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Differential toxicity of antimonial compounds and their effects on glutathione homeostasis in a human leukaemia monocyte cell line

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Abbreviations:
7-AAD, 7-aminoactinomycin D
H_2DCFDA, dichlorodihydrofluorescein diacetate
PMA, phorbol 12-myristate 13-acetate
PtdSer, phosphatidylserine
ROS, reactive oxygen species
Sb^{III}, trivalent antimony
Sb^{V}, pentavalent antimony
TCEP, tris(2-carboxyethyl) phosphine

ABSTRACT

Trivalent antimonial compounds (Sb^{III}), originally used in the treatment of leishmaniasis, are now being proposed as a novel therapy for acute promyelocytic leukaemia (APL). Here, we examine the effects of Sb^{III} and pentavalent antimonial drugs (Sb^{V}) on glutathione homeostasis, oxidative stress and apoptosis in the human leukaemia monocyte cell line, THP-1. Although growth of THP-1 macrophages is unaffected by Sb^{V}, macrophages are extremely sensitive to Sb^{III}. On exposure to Sb^{III}, intracellular free glutathione (GSH) levels in macrophages decrease linearly by 50% over 4 h, associated with efflux of both GSH and accumulation of intracellular glutathione disulphide (GSSG). Together these effects increase the redox potential of the GSSG/GSH couple from −282 to −225 mV. Sb^{III}-induced GSH efflux from THP-1 macrophages is accompanied by the concomitant efflux of Sb^{III} at a constant molar ratio of 3 (GSH) to 1 (Sb^{III}), respectively. Sb^{III} directly inhibits glutathione reductase activity in macrophages, significantly retarding the regeneration of GSH from GSSG, following diamide oxidation. Sb^{III}-treated THP-1 macrophages go on to exhibit elevated levels of reactive oxygen species and show the early signs of apoptosis. The absence of these effects in Sb^{V}-treated THP-1 cells suggests that macrophages do not efficiently reduce Sb^{V} to Sb^{III}. Collectively, these findings suggest that Sb^{III} seriously compromises thiol homeostasis in THP-1 macrophages and that this may be an early defining event in the mode of action of antimonials against leukaemia cells.

1. Introduction

Arsenic trioxide (Trisenox®, As_2O_3) is used in the treatment of acute promyelocytic leukaemia (APL) and is undergoing clinical trials against other haematological malignancies and solid tumours [1,2]. The mechanisms by which As_2O_3 acts upon APL cells are manifold, ultimately resulting in the triggering of apoptosis or cell cycle arrest [3]. As_2O_3-triggered apoptosis is p53 and caspase-dependent [4], characterized by nuclear condensation, DNA fragmentation, mitochondrial

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transmembrane potential collapse and increased production of reactive oxygen species (ROS) [5,6].

However, clinical use of As_2O_3 is likely to be limited by its toxicity and carcinogenic effects [7]. Antimony (Sb), which belongs to the same group of the periodic table (Group V), has emerged as a less toxic, alternative therapy for several malignancies. While arsenite-mediated apoptosis in mammalian cells has been extensively studied, comparatively little is known about the mechanisms of apoptosis mediated by antimonite [8,9]. Indeed, there are several reported differences in the biological features and therapeutic aspects of As^{III} and trivalent antimony (Sb^{III}) [10]. Thus, further study on the action of antimonials against leukaemia cells is clearly warranted.

The use of antimonials in the treatment of disease is far from novel. Trivalent antimonials were extensively used for treating sleeping sickness in the first half of the 20th century and pentavalent antimonials have been the front-line drugs for the treatment of leishmaniasis for the past 50 years, despite scant knowledge concerning their mode of action. Recently, we demonstrated that Sb^{III} profoundly interferes with the thiol metabolism of *Leishmania* [11]. In these parasites, thiol metabolism is uniquely dependent upon trypanothione (N^1,N^8-bis(glutathionyl) spermidine) in contrast to most other organisms (including their mammalian hosts), which utilise glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) as their principal thiol. Sb^{III} decreases thiol buffering capacity in drug-sensitive *Leishmania* parasites by inducing rapid efflux of intracellular trypanothione and glutathione; it also inhibits trypanothione reductase in intact cells, resulting in the accumulation of the disulphide forms of both trypanothione and glutathione (GSSG). These two effects combine to profoundly compromise thiol redox potential in *Leishmania* parasites and may constitute the primary mode of action of antimonial drugs. These studies have posed the following question: is perturbation of thiol homeostasis a major contributing factor in the mode of action and toxicity of antimonial drugs against other rapidly dividing cells such as leukaemia? Certainly Sb^{III} reversibly inhibits isolated human glutathione reductase as well as inhibiting the complementary *Leishmania* enzyme, trypanothione reductase [12]. Should this inhibition occur in vivo it can be predicted that many of the Sb-induced effects observed on *Leishmania* thiol metabolism would be duplicated in leukaemia cells.

Current therapies for leishmaniasis are based upon the pentavalent form of antimony (Sb^V). However, Sb^V is generally regarded as a pro-drug that first has to be activated by conversion to Sb^{III} before becoming biologically active against *Leishmania* parasites. The site of Sb^V reduction (host macrophage cell, parasite, or both) and mechanism of reduction (enzymatic or non-enzymatic) are not fully understood and remain an area of some controversy within the research community. Largely based on data showing that Sb^{III} is considerably more toxic for *Leishmania* than Sb^V [13], it has been hypothesized that Sb^V is in fact reduced by the macrophage host cell [13,14]. If so, it is possible that Sb^V-based therapies may provide a less toxic alternative to As_2O_3-based treatment of APL. With this in mind, we have undertaken a comparative study on the susceptibility of the human leukaemia cell line, THP-1, as undifferentiated monocytes and after differentiation to macrophages, to both the pentavalent and trivalent forms of antimony. The findings presented here have implications for both the treatment of leukaemia and leishmaniasis.

2. Materials and methods

2.1. Cell lines and culture conditions

The human leukaemia monocyte cell line, THP-1 (American Type Culture Collection, MD, USA), was cultured in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) foetal calf serum. Cultures were initiated at 1×10^5 cells ml^{-1} and grown at 37 °C in 5% CO_2. Mid-log THP-1 cells were differentiated by a 48 h incubation in medium containing a final concentration of 10 ng ml^{-1} phorbol 12-myristate 13-acetate (PMA; Sigma). Adherent cells were washed in fresh culture medium in the absence of PMA. Mid-log THP-1 cells which were not treated with PMA were also analysed in this study.

In order to examine the effects of antimonial drugs on growth, triplicate cultures containing Sb^{III} (as potassium antimony tartrate) or Sb^V (as sodium stibogluconate, a gift from Glaxo-Smith-Kline) were seeded with 5×10^5 cells ml^{-1}. Cell densities were determined microscopically after culture for 72 h and IC_{50} values determined using the IC_{50} four-parameter equation provided with GraFit.

2.2. Analysis of intracellular GSH

Differentiated THP-1 cells were centrifuged (1600 × *g*, 10 min, 4 °C), re-suspended in fresh culture medium at 1×10^6 ml^{-1} and incubated with Sb. The viability of cells was monitored microscopically and by the LIVE/DEAD™ Viability/Cytotoxicity assay (Molecular Probes). At intervals, 1×10^6 cells were collected by centrifugation and derivatised with monobromobimane, as described previously [11]. Acid soluble thiols were separated by ion-paired, reverse phase HPLC on a Beckman Ultrasphere C_{18} column using a Beckman System Gold instrument fitted with a Gilson-121 fluorometer. Control experiments confirmed that Sb^{III} does not interfere with this assay. Varying concentrations of Sb^{III} were assessed for their effect on intracellular GSH levels within THP-1 macrophages. In these experiments, THP-1 macrophages were incubated with Sb^{III} for 4 h before being processed for thiol analysis, as described.

2.3. Sb-induced thiol efflux

Differentiated THP-1 cells were re-suspended in fresh culture maintenance medium (44 mM NaCl, 56 mM glucose, 56 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 8) at 1.5×10^7 ml^{-1} in either the presence or absence of Sb^{III} (105 μg ml^{-1}). Following incubation (4 h, 37 °C), 1 ml aliquots of culture were pelleted by centrifugation and the supernatant removed. Duplicate sets of supernatants were collected and GSH identified by HPLC as above. However, in this instance, one set of samples was reduced using a 4–5 M excess of tris(2-carboxyethyl)phosphine (TCEP) prior to derivatisation while a duplicate set was not reduced.

2.4. Determination of Sb^{III} to GSH efflux in THP-1 macrophages

THP-1 macrophages (2.5×10^7 ml^{-1}) were incubated with 105 μg ml^{-1} Sb^{III} (including 10 μg of $^{124}Sb^{III}$, 15 MBq mg^{-1}, Polatom) for 1 h. Cells were then washed twice with phosphate-buffered saline (PBS) at 4 °C, re-suspended in fresh maintenance medium and incubated at 37 °C in an atmosphere of 5% CO_2. At intervals 2 ml of culture was removed and pelleted by centrifugation. Levels of $^{124}Sb^{III}$ were analysed in 0.5 ml of the supernatant by scintillation counting and the total Sb^{III} effluxed calculated. The remaining supernatant (1.5 ml) was added to a 1 cm^3 Sep-Pak C_{18} column in the presence of 5% propan-1-ol. GSH was eluted from the column using 80% propan-1-ol, fractions were collected, dried down and re-suspended in HEPES buffer containing TCEP in preparation for analysis by HPLC, as previously described [11].

2.5. Regeneration of intracellular GSSG

Diamide (diazine dicarboxylic acid bis(*N*,*N*-dimethylamide)), from Sigma, was used to oxidize intracellular thiols. Cultures were pre-incubated at 10^6 ml^{-1} at 37 °C for 1 h in fresh culture medium (controls) or in medium containing either Sb^{III} (105 μg ml^{-1}) or Sb^V (1000 μg ml^{-1}). Cells were centrifuged (1600 × *g*, 10 min, 4 °C), and re-suspended at 1×10^6 ml^{-1} in ice-cold maintenance medium containing 5 mM diamide and Sb. Following incubation (20 min, 4 °C), cells were pelleted by centrifugation (1600 × *g*, 10 min, 4 °C) and re-suspended in fresh culture medium with or without Sb. The viability of cells following treatment with diamide was monitored as described above. At intervals, aliquots were removed and analysed for thiol content as above.

2.6. Analysis of glutathione reductase activity in THP-1 macrophage cell lysates

THP-1 macrophages (1×10^6 ml^{-1}, 1 l) were pelleted by centrifugation (1600 × *g*, 10 min, 4 °C), washed twice in PBS and re-suspended in cell lysis buffer (10 mM phosphate, pH 7.0, 0.7 mM 2-mercaptoethanol and 2.7 mM EDTA). Cells were then lysed under pressure (30 kpsi) using a one shot cell disruptor (Constant Systems). Following centrifugation (100,000 × *g* for 1 h), cell supernatant was collected and protein concentration determined. The glutathione reductase activity of the THP-1 macrophage cell lysate was measured spectrophotometrically at 340 nm [12]. Prior to initiation of the glutathione reductase assays by the addition of GSSG, lysates were pre-incubated with varying concentrations of Sb^{III} or Sb^V for 15 min.

2.7. Determination of redox potential

At intervals following incubation with Sb^{III} (105 μg ml^{-1}), duplicate sets of 1×10^6 THP-1 cells were harvested. In one set, the free GSH levels were determined, in the other set the total glutathione content was determined by reduction with tris(2-carboxyethyl)phosphine, prior to derivatisation. Levels of GSSG were calculated from the difference between these measurements ([total − free]/2). Electron potentials for GSSG/2GSH (−240 mV) were calculated assuming an intracellular pH of 7.0 and using the appropriate Nernst equation [15]. For GSSG/2GSH (in millivolts at 37 °C, pH 7.0):

$$E = E'_0 - \left(\frac{59.1}{n}\right) \log\left(\frac{[GSH]^2}{[GSSG]}\right)$$

The cell volumes of THP-1 macrophages used in this calculation were measured using a Schärfe Systems CASY1 cell counter yielding 20 ± 0.8 μl [10^6 cells]$^{-1}$.

2.8. H_2DCFDA labelling of THP-1 macrophages

The cell permeant probe dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes) was used to detect the generation of H_2O_2 within Sb-treated THP-1 cells. H_2DCFDA readily diffuses into cells, where it is hydrolysed to H_2DCF. In the presence of ROS, primarily H_2O_2 and $^{\bullet}OH$, H_2DCF is oxidized to the fluorescent product 2,7-dichlorofluorescein. THP-1 macrophages, grown and differentiated in 8-well chamber slides, were incubated with either Sb^{III} (105 μg ml^{-1}) or Sb^V (1000 μg ml^{-1}) for 4 h. H_2DCFDA was added to the culture medium to a final concentration of 5 μM. Following a 15 min incubation, cells were washed three times in PBS, air dried and fixed in 4% (w/v) paraformaldehyde for 30 min. Images were collected using a fluorescein isothiocyanate filter on a Zeiss Axiovert 200 M fluorescence microscope. Each image was generated in an identical manner using identical exposure times and image processing parameters.

2.9. Detection of phosphatidylserine surface exposure

Annexin V labelling was performed using the Vybrant apoptosis assay kit #2 (Molecular Probes), with minor modifications. Briefly, differentiated THP-1 macrophages were incubated for 2 h in fresh culture medium containing Sb^{III} (105 μg ml^{-1}) or Sb^V (1000 μg ml^{-1}). Control cells were incubated in the absence of Sb. Cells were then washed in ice-cold phosphate buffer and re-suspended in annexin V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM and $CaCl_2$, pH 7.4). Annexin V conjugated to Alexa Fluor 488 and 7-aminoactinomycin D (7-AAD; Molecular Probes) were incubated with cells. Following a 15-min incubation, cells were analysed on a FACSort flow cytometer (Becton Dickinson) and CELLQuest software.

3. Results

3.1. The effects of trivalent and pentavalent antimony on the intracellular GSH levels of THP-1 macrophages

Initially, the sensitivity of PMA-differentiated THP-1 macrophages for Sb was established by determining the effects of growth with either Sb^{III} (potassium antimony tartrate) or Sb^V (sodium stibogluconate) (Fig. 1). In keeping with published data (31.5 μg ml^{-1} [16]), we obtained an IC_{50} value of 25 ± 3 μg ml^{-1} for Sb^{III} in THP-1 macrophages. In contrast, Sb^V concentrations up to 1000 μg ml^{-1} had no effect on the growth or viability of THP-1 cells.

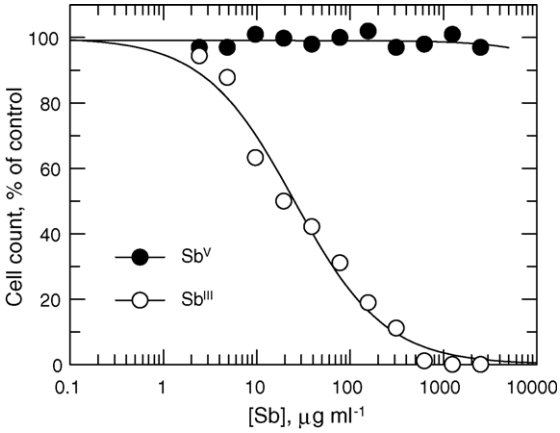

Fig. 1 – Effects of Sb^{III} and Sb^{V} on the growth of THP-1 cells. The dose–response curve of THP-1 human leukaemia macrophages treated for 72 h in culture medium with Sb^{III} (potassium antimony tartrate) is shown in open circles and for cells treated with Sb^{V} (sodium stibogluconate) shown in closed circles. Data for Sb^{III} are fitted by non-linear regression, as described in Section 2. Data for Sb^{V} show the fit for linear regression. Data are the mean of triplicate measurements.

The time-dependent effects of fixed concentrations of Sb^{III} and Sb^{V} on THP-1 cell intracellular GSH levels were examined in detail (Fig. 2). In the absence of Sb, levels of GSH remained stable at approximately 5.5 nmol $[10^6 \text{ cells}]^{-1}$ throughout a 4-h time course (Fig. 2A, open squares), identical to previously published values for intracellular GSH in macrophages [17]. In contrast, treatment of THP-1 macrophages with Sb^{III} (105 μg ml^{-1}, ~4 times the IC_{50} value) resulted in a significant, time-dependent decrease in the level of intracellular GSH (Fig. 2A). Overall disappearance of GSH was essentially linear throughout the experiment, such that half (2.85 nmol $[10^6 \text{ cells}]^{-1}$) was lost over the 4-h incubation at a rate of 12 pmol min^{-1} $[10^6 \text{ cells}]^{-1}$. However, treatment of THP-1 cells with Sb^{V} (1000 μg ml^{-1}) had no effect on intracellular GSH levels (Fig. 2A). In a parallel experiment the loss of GSH from THP-1 macrophages was found to be dose-dependent with an IC_{50} value of 55 ± 8 μg ml^{-1} after 4 h exposure to Sb^{III} (Fig. 2B), approximately double the IC_{50} value for growth inhibition after 72 h exposure (Fig. 1). Identical results were obtained with THP-1 monocytes that had not been differentiated by treatment with PMA. Likewise, the murine macrophage-like cell line, J774.2, showed similar changes in GSH on exposure to Sb^{III} (not shown). Thus, these effects are independent of PMA induction and common to monocyte cell lines.

3.2. *Antimony-induced GSH efflux from THP-1 macrophages*

The fate of cellular GSH following treatment with Sb was monitored in the supernatants of Sb^{III}-treated and control THP-1 macrophages. Following reduction with TCEP and derivatisation with monobromobimane, extracts of cell-free medium were analysed by HPLC (Fig. 2C). Supernatants from cells treated with Sb^{III} contained a major thiol peak (0.32 ± 0.03 nmol $[10^6 \text{ cells}]^{-1}$) which co-eluted with the bimane derivative of a GSH standard. No peaks corresponding to cysteine or cysteinylglycine were observed. In contrast, the TCEP-reduced supernatants of untreated control cells contained virtually no GSH (0.015 ± 0.003 nmol $[10^6 \text{ cells}]^{-1}$). Throughout the 4 h of the experiment the viability of cells was monitored microscopically and by the LIVE/DEAD™

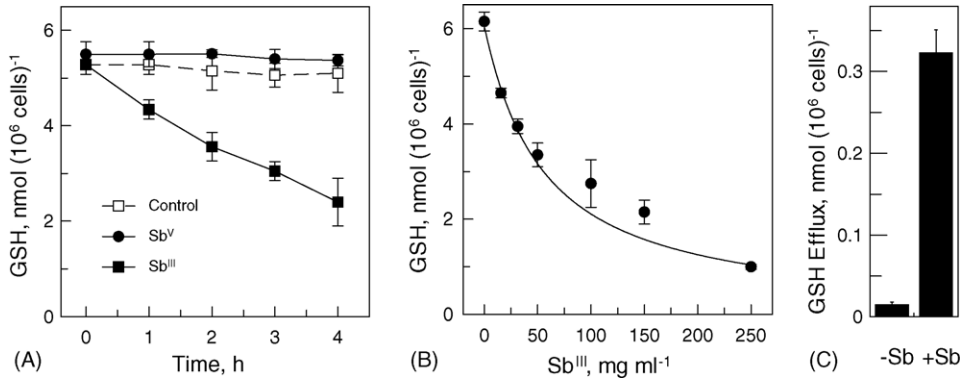

Fig. 2 – Effects of Sb on intracellular GSH in THP-1 cells. (A) Time-dependent effects of Sb. THP-1 cells were incubated with or without Sb (105 μg ml^{-1} Sb^{III} or 1000 μg ml^{-1} Sb^{V}) and GSH content determined by HPLC as described in Section 2. Control, no treatment: open squares, dashed lines; plus Sb^{III}: closed squares, solid lines; plus Sb^{V}: closed circles, solid lines. Each value represents the mean ± S.D. of triplicate determinations. (B) Effects of varying concentrations of Sb^{III} on the total GSH content of THP-1 macrophages. THP-1 macrophages were incubated for 4 h in culture medium containing Sb^{III} (0–250 μg ml^{-1}) before thiol analysis. Data are fitted by non-linear regression to the IC_{50} (background corrected) equation in GraFit and each data point is the mean of triplicate measurements. (C) Sb^{III}-induced GSH efflux. THP-1 cells were re-suspended in a minimal maintenance medium lacking cysteine and methionine in the presence or absence of Sb^{III} (105 μg ml^{-1}). Supernatants were collected at 4 h and reduced by the addition of TCEP prior to derivatisation with monobromobimane. Equal volumes of the supernatants were then analysed by HPLC and the GSH levels determined in both the Sb-treated and control supernatants.

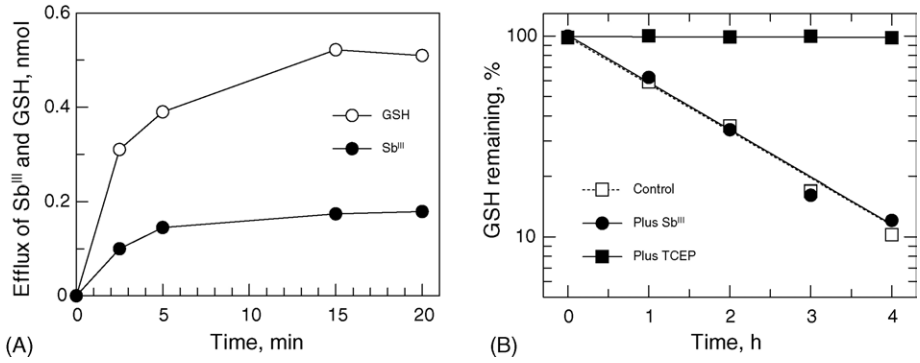

Fig. 3 – Efflux of Sb^{III} and GSH from Sb^{III}-treated THP-1 macrophages and stability of GSH in medium. (A) Efflux. THP-1 macrophages were incubated with 105 μg ml^{-1} Sb^{III} (including 10 μg of $^{124}Sb^{III}$) for 1 h, washed and re-suspended in maintenance medium. At intervals aliquots were removed and supernatants analysed for GSH and Sb^{III}, as described in Section 2. GSH: open circles; Sb^{III}, closed circles. (B) Stability of GSH in medium. GSH was incubated in maintenance medium in the presence or absence of Sb^{III} at 37 °C, 5% CO_2. At intervals samples were removed and analysed for GSH, as described in Section 2. Control, no treatment: open squares, dotted line; plus Sb^{III}: closed circles; control after reduction with TCEP: closed squares. Each data point is the mean of triplicate measurements and the lines represent the best fit to a single exponential decay.

Viability/Cytotoxicity assay to ensure that Sb-induced cell lysis was not responsible for any thiol released into the supernatant.

3.3. *Stoichiometry of Sb^{III} to GSH efflux in Sb^{III}-treated THP-1 macrophages*

Sb^{III} forms a stable 3:1 complex with GSH at physiological pH [18] and is excreted in a glutathione-dependent manner from the liver into the bile duct in rats [19]. To determine whether a similar mechanism occurs in THP-1 macrophages the efflux of both Sb^{III} and GSH from Sb-treated macrophages was measured in a minimal medium lacking GSH and its amino acid precursors (Fig. 3A). The efflux of GSH and Sb^{III} from Sb-treated macrophages was found to be time-dependent. Indeed, the molar ratio of Sb^{III} to GSH effluxed was found to remain constant throughout the time course with a mean of 2.9 ± 0.2 suggesting that for every mole of Sb^{III}, 3 mol of GSH is effluxed. Efflux was completed by 20 min with 10% of $^{124}Sb^{III}$ remaining in the cells.

The supernatants of Sb^{III}-treated macrophages that were not reduced with TCEP prior to derivatisation with monobromobimane appeared to contain no GSH. This observation suggests that glutathione is either effluxed from cells in the form of GSSG or that GSH is oxidized in the extracellular medium over the time course of this experiment. Control experiments demonstrated that GSH in media alone and in a 3× molar excess to Sb^{III} was oxidized to GSSG with a half life of 1.5 h (Fig. 3B). Following reduction of samples with TCEP, $99.1 \pm 0.9\%$ of the GSH at zero time was recovered.

3.4. *Trivalent antimony inhibits glutathione reductase activity and thiol regeneration in THP-1 macrophages*

The specific activity of glutathione reductase in THP-1 macrophage extracts was 66 ± 0.5 nmol min^{-1} mg^{-1} (0.9 nmol min^{-1} $[10^6$ cells$]^{-1}$), similar to 86 ± 0.7 nmol min^{-1} mg^{-1} obtained for J774.2 macrophages. These cell extracts were treated with varying concentrations of Sb^{III} or Sb^{V} and glutathione reductase activity directly measured (Fig. 4A). As expected, Sb^{III} potently inhibited glutathione reductase in THP-1 cell lysates with an IC_{50} of 8.5 ± 0.8 μg ml^{-1}. However, Sb^{V} (>1000 μg ml^{-1}) had no inhibitory effect on glutathione reductase activity.

Cellular regeneration of thiols from disulphides, formed by transient exposure to the oxidant diamide, has been used as an indirect method of assessing disulphide reductase activity within intact cells [20–22]. Using this method, we have examined the effect of Sb on recovery of GSH in THP-1 macrophages (Fig. 4B). GSH levels were below the limits of detection immediately following diamide treatment. After re-suspension of cells into fresh minimal medium, in the absence of Sb, THP-1 cells were able to rapidly regenerate GSH to 60% of control values within 10 min with a linear rate of 0.4 nmol min^{-1} $[10^6$ cells$]^{-1}$ (Fig. 4B) in good agreement with the specific activity determined above in whole cell extracts. In keeping with the observation that glutathione reductase is not inhibited by Sb^{V}, the rate of recovery of GSH in cells treated with Sb^{V} (1000 μg ml^{-1}) was essentially identical to untreated control cells (Fig. 4B). In contrast, in cells exposed to Sb^{III} throughout the experiment, both the rate of recovery and the final thiol levels were impaired. The initial rate of regeneration of GSH was approximately two-fold slower (0.2 nmol min^{-1} $[10^6$ cells$]^{-1}$) than in control cells and then fell to zero such that the final thiol level was only 23% of control levels compared to 80% in untreated control cells. Collectively, these data suggest that Sb^{III} impairs the function of glutathione reductase within intact cells while Sb^{V} has no apparent effect.

3.5. *Effect of trivalent and pentavalent antimony on levels of GSSG within THP-1 macrophages*

If Sb^{III} inhibits glutathione reductase in intact cells, then the levels of GSSG might be expected to rise in the face of either endogenous or exogenous oxidant stress as well as other

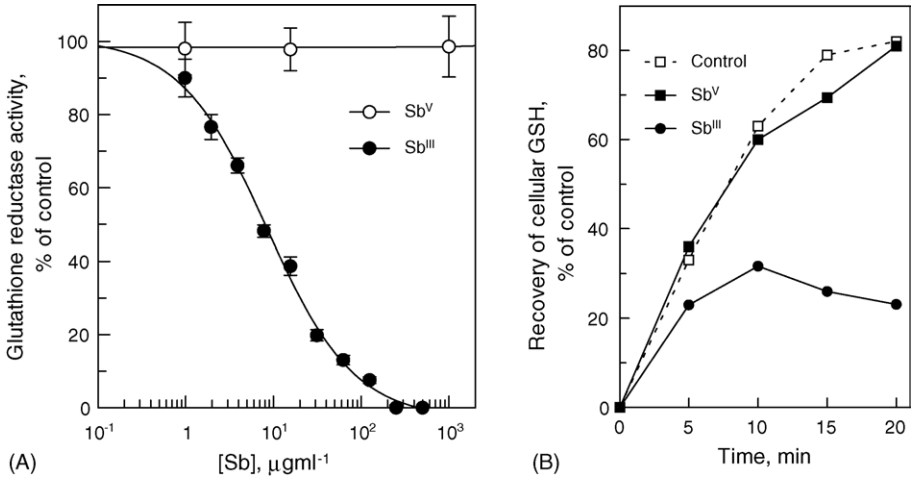

Fig. 4 – Effects of Sb^{III} and Sb^{V} on glutathione reductase activity and the regeneration of GSH in THP-1 macrophages. (A) THP-1 cell lysates were pre-treated with varying concentrations of either Sb^{III} or Sb^{V} (0–1000 μg ml^{-1}) and then assayed for relative glutathione reductase activity, as described in Section 2. Sb^{III}: closed circles; Sb^{V}: open circles. All data presented are means of triplicate measurements ± S.D. (B) Cells were pre-treated for 1 h with or without Sb (Sb^{III} at 105 μg ml^{-1} or Sb^{V} at 1000 μg ml^{-1}) in culture medium prior to diamide oxidation of intracellular thiols as described in Section 2. Cells were then re-suspended in fresh culture medium (±Sb) and the recovery of GSH was monitored as in Fig. 2. Each time point is expressed as a percentage of the GSH present at the equivalent time in control incubations (±Sb) treated in an identical manner, but not exposed to diamide oxidation. Controls: open squares, dashed lines; Sb^{III}: closed circles, solid lines; Sb^{V}: closed circles, solid lines.

thiol-dependent metabolic processes. To determine whether this was indeed the case, levels of GSSG were monitored throughout a time course of Sb treatment. In the absence of Sb, 98% of the total glutathione pool (0.27 mM) was recovered as the free thiol (Fig. 5), and remained constant over the 4-h incubation (data not shown). In contrast, Sb^{III} treatment induced a time-dependent decrease in GSH and a concomitant increase in GSSG, such that free GSH decreased by ~50% after 4 h and represented only 68% of the total remaining glutathione pool.

Ratios of GSSG/GSH levels in THP-1 macrophages were monitored throughout a time course of either Sb^{III} or Sb^{V} incubation (Fig. 6A). GSSG/GSH levels increased exponentially in Sb^{III}-treated cells while the ratios of control and Sb^{V}-treated cells remained unchanged. Absolute concentrations of GSH and GSSG within Sb^{III}-treated were used to calculate half-cell reduction potentials for the GSSG/2GSH couple at pH 7.0 (Fig. 6B). After exposure to Sb^{III} for 4 h, the redox potentials for couples became markedly less electro-negative (i.e. indicative of a more oxidizing environment), increasing by +57.8 mV. Changes in electron potential of this magnitude are consistent with cells entering into an apoptotic-like state [15]. An equivalent incubation with Sb^{V} left reduction potentials relatively unchanged (Fig. 6B).

3.6. *Sb^{III}-induced generation of ROS*

Having established that Sb^{III} induces both loss of GSH and build-up of GSSG in THP-1 cells, we monitored any corresponding changes in the levels of ROS within the cells using the ROS-specific fluorescent probe H_2DCFDA (Fig. 7). Following a 4-h incubation with Sb^{III} (105 μg ml^{-1}), THP-1 macrophages

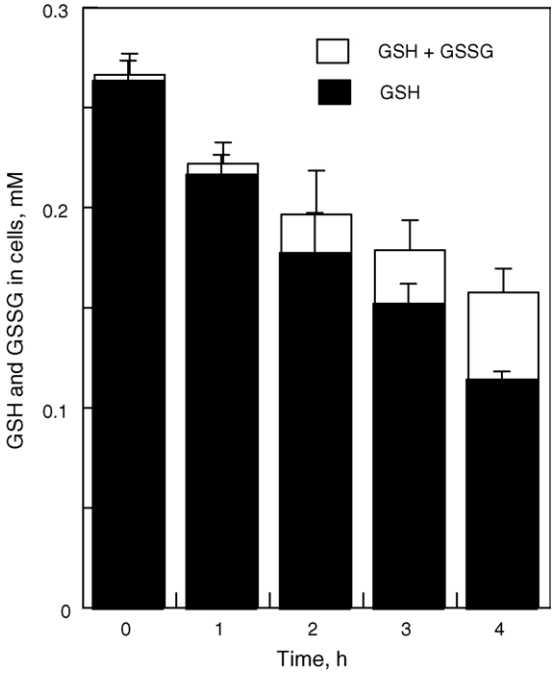

Fig. 5 – Effects of Sb^{III} on levels of GSH and GSSG within THP-1 cells. Intracellular GSH concentrations were calculated in THP-1 cells treated with Sb^{III} using a cell volume of 20 ± 0.8 μl [10^{6} cells]$^{-1}$ (see Section 2). Free GSH is shown by black bars overlaid on total glutathione (white bars). All data presented are means of triplicate measurements ± S.D.

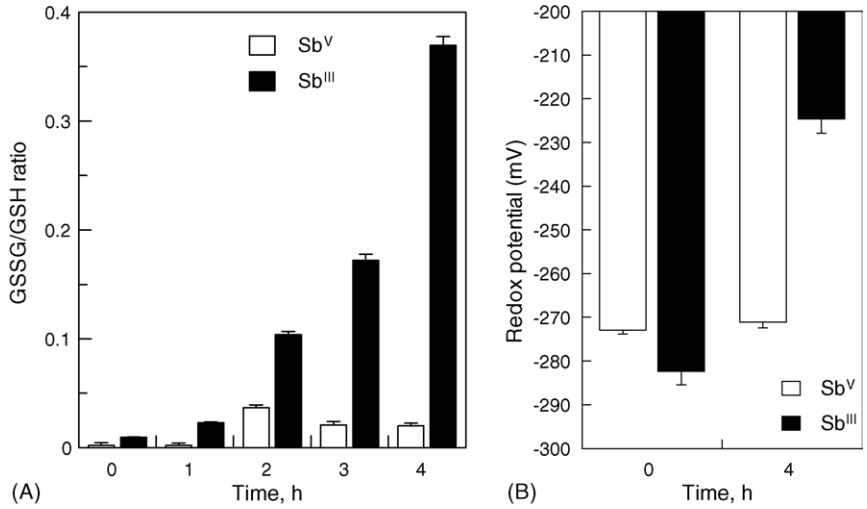

Fig. 6 – Effects of Sb^{III} and Sb^V on the ratio of GSSG to GSH and the glutathione redox potential in THP-1 cells. Total and free thiol content was measured at defined intervals throughout a 4-h incubation with either Sb^{III} or Sb^V, as described in Section 2. (Panel A) Ratio of GSSG/GSH. These were calculated in THP-1 cells from total glutathione (T) and free GSH (F), where GSSG = (T − F)/2. Sb^V: white bars; Sb^{III}: black bars. (Panel B) Glutathione redox potential. These were calculated using the Nernst equation for GSH at 37 °C, at pH 7.0, as described in Section 2. Redox potentials for cells treated with Sb^V shown in white and cells treated with Sb^{III} are shown in black. All data presented are means of triplicate measurements ± S.D.

loaded with H_2DCFDA exhibited increased levels of fluorescence (Fig. 7A and B), as compared to cells incubated in the absence of Sb (data not shown). The increased levels of fluorescence demonstrate an Sb^{III}-induced increase in the levels of $^{\bullet}OH$ and H_2O_2, primarily detected by H_2DCFDA. In contrast, incubation of THP-1 cells with Sb^V (1000 μg ml^{-1}) did not result in any apparent increase in fluorescence (Fig. 7C and D).

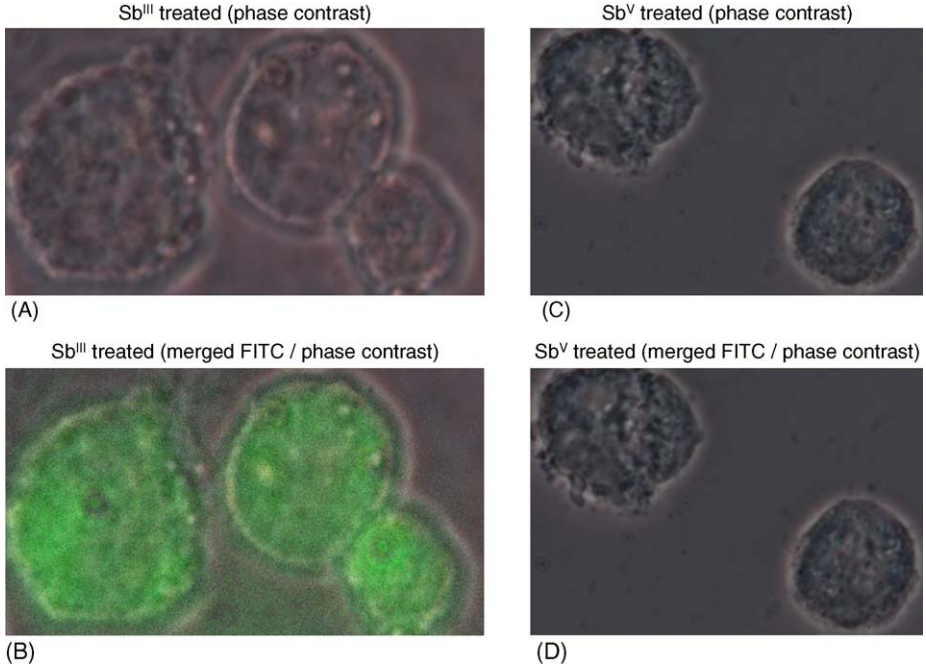

Fig. 7 – Effects of Sb^{III} and Sb^V on the levels of ROS within THP-1 cells. THP-1 cells were treated for 4 h with either Sb^{III} (105 μg ml^{-1}) or Sb^V (1000 μg ml^{-1}). Cells were then washed and loaded with 5 μM H_2DCFDA, a cell permeant probe used to detect the generation of ROS, as described in Section 2. Images were collected using a FITC filter on a Zeiss Axiovert 200 M fluorescence microscope. Live images of Sb^{III}- and Sb^V-treated cells are shown in panels A and C, respectively, and merged FITC/live images of Sb^{III}- and Sb^V-treated cells are shown in panels B and D. Note that each image was generated in an identical manner using identical exposure times and image processing parameters.

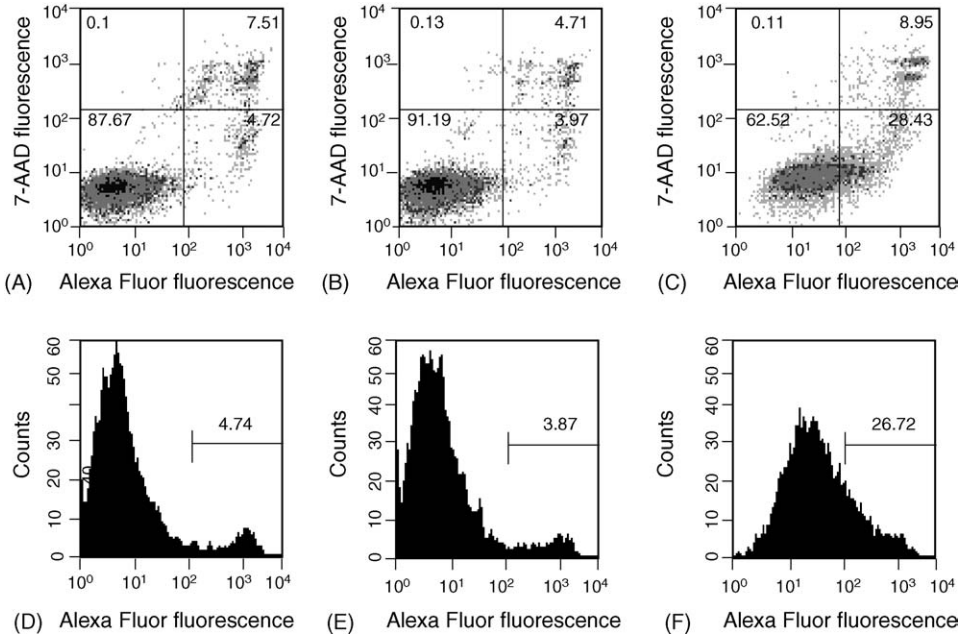

Fig. 8 – Phosphatidylserine exposure on the cell surface of THP-1 cells treated with Sb^{III} and Sb^{V}. THP-1 cells were pre-treated with either Sb^{III} (105 μg ml^{-1}), Sb^{V} (1000 μg ml^{-1}) or left untreated for 4 h. Cells were then labelled with annexin V conjugated to Alexa Fluor and 7-AAD, as described in Section 2, and analysed by flow cytometry. Note that apoptotic cells should become singly labelled with annexin V, necrotic cells should become doubly labelled with both annexin V and the nuclear stain 7-AAD while viable cells should remain unlabelled. The relative staining of the THP-1 control cell population is shown in panel A. Relative staining of cells treated with Sb^{V} and Sb^{III} is shown in panels B and C, respectively. The percentage of the cell populations in each quadrant of the above panels is noted and apoptotic cells are those cells in the bottom right of each panel. Histogram analyses of annexin V labelling in untreated, Sb^{V}- and Sb^{III}-treated cells are shown in panels D–F, respectively. The percentage of apoptotic cells in each population is noted. The data are representative of at least three independent experiments.

3.7. *Apoptosis*

One of the earliest events in apoptosis is the translocation of phosphatidylserine (PtdSer) from the inner to the outer leaflet of the cell membrane. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PtdSer, is routinely used in a fluorescent-conjugated form to label externalized PtdSer. However, since annexin V can also label necrotic cells following membrane disruption by binding to the inner leaflet of the plasma membrane, 7-AAD, a cell impermeable nuclear stain was added to the assay to help distinguish between apoptotic (annexin V labelled), necrotic (annexin V and 7-AAD labelled) and live cells (unlabelled). PtdSer exposure was measured on the surface of THP-1 macrophages following a 4-h incubation in the presence or absence of Sb. Flow cytometric analysis revealed that only 4.7% of the total cell population of untreated control cells were heavily labelled with annexin V (Fig. 8A and D). Similarly, only 4% of THP-1 macrophages treated with Sb^{V} (1000 μg ml^{-1}) became labelled with annexin V (Fig. 8B and E). However, 28.4% of THP-1 cells treated with Sb^{III} became heavily labelled with annexin V and were consequently designated apoptotic (Fig. 8C and F).

4. Discussion

Trivalent antimonial compounds (Sb^{III}), originally, but no longer used in the treatment of leishmaniasis, are now being proposed as novel therapies for acute promyelocytic leukaemia and several other malignancies. The results of this study indicate that Sb^{III} is highly toxic for human THP-1 monocytes and macrophages, as well as the murine macrophage-like J774 line, while Sb^{V} has little effect on cell viability at concentrations up to 1000 μg ml^{-1}. The tolerance of THP-1 macrophages for Sb^{V}, in comparison to their sensitivity to Sb^{III}, suggests that macrophage cells do not reduce Sb^{V} to any significant amount. Even if the efficiency of any macrophage-dependent reduction of Sb^{V} was extremely low, the host cell would only need to reduce approximately 0.1% of the pro-drug over the 72 h for growth effects to be observed. Evidently, this does not happen.

Sb^{III}, but not Sb^{V}, has profound effects on the thiol metabolism of THP-1 macrophages promoting the specific efflux of GSH with a constant stoichiometry of 3:1 GSH to Sb^{III}. Although GSH was not detectable in cell free supernatants without prior reduction by TCEP, we attribute this to rapid oxidation of GSH to GSSG. The ready formation of

$Sb^{III}(SG)_3$ ternary complexes and their inherent thermodynamic stability (log K 25) has previously been demonstrated by NMR and electrospray ionization–mass spectrometry [18]. In addition, biliary excretion of Sb^{III} from the liver in rats is accompanied by the co-transport of three molecules of GSH, consistent with the excretion of a $Sb^{III}(SG)_3$ ternary complex [19]. Together with our experimental findings, these observations suggest that $Sb^{III}(SG)_3$ ternary complexes may be the preferred major substrate for efflux in THP-1 macrophages. The transporters involved in detoxifying $Sb^{III}(SG)_3$ complexes from leukaemia cells remain to be determined, however, efflux may well involve the ATP-dependent MRP/GS-X pump or the multi-drug resistance associated protein, MRP1. MRP-GS-X has been shown to transport cytotoxic GSH–platinum complexes from tumour cells, thereby modulating GSH-associated resistance to the platinum-based drug, cisplatin [23] and overexpression of MRP1, in a human lung cancer cell line, resulted in resistance to potassium antimony tartrate and enhanced efflux of the drug [24]. Further studies are required to determine the possible roles of these and other transporters in efflux of Sb^{III}.

A second effect of Sb^{III} on human THP-1 leukaemia monocytes is a marked increase in intracellular GSSG concentration. In part, this must occur through inhibition of glutathione reductase as demonstrated on the isolated enzyme [12] and, as shown here, in whole cell lysates and in intact cells. The reason for the partial recovery of GSH in diamide oxidized and Sb^{III}-treated cells is not fully understood, but has also been observed in *Leishmania* parasites [11]. Regeneration of GSSG via thiol-disulphide exchange with protein thiol groups deserves investigation. Nonetheless, the build-up of GSSG, combined with the loss of free GSH through efflux, results in a significant alteration of the redox potential of Sb^{III}-treated cells and, as a consequence, increased ROS production and induction of apoptosis. It is somewhat surprising that after only 4-h of treatment with Sb^{III}, and the loss of ~50% of cellular free GSH, levels of ROS should have increased to such an extent. Macrophage cells are dependent on a variety of peroxidases for the metabolism of peroxides [25,26], and it is possible that Sb^{III} may also inhibit these enzymes. In addition, the rate of glutathione peroxidase activity is known to be directly proportional to free GSH concentrations and the loss of 50% of cellular GSH would presumably lead to a 50% decrease in glutathione peroxidase activity [27]. In fact, the effective loss of free glutathione within Sb^{III}-treated macrophages may be even more substantial than the amounts observed in our studies. As we have demonstrated in this study, intracellular Sb^{III} is likely to form $Sb(SG)_3$ complexes in vivo which cannot be distinguished from free GSH by our analytical method, thereby effectively reducing the intracellular pool of free GSH even further. This would undoubtedly exacerbate any increase in ROS production as well as increasing the thiol redox potential. Leukaemia cells are already under increased intrinsic oxidative stress and vulnerable to free radical-induced apoptosis. Interestingly, trivalent arsenic has been reported to increase ROS production in myeloid leukaemia cells either by inhibition of mitochondrial respiration [28] or by induction of NADPH oxidase activity [29]. Were Sb^{III} to induce similar effects, then this would further increase the burden of oxidant stress on these cell lines.

In addition to our own work, several studies have also established an association between glutathione metabolism and the use of Sb^{III} in the treatment of leukaemia [30–32]. Co-treatment with buthionine sulphoximine, an inhibitor of glutathione biosynthesis, has been shown to enhance Sb^{III}-induced cytotoxic effects while *N*-acetyl-cysteine, which increases GSH synthesis, abolishes these effects [30]. These observations, combined with the results presented in this study, suggest that the Sb^{III}-induced alterations in cell redox status may provide the trigger for apoptosis in leukaemia cells as in THP-1 macrophages. Recent studies from Park et al. have reported a similar mechanism in acute myeloid leukaemia cells treated with L-ascorbic acid [33]. Incubation of cells with L-ascorbic acid leads to the oxidation of GSH to GSSG, an accumulation of intracellular H_2O_2 and finally apoptosis. Significantly, leukaemia cells with relatively high basal levels of GSH and relatively high basal activities of glutathione peroxidase were not susceptible apoptosis. Furthermore,

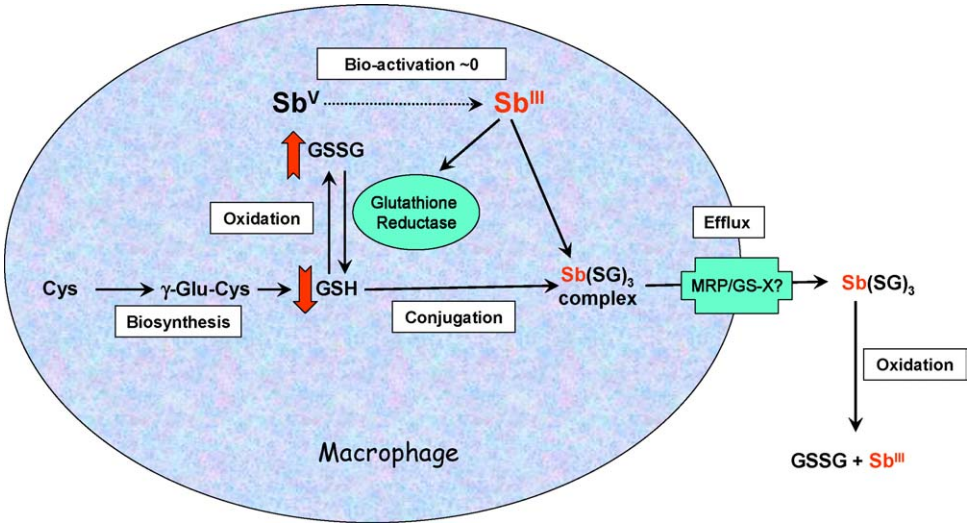

Fig. 9 – Model for the mode of action of antimonial drugs on THP-1 macrophages.

Friesen et al. reported on the key role of glutathione in determining apoptosis sensitivity and resistance in leukaemia cells [34]. Our studies establish a primary effect on GSH metabolism that could account for many of the pleiotropic consequences of Sb^{III} treatment. Thus, the selective toxicity for Sb^{III} treatment of leukaemia is, at least in part, linked to the intrinsic levels of glutathione, its rate of synthesis and degradation, the ratio of GSSG/GSH and glutathione redox potential, as well as the cell's susceptibility to oxidant stress. However, since Sb^{III}, like trivalent arsenic, has a general propensity to react with other protein sulphydryl groups, it could also affect other positive and negative regulators controlling cell growth, differentiation and cell death. Further work is required to resolve these issues.

A previous study demonstrated that radiolabelled Pentostam is rapidly taken up from the external medium by uninfected J774 macrophages [35] thereby ruling out failure of drug uptake as an explanation for the absence of cytotoxicity and perturbation of thiol metabolism in Sb^{V}-treated macrophages. These observations can be more readily explained by the failure of these cells to reduce Sb^{V} to Sb^{III} to any great extent. Therefore, it is unlikely that drugs such as Pentostam and Glucantime, known to act as pro-drugs in the treatment of leishmaniasis, could be used as a less toxic, alternative therapy for leukaemia. It should be noted, however, that several studies have reported that the pentavalent antimonial drug, sodium stibogluconate, acts as a potent inhibitor of protein tyrosine phosphatases in myeloid leukaemia cell lines, inducing irreversible differentiation [36]. In this case, it would appear that the mode of action of sodium stibogluconate against these cells is independent from the Sb^{III}-related mechanism detailed in this study.

In conclusion, our findings demonstrate that Sb^{III}, but not Sb^{V}, is toxic for macrophage cells and has significant effects on macrophage glutathione metabolism (Fig. 9). Sb^{III} profoundly perturbs glutathione homeostasis in THP-1 macrophages and this action is likely to be an early but definitive event in the mode of action of antimonials against leukaemia cells.

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REFERENCES

[1] List A, Beran M, DiPersio J, Slack J, Vey N, Rosenfeld CS, et al. Opportunities for Trisenox (arsenic trioxide) in the treatment of myelodysplastic syndromes. Leukemia 2003;17:1499–507.
[2] Murgo AJ. Clinical trials of arsenic trioxide in hematologic and solid tumors: overview of the National Cancer Institute Cooperative Research and Development Studies. Oncologist 2001;6(Suppl. 2):22–8.
[3] Hyun PW, Hee CY, Won JC, Oh PJ, Kim K, Hyuck IY, et al. Arsenic trioxide inhibits the growth of A498 renal cell carcinoma cells via cell cycle arrest or apoptosis. Biochem Biophys Res Commun 2003;300:230–5.
[4] Liu Q, Hilsenbeck S, Gazitt Y. Arsenic trioxide-induced apoptosis in myeloma cells: p53-dependent G1 or G2/M cell cycle arrest, activation of caspase-8 or caspase-9, and synergy with APO2/TRAIL. Blood 2003;101:4078–87.
[5] Wang TS, Kuo CF, Jan KY, Huang H. Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. J Cell Physiol 1996;169:256–68.
[6] Dai J, Weinberg RS, Waxman S, Jing Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 1999;93:268–77.
[7] Leonard A, Gerber GB. Mutagenicity, carcinogenicity and teratogenicity of antimony compounds. Mutat Res 1996;366:1–8.
[8] Muller S, Miller Jr WH, Dejean A. Trivalent antimonials induce degradation of the PML-RARα oncoprotein and reorganization of the promyelocytic leukemia nuclear bodies in acute promyelocytic leukemia NB4 cells. Blood 1998;92:4308–16.
[9] Lecureur V, Lagadic-Gossmann D, Fardel O. Potassium antimonyl tartrate induces reactive oxygen species-related apoptosis in human myeloid leukemic HL60 cells. Int J Oncol 2002;20:1071–6.
[10] Yan S, Jin L, Sun H. ^{51}Sb antimony in medicine. In: Gielen M, Tiekink ERT, editors. Metallotherapeutic drugs & metal-based diagnostic agents, the use of metals in medicine. 2005. p. 441–61.
[11] Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. J Biol Chem 2004;279:39925–32.
[12] Cunningham ML, Zvelebil MJJM, Fairlamb AH. Mechanism of inhibition of trypanothione reductase and glutathione reductase by trivalent organic arsenicals. Eur J Biochem 1994;221:285–95.
[13] Detke S, Katakura K, Chang K-P. DNA amplification in arsenite-resistant *Leishmania*. Exp Cell Res 1989;180:161–70.
[14] Borst P, Ouellette M. New mechanisms of drug resistance in parasitic protozoa. Annu Rev Microbiol 1995;49:427–60.
[15] Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001;30:1191–212.
[16] Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouaissi A, Lemesre JL. Axenically grown amastigotes of *Leishmania infantum* used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob Agents Chemother 1998;42:3097–102.
[17] Kosower NS, Kosower EM. The glutathione status of cells. Int Rev Cytol 1978;54:109–60.
[18] Sun HZ, Yan SC, Cheng WS. Interaction of antimony tartrate with the tripeptide glutathione—implication for its mode of action. Eur J Biochem 2000;267:5450–7.
[19] Gyurasics A, Koszorus L, Varga F, Gregus Z. Increased biliary excretion of glutathione is generated by the glutathione-dependent hepatobiliary transport of antimony and bismuth. Biochem Pharmacol 1992;44:1275–81.
[20] Hothersall JS, Cunha FQ, Neild GH, Norohna-Dutra AA. Induction of nitric oxide synthesis in J774 cells lowers intracellular glutathione: effect of modulated glutathione redox status on nitric oxide synthase induction. Biochem J 1997;322:477–81.
[21] Kosower NS, Kosower EM, Koppel RL. Sensitivity of hemoglobin thiol groups within red blood cells of rat during oxidation of glutathione. Eur J Biochem 1977;77:529–34.

[22] Tovar J, Cunningham ML, Smith AC, Croft SL, Fairlamb AH. Down-regulation of *Leishmania donovani* trypanothione reductase by heterologous expression of a trans-dominant mutant homologue: effect on parasite intracellular survival. Proc Natl Acad Sci USA 1998;95:5311–6.
[23] Ishikawa T, Bao J-J, Yamane Y, Akimaru K, Frindrich K, Wright CD, et al. Coordinated induction of MRP/GS-X pump and gamma-glutamylcysteine synthetase by heavy metals in human leukemia cells. J Biol Chem 1996;271:14981–8.
[24] Vernhet L, Courtois A, Allain N, Payen L, Anger JP, Guillouzo A, et al. Overexpression of the multidrug resistance-associated protein (MRP1) in human heavy metal-selected tumor cells. FEBS Lett 1999;443:321–5.
[25] Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, Schomburg D, et al. Diversity of glutathione peroxidases. Methods Enzymol 1995;252:38–53.
[26] Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. Free Radic Biol Med 1999;27: 951–65.
[27] Flohé L, Günzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114–26.
[28] Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resist Updat 2004;7: 97–110.
[29] Chou WC, Jie C, Kenedy AA, Jones RJ, Trush MA, Dang CV. Role of NADPH oxidase in arsenic-induced reactive oxygen species formation and cytotoxicity in myeloid leukemia cells. Proc Natl Acad Sci USA 2004;101:4578–83.
[30] Lecureur V, Le Thiec A, Le Meur A, Amiot L, Drenou B, Bernard M, et al. Potassium antimonyl tartrate induces caspase- and reactive oxygen species-dependent apoptosis in lymphoid tumoral cells. Br J Haematol 2002;119:608–15.
[31] Salerno M, Petroutsa M, Garnier-Suillerot A. The MRP1-mediated effluxes of arsenic and antimony do not require arsenic–glutathione and antimony–glutathione complex formation. J Bioenerg Biomembr 2002;34:135–45.
[32] Rappa G, Lorico A, Hildinger M, Fodstad O, Baum C. Novel bicistronic retroviral vector expressing gamma-glutamylcysteine synthetase and the multidrug resistance protein 1 (MRP1) protects cells from MRP1-effluxed drugs and alkylating agents. Hum Gene Ther 2001;12:1785–96.
[33] Park S, Han SS, Park CH, Hahm ER, Lee SJ, Park HK, et al. L-Ascorbic acid induces apoptosis in acute myeloid leukemia cells via hydrogen peroxide-mediated mechanisms. Int J Biochem Cell Biol 2004;36:2180–95.
[34] Friesen C, Kiess Y, Debatin KM. A critical role of glutathione in determining apoptosis sensitivity and resistance in leukemia cells. Cell Death Differ 2004;11(Suppl. 1):S73–85.
[35] Berman JD, Gallalee JV, Hansen BD. *Leishmania mexicana* uptake of sodium stibogluconate (pentostam) and pentamidine by parasite and macrophages. Exp Parasitol 1987;64:127–31.
[36] Pathak MK, Hu X, Yi T. Effects of sodium stibogluconate on differentiation and proliferation of human myeloid leukemia cell lines in vitro. Leukemia 2002;16:2285–91.