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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.