

A putrescine–anthracene conjugate: a paradigm for selective drug delivery

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Increased polyamine concentrations play an important role in the development of cancer at all stages, from initiation through to maintenance of the transformed phenotype. One way cancer cells accumulate increased concentrations of polyamines is by increased uptake of preformed polyamines via their PTS (polyamine transport system). The PTS is promiscuous and will transport a range of polyamine-based molecules. Therefore it may be that cytotoxic drugs could be attached to polyamine vectors and targeted selectively to cancer cells by utilizing the PTS. The aim of the present study was to investigate the potential of Ant 4, a putrescine–anthracene conjugate, to target cytotoxic agents to human cancer cells as a paradigm for a novel method of selective drug delivery. Ant 4 induced cytotoxicity after only 24 h exposure. Apoptosis was the predominant type of cell death, with mechanistic studies revealing that oxidative stress and DNA

damage may have a part to play. For the first time, uptake of Ant 4 via the PTS was demonstrated both directly and indirectly in human cell lines. In addition, Ant 4 significantly reduced putrescine uptake, demonstrating that this conjugate not only used the PTS, but also could successfully compete with its native polyamine for uptake. However, the most interesting finding was the intracellular depletion of the polyamine pools, providing an additional mode of toxicity for Ant 4 and the possibility that this molecule may act as a ‘double-edged sword’: preventing cell growth by delivery of the toxic moiety and by depletion of intracellular polyamine content.

Key words: apoptosis, cancer, DNA damage, drug conjugate, leukaemia, polyamine.

INTRODUCTION

The development of a ‘magic bullet’ for cancer therapy would revolutionize current treatment. Although some headway has been made, the majority of chemotherapeutic agents are limited by their non-specific actions and side effects. As a result, the maximum therapeutic dose which can be given and, therefore, the maximum anti-cancer effect these agents can exert is restricted. This latter point is a particular disappointment, given that many agents possess potent anti-cancer activity *in vitro*. Thus if these agents could be delivered selectively to tumour cells, the ‘holy grail’ of an effective anti-cancer drug with minimal peripheral toxicity would be a reality. One possibility is that drugs could be targeted to cancer cells via selective transport systems such as the polyamine pathway.

The polyamines have a multitude of roles within the cell. Most important, however, is that tight regulation of their intracellular concentration is required for normal cell growth [1]. Indeed, it is well known that increased polyamine concentrations play an important role in the development of cancer at all stages from initiation through to maintenance of the transformed phenotype [2]. One way cancer cells accumulate increased intracellular polyamine concentrations is by increased uptake of preformed polyamines from the extracellular environment via their PTS (polyamine transport system) [3].

In comparison with normal cells, it has been shown that transformed cells accumulate exogenous polyamines at an enhanced rate [4,5]. *In vivo*, radiolabelled putrescine accumulates in brain tumours at a rate 30-fold greater than the surrounding normal brain parenchyma [6]. In addition, the human promyelocytic leukaemia cell line HL-60 has an affinity for putrescence,

spermidine and spermine between 10- and 200-fold greater than normal human PMNs (polymorphonuclear leucocytes) [7]. Furthermore, uptake of putrescine is 3.6-fold greater in the HL-60 cell line in comparison with normal PMNs [7]. The importance of the PTS in cancer cells is further underlined when in PTS-deficient cell models, the curative effect of DFMO (α -difluoromethylornithine), a polyamine synthesis inhibitor, is increased due to the PTS-deficient cell model being unable to utilize extracellular polyamines to compensate for the loss of biosynthesis [8]. Most importantly, however, is the promiscuous nature of the PTS, with it being unable to differentiate between natural polyamines and a range of structural analogues [9]. Indeed, it has been shown that the PTS can tolerate a wide range of additions to the basic polyamine molecule. Interestingly, specific depletion of one polyamine does not result in selective uptake of that depleted polyamine [10].

By using these features of the PTS, it has been proposed that the transport system can be exploited to deliver cytotoxic drugs to cancer cells. With cancer cells demonstrating high transport activity and an increased demand for polyamines, the attachment of cytotoxic compounds to polyamines would allow specific targeting of cancer cells [11]. The polyamine can be attached to the toxin to provide a vector for delivery through the PTS and, in doing so, reduce the toxic effect on normal cells associated with conventional chemotherapy and therefore decrease peripheral toxicity. With this in mind, a series of compounds combining the DNA-intercalating agent, anthracene, with a polyamine side chain were synthesized [9].

The aim of the present study was to investigate the potential of Ant 4, a putrescine–anthracene conjugate, to target anthracene to human cancer cells as a paradigm for a novel method of selective

Abbreviations used: Ant 4, a putrescine–anthracene conjugate; AZP, azirdine–putrescine conjugate; CHO, Chinese-hamster ovary; DAPI, 4',6-diamidino-2-phenylindole; DFMO, α -difluoromethylornithine; FBS, foetal bovine serum; HDAC, histone deacetylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; ODC, ornithine decarboxylase; OPA, o-phthalaldehyde; PCA, perchloric acid; PMN, polymorphonuclear leucocyte; PTS, polyamine transport system; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid.

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drug delivery using the human leukaemia HL-60 cell line as an *in vitro* model system.

MATERIALS AND METHODS

Materials

RPMI 1640 growth medium, penicillin and streptomycin were from Life Technologies. Acrodisc 0.2 μM pore syringe filters, BSA, copper sulfate, cytofilter cards, dansyl chloride $[(\text{CH}_3)_2\text{NC}_{10}\text{H}_6\text{SO}_2\text{Cl}]$, DAPI (4',6-diamidino-2-phenylindole), DMSO, disodium hydrogen orthophosphate, ethanol, EDTA, Folin and Ciocalteu's phenol reagent, formaldehyde (38% w/v), glass coverslips (22 mm²) thickness number 1, glutathione (reduced form), glycerol, propan-2-ol, slides (SuperFrost 1 mm), 70% (v/v) PCA (perchloric acid), sodium carbonate, sodium chloride, sodium hydroxide and sodium potassium tartrate were obtained from the Sigma Chemical Company. Goldstar scintillation fluid was obtained from Canberra Packard. ¹⁴C-labelled polyamines were obtained from Amersham International.

All cell-culture plastics which included cuvettes, cryovials (1.8 ml), 96-well microtitre plates, 10 ml sterile syringes, 19-gauge needles, cell-culture flasks (25 cm² and 75 cm²), cell-culture plates (3.5 cm, 5 cm and 10 cm), pipettes (digital P20, P200, P1000 and P5000), universal containers (15 ml, 30 ml and 50 ml), stripette pump and sterile stripettes (5 ml, 10 ml and 25 ml) were obtained from Nunc (Nalge Nunc International). FBS (foetal bovine serum) was from PAA Laboratories. Trypan Blue and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] were obtained from the Sigma Chemical Company. Acetonitrile (HPLC Grade) was obtained from Refer Scientific. HA nitrocellulose filter (pore size, 0.45 μm) was from Millipore. HPLC vials and caps were obtained from Anachem. OPA (*o*-phthalaldehyde) was from Fisher Scientific.

Ant 4 was synthesized by Professor Otto Phanstiel's group at the University of Central Florida, Orlando, FL, U.S.A. Synthesis of this compound has been previously described [12].

Cell-culture methods

HL-60 human promyelogenous leukaemic cells were grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin (termed RFC₁₀). The cells were grown at 37 °C in a humidified atmosphere flushed with 5% CO₂. Owing to the susceptibility of HL-60 cells to differentiate spontaneously beyond the promyelocytic stage with increasing time in culture, cells were routinely subcultured every 2–3 days, reseeded at a ratio of 1:6 and kept for a maximum of 6 weeks before being replaced with freshly recovered stocks. For experiments, cells were seeded at 6.8×10^4 cells/ml and grown for 48 h prior to treatment.

Measurement of cytotoxicity

Cytotoxicity was measured using the MTT assay, based on the method of Denzot and Lang [13], from the original method described by Mosmann [14]. In summary, HL-60 cells were plated on to 96-well plates and grown for 48 h, after which time cells were treated with various concentrations of drug. After 48 h exposure, MTT was added to the plates at a concentration of 5 mg/ml as a sterile solution dissolved in complete PBS. Plates were then left for 4 h in a humidified atmosphere of 5% CO₂ at 37 °C. Metabolism of MTT by actively respiring cells produces an insoluble formazan salt which was then dissolved using 100% DMSO. Absorbance values were measured at wavelengths of

540 and 690 nm and expressed as a percentage of the untreated controls.

Determination of conjugate uptake

To determine whether Ant 4 was gaining entry into the cell, deconvolution microscopy was used. In summary, cells were seeded at the appropriate density on 3.5-cm plates and grown for 48 h. After 48 h, cells were incubated with vehicle (0.9% saline) or Ant 4 for 30 min before being harvested. Cells were centrifuged for 5 min at 2800 g_{av} . The supernatant was discarded and the cells were washed twice in 1 ml of complete PBS. Then, 100 μl of cells were fixed on to a microscope slide before being coated with 150 μl of poly-lysine (1 mg/ml) and covered with a dry coverslip. Slides were then incubated for 1 h at room temperature (20 °C). After this time, slides were viewed using a DeltaVision Core machine with an excitation wavelength of 364 nm and an emission wavelength of 417 nm.

Conjugate activity on cell growth

HL-60 cell growth was determined using the Trypan Blue exclusion assay. To determine cell number, 100 μl of cell suspension was mixed with 900 μl of Trypan Blue and cells were counted using an improved Neubauer haemocytometer. This method also allowed viability to be determined, since Trypan Blue penetrates non-viable cells, staining them blue. A modification of the method described by Lowry et al. [15] was used to determine total cellular protein. Samples were quantified against standards, prepared from a stock solution of 500 $\mu\text{g}/\text{ml}$ BSA, to provide a range of standards from 0 to 250 $\mu\text{g}/\text{ml}$ BSA.

Determination of intracellular polyamine concentrations

After the appropriate time interval, HL-60 cells were harvested by the removal of cells and medium to a sterile 15 ml centrifuge tube. To ensure that all cells had been removed from the plate, the plate was rinsed with an equal volume of RFC₁₀ which was added to the tube. Next, the cell suspension was centrifuged at 2800 g_{av} for 5 min. The supernatant was discarded and the pellet was washed twice in 1 ml of complete PBS before being transferred to a clean microtube. The cell suspension was centrifuged again at 7500 g_{av} for 5 min and the supernatant discarded before resuspending the pellet in 300 μl of 0.2 M PCA. This was placed on ice for 30 min in order to extract the acid-soluble fraction. After this time each tube was centrifuged at 7500 g_{av} for 5 min and the supernatant was removed to a clean Eppendorf tube. This was stored at –20 °C until analysis by HPLC. The method of HPLC used was that of pre-column dansylation. Samples were dansylated at 25 °C overnight, extracted in toluene, blown to dryness in a nitrogen stream, and then reconstituted in 200 μl of acetonitrile. Samples were analysed by reverse-phase HPLC on a Hichrom RPB 5 μm column using a gradient of 100% acetonitrile to 40:60 (v/v) acetonitrile/water.

Determination of polyamine uptake

Polyamine uptake was determined using radiolabelled polyamines. In summary, cells were seeded at $6.8 \times 10^4/\text{ml}$ in triplicate on 24-well plates for 48 h. After this time, cells were incubated with 5 nCi/ml of the appropriate ¹⁴C-labelled polyamine with or without 15 μM Ant 4 for 10, 20, 30 and 60 min. After the appropriate exposure, the cell suspension was removed, placed in a microtube and centrifuged for 5 min at 2800 g_{av} . The supernatant was discarded and the cells were washed in 1 ml of complete PBS before being centrifuged for 4 min at 2800 g_{av} . The supernatant

was again discarded before resuspending the cells in 300 μl of 0.2 M PCA and storing on ice for 30 min in order to allow acid extraction and protein precipitation. After this time, the cells were centrifuged for 5 min at 2800 g_{av} and the acid-soluble fraction was removed to a clean microtube. Then, 300 μl of 0.3 M NaOH was added to the remaining pellet which was left to dissolve overnight at 37°C before protein content was measured using the Lowry method [15]. To analyse the uptake of polyamine, 50 μl of the acid-soluble fraction was added to a scintillation vial containing 4 ml of scintillation fluid. Finally the amount of radioactive polyamine taken up by cells was counted using a Canberra Packard 1900A Scintillation spectrophotometer with a protocol for ^{14}C . Samples were counted for 10 min or 10000 counts, with a 2% σ error.

Determination of glutathione content

Intracellular glutathione was extracted in 0.2 M PCA and stored at -20°C until measurement. The method used was based on the method of Cohn and Lyle [15a], as modified by Hissin and Hilf [15b]. In summary, a range of standards (0, 10, 25, 50, 75 and 100 μM) were prepared from a 1 mM stock of reduced glutathione dissolved in deionized water. Sample or standard (25 μl) was then added to 2.3 ml of glutathione buffer, followed by 100 μl of OPA solution (1 mg/ml in methanol) and left for 15 min. After this time, each tube was decanted into a fluorimetric cuvette and read in turn using a PerkinElmer LS-50B luminescence spectrophotometer set at an excitation wavelength of 350 nm, with a slit width of 5 mm and emission wavelength of 420 nm. A standard curve was plotted and this was used to determine the amount of reduced glutathione (in nmol) present in the samples.

Determination of DNA damage

DNA damage was detected using the Comet assay. The method used was based on the method of Singh et al. [16]. Fully frosted slides were coated with 100 μl of 1% standard agarose in PBS and covered with a glass coverslip. Next, the slides were incubated at 4°C for 5 min to allow the agarose to solidify. Next, cell suspensions were centrifuged at 2800 g_{av} for 5 min at 4°C. The supernatant was discarded and the pellet was washed twice in 1 ml of PBS. The supernatant was then removed and the cells were dispersed in the small volume of medium remaining in the tube. Next, 85 μl of 1% LMP (low melting point) agarose in PBS at 37°C was added and immediately pipetted on to a frosted slide. The agarose was allowed to set for 10 min at 4°C. After solidification, the slides were incubated in lysis solution [2.5 M NaCl, 10 mM Tris, 100 mM EDTA (NaOH to pH 10) with 1% (v/v) Triton X-100] for 1 h at 4°C to remove cellular protein. This leaves the DNA as distinct nucleoids. After lysis, the slides were washed three times in endonuclease III buffer [40 mM Hepes/KOH, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml BSA (pH 8.0)]. After rinsing, the slides were blotted dry and incubated with endonuclease III (1 $\mu\text{g}/\text{ml}$) for 45 min. After this time, slides were placed side-by-side in a horizontal electrophoresis chamber. The chamber was filled with running buffer to approx. 2 mm above the agarose layer, and the slides were subjected to electrophoresis at 25 V for 30 min at 4°C. Following electrophoresis, the slides were rinsed three times for 5 min in neutralizing buffer [0.4 M Tris/HCl (pH 7.5)] and allowed to dry. Slides were then stained with DAPI (5 $\mu\text{g}/\text{ml}$) just before examination under an Olympus BX40 microscope with an Olympus U-RFL-T fluorescent burner and DAPI filter.

Detection of apoptosis

In order to assess the changes in morphology associated with apoptosis, the nuclear chromatin of the cells was stained with the

fluorogenic compound DAPI. In summary, cells were fixed in 0.4% formaldehyde before being centrifuged (200 g for 5 min) on to glass slides and stained with DAPI. The morphology of the nuclei of the cells was then observed using a fluorescence microscope, which was an Olympus BX40 microscope with an Olympus U-RFL-T fluorescent burner and DAPI filter. Nuclei were considered to have a normal phenotype by their more diffuse and grey staining. Apoptotic nuclei were identified by the fact that they were brightly stained due to fragments of DNA that had DAPI stain bound. For quantification, a blinded observer counted 100 cells five times from non-overlapping views.

Flow cytometry

A cell-cycle profile was obtained and apoptosis detected by flow cytometry. In summary, HL-60 cells were grown on 10-cm plates for 48 h before treatment with 15 μM Ant 4 or vehicle for 48 h. After this time, cells were harvested and centrifuged for 5 min at 800 g_{av} . The medium was then discarded, and the cell pellet was resuspended in the small volume of medium remaining in the tube. The cells were then resuspended in 1 ml of Dulbecco's PBS, transferred to microtubes and centrifuged at 800 g_{av} for 5 min. The supernatant was again discarded, and the pellet was again resuspended in the small volume remaining by knocking. Next, cells were suspended in 1 ml of ice-cold 70% (v/v) ethanol in deionized water and stored overnight at -20°C to allow quantification of sub- G_1 cells. Cells were then resuspended in 1 ml of staining buffer (50 $\mu\text{g}/\text{ml}$ propidium iodide, 50 $\mu\text{g}/\text{ml}$ ribonuclease A and 0.1% Triton X-100 in PBS), before being incubated at room temperature in the absence of light for 20 min. Samples were then analysed by flow cytometry using a Coulter Flow Cytometer until sufficient events (>10000) were obtained.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.01. All values shown are means \pm S.E.M. with each experiment performed independently a minimum of three times with replicates (unless otherwise stated).

RESULTS

The range of concentrations for Ant 4 and the time exposures were based on previously published work on this drug [9]. Ant 4 was found to be cytotoxic to the HL-60 cell line, demonstrating dose-dependent cytotoxicity (Figure 1), with a significant cytotoxic response at concentrations of 15 μM and above ($P < 0.01$).

The IC_{50} value, at which 50% inhibition of cell growth was observed, was calculated to be 20 μM after 48 h exposure. Multiple studies from our laboratory have shown that IC_{50} values calculated from MTT curves give an overestimate of the value (F.R. Saunders and H.M. Wallace, unpublished work). Therefore in order to examine the response to Ant 4 over time in greater detail, a concentration of 15 μM was chosen ($\sim\text{IC}_{30}$ value). At this concentration, inhibition of growth was observed by decreases in both cell number (Figure 2) and protein content (result not shown). Cytotoxic effects were observed after 24 h exposure to Ant 4. Despite the low cell numbers at longer times, cell viability remained high (86.5%), a feature common to apoptotic, rather than necrotic, cell death [17].

As the anthracene conjugates contain polyamine-like molecules they could be considered similar to the polyamine analogues; however, it is not known whether these conjugates affect the polyamine content of cells. One of the ways that polyamine analogues induce apoptosis is by causing depletion of intracellular

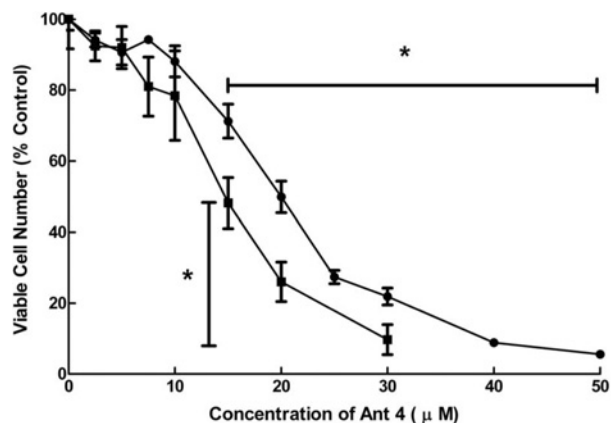


Figure 1 Cytotoxicity of Ant 4 and the effect of DFMO on the cytotoxicity of Ant 4 in HL-60 cell line

HL-60 cells were grown in RFC₁₀ and seeded at 6.8×10^4 cells/ml in 96-well round-bottomed microtitre plates and grown for 48 h either with (●) or without (■) 1 mM DFMO. After this time, both pre-treated and untreated cells were exposed to increasing concentrations of Ant 4 for 48 h. For the DFMO pre-treated cells, results shown are the means \pm S.E.M. ($n=3$), with three replicates per experiment. For the control, i.e. no DFMO pre-treatment, results shown are the means \pm S.E.M. ($n=8$); three with six replicates per experiment and five with three replicates per experiment. Results were analysed statistically using ANOVA with Bonferroni's multiple comparison post-hoc test ($*P < 0.05$).

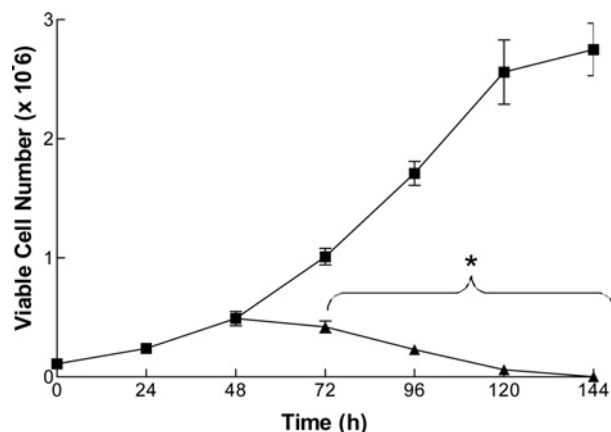


Figure 2 HL-60 viable cell number in the presence of Ant 4

HL-60 cells were seeded at 6.8×10^4 cells/ml in 3.5-cm plates and grown for up to 144 h. After 48 h, cells were treated with vehicle (■) or 15 μ M Ant 4 (▲). Duplicate plates were harvested at 0 h and then every 24 h. Viable cell number and cell viability were determined by Trypan Blue exclusion and microscopy. Protein content was determined using the Lowry assay. Results are the means \pm S.E.M. ($n=4$) with two replicates per experiment. Results were analysed statistically using ANOVA with Bonferroni's multiple comparison post-hoc test ($*P < 0.01$; $**P < 0.001$).

polyamine pools [17]. Therefore to determine whether Ant 4 was having additional analogue-type actions, the intracellular polyamine concentrations in response to Ant 4 treatment were measured over time.

Compared with the controls, HL-60 total polyamine content (Figure 3a) decreased significantly by 53.8% at 48 h ($P < 0.001$), 50% at 72 h ($P < 0.05$) and 68.7% at 96 h ($P < 0.01$) after addition of Ant 4. This was predominantly made up by decreases in spermidine content (Figure 3b). Compared with the controls, decreases in spermidine were 81.9% at 48 h ($P < 0.001$), 74.0% at 72 h ($P < 0.01$) and 76.3% at 96 h ($P < 0.05$) after addition of Ant 4. Spermidine was also lower 24 h after addition of Ant 4, but this was compensated by an increase in the concentration

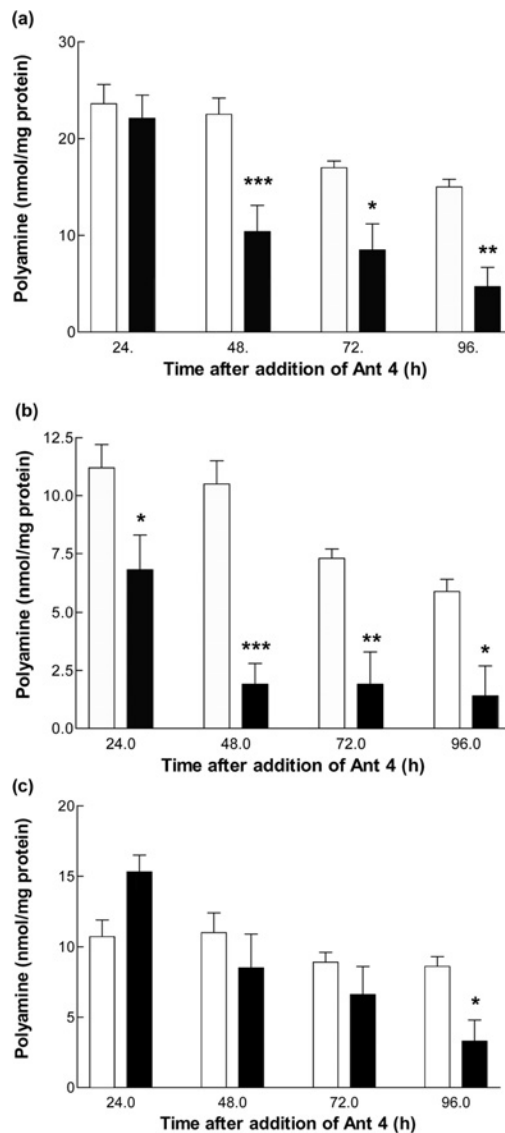


Figure 3 (a) Total polyamine content in response to Ant 4 treatment; (b) spermidine content in response to Ant 4 treatment; and (c) spermine content in response to Ant 4 treatment

HL-60 cells were seeded at 6.8×10^4 cells/ml on 3.5-cm plates and grown for up to 144 h. After 48 h growth, plates were exposed to vehicle (open bars) or 15 μ M Ant 4 (closed bars). Plates were harvested at 0 h and then every 24 h, with the polyamine fraction extracted in 0.2 M PCA and stored at -20°C until its measurement. Samples were then dansylated and the polyamine content determined by HPLC. Results shown are the means \pm S.E.M. ($n=3$) with two replicates per experiment. Results were analysed statistically using ANOVA with Bonferroni's multiple comparison post-hoc test ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

of spermine relative to the control. Compared with the controls, spermine content was significantly depleted 96 h after addition of Ant 4 ($P < 0.05$) (Figure 3c). Consistent with published [17] and unpublished observations (H.M. Wallace, unpublished work) from our laboratory, the intracellular polyamine concentrations of the controls decreased with increasing time in culture.

In the absence of a specific inhibitor of transport, several approaches were used to determine whether Ant 4 utilized the PTS. DFMO is a suicide inhibitor of ODC (ornithine decarboxylase), the first and rate-limiting enzyme in polyamine synthesis. Treatment of HL-60 cells with DFMO has been shown to result in increased uptake of preformed polyamines via the PTS

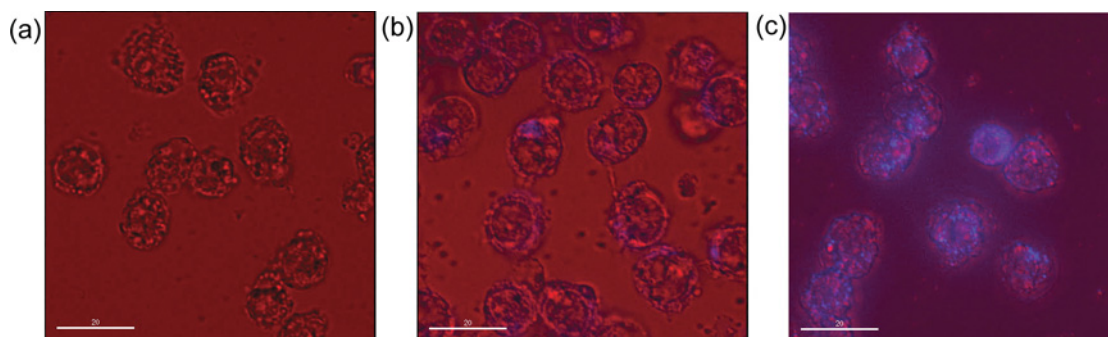


Figure 4 Deconvolution microscopy of (a) untreated HL-60 cells, (b) Ant 4-treated HL-60 cells and (c) DFMO- and Ant 4-treated HL-60 cells

HL-60 cells were seeded at 6.8×10^4 cells/ml on 3.5-cm plates and grown for 48 h with (c) and without (a and b) 5 mM DFMO pre-treatment. Following this, cells were left untreated (a) or treated (b and c) with $15 \mu\text{M}$ Ant 4 for 30 min. After this time, cells were harvested and examined by deconvolution microscopy. Ant 4 localization is shown by the fluorescent purple colour. The experiment was repeated ($n = 3$) with no replicates. Scale bar, $20 \mu\text{m}$.

[7]. HL-60 cells were pre-treated with 1 mM DFMO to increase uptake before exposure to Ant 4. DFMO pre-treatment had a significant effect on the cytotoxicity of Ant 4 from concentrations of $15 \mu\text{M}$ and above ($P < 0.05$) in comparison with the control (no DFMO pre-treatment). The IC_{50} value was decreased from $20.0 \mu\text{M}$ to $14.6 \mu\text{M}$ in the DFMO-treated cells (Figure 1), suggesting increased uptake.

In addition to inhibition of topoisomerase II, the anthracene component of Ant 4 has been shown to fluoresce with a wavelength maximum near 364 nm and emission maximum near 417 nm [12]. As such, the fluorescent properties of Ant 4 can be used to track its cellular localization. Therefore to see whether the decrease in the IC_{50} value was due to increased uptake of the conjugate and not due to synergy of DFMO and Ant 4, deconvolution microscopy was used to see whether Ant 4 gained entry to the HL-60 cells. Ant 4 was shown to localize within HL-60 cells (Figure 4b) after 30 min exposure in comparison with the untreated controls (Figure 4a). Furthermore, HL-60 cells grown for 48 h in 5 mM DFMO prior to Ant 4 exposure demonstrated greater accumulation of Ant 4 (Figure 4c) in comparison with cells treated with Ant 4 only (Figure 4b).

To provide further evidence that Ant 4 utilized the PTS in the HL-60 cell line, uptake studies were conducted. Uptake of [^{14}C]putrescine was significantly reduced by Ant 4 at 10 ($P < 0.001$), 20 ($P < 0.001$), 30 ($P < 0.001$) and 60 ($P < 0.001$) min (Figure 5). This result suggests that Ant 4 successfully competes with putrescine for the PTS in the HL-60 cell line. Furthermore, Ant 4 had no inhibitory effect on spermidine or spermine uptake (results not shown), suggesting that in the HL-60 cell line, different transporters exist for the transport of the individual polyamines.

With induction of apoptosis, the favoured cell death pathway for any effective chemotherapeutic drug, the type of cell death induced by Ant 4 was determined by a number of different methods. DAPI staining of the cellular chromatin showed there to be significant induction of apoptosis ($P < 0.001$) 48 h after exposure to Ant 4, with 72.6% of cells displaying characteristic morphological features of this type of cell death (Table 1). This was in comparison with the controls where 3.1% of cells displayed characteristic morphological features of apoptosis.

Analysis of the cell cycle (Table 2) also showed a statistically significant ($P < 0.05$) increase in the apoptotic population (also known as G_0 or sub- G_1). The G_0 population was found to be 43.3% in the Ant 4-treated cells. This was in comparison with the controls where it was found to be 12.4%.

Table 1 Apoptosis in response to Ant 4 treatment

HL-60 cells were seeded at 6.8×10^4 cells/ml in 3.5-cm plates and grown for 48 h. After 48 h, cells were treated with vehicle or $15 \mu\text{M}$ Ant 4 for 48 h. After this time plates were harvested, and after their preservation in 0.4% formaldehyde, cells were cytospun on to glass slides, stained with DAPI and analysed by fluorescence microscopy to allow the identification of apoptotic cells. The results show the viable cell number ($\times 10^{-6}$) and the percentage of apoptotic cells. Values are the means \pm S.E.M. ($n = 3$) with two replicates per experiment. Results were analysed statistically using a paired Student's *t* test ($*P < 0.05$; $**P < 0.0001$).

Treatment	Viable cell number ($\times 10^{-6}$)	Apoptosis (percentage of the total cells counted)
Control	3.70 ± 0.9	3.1 ± 0.3
Ant 4	$0.5 \pm 0.1^*$	$72.6 \pm 4.1^{**}$

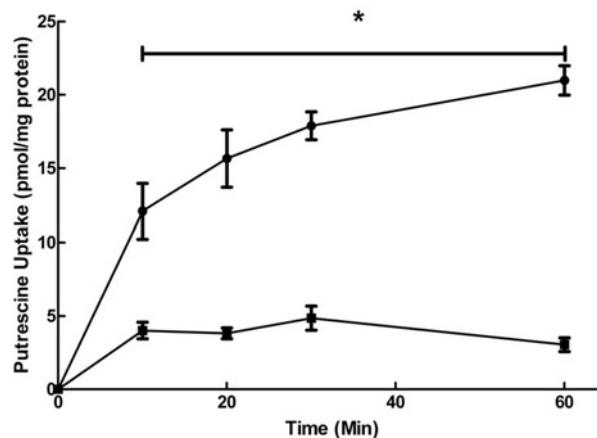


Figure 5 Putrescine uptake in response to Ant 4

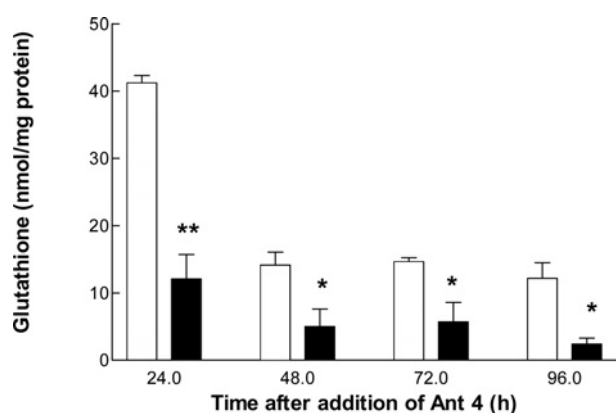
HL-60 cells were seeded at 6.8×10^4 cells/ml on 24-well plates for 48 h. After this time, cells were incubated with [^{14}C]putrescine for 10, 20, 30 and 60 min with (●) or without (■) $15 \mu\text{M}$ Ant 4. Plates were harvested at the appropriate timepoint and the polyamine fraction extracted in 0.2 M PCA. Samples were then analysed using a Canberra Packard 1900A scintillation spectrophotometer for 10 min or 10000 counts, with a 2% error. Results shown are the means \pm S.E.M. ($n = 3$) with three replicates per experiment. Results were analysed statistically using ANOVA with Bonferroni's multiple comparison post-hoc test ($*P < 0.001$).

In order to provide a possible explanation for the mechanism of cell death, the glutathione content of the cells and the DNA damage was measured after exposure to Ant 4.

Table 2 Cell-cycle distribution in response to Ant 4 treatment

HL-60 cell were seeded at 6.8×10^4 cells/ml and grown for 48 h prior to treatment with vehicle or $15 \mu\text{M}$ Ant 4 for 48 h. This Table details the percentage of cells from the total measured by flow cytometry that were classified in G_0 . The remaining phase of the cell cycle was adjusted to 100%, and their distribution adjusted to a percentage of the total. The Table shows the percentage distribution of three experiments ($n=3$) with one replicate per experiment. Data were analysed statistically using a paired Student's *t* test ($*P < 0.05$).

Treatment	Relative percentage of cells in each phase			
	G_0	G_1	S	G_2/M
Control	12.4 ± 0.4	22.6 ± 6.5	38.5 ± 1.6	26.5 ± 2.6
Treated	$43.3 \pm 3.9^*$	10.6 ± 0.4	26.2 ± 2.0	19.2 ± 1.2

**Figure 6 Glutathione content in response to Ant 4 treatment**

HL-60 cells were seeded at 6.8×10^4 cells/ml in 3.5-cm plates and grown for up to 144 h. After 48 h, cells were treated with vehicle (open bars) or $15 \mu\text{M}$ Ant 4 (closed bars). Duplicate plates were then harvested every 24 h. Glutathione was extracted in 0.2 M PCA and stored at -20°C until its measurement. Results shown are the means \pm S.E.M. ($n=3$) with two replicates per experiment. Results were analysed statistically using ANOVA with Bonferroni's multiple comparison post-hoc test ($*P < 0.05$; $**P < 0.001$).

In response to Ant 4 treatment, HL-60 cells showed a hugely significant decrease in glutathione content compared with the controls at each 24 h time point after addition of Ant 4 (Figure 6). These decreases were 70.6% at 24 h ($P < 0.001$), 65.8% at 48 h ($P < 0.05$), 60.0% at 72 h ($P < 0.05$) and 80.1% at 96 h ($P < 0.05$). Furthermore, after 48 h exposure to Ant 4 there was over a 3-fold increase in the DNA damage compared with the controls ($P < 0.01$) (results not shown). Consistent with observations in our laboratory (A.V. Fraser and H.M. Wallace, unpublished work), the glutathione content of the controls decreased with increasing time in culture.

DISCUSSION

To date, the cytotoxicity of Ant 4 has been established in CHO (Chinese-hamster ovary) cells [11,12,18], L1210 murine leukaemia cells [11,12,18,19], B16 murine melanoma cells [19] and the human cervical cancer cell line, HeLa [19]. We found the HL-60 cell line to display dose-dependent cytotoxicity. The requirement of low amounts of Ant 4 to bring about these changes, and the short time exposure, compares favourably with the doses and exposure times used in the previously mentioned *in vitro* studies.

In an effort to show that Ant 4 utilized the PTS to gain entry into the cell, we compared the cytotoxicity of Ant 4

in untreated and DFMO-treated cells respectively. DFMO is a suicide inhibitor of ODC, the first and rate-limiting enzyme in polyamine synthesis, and it has been shown that treatment of HL-60 cells with DFMO results in depletion of intracellular polyamines which is compensated for by increased active uptake of preformed polyamines via the PTS of the cell [7]. As it has been demonstrated with the anthracene conjugates that cytotoxicity is directly related to cellular import [9,11], we used the increased polyamine uptake induced by DFMO and the subsequent increase in cytotoxicity as indirect evidence for Ant 4 using the PTS to gain entry into the cell. In addition, two further observations support our hypothesis that Ant 4 is taken up by the PTS. First, deconvolution microscopy demonstrated that DFMO resulted in increased uptake of Ant 4 in comparison with cells treated with Ant 4 only. This is the first time increased cytotoxicity has been directly related to increased uptake in human cell lines with these conjugates, having only previously been demonstrated using CHO cells [9,11]. Secondly, Ant 4 significantly reduced the uptake of putrescine, demonstrating that this conjugate not only used the PTS, but that it could also successfully compete with its native polyamine for uptake. Interestingly, spermidine and spermine did not compete with Ant 4 for uptake, suggesting that more than one transporter exists in the HL-60 cell line.

However, utilization of the PTS by Ant 4 goes against other findings in the literature. Ant 4 has been shown to be no more cytotoxic in DFMO-treated L1210 murine leukaemia cells when compared with non-DFMO treated cells [12]. Work conducted on a CHO and a PTS-deficient CHO (MG-CHO) cell line has also shown no difference in the cytotoxicity of Ant 4 [9,12]. However, this can be explained by different PTS existing in different cell types. In mammalian cells it has been shown that some cell types have a single carrier for all three polyamines. This is the case for L1210 murine leukaemia cells [20]; however, most cell types appear to have two classes of carrier: one with a preference for putrescine, and one for spermidine and spermine [1,10]. Work conducted by others suggests this is the case for the HL-60 cell line [7]. Indeed, two transporters existing in the HL-60 cell line would support our finding that Ant 4 cannot inhibit the uptake of spermidine and spermine. Furthermore, multiple transporters are thought to exist in CHO cells [21]. Therefore it is likely that different vectors work better on different cell types. For example, an AZP (azirdine-putrescine) conjugate has been shown to utilize the PTS in the human prostate, PC3, cell line. This was shown by demonstrating an 8.6-fold increase in its cytotoxicity and a 3.7-fold increase in the intracellular accumulation of AZP with DFMO pre-treatment [22].

An important point to address with respect to DFMO-induced polyamine depletion is that it has been shown to decrease intracellular concentrations of calcium resulting in increased membrane permeability in the normal intestinal cell line IE-6 [23]. Therefore it could be argued that the increased absorption of Ant 4 we observed was due to an increase in membrane permeability and not increased transport. However, in the HL-60 cell line, it has been shown that polyamine depletion with DFMO has no effect on calcium homeostasis [24]. Therefore if DFMO is causing increased membrane permeability in the HL-60 cell line, it is not via alterations in intracellular calcium concentrations. As such, we believe that the increased uptake of Ant 4 observed is due to increased transport.

Another novel finding was the effect of these molecules on polyamine metabolism. Indeed, it seems that these molecules may have an additional mode of toxicity by depleting intracellular polyamines, an event associated with induction of apoptosis via the activation of p53 [25]. The depletion of spermidine is particularly interesting, given its importance in the post-translational

modification of eIF5A (eukaryotic initiation factor 5A), a ubiquitous protein essential for cell growth [26].

However, why should these molecules be having such an effect? One possibility is chain elongation of the polyamine. For example, the putrescine part of Ant 4 may allow it to act as a substrate for spermidine synthase resulting in chain elongation and the formation of Ant 4,3. Bergeron et al. [27] observed this type of chain extension event *in vitro* with N¹-substituted spermidine. The formation of Ant 4,3 would result in the consumption of the free aminopropyl units available and therefore hinder spermidine synthesis resulting in a decreased intracellular concentration of spermidine. However, analysis of our HPLC traces revealed no evidence of any larger Ant 4 derivatives.

Another possible explanation is, given that these molecules are polyamine-like, one could consider them synonymous with the polyamine analogues, and as such, may have analogue-type actions. With the topoisomerase II inhibitory effects of anthracene already established [28], this observation allows one to speculate on the possibility of a ‘double-edged sword’ effect. Therefore we postulate that Ant 4 may be recognized by the cell as a polyamine and induce their breakdown; however, like the analogues, Ant 4 will be sufficiently different so as not to function as a polyamine in cells. With the effect of these molecules on the enzymes in polyamine metabolism unknown, this is an interesting avenue for future work.

Furthermore, with intracellular polyamines preferentially binding to anionic DNA [1], polyamine depletion by Ant 4 would be desirable as this would allow Ant 4 to be targeted to topoisomerase II, which is inhibited by anthracene. Indeed, the inert nature of anthracene means the point charges on the putrescine vector are not disturbed, thus allowing it to behave as a native polyamine and therefore making it an ideal DNA-targeting vector. Such a DNA-targeting effect is supported by work conducted on the second-generation HDAC (histone deacetylase) inhibitor SAHA (suberoylanilide hydroxamic acid; trade name, Vorinostat) by Patrick Woster’s group [29]. In some cancers, hypoacetylation of histones due to overactive HDAC results in the underexpression of growth regulatory factors such as p21^{Waf1}. Woster’s group attached SAHA to a number of polyamine vectors to increase its affinity for chromatin and demonstrated an increase in the expression of p21^{Waf1} in comparison with SAHA alone [29]. In addition, they also demonstrated that one of these SAHA conjugates utilized the PTS for uptake [29].

For these anthracene conjugates many studies have looked at their ability to interact with the PTS, but have not looked in any detail at their effect on cancer cell growth and death.

In the HL-60 cell line, Ant 4 induced a significant cytotoxic response after 24 h exposure. Given such a cytotoxic response, it was important to distinguish whether this was due to apoptotic or necrotic causes. The DAPI-staining results demonstrated that the HL-60 cell line displayed characteristic morphological features of apoptosis after Ant 4 exposure. Induction of apoptosis is the favoured cell-death pathway for any effective chemotherapeutic drug because apoptosis occurs without involvement of an immune response. In fact, this result compares favourably with the established chemotherapeutic agent etoposide, a potent inducer of apoptosis and known inhibitor of topoisomerase II [17].

This finding is also in keeping with data published on another of the conjugates, Ant 4,4 [19,30]. This compound has been shown to induce an accumulation of cells in the G₀/G₁- phase of the cell cycle in both the B16 murine melanoma [24] and the HL-60 [30] cell lines. We also demonstrated a significant increase in the G₀ population after 48 h exposure to Ant 4. Furthermore, Ant 4,4 has been shown to cause a loss in mitochondrial membrane potential and subsequent release of cytochrome *c* in the HL-60 cell line

[30], indicating that apoptosis is likely to be occurring via the mitochondrial-dependent pathway.

Our novel finding of oxidative stress occurring provides a possible mechanism for the triggering of apoptosis via the mitochondrial pathway. In cells, glutathione functions to protect cells from potentially damaging cellular stresses caused by electrophilic compounds such as ROS (reactive oxygen species). Therefore glutathione depletion acts as a measure of cellular stress. Indeed, ROS have been implicated in apoptotic cell death with mitochondria-generated ROS playing an important role in the release of cytochrome *c* and other pro-apoptotic proteins which trigger caspase activation and apoptosis [31]. Furthermore, we also demonstrated that these compounds induce significant DNA damage, another well known signal for activation of the mitochondrial pathway and a well known response to oxidative stress.

In summary, the overall aim of the present study was to investigate the potential of Ant 4 as a paradigm for the delivery of cytotoxic substances via the PTS. Most excitingly, we have demonstrated that these conjugates seem to have additional effects over and above their original design (delivery) by also depleting intracellular polyamines. In addition, the triggering of apoptotic pathways, oxidative stress and DNA damage are important mechanisms of action for these compounds. Finally, we showed the successful utilization of the PTS for the first time in human cancer cells by one of these conjugates. Taken together the findings of the present study demonstrate great promise for the delivery of cytotoxic agents via the PTS.

AUTHOR CONTRIBUTION

Andrew Palmer performed the majority of scientific experiments and writing of the paper. Radiah Abdul Ghani performed some of the experiments. Otto Phanstiel and Naveet Kaur synthesized and provided the conjugates. Heather Wallace had the original idea, secured finance for the study, and was involved in writing and editing the paper.

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