma is a cysteinyl residue at position 34 on albumin [8,9]. Unfortunately, there is no correlation between blood plasma gold concentration and clinical outcome in patients [3,10,11].



Auranofin (AF)
(orally administered drug)



Myochrysine (AuTm)
(Gold(I) sodium thiomalate, injectable)

Fig. 1. Structures of auranofin and myochrysine.

☆ This paper is dedicated to Professor F.A. Cotton on the occasion of his 65th birthday.

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So far, the most widely used gold-based therapeutic agent for RA is myochrysine, a polymer of gold(I) with thiomalic acid in which the gold is bound to the sulfur of the ligand (Fig. 1). This compound reacts readily with sulfhydryl groups and more slowly with disulfides [12]. Although the active site for gold is not established, the general nature of the reactions of gold drugs in vivo may be predicted on the basis of in vitro studies with proteins and cells, and studies of gold distribution in tissue compartments [13]. Recently more attention has been paid to specific gold levels in cells [3,14]. Detection and speciation of the low-molecular weight gold transformation products have been emphasized [15]. The effect of a patient's smoking habits on gold distribution in blood fractions has also been noticed [16,17]. A correlation has been suggested between the gold concentration in erythrocytes (red blood cells, RBCs) and the incidence of toxic reactions [18], although some authors deny this relationship [19]. The interesting observation was that smokers, with a high blood level of cyanide (CN^-), usually showed a toxic reaction earlier than non-smokers [16]. It has been confirmed that there are higher gold levels in the RBCs of smokers than in non-smokers [17]. It is also known that cellular uptake of gold is dependent on cyanide. In vivo cyanide can be produced in two different ways: one is by smoking, through the inhalation of hydrocyanic acid in tobacco smoke [20]; another is the result of secretion of CN^- by polymorphonuclear leukocytes (PMN) in conjunction with phagocytosis [21]. One proposed mechanism was that cyanide acts as a shuttle to carry gold into RBCs [20]. The effect of CN^- was demonstrated in vitro to be due to the conversion of aurothiomalate to the complex ion: aurocyanide or dicyanogold(I), which is rapidly taken up by RBCs. Although we recently found that dicyanogold(I) is the common metabolite of several gold drugs [22], it is not known yet how the dicyanogold(I) distributes in and reacts with whole blood and RBCs.

Smith et al. [2] suggested that gold reacts with the sulfhydryl groups available on the surface of cells, but it is not clear how gold behaves once inside RBCs. Little effort has been made to quantify the gold concentration and distribution in cells due to lack of a powerful and sensitive analytical method. In this paper, a systematic method is described to measure gold distribution and binding sites in blood, especially in RBCs. High-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectroscopy (ICP-MS) has been used to provide not only efficient separation but also a very low detection limit and element specific detection [23]. This study leads to new and additional insights regarding the applications of chrysotherapy, providing a preliminary assessment of the distribution of gold in blood, especially in RBCs.

2. Experimental

2.1. Instrumentation

The liquid chromatography (LC) system included a Spectra-Physics 8800 ternary HPLC pump, a Rheodyne 7125 injector with 10 or 100 μl sample loops, a variable wavelength UV detector (Applied Biosystems 757 absorbance detector) and a strip chart recorder (Houston Instruments, Austin, TX). A Sciex Elan 250 inductively coupled plasma mass spectrometer was used for detecting gold-containing complexes in the HPLC effluent. Gold (*m/z* 197) was monitored. The RF power was 1.4 kW, the nebulizer Ar flow rate was 1 l min^{-1} and the nebulizer spray chamber was cooled at -10 °C to condense most of the organic vapor. The column was connected to the nebulizer by means of PTFE capillary tubing (100 cm, 0.1 mm i.d.). The tubing had a volume of approximately 0.08 μl cm^{-1} and gave a minimal effect on extra-column peak broadening. Data were collected in a multielement mode with an IBM PS/2 computer. An IEC Centra-4B centrifuge (DAMON, USA) was used to separate RBCs from plasma. A Precision Scientific shaking water bath 25 was used for incubation experiments. A Perkin-Elmer Lambda 5 UV/VIS spectrophotometer was used for UV–Vis scanning spectroscopy.

2.2. Chemicals

All chemicals used for mobile phases were obtained from commercial sources, and were of analytical grade. All water used was purified (18 MΩ resistance reading) through a Barnstead (Milford, MA) Nanopure system equipped with a 2 μm filter. Prior to use, the mobile phase was filtered through a 0.45 μm Nylon–66 membrane filter. AuTm (sodium gold(I) thiomalate) and DIDS (4,4′-diisothiocyanatostilbene-2,2′-D,L-sulfonic acid) were purchased from Aldrich Chemicals (Milwaukee, WI). Auranofin (AF) was provided by SmithKline Beecham (Philadelphia, PA). Potassium dicyanogold(I) and tetrabutylammonium chloride (TBAC) were purchased from Sigma Chemical Co. (St. Louis, MO). A standard protein mixture was purchased from Bio-Rad Laboratories (Hercules, CA). Sodium dodecyl sulfate (SDS) was purchased from Fluka Biochemika (Switzerland). The phosphate buffered saline was 0.8% NaCl, 0.02% KH_2PO_4 and 0.116% Na_2HPO_4 (wt./vol.).

2.3. Columns and chromatographic conditions

An analytical C_{18} column (Adsorbosphere C_{18} from Alltech, 5 μm, 300×4.1 mm) was used for analyses of low molecular weight gold metabolites in biological samples. The mobile phase was composed of 1:1 meth-

anol–water, 5 mM phosphate buffer (pH = 7.3) and 10 mM ion-pairing reagent (TBAC). The column was equilibrated with the desired mobile phase for at least 1 h before injection. All analyses were performed at a flow rate of 1.0 ml min^{-1} in an isocratic mode at room temperature. Size exclusion chromatography was performed by using a Bio-Sil 250-5 column (300×7.8 mm, nominal molecular weight range: 10 000–300 000 Da). The mobile phase contained 0.05% NaN_3, 0.05 M Na_2SO_4 (or NaCl) and 0.01 M phosphate buffer (pH = 7.3). Flow injection analysis (FIA) was used to measure total gold levels in serum fractions and in RBCs.

2.4. Patients' samples

Clinical blood samples were obtained from RA patients being treated with myochrysine in the Arthritis Clinic, University of Cincinnati Medical Center, under a protocol approved by the Institutional Review Board. Samples were taken weekly, just before the drug was injected while the patients were undergoing routine evaluation. Naive serum and RBCs were donated from a healthy volunteer. EDTA was used as anticoagulant. Serum was frozen and RBCs were kept at 4 °C until used. All plasma and RBC lysate solutions were centrafiltered at 4000 rpm for 2 h using a membrane filter with a molecular weight cutoff of 10 000 Da (Alltech) before they were injected onto the reversed-phase column.

2.5. Incubation experiments

Incubation experiments were performed with either whole blood (3 ml) or RBCs (about 1.7×10^{10} total cells from 3 ml whole blood, serum was removed by centrifuging at 1500*g* for 5 min and the RBCs were washed five times with phosphate buffered saline solution). Samples were incubated with dicyanogold(I), AuTm and AF for various times and different concentrations at fixed temperatures. After incubation, the reaction was stopped by centrifugation and the serum or supernatant (if incubated with RBCs) was separated from RBCs to test the gold level outside the cells by FIA with ICP-MS detection (FIA-ICP-MS). The treated RBCs were washed five times with phosphate buffered saline solution and then were lysed in distilled water or by ultrasound. Membranes were collected by centrifuging at 15 000*g* for 30 min. The lysate solutions were measured for gold by FIA-ICP-MS. The membranes were washed five times with deionized water and then dissolved in 0.01 M SDS solution for gold measurement. Gold distribution in the patients' blood samples was measured in a similar fashion. To study inhibition effects on gold uptake, RBCs were first incubated with the desired inhibitors before exposure to the gold compounds.

3. Results and discussion

3.1. Gold levels in patients' blood

Gold is widely distributed in the patient's body. The highest concentration of gold has been found in the lymph nodes, followed by the adrenal gland, renal cortex, liver, bone marrow, lung, spleen, renal medulla, thyroid, muscle, skin and brain, respectively [24,25]. Among the body fluids, blood plasma contains the greatest quantities of gold followed by pericardial and synovial fluids. Therefore it is very important to understand gold metabolism in the blood stream to appreciate the efficacy or toxicity of the drug. An experiment was designed to measure gold levels in a patient's serum and RBCs by means of FIA-ICP-MS.

Fig. 2 shows the gold distribution in the blood from two different patients undergoing myochrysine therapy. Patients basically received the same drug regimen. The results in Fig. 2, in terms of gold concentration in patients' sera and RBC lysate solutions, show that although the gold levels in plasma are similar, the gold levels in RBCs are very different between the two

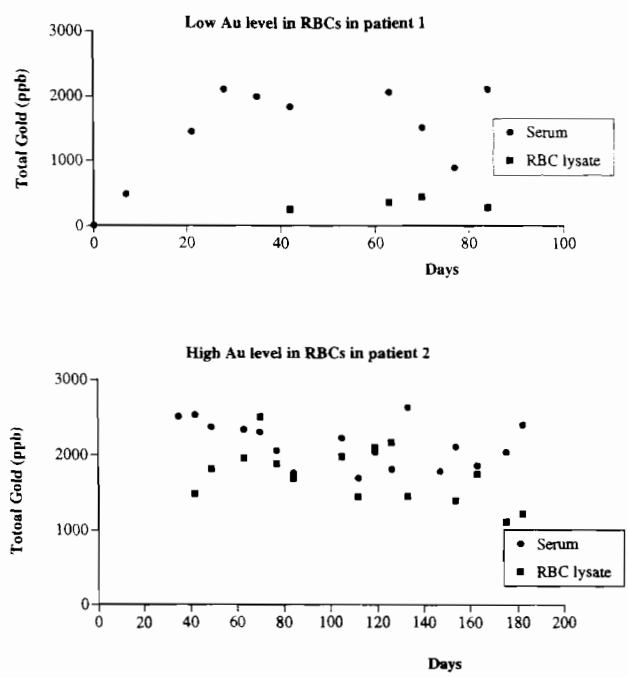

Fig. 2. Gold levels in patients' blood sera and lysate fractions. Patients received myochrysine weekly and the therapy started at 0 days in the figure. Gold concentration was determined by FIA of serum and RBC lysate solutions from patients' blood samples. For patient 1, no blood samples were obtained on the 14th, 49th, and 56th days (however, the patient still received the drug), on the 63rd day patient 1 donated a sample but did not receive a drug shot.

patients. Gold levels in serum can be as high as 2.5 ppm and reach 'equilibrium' values after about 30 days therapy. In patient 1 the gold level in the RBCs is only about 15% of that in serum, but in patient 2 the gold levels in both fractions are quite comparable. Differences such as these were also observed by Smith et al. [26]. Generally, very low gold levels are found in RBCs for myochrysine therapy patients [3,6,14]. There is no known explanation for the differences seen here (both patients reported that they smoked occasionally). This result emphasizes the fact that different people have different responses to the gold drugs and suggests that it is worthwhile to study gold behavior in RBCs.

3.2. Gold distribution in patients' RBCs

Protein-bound gold was studied by size-exclusion chromatography. By coupling HPLC with ICP-MS, we can measure gold-bound protein peaks eluted from the column. Fig. 3 is a typical size-exclusion chromatogram of the RBC lysate solution from the patient 2 sample. For comparison, the chromatogram of serum is also shown in the figure. In the serum chromatogram only one main albumin-bound gold peak is observed, which is expected. In the RBC lysate solution, if most of the gold were bound to hemoglobin, there should be a major gold peak eluted at a similar retention time to that for albumin since they have similar molecular weights. The chromatogram shows, however, that the main gold peak is eluted at about 7 min instead of around 8.5 min for hemoglobin. This retention time suggests that most of gold is bound to a much larger protein instead of hemoglobin. There are overlapping peaks in the hemoglobin region. Apparently only some of the gold is bound to hemoglobin.

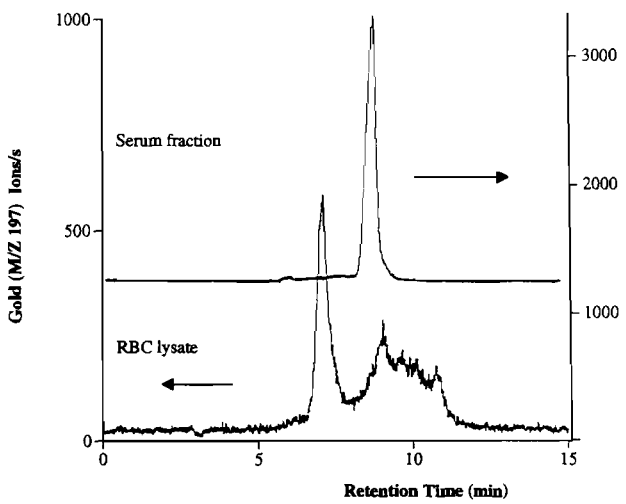

Fig. 3. Size-exclusion chromatograms of 10 μl RBC lysate solution and 10 μl serum from patient 2. Bio-Sil 250-5 (300×7.8 mm) column. Mobile phase contained: 0.05% NaN_3, 0.05 M Na_2SO_4, 0.01 M NaH_2PO_4, pH = 7.3. The first peak on the bottom chromatogram is the same as peak 1 in Fig. 7.

Gold(I)([Xe]$4f^{14}5d^{10}$) is a soft Lewis acid, which reacts readily with soft bases such as CN^-, RSH and R_3P groups. This property gives gold a strong affinity for thiolate sulfur and, in turn, determines the gold biochemistry in a biological environment. It has been shown that the order of thiol affinity for Au(I) is albumin > thioglucose > glutathione, cysteine > thiomalate > hemoglobin > ergothionine [27,28]. In RBCs, 90% of the sulfhydryl groups is located on hemoglobin molecules, while the remainder is approximately equally divided between glutathione and the membrane [29]. After gold enters into RBCs it can recombine with other thiol-containing species. It is well known that gold in the plasma is mainly bound to the Cys-34 of albumin [27], but little is known about the gold binding sites in RBCs. It has been suggested that the distribution of gold in RBCs should correspond closely with the distribution of sulfhydryl groups [16] and, thus, hemoglobin is proposed to be the main protein target for gold due to its high concentration and thiol content. The results from our experiments do not confirm this assumption. Instead, most of the gold in RBCs is bound to a larger protein. This major gold-containing protein peak has been partly characterized in incubation studies described later.

Low molecular weight, gold metabolites were studied by reversed-phase chromatography. The identification of these low molecular weight, gold-containing metabolites in the blood of patients undergoing chrysotherapy should be a significant advance toward understanding how the drugs are metabolized and, ultimately, how they work. As mentioned before [30], glutathione is a thiol-containing molecule that can possibly bind with a significant amount of the gold present because of its high concentration (4–5 mM) in RBCs and formation of stable complexes with gold, generally as the bis(glutathione)gold(I) complex. Cyanide is a strongly bound ligand of gold. Although the concentration of cyanide is very low in RBCs (0.3–1 μM) [31,32], there is a high formation constant between cyanide and gold(I) [33]:

$$Au^+ + 2CN^- \longrightarrow [Au(CN)_2]^- \qquad K_f = 10^{39}$$

and therefore bis(glutathione)gold(I) and dicyanogold(I) are likely to be found in RBCs.

C_{18} reversed-phase chromatography was used for this analysis due to its high separation efficiency. Before injection, proteins in the samples were removed by ultrafiltration with a 10 000 Da molecular weight cutoff membrane. Fig. 4 shows a typical chromatogram of the treated RBC sample. The two uppermost chromatograms show, at the top, a mixture of 2 μM AuTm and 40 μM glutathione, and, below, a mixture of 2 μM dicyanogold(I) and 40 μM glutathione. Since glutathione is in excess compared to cyanide in the medium and gold has a stronger affinity for glutathione than for

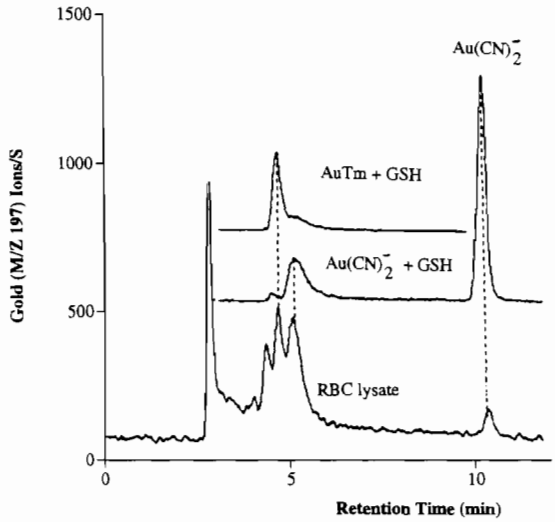

Fig. 4. Reversed-phase chromatograms of a 100 μl sample of a patient's RBC lysate and 10 μl samples of Au–glutathione complexes with ICP-MS detection. Alltech C_{18} (300×4.1 mm) column. Mobile phase contained 50% MeOH, 10 mM TBAC and 5 mM NaH_2PO_4 at pH = 7.3.

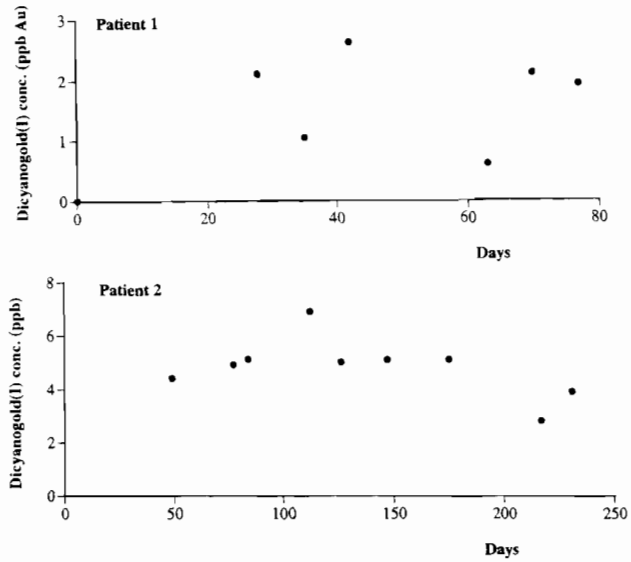

Fig. 5. Dicyanogold(I) levels in patients' blood serum fractions. Patients received myochrysine in a weekly dose of 50 mg gold and the therapy started at 0 days in the figure. Dicyanogold(I) concentration was determined by reversed-phase chromatography. Serum was filtered using 10 000 MW cutoff membrane before injection onto the column.

thiomalate, glutathione may replace thiomalate or cyanide to form glutathione-containing complexes or even bis(glutathione)gold(I). In the chromatogram in Fig. 4, there are some new gold-containing peaks around 5 min arising from these products. The bottom chromatogram is of the lysate solution from the blood sample of patient 2. The first peak in that chromatogram is from non-retained gold-containing species which probably include ionic and polar molecules. Between 4.2 and 5.3 min, there is a group of peaks which overlap each other. Comparison with the top two chromatograms shows that the retention times for the peaks in this group are similar to those for gold–glutathione complexes. It seems likely that one or two of the peaks in the patient's sample are due to gold–glutathione complexes, including bis(glutathione)gold(I). Further characterization will be necessary to establish whether it is in fact present.

The last peak in the chromatogram is a single peak. Comparing this peak with the standard dicyanogold(I) peak above, it is easy to identify this peak as from dicyanogold(I). It has been shown in our laboratory [22,34] that dicyanogold(I) is one of the metabolites in the blood serum fraction and urine of RA patients. Here dicyanogold(I) is also found in RBCs. Approximately 4% of the total gold measured in the low molecular weight, gold-specific chromatogram is bound to cyanide as dicyanogold(I).

The work of Graham and co-workers [35] indicates that dicyanogold(I) can inhibit the oxidative burst of PMN and thus may slow the progression of RA. Thus, we wished to measure also dicyanogold(I) in blood serum fractions for patients 1 and 2 by using HPLC with ICP-MS detection (Fig. 5). The dicyanogold(I) can be separated from other species and quantified by using a calibration curve. The recovery of dicyanogold(I) after membrane filtration was about 95%. The dicyanogold(I) level is found to be only several parts per billion. There was about twice as much dicyanogold(I) in the serum of patient 2 as in patient 1 (about 5 ppb versus 2.5 ppb). Although this small difference may not explain the higher gold levels in the RBCs of patient 2, we wanted to see how dicyanogold(I) reacts with RBCs and where it binds. This was studied by in vitro incubation.

3.3. *Incubation studies of RBCs and gold complexes*

Naive blood, i.e. not exposed to gold drugs, was obtained from a healthy volunteer. Cleaned RBCs were obtained by centrifugation from the blood and washed with phosphate buffered saline solution. Either the RBC fraction or whole blood was incubated with various amounts of dicyanogold(I) at fixed temperatures and times. Then gold levels in serum, lysate and membranes were measured by FIA-ICP-MS. Table 1 shows the results from whole blood and RBC incubation experiments with dicyanogold(I). After RBCs were incubated with dicyanogold(I), most of the gold (over 90% of total gold) was found inside the cells rather than outside. This uptake of gold by RBCs was found to be quite rapid, usually reaching constant values within a few minutes. Increasing the amount of dicyanogold(I) appears to increase the percentage gold uptake by the RBCs. The membrane fraction usually contained about 5% of the gold associated with RBCs. When whole blood was incubated with dicyanogold(I), the albumin

Table 1
Gold distribution after dicyanogold(I) incubation

Dicyanogold(I) incubated with RBCs						
$Au(CN)_2^-$ (μg)	0.4	0.4	0.4	0.4	0.4	4
Time (min)	0[a]	0[a]	5	20	25	25
Temp. (°C)	2	25	37	37	37	37
Supernatant (%)	1.2	1.3	0.8	1.4	1.1	0.5
Lysate (%)	92.4	91.5	92.5	92.3	92.4	95.1
Membranes (%)	6.4	7.2	6.7	6.3	6.5	4.4
Dicyanogold(I) incubated with whole blood						
$Au(CN)_2^-$ (μg)	4	4	4	4	4	0.4
Time (min)	0[a]	0[a]	25	360	1440	25
Temp. (°C)	2	25	37	37	37	37
Serum (%)	38.1	25.5	6.0	4.6	6.3	16.7
Lysate (%)	59.6	70.8	89.8	92.3	90.0	77.9
Membranes (%)	2.2	3.6	4.2	3.1	3.7	5.4

[a] 0 min incubation time means that the sample was immediately centrifuged after mixing 3 ml of whole blood or the RBCs from 3 ml of whole blood.

in serum did not compete significantly with the RBCs for dicyanogold(I). After a 25 min incubation at body temperature, 90% of the gold was in the RBCs. Serum only contained about 6% of total gold. Although dicyanogold(I) is taken up by RBCs, how the dicyanogold(I) crosses the membrane is unknown. The experiments below were designed to examine dicyanogold(I) transport.

3.4. Transport of dicyanogold(I) through the RBC membrane

Dicyanogold(I) is an anion in aqueous solution. It is known that there are anion channels in the RBC membrane and they are used to transport anions across the lipid bilayer [36]. Experiments were designed to test whether dicyanogold(I) will go through those channels. DIDS is known to be an anion channel blocker [37,38]. Thus, if dicyanogold(I) enters through anion channels, gold uptake by RBCs should be reduced if cells are pre-incubated with DIDS. For comparison, two groups of RBCs were tested with dicyanogold(I). One group was pre-incubated with DIDS before the addition of dicyanogold(I). A control was without DIDS. To obtain results having statistical significance, the experiments were repeated several times for each group and standard deviations were calculated.

The results are shown in Table 2. The first data column is the control experiment without DIDS. The second data column is with DIDS. It is obvious that there is no significant difference of gold uptake by RBCs between the two groups and the DIDS did not inhibit gold entering the RBCs. Experiments confirmed that a high DIDS concentration in the medium did not block uptake even when the DIDS concentration reached 2 mM. Thus, dicyanogold(I) does not go through the anion channels.

Another mechanism for transport of gold complexes has been proposed [39]. It utilizes the so-called 'sulfhydryl shuttle' in which gold(I) complexes undergo ligand exchange to bind to sulfhydryl groups immobilized on or in the membrane. A series of such exchange reactions then passes the gold(I) across the membrane and into the cells, where it may then be released into the cytosol by reaction with some proteins and low molecular weight sulfhydryl groups such as glutathione. Lewis and Shaw [40] have studied the reaction envisioned for dicyanogold(I) in model systems where the reaction may be written:

$$[Au(CN)_2]^- + RSH \longleftrightarrow [RSAuCN]^- + HCN$$

Clearly, this reaction should be repressed by additional CN^-.

We tested the effect of incubation with additional cyanide before the addition of dicyanogold(I) as shown in the third data column of Table 2. There is clearly a marked reduction of the amount of gold found in either the cell lysate or the cell membrane. Most of the gold is now found in the supernatant. Thus, inhibition of cyanide loss by additional cyanide present in the medium strongly inhibits uptake of dicyanogold(I). The system is complex, as some of the cyanide is taken up by the RBCs [32]. However, under the incubation conditions used here, there appears to be enough free cyanide still in the medium to have a dramatic effect on dicyanogold(I) uptake.

3.5. Incubations with auranofin and myochrysine

Auranofin (AF) and myochrysine (AuTm) uptakes were investigated as well. The results are summarized in Table 3. AF showed similar properties to that of dicyanogold(I) in terms of gold uptake. When incubated with RBCs only, over 90% of gold was found in the RBC lysate solution. When incubated with whole blood, over 60% of gold is bound to or in the RBCs. The gold content in the membrane was also about 5% of total gold. In the case of incubation with whole blood, albumin in serum has strong affinity for gold and competes with the RBCs for AF. The result is that some of the AF reacts with albumin and less gold is found in the RBCs compared to incubation with RBCs only. Contrary to dicyanogold(I) and AF, AuTm did not cross the cellular membrane easily and most of it stayed outside the cells. Only about 5% of total gold was in the lysate solution regardless whether the incubation was with RBCs or whole blood. Also, there is more gold bound in the membranes than in the lysate solution (Table 3). This result is consistent with previous NMR studies [2].

Table 2
Effect of DIDS and NaCN on uptake of dicyanogold(I) by RBCs [a]

Inhibitors	Phosphate buffered saline	0.07 mM DIDS	0.67 mM NaCN
Supernatant (%)	1.8±1.3	0.8±0.1	81.3±0.3
Lysate (%)	94.4±1.8	94.4±0.9	18.4±0.4
Membranes (%)	4.7±1.6	4.9±1.0	0.4±0.2

[a] Incubation conditions: 20 min at 30 °C with DIDS or NaCN, and 20 min at 30 °C with 0.9 ppm potassium dicyanogold(I). The total incubation volume was 4.5 ml, including the RBCs. The standard deviations are listed after each number.

Table 3
Gold drugs uptake by RBCs [a]

Drugs	1.2 ppm AuTm		3.1 ppm AF	
Incubation condition	Whole blood	RBCs only	Whole blood	RBCs only
Supernatant or serum (%)	90	84	39.4	3.1
Lysate (%)	4.6	6.1	55.9	91.7
Membranes (%)	5.6	9.9	4.8	5.3

[a] Supernatant refers to the solution outside the cells when incubated with RBCs only. AuTm and AF were incubated at 30 °C for 30 min with 3 ml of either whole blood or RBCs from 3 ml whole blood. The total incubation volume was 3.2 ml, including the RBCs.

3.6. *Characterization of high molecular weight, gold-containing proteins in the RBCs*

The majority of the gold binds to proteins once it enters the RBCs. These proteins can be separated by size-exclusion chromatography and gold-bound protein peaks eluted from the column can be measured by using HPLC with UV and ICP-MS detection. Fig. 6 (with UV detection) and Fig. 7 (with ICP-MS detection) are typical size-exclusion chromatograms of the RBC lysate solution from an in vitro incubation sample. For comparison, UV and ICP-MS detections were on line (in series) to monitor UV absorption and gold signal simultaneously. With UV detection (Fig. 6), the hemoglobin peak (peak 3 with very high absorbance) can be easily identified. Although there are many UV peaks in the chromatogram, there is no indication which peaks contain gold. With ICP-MS detection in Fig. 7, however, all the gold-containing peaks (such as peaks 1–3, etc.) can be seen. At the bottom of Fig. 7 is the chromatogram of the lysate solution. There are three main gold peaks.

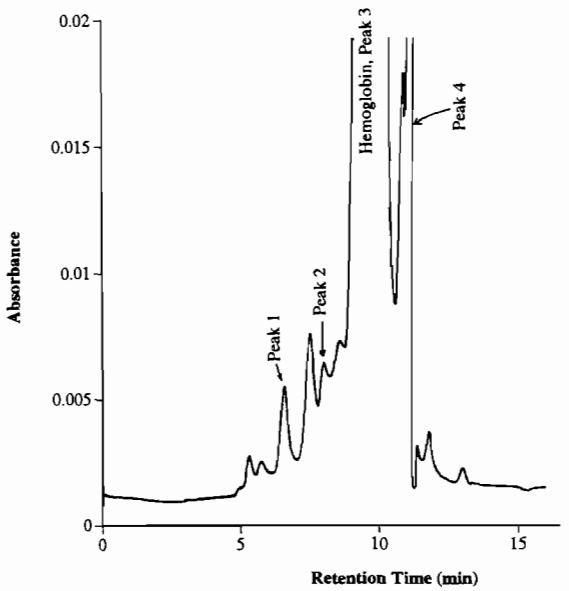

Fig. 6. Size-exclusion chromatogram of RBC lysate solutions with UV (254 nm) detection. The lysate solution was from RBCs incubated with dicyanogold(I) in vitro. Bio-Sil 250-5 (300×7.8 mm) column. Mobile phase contained: 0.05% NaN_3, 0.05 M Na_2SO_4, 0.01 M NaH_2PO_4, pH = 7.3.

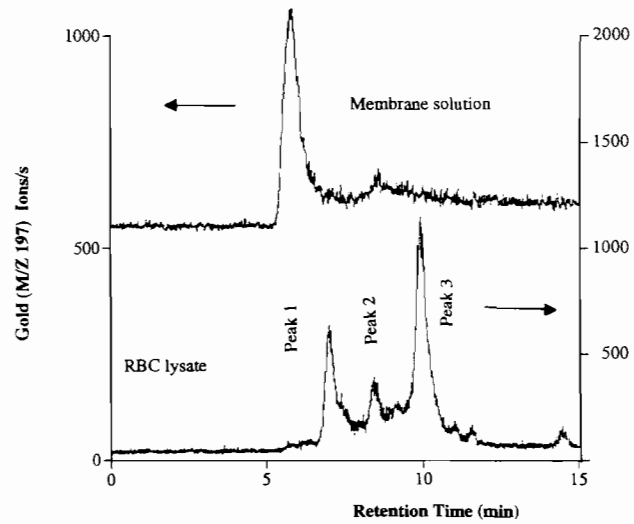

Fig. 7. Size-exclusion chromatograms of 20 μl RBCs lysate and 100 μl membrane solutions with ICP-MS detection. The lysate solution was from RBCs incubated with dicyanogold(I) in vitro. Bio-Sil 250-5 (300×7.8 mm) column. Mobile phase contained: 0.05% NaN_3, 0.05 M Na_2SO_4, 0.01 M NaH_2PO_4, pH = 7.3. Membrane was dissolved in SDS solution.

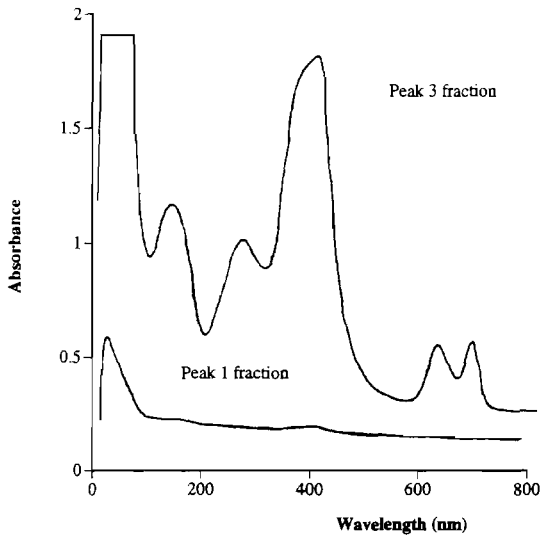

Fig. 8. UV–Vis spectra for peaks 1 and 3. The peaks were collected in mobile phase and mobile phase was used as the blank.

Peak 1 at about 7 min is from a large protein with a very low absorbance at 254 nm. Peak 3, close to 10 min, arises from hemoglobin and has significant absorbance at 420 nm in these experiments. Depending on the lysing method, hemoglobin can dissociate into dimers or even monomers at low ionic strength [41]. The retention time for peak 3 is somewhat longer than that expected for hemoglobin and this is very likely due to its dissociation. The spectrum of peak 3 (top spectrum in Fig. 8) is characteristic of that for hemoglobin. It was reported previously that gold binds to Cys-93 of hemoglobin ß subunit [42] and the product AuHb is a high-spin Fe(III) heme protein [43].

Peak 1 is interesting in that it contains much gold. Its UV–Vis spectrum is given in Fig. 8. Peak 1 shows a very weak UV–Vis absorption and, therefore, it is unlikely to represent an aggregation of hemoglobin. We also found that the gold content in peak 1 increases and the gold amounts in the other peaks (including the hemoglobin peak) decrease with the sample aging time. Since the protein of peak 1 is not formed due to gold (an experiment confirmed that this protein peak was found in the chromatogram of a naive blood sample), there must be a gold equilibrium among these proteins, i.e., the peak 1 protein, hemoglobin and others. It seems that the gold complex with the peak 1 protein is relatively thermodynamically stable. To compare with Fig. 3, the first peak in that chromatogram of the patient's RBC lysate solution is the same as peak 1 in Fig. 7. Interestingly, the highest gold level is found in the peak 1 fraction from the lysate solution of a patient's blood.

To characterize the peak 1 protein, a standard protein mixture solution was used to create a linear calibration curve. The standard protein mixture contained thyroglobulin (670 000 Da), γ-globulin (158 000 Da), human albumin (68 500 Da), ovalbumin (44 000 Da) and myoglobin (17 000 Da). The molecular weight of the peak 1 protein was estimated to be 330 000 Da. So far, we have not identified this protein. Peaks 2 and 4 in Figs. 6 and 7 are other gold-bound proteins. Using the same calibration, the molecular weights for peaks 2 and 4 proteins are 85 000 and 6000 Da, respectively. The last peak in Fig. 7 at 14.3 min is due to low molecular weight gold species that are totally included on the column. According to the peak areas, gold bound to low molecular weight species represents about 3% of the total gold in the RBC lysate solution.

Gold-containing proteins in the dissolved membranes were also tested by using size-exclusion chromatography. After the membranes were cleaned, they were dissolved in 0.01 M SDS solution and the solution injected onto the column. It was found that the proteins containing most of the membrane gold were not retained by this column due to their large size (Fig. 7).

The differences between patients' samples and incubation samples in terms of gold amount in peak 1 are partly due to sample aging time (the patient's sample tested here was stored for a month). But the common observation is that there is a high molecular weight protein which contains a great amount of gold in both samples. This is the first time that this protein has been reported and its identification may be important in understanding gold metabolism in RBCs.

3.7. Possible use of dicyanogold(I) as an AIDS therapeutic

Previously, various gold complexes have been found to inhibit the replication of HIV, the AIDS virus [44,45]. The difficulty has been that the compounds examined, such as solganol, gold(I)thioglucose, have minimal uptake into cells and appear likely to be rather toxic for human patients at the required dose levels. Our own experience with RA patients on chrysotherapy shows that some of them excrete high levels of dicyanogold(I) and show no apparent signs of toxicity [22]. The finding here of the rapid and nearly complete uptake of dicyanogold(I) by RBCs suggests that this material, which was once used to treat tuberculosis [46], might provide a form of gold(I) with high cellular uptake and relatively low toxicity capable of generating the necessary intracellular levels of gold to provide a useful therapeutic for the treatment of AIDS.

4. Conclusions

Gold distribution, transport and possible binding sites in RBCs have been investigated. Samples from RA patients and from in vitro incubation experiments have been studied by using FIA, ion-pairing, reversed-phase

and size-exclusion chromatography with gold-specific and sensitive ICP-MS detection.

It was found that total gold levels in patients' sera can be as high as 2.5 ppm and reach 'equilibrium' values after about 30 days therapy. The major difference between the patients in terms of total gold level is found in their RBCs. Different patients have very different RBC gold levels. These differences emphasize the fact that patients will have different responses to the drugs. Using size-exclusion chromatography, we found that most of the gold is bound to a high molecular weight protein (330 000 Da) rather than hemoglobin. Possible low molecular weight gold metabolites have been characterized by using reversed-phase chromatography. Dicyanogold(I) is found in the patients' RBCs. It represents 4% of the total gold in low molecular weight, gold-containing species. Gold–glutathione complexes may also occur in the lysate solution of patients' samples.

From in vitro incubation experiments, it was found that dicyanogold(I), one of the metabolites, is taken up by RBCs quickly and completely (over 90% of total gold). The gold will recombine with other species once dicyanogold(I) enters the RBCs. Albumin in serum seemed not to compete with RBCs for dicyanogold(I). The pathway for this uptake is not through the anion channels in the membranes since the uptake is not inhibited by DIDS (an anion channel blocker). NaCN appeared to inhibit significantly the uptake of dicyanogold(I) by RBCs. Gold levels in the membrane also decreased if RBCs were pre-incubated with NaCN before dicyanogold(I) incubation. Since the 'sulfhydryl shuttle' is proposed to transport gold, the result here suggests that the loss of cyanide from dicyanogold(I) is very important in dicyanogold(I) uptake by RBCs. Additional free cyanide will inhibit this loss and in turn inhibit the dicyanogold(I) uptake. The uptake of myochrysine by RBCs is very limited and there is relatively more gold on the membranes than in the lysate solution. Auranofin and dicyanogold(I) show similar uptake behavior by RBCs. However, albumin seems to compete with RBCs more efficiently for AF than for dicyanogold(I).

Size-exclusion chromatography has confirmed, from in vitro experiments, that hemoglobin is not the principal protein target for gold in RBCs. A larger protein than hemoglobin is found to bind at least half of the gold in RBCs. The amount of gold in this protein will increase with sample aging time. The approximate molecular weight of this protein is 330 000 Da. UV–Vis spectroscopy shows that this protein has a small UV absorbance and is not an aggregation of hemoglobin. This is the first time that this gold-containing protein, which has yet to be identified, has been reported in RBCs.

Finally, based on observations made here and elsewhere, we propose that dicyanogold(I) may be a useful therapy in the treatment of AIDS.

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