Cultured human cells with high levels of gold-binding cytosolic metallothionein are not resistant to the growth inhibitory effect of sodium aurothiomalate

A GLENNÅS1 2 AND H E RUGSTAD1

From the 1Division of Clinical Pharmacology, Institute for Surgical Research, Rikshospitalet, the National Hospital, and 2Oslo Sanitetsforening Rheumatism Hospital, Oslo, Norway

SUMMARY We have previously shown that cells with a high content of the gold-binding protein metallothionein (MT) are resistant to the growth inhibitory effects of gold(III) chloride and auranofin. To investigate whether MT confers resistance to sodium aurothiomalate two cell lines of cultured human epithelial (HE) cells were used; the parental cell line (HE) and a substrain (HE100) containing high levels of cytosolic MT. Sodium aurothiomalate and thiomalic acid without gold both caused a dose-dependent growth inhibition of both cell lines when used in the concentration range 25–300 μmol/l culture medium and for four days’ exposure. MT, despite binding about one third of the cytosolic gold, did not protect against the antiproliferative effect of sodium aurothiomalate. The gold and the thiomale moieties were distributed differently within the cells; 30% of the cellular gold and 80% of the thiomalic acid were recovered in the cytosol. Gold was mainly protein bound in both cell lines, as shown by G75 Sephadex gel filtration of the cytosols. In the HE100 cells about 30% of the gold eluted with MT. The thiomalic eluted mainly with substances with molecular weights less than 1000. Cellular synthesis of MT was not observed during sodium aurothiomalate treatment. The results indicate that the sodium aurothiomalate molecule dissociates and support the suggestion that the thiomale moiety is, in part, responsible for the antiproliferative effect of the drug.

Key phrase: drug resistance.

Metallothionein (MT) is the name given to a group of low molecular weight isoproteins (6000–7000) with a unique amino acid composition, in that 30% of the 61 amino acid residues are cysteine. MT has the capacity to bind certain heavy metals.1 The protein is widely distributed among different animal species and seems to occur in all tissues but is found mainly in the cytosol of liver and kidney cells.1 Cadmium (Cd) is the most potent MT inducer metal known at present, and MT is probably important in the detoxification of chronic Cd intoxications.1 Sharma and McQueen2 and Moglinicka and Webb3 have shown the occurrence of low molecular weight gold-binding proteins in the liver and kidneys of rats after intramuscular administration of the gold-containing drug, sodium aurothiomalate, to the animals. These proteins have not been fully characterised, but their behaviour on gel filtration columns suggests that they may be of MT nature. The biological functions of the proteins have not yet been studied.

We have previously shown that cultured human cells containing high amounts of cadmium-induced cytosolic MT are resistant to the antiproliferative effects of 350 μmol gold(III) chloride/l culture medium4 and 2 μmol/l of the antiarthritic drug, auranofin.5 These concentrations when given continuously were lethal to the parent cell line with undetectable levels of cytosolic MT. The demonstration of the binding of gold to the MT within the

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Correspondence to Dr Anne Glennås, Division of Clinical Pharmacology, Institute for Surgical Research, Rikshospitalet, N-0027 Oslo 1, Norway.
resistant cells led to the conclusion that MT may act as one protective factor against the growth inhibitory effect of the two gold-containing compounds.

The purpose of the present study was to investigate the possible protective role of MT against the antiproliferative effect of sodium aurothiomalate. Cultured human cells with and without detectable amounts of cytosolic MT were used. The cellular concentrations and cytosolic distribution of the gold and the thiomalate moieties of the drug were investigated. For comparison, the cells were also exposed to thiomalic acid without gold.

Materials and methods

Cells and cell culturing

The cells were human epithelial cells (HE) derived from normal skin (NCTC 2544) and obtained from the American Type Culture Collection, Maryland, USA. They were grown as monolayers in Dulbecco’s modification of Eagle’s medium (Flow Laboratories, Irvine, Scotland) supplemented with serum, antibiotics, and mycostaticum.1 A substrain (HE100) had previously been adapted to 100 μmol CdCl₂/l culture medium, an otherwise lethal concentration for the cells.9 There is considerable evidence that the Cd resistance of these cells is due to induction of MT synthesis and the binding of Cd to MT. About 2–3% of the cytosolic proteins in these cells are estimated to be MT.10 Characterisation of the cells by methods including transmission electron microscopy, surface antigen typing, and isoenzyme analysis6 has confirmed their epithelial and human nature. Flow cytometric studies, cellular glutathione determinations,7,8 two-dimensional polyacrylamide gel electrophoresis of cellular proteins, and preliminary studies on DNA repair capacity9 have shown no major differences between the HE and HE100 cells, apart from the Cd-MT content.

Drugs and chemicals

Disodium auro[2,3,14C₂]thiomalate (specific activity 0-31 mCi/mmol) and [2,3,14C₂]thiomalic acid (specific activity 0-88 mCi/mmol) were kindly supplied by Rhône-Poulenc (May & Baker Ltd, Dagenham, Essex, England). The radiochemical purity of the 14C-labelled disodium aurothiomalate was determined at the start of the experiments by thin layer chromatography (TLC) with n-butanol/glacial acetic acid/water 80:20:20 on silica gel plates (Polygram Sil G/UV 254, Schleicher & Schull GmbH, Dassel, W Germany). Subsequent determination of radioactivity in sections of the plates indicated a radiochemical purity of 90%. The purity of the labelled thiomalic acid was 68% as estimated by TLC with toluene/methanol/glacial acetic acid 45:8:4 on silica gel. Batches of sodium [199Au] aurothiomalate (specific activity 123.5 mCi/mmol and 163.0 mCi/mmol) were synthesised at the Department of Biophysics, University of Manchester. Sodium aurothiomalate (Myocrin) was the commercially available drug, and thiomalic acid was purchased from Sigma Chemical Company, Mo, USA. The thiomalic acid was stored under nitrogen until use, and all compounds were protected from light. Fresh solutions of the drugs were prepared for each experiment. Cadmium chloride purum was obtained from Fluka AG, Switzerland.

Cell growth studies

Cells from both cell lines (HE and HE100) were subcultured into Costar tissue culture wells (16 mm) (Costar, Maryland, USA) at a density of 1.0×10⁴ cells per well. The HE100 cells were removed from the Cd-containing medium, and all cells were grown without metal supplement for 24 h. Cultures were then exposed to medium containing 25, 50, 100, 200, or 300 μmol of sodium aurothiomalate or thiomalate derived from thiomalic acid per litre of culture medium. Controls were grown without drug supplementation. Cells from six replicate wells of each cell line, drug, and drug concentration were harvested by trypsinisation and counted in a Coulter counter at the start of treatment and daily until day 4. Media with and without the drug supplements were changed on day 2. On day 4 the viability of the cells was tested by the dye exclusion test (trypan blue).10 Differences in sensitivity to the drugs between the cell lines were calculated by means of an area method (see Appendix).

Cellular concentration and distribution of 199Au and 14C

After removal of the Cd supplement for 24 h exponentially growing HE and HE100 cells were exposed to 300 μmol/l of sodium [199Au] aurothiomalate, disodium auro[2,3,14C₂]thiomalate, or [2,3,14C₂]thiomalic acid for another 24 h. The cell cytosols were then obtained by ultrasound sonication and ultracentrifugation (105 000 g, 1 h, 4°C). Aliquots of sonicates and cytosols were analysed for radioactivity and for protein content (Abbot bichromatic analyser 100) with human albumin (Behringwerke AG, W Germany) as a standard. Differences in concentrations and the distribution of 199Au and 14C were tested by Student’s t test, taking significance as p<0.05. The remaining cytosols were chromatographed on a G75 Sephadex column (1·8×90 cm) with 10 mM ammonium formate buffer pH 8 as eluent. The elution rate was 18 ml/h and the fraction volume 3 ml. The concentrations of Cd, Zn, and Cu in the eluted fractions
were determined by atomic absorption spectrophotometry (Instrumentation Laboratories a/a spectrophotometer 257). The $^{199}$Au concentration was determined with a gamma scintillation spectrometer (Packard 5221). The counting efficiency was 35% with a 120–185 keV window. The $^{14}$C activity was determined in a Mark II liquid scintillation system (Tracor Analytic Inc., Ill., USA). Each fraction showed a similar degree of quenching. The counting efficiency determined by an external standard ratio method for $^{14}$C was 89%. The coefficients of variation (standard deviation corrected for background as a percentage of net counts) of the counts per minute had a range of 0.2–1.1% ($^{199}$Au) and 0.5–2.7% ($^{14}$C) for the gel filtration peaks and 1.0–3.3% ($^{199}$Au) and 20.8–32.3% ($^{14}$C) for the regions between the peaks.

**IN-VITRO INCUBATION OF Cd-MT AND ISOTOPE-LABELLED SODIUM AUROTHIOMALATE**

Cd-induced MT, obtained after G75 Sephadex gel filtration of cytosols from HE$_{100}$ cells as described above, was dissolved in 10 mM ammonium formate buffer pH 7.5. The concentration of MT was approximately 10 $\mu$mol/l. Sodium [$^{199}$Au]auromalate or disodium auro[2,3-$^{14}$C$_2$]thiomalate was added to a final drug concentration of 20 $\mu$mol/l and incubated for 1 h at 20°C. The drug concentration used was equal to the cytosolic gold concentration after drug exposure of living cells. After a second G75 Sephadex gel filtration run the concentrations of $^{199}$Au, $^{14}$C, Cd, and Zn in the eluted fractions were determined.

**INCUBATION OF $^{14}$C-LABELLED SODIUM AUROTHIOMALATE WITH CULTURE MEDIUM**

To examine the distribution of Au and $^{14}$C the culture medium was supplemented with 300 $\mu$mol/l of disodium auro[2,3-$^{14}$C$_2$]thiomalate and incubated for 24 h in tissue culture flasks without cells. After G75 Sephadex chromatography of the medium the $^{14}$C concentration in the eluted fractions was determined as above. The Au concentration was determined by flame atomic absorption spectrophotometry.

**Results**

**EFFECT OF SODIUM AUROTHIOMALATE AND THIOMALIC ACID ON CELL GROWTH**

The growth of HE and HE$_{100}$ cells during exposure to 25–300 $\mu$mol/l of sodium aurothiomalate for four days is shown in Fig. 1. Fig. 2 shows the cell growth during exposure to equimolar concentrations of...
thiomalate derived from thiomalic acid. Both compounds caused a dose-dependent growth inhibition of both cell lines. No significant differences between the HE and HE\textsubscript{100} cells were detected in the responses when grown with sodium aurothiomalate or thiomalate. On day 4 of treatment with 300 \( \mu \text{mol} \) sodium aurothiomalate/l 6.5\% (percentage of median values for the cell counts) of the HE cells and 5.4\% of the HE\textsubscript{100} cells remained adherent to the wells compared with the control cells. On treatment with 300 \( \mu \text{mol} \) thiomalate/l 0.7\% of the HE cells and 0.7\% of the HE\textsubscript{100} cells remained attached. The viability of the remaining cells was more than 85\%. At concentrations of sodium aurothiomalate or thiomalate higher than 100 \( \mu \text{mol} \)/l all cells detached from the wells and died within about two weeks.

**Cellular Concentrations of \textsuperscript{199}Au and \textsuperscript{14}C**

The concentrations of \textsuperscript{199}Au and \textsuperscript{14}C in sonicates and cytosols after 24 h exposure of the cells to 300 \( \mu \text{mol}/l \) of either sodium [\textsuperscript{199}Au]aurothiomalate, disodium auro[2,3,\textsuperscript{14}C\textsubscript{2}]thiomalate, or [2,3,\textsuperscript{14}C\textsubscript{2}]thiomalic acid are shown in Table 1. After exposure to the \textsuperscript{199}Au- or \textsuperscript{14}C-labelled aurothiomalate \textsuperscript{199}Au and \textsuperscript{14}C were recovered in the cells (sonicates) in equimolar concentrations, 9.5–13.2 nmol/mg sonicate protein. No significant differences were found between the HE and HE\textsubscript{100} cells in either \textsuperscript{199}Au or \textsuperscript{14}C content. About 30\% of the cellular \textsuperscript{199}Au and 80\% of the cellular \textsuperscript{14}C derived from the thiomalate moiety were recovered in the cytosol. Accordingly, the cytosolic \textsuperscript{199}Au concentrations were lower than those of \textsuperscript{14}C, 5.0–6.1 nmol \textsuperscript{199}Au/mg cytosol protein and 18.0–23.3 nmol \textsuperscript{14}C/mg cytosol protein respectively. No significant differences in the cytosolic concentrations of \textsuperscript{199}Au and \textsuperscript{14}C between the HE and HE\textsubscript{100} cells were detected. When the cells were exposed to the \textsuperscript{14}C-labelled thiomalate derived from thiomalic acid, similar or slightly higher concentrations and a similar distribution of \textsuperscript{14}C between the sonicates and cytosols was found compared with that found after exposure of the cells to the \textsuperscript{14}C-labelled disodium aurothiomalate.
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Table 1  Concentrations of $^{199}$Au or $^{14}$C in sonicates and cytosols of HE and HE$_{100}$ cells*$

<table>
<thead>
<tr>
<th>Isotope/drug</th>
<th>HE cells</th>
<th>HE$_{100}$ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sonicate</td>
<td>Cytosol</td>
</tr>
<tr>
<td>$^{199}$Au from sodium $[^{199}$Au] aurothiomalate</td>
<td>9.5±6.1</td>
<td>6.1±5.0</td>
</tr>
<tr>
<td>$^{14}$C from disodium auro[2,3-$^{14}$C$_2$]thiomalate</td>
<td>12.0±1.6</td>
<td>18.0±6.4</td>
</tr>
<tr>
<td>$^{14}$C from [2,3-$^{14}$C$_2$]thiomalic acid</td>
<td>20.4±3.4</td>
<td>34.8±0.7</td>
</tr>
</tbody>
</table>

*Concentrations (nmol/mg protein) are means±SD from three ($^{199}$Au) or two ($^{14}$C) experiments. See methods section for details.

![Fig. 3 Elution profiles of $^{199}$Au (---), Cd(--), Zn(--), and Cu (....) obtained after G75 Sephadex gel filtration of cytosols from HE and HE$_{100}$ cells after exposure to sodium $[^{199}$Au]aurothiomalate (panel A-B). Elution profiles of $^{14}$C(---), Cd(--), Zn(--), and Cu (....) obtained in the same way and after treatment of HE and HE$_{100}$ cells with disodium auro[2,3-$^{14}$C$_2$]thiomalate (panel C-D).](image-url)
The G75 Sephadex elution profiles of $^{199}$Au, $^{14}$C, Cd, Zn, and Cu after gel filtration of cytosols from HE and HE100 cells treated with sodium $[^{199}$Au]aurothiomalate, disodium auro[2,3-$^{14}$C$_2$]thiomalate, or [2,3-$^{14}$C$_2$]thiomalic acid are shown in Figs 3 and 4. The fraction of $^{199}$Au eluting with the void volume proteins (peak I, $V_e/V_0=1$, mol.wt $> 75,000$) was 72% in HE and 46% in the HE100 cytosols. In the HE100 cytosols 36% of the $^{199}$Au coeluted with the Cd-induced MT (peak II, $V_e/V_0=2$, mol.wt $\approx 10,000$). No such peak was observed in the HE cytosols, as indicated by the profiles of $^{199}$Au, Zn, and Cu. 7% of the $^{14}$C eluted with the void volume proteins, whereas 88% eluted with substances of molecular weights less than 1000 (peak III, $V_e/V_0=3$). This pattern was the same in both lines and after treatment with both of the $^{14}$C-labelled compounds. No $^{14}$C activity was detected in the MT-containing fractions of the HE100 cytosols.

**Distribution of $^{199}$Au and $^{14}$C within the cytosols**

**In-vitro binding of isotope-labelled sodium aurothiomalate to Cd-MT**

Fig. 5 shows the coelution of $^{199}$Au derived from sodium $[^{199}$Au]aurothiomalate and Cd derived from MT after in-vitro incubation of the drug with MT and subsequent gel filtration. About 29% of the Cd bound to MT was exchanged with $^{199}$Au under the conditions described. When MT was incubated with disodium auro[2,3-$^{14}$C$_2$]thiomalate, $^{14}$C and Cd derived from MT eluted separately on the gel filtration column.

**Distribution of Au and $^{14}$C in the culture medium**

After G75 Sephadex gel filtration of medium incubated with disodium auro[2,3-$^{14}$C$_2$]thiomalate about 65% of the Au and 35% of the $^{14}$C distributed with the medium proteins. The major part of the $^{14}$C eluted with fractions containing substances of molecular weights less than 1000 (profiles not shown).
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Discussion

The present study shows that cellular Cd-induced MT has the capacity to bind gold when human cells are cultured in the presence of sodium aurothiomalate. Cd-MT also binds gold after in vitro incubation with sodium aurothiomalate. The degree of Cd exchange by Au was somewhat higher (29%) after in-vitro incubation than after treatment of living cells (16%) (Fig. 3B). An almost complete exchange of MT-bound Cd by Au was achieved in vitro when an even higher concentration of sodium aurothiomalate (9 mmol/l, data not shown) was used. MT does not confer resistance to the antiproliferative effect of the drug, despite binding about 36% of the cytosolic gold in living cells. This contrasts with the results obtained when auranofin was given to the same cells. The concentration of 2 μmol auranofin/l culture medium caused an almost complete arrest of growth of the HE cells, whereas the HE100 cells continued to grow exponentially. About 15% of the cytosolic gold derived from auranofin was bound to the MT in the HE100 cells.

The gel filtration profiles (Figs 3 and 4) gave no indication of induced MT synthesis in the HE cells after 24 h of sodium aurothiomalate treatment. This does not rule out the possible induction of MT, which has been observed after sodium aurothiomalate administration to rats. In-vivo alterations in tissue metal distribution caused by gold treatment may indirectly induce MT via zinc or copper, as suggested by Mogilnicka and Webb.

The gold and the thiomalate derived from sodium aurothiomalate have different intracellular distributions. In the present study the thiomalate distributes similarly regardless of whether it originates from sodium aurothiomalate or thiomalic acid without gold. The thiomalate was mainly present in the cytosol, whereas the gold was recovered mainly in the pellet after ultracentrifugation. Furthermore, most gold was protein bound within the cytosol, while the thiomalate obviously appeared as the thiomalate anion, as its own disulphide, or as mixed disulphides with sulphhydryl-rich oligopeptides. This indicates a dissociation of the sodium aurothiomalate molecule in the cells. Evidence was also obtained that this dissociation takes place even in the culture medium, before the drug penetrates the cells. Preliminary data indicate that the major part of the cytosolic thiomalate is free thiomalate and not its corresponding disulphide (unpublished results). The present results are in accordance with the

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Fig. 5  Elution profiles of 199Au(—), Cd(— —), and Zn(— —) (panel A) and 14C(—), Cd(— —), and Zn(— —) (panel B) after G75 Sephadex gel filtration of Cd-induced MT incubated in vitro with sodium [199Au]thiomalate or disodium auro[2,3-14C2]thiomalate. The Cd-MT was obtained from a previous G75 Sephadex run of cytosols from HE100 cells.
studies of Jellum et al., who showed that the gold and thiomalate moieties separate in vivo after intramuscular administration of $^{195}$Au-14C]aurothiomalate to mice and rats, resulting in protein-bound gold and release of thiomalate. Other in vivo studies have confirmed the different distribution of the gold and thiomalate both in animals and humans. The present study shows that the gold and the thiomalate moieties behave differently even within cells.

The results discussed above and the closely similar growth inhibitory effects of sodium aurothiomalate and thiomalate derived from thiomalic acid raise the question of which part of the sodium aurothiomalate molecule is responsible for the antiproliferative effect observed. The possibility that the thiomalate moiety in part mediates the effect ascribed to sodium aurothiomalate cannot be excluded. Other studies have shown that thiomalic acid exerts effects similar to those of D-penicillamine, a thiol-containing antiarthritic drug, on various models of immunologically mediated inflammation in vivo, and in vitro on the responsiveness of lymphocytes to concanavalin A in the presence or absence of macrophages. Munthe and Jellum, in a preliminary study on five patients, suggested that thiomalic acid may have an antiarthritic effect when used in the treatment of rheumatoid arthritis.

In our experiments the concentration range of sodium aurothiomalate used was 25–300 μmol/l culture medium. Thus the lower concentrations correspond to the serum gold levels obtained when the drug is used clinically. The peak serum gold concentration after the intramuscular administration of 50 mg sodium aurothiomalate is reported to be 30–45 μmol/l, and the trough values to be 4–25 μmol/gd/l at steady state conditions on chronic treatment with 50 mg every fourth week or every week. The maximum concentration used was that which caused complete growth arrest of the HE cells without causing significant cell death within the experimental period. When compared on this basis the concentration of auranofin equally is effective to 300 μmol/l of sodium aurothiomalate is about 2 μmol/l. This probably reflects the differences in cellular affinity and in toxicity between the two drugs. It may also support the suggestion that the antiproliferative effect of gold-containing drugs may be ascribed to drug components other than the gold moiety or in addition to it.

In conclusion, we have shown that human cells containing high amounts of cytosolic MT are not resistant to the antiproliferative effect of sodium aurothiomalate, though MT traps about one third of the cytosolic gold. Sodium aurothiomalate exposure does not induce the synthesis of detectable amounts of MT. Sodium aurothiomalate and thiomalic acid have similar growth inhibitory effects. The observed differences in the cellular and cytosolic distributions of gold and thiomalate indicate a dissociation of the sodium aurothiomalate molecule and raise questions about the effector moiety of the molecule.

We are grateful to Mrs K R Modalsli, Miss K Moestue, and Miss A K Syversen for excellent technical assistance; to Dr H L Sharma (Department of Medical Biophysics, University of Manchester, England) for synthesising sodium $^{199}$Au[aurothiomalate, and May & Baker Ltd who provided the 14C-labelled compounds. Dr L Mørkrid provided statistical advice. A Glennås is a research fellow sponsored by the Norwegian Hydro Company.

Appendix

The relative sensitivity of the two cell lines HE and HEK100 to sodium aurothiomalate or thiomalic acid was calculated as follows: Cell growth was expressed by the formula:

$$N(t) = N_0 \exp \{\int (t; c)\}$$

where N is the cell number at time (t) and c is the drug concentration used.

The following expression was obtained for the area under the curves (AUC) (Figs 1 and 2) by integration from t=0 to t=T:

$$AUC = \int \ln N(t) \, dt = T \ln N_0 + \frac{1}{2} \int (t; c) \, dt$$

AUCc = T In N0 was the same for all values of c, thus

$$\Delta AUC = AUC_T - AUC_0$$

is independent of N0, the cell number on day 0.

The relative sensitivity to various concentrations of the drug, Y(c), of one cell line was expressed as:

$$Y(c) = \frac{\Delta AUC(c)}{\Delta AUC(c=0)}$$

$\Delta Y(\mathcal{Y} = (Y' - Y) \mathcal{Y}' + Y) = \mathcal{Y}' + Y$ and $\mathcal{Y}'$ is a linear first-order function of log c, Y denoting the values for the HE cells and $Y'$ those for the HE100 cells. Differences in sensitivity to the drugs were tested by a significant correlation between the $\Delta Y(\mathcal{Y} + Y)$ and log c, taking significance as $p<0.05$.

References

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