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ORIGINAL ARTICLE

Ant 4,4, a polyamine-anthracene conjugate, induces cell death and recovery in human promyelogenous leukemia cells (HL-60)

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Abstract One of the major problems in cancer therapy is the lack of specificity of chemotherapeutic agents towards cancer cells, resulting in adverse side effects. One means to counter this is to selectively deliver the drug to the cancer cell. Cancer cells accumulate increased concentrations of polyamines compared to normal cells, mainly through an increased uptake of preformed polyamines via the polyamine transport system (PTS). Furthermore, the non-stringent structural requirements of the PTS enable the transport of a range of polyamine-based molecules. Thus, the PTS can be used to transport compounds linked to polyamines selectively to cancer cells. In our laboratory, polyamine–anthracene conjugates have shown potent anti-tumour activity towards HL-60 cells. The aim of this study was to determine the cytotoxicity of Ant-4,4, a homospermidine–anthracene conjugate, and assess the long-term effects by determining whether cancer cells were able to recover from treatment. During exposure, Ant-4,4 was an effective growth-inhibitory agent in HL-60 cells decreasing viable cell number, protein and polyamine content. Evidence indicates concomitant cell-cycle arrest and increased apoptosis. Once the drug was removed, HL-60 cells recovered gradually over time. Increasing cell number, protein content and polyamine content, as well as diminished effects on cell-cycle and apoptotic stimuli were observed over time. These data suggest that, despite being an effective way of delivering anthracene, these polyamine conjugates do not exert long-lasting effects on HL-60 cells.

Keywords Cancer · Targeted drug delivery · Polyamine conjugate · Recovery of cancer cells

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Introduction

A wide range of chemotherapeutic drugs have high incidence of adverse side effects as a result of the lack of specificity towards malignant cells (Palmer and Wallace 2010). The clinical use of these anti-cancer agents is therefore limited, despite most of them having potent anti-tumour activity in vitro. Targeting tumour cells more selectively than the healthy cells and overcoming the non-specific actions are therefore major challenges facing cancer therapy. Prevailing approaches to develop tumour-specific drugs are based on targeting a single-deregulated pathway or overexpressed receptor. Although a number of successful targeting molecules validate this strategy, including monoclonal antibodies, peptides, hormones and growth factors, only a few are useful in clinical therapy since most of them have been shown to be highly selective but with modest anti-tumour activity (Chari 2008).

An attractive alternative is to develop vector systems which have an enhanced activity towards cancer cells, increasing the cytotoxicity of already established chemotherapeutic agents via preferential uptake and diminished secondary effects on healthy cells (Palmer et al. 2009).

Over the last few years, conjugates between known cytotoxic agents and polyamines have been developed as such a vector system, aiming to take advantage of the upregulated polyamine transport system (PTS) in cancer cells for selective drug delivery (Wang et al. 2003a).

The natural polyamines, putrescine (Put), spermidine (Spd) and spermine (Spm) have a multitude of functions within the cell, including regulation of normal cell proliferation, differentiation and survival. It is well established that tight regulation of polyamine intracellular content is required for normal cell growth (Wallace et al. 2003; Thomas and Thomas 2001). In cancer cells, however, polyamines are accumulated at an enhanced rate compared to healthy cells, and one way this is achieved is through an increase in uptake of polyamines from external sources (Seiler et al. 1990). Extensive studies in a range of tumour cell lines have supported this, including neuroblastoma cells, B16 melanoma cells, human colonic and lung tumour cells lines (Xie et al. 2010). In addition, the human promyelogenous leukaemic cell line (HL-60) has shown an affinity for putrescine, spermidine and spermine between 10- and 200-fold greater than normal human polymorphonuclear leucocytes (Walters and Wojcik 1994).

The upregulation of the PTS in cancer cells is absolutely crucial to the idea of using polyamines as vectors for selective delivery of the cytotoxic drugs, however, it is not the only parameter that must be taken into account. Most important is whether the structural requirements of the PTS enable the transport of polyamine-like molecules. It is now well known that the PTS is not restricted to the natural polyamines, but it allows various polyamine analogues as well as many polyamine–drug conjugates that structurally mimic the natural polyamines to be transported (Phanstiel et al. 2007).

Bearing these features in mind, polyamine skeletons have been considered as potent cell-delivery vectors, allowing selective uptake of anti-cancer agents by tumour cells by means of the PTS. Previous studies performed in our laboratory with polyamine–anthracene conjugates have shown promising results regarding this novel method for selective drug delivery. A putrescine–anthracene conjugate (Ant-4), consisting of an anthracene nucleus covalently bound to a putrescine molecule, has shown potent antitumour activity towards a human promyelogenous leukaemic cell line (Palmer et al. 2009).

Among other polyamine backbones, the homospermidine and homospermine have also been linked to anthracene (Ant-4,4 and Ant-4,4,4, respectively) (Wang et al. 2003b, c). The aim of the present study was to assess the cytotoxicity of Ant-4,4 and determine whether cancer cells were able to recover from treatment. In this way, we aimed to assess the long-term effects using a human promyelogenous leukaemia (HL-60) cell line as in vitro model system.

Materials and methods

Materials

The HL-60 cell line was purchased from the European Collection of Animal and Cell Cultures (ECACC). RPMI 1640 growth medium, penicillin, streptomycin and PBS were purchased from Lonza (Belgium). MTT [3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyl-2*H*-tetrazolium bromide], dimethylsulphoxide (DMSO), bovine serum albumin (BSA), Trypan Blue and foetal bovine serum (FBS) were purchased from Sigma-Aldrich Inc. (USA). Cell culture flasks (25 and 75 cm^2), plates (3.5, 5 and 10 cm diameter) and 2 ml cryovials were purchased from Greiner bio-one (Germany); 96-well microtitre plates were from NUNCLON^{TM} (Denmark); 5 ml syringe and 0.22 μM syringe filters were purchased from Becton–Dickinson (UK) and sterile stripettes (5, 10 and 25 ml) were from Sigma Chemical Company Ltd. (Poole, UK). The Vectashield Mounting Medium with DAPI was purchased from Vector Laboratories (CA, USA), formaldehyde 37 % (v/v) from Acros Organics (Belgium) and Folin–Ciocalteau's reagent from VWR International (Poland). Ant-4,4 was a kind gift from Professor Otto Phanstiel, University of Central Florida (Orlando, USA). Synthesis of Ant-4,4 has been previously described (Wang et al. 2003b).

Cell culture

HL-60 human promyelogenous leukaemic cells were grown in suspension in RPMI 1640 medium supplemented with 10 % (v/v) FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C. Cells were routinely subcultured every 3 days and reseeded at a ratio of 1:6. A seeding of 6.8×10^4 cells/ml was used for all experiments, except for recovery assay (see below), and grown for 48 h prior to treatment. Owing to the susceptibility of HL-60 to differentiate beyond the promyelocytic stage with increasing time in culture, cells were kept for a maximum of 6 weeks before being replaced with fresh stocks recovered from −135 °C.

Cytotoxicity assay

Cytotoxicity was quantified according to the Denizot and Lang (1986) modification of Mosmann's (1983) original method. Cells were grown on 96-well microtitre plates for 48 h and exposed to the appropriate concentration of drug. After 48 h exposure, 10 μL of a 5 mg/ml sterile solution of MTT in complete PBS was added to the cells. Plates were then left for 4 h in a humidified atmosphere of 5 % CO_2 at 37 °C. Actively respiring cells metabolise MTT to an

insoluble formazan salt which was then dissolved using 100 % DMSO. After 20 min, the plate was read at a reference wavelength of 540 nm and a test wavelength of 690 nm and the results were expressed as a percentage of control values.

Recovery experiments

Cultures were seeded at 1.5×10^6 cells/ml into T75 cm^2 flasks and grown for 48 h, after which time they were exposed to the appropriate concentration of drug. After 48 h treatment, the drug was removed thoroughly by washing the cells twice with fresh RPMI 1640 culture medium without FBS. The cells were then resuspended in RPMI 1640 growth medium with FBS and counted on a Neubauer haemocytometer via Trypan Blue exclusion assay. Cultures were plated in 3.5 cm plates at 6.8×10^4 cells/ml and their recovery capability from drug treatment was evaluated at several time points and parameters (see below).

Cell growth determination

HL-60 cell growth after drug removal was determined using the Trypan Blue exclusion assay. Cells were harvested at different time points and counted using an improved Neubauer haemocytometer. Cells that are stained blue were counted as non-viable whereas those excluding the dye were considered viable. Results were expressed as total viable cell number.

Protein content determination

Total cellular protein content was determined at several time points after drug removal using a modification of the method described by Lowry et al. (1951). Standards were prepared from a stock solution of 500 μg/ml BSA by serial dilution in 0.3 M NaOH to give standards within the range of 0–250 μg/ml. Absorbance values were measured at a wavelength of 690 nm and results were expressed as total protein content (mg/culture).

Measurement of polyamine intracellular content

Intracellular content of polyamines was quantified at several time points after drug removal with a pre-column derivatisation method for HPLC determination. In summary, cells were harvested and centrifuged at $200g_{av}$ for 5 min at 5 °C. After resuspending the pellet in 1 ml of PBS, the cell suspension was transferred into clean eppendorf tubes and centrifuged at $7{,}500g_{av}$ for 5 min. The supernatant was then discarded and the pellet was resuspended in 300 μL of 0.2 M $HClO_4$ and placed on ice for 20 min. Samples were centrifuged again at $7{,}500g_{av}$ for 5 min and the supernatant containing the polyamine fraction stored at −20 °C until its measurement by HPLC. At this time 200 μL aliquots of each sample were dansylated overnight at 37 °C, extracted in toluene, evaporated to dryness in a nitrogen stream, and then reconstituted in 200 μL of methanol. These, in turn, were centrifuged at $7{,}500g_{av}$ for 3 min. The reconstituted polyamine samples were analysed by a reverse-phase HPLC on a HIRPB-2922 column using a gradient of 100 % deionised water to 100 % methanol.

Flow cytometry

A cell-cycle profile was obtained after removing the drug by flow cytometry. Cells were classified into phases of cell cycle based on the amount of DNA they contained. In summary, cells were grown in 5 cm plates for 48 h before being exposed to the appropriate drug concentration for a further 48 h. After treatment cultures were washed twice thoroughly to remove the drug, they were resuspended in RPMI 1640 growth medium and incubated at 37 °C. At each time point, cells were harvested and centrifuged at $800g_{av}$ for 5 min, the medium discarded and the pellet resuspended in 2 ml of cold PBS. Cells were counted through Trypan Blue exclusion assay and adjusted to 1×10^6 each sample, transferred to microtubes and centrifuged again at $200g_{av}$ for 5 min at 4 °C. The cell pellet was then resuspended in 2 ml of ice-cold 70 % (v/v) ethanol in PBS. Samples were stored at −20 °C until analysis, at which time they were centrifuged twice for 5 min at $200g_{av}$ to remove all traces of ethanol. Cells were then resuspended in 1 ml of staining buffer (50 μg/ml propidium iodide, 50 μg/ml ribonuclease A, 0.1 % (v/v) triton x-100 in PBS) and incubated at room temperature in the absence of light for 20 min. Samples were analysed by flow cytometry using a FACS Calibur until sufficient events were obtained.

Morphological determination of apoptosis

The fluorigenic compound DAPI was used to assess morphological changes associated with apoptosis. For this purpose, cells were seeded in 10 cm diameter plates and grown for 48 h, after which time they were exposed to the appropriate concentration of drug for a further 48 h. After treatment, cultures were washed twice in pre-warmed RPMI 1640 medium without FBS to remove the drug, resuspended in RPMI 1640 growth medium with serum and incubated at 37 °C. From this point forward, cells were harvested at each time point and washed in cold PBS to remove medium. When the pellet was medium free cells were fixed in 0.4 % (v/v) of formaldehyde per million cells

before being spun at $28g_{av}$ for 5 min on to glass slides and stained with Vectashield Hard-set Mouting medium with DAPI solution. The morphology of the nuclei of the cells was observed using an Olympus BX40 microscope with an Olympus U-RFL-T fluorescent burner. Nuclei were considered to have a normal phenotype by their more diffuse and grey staining, whereas apoptotic nuclei were identified as being brightly stained due to fragments of DNA that had DAPI stain bound.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. Results are displayed as mean ± SEM and were analysed by either Student's unpaired *t* test or two-way ANOVA with Bonferroni's post-test. Results were considered to be significant when a *p* value of <0.05 was observed.

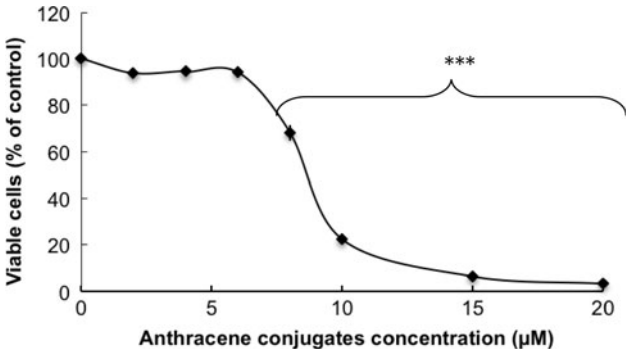


Fig. 1 Cytotoxicity of Ant-4,4 in HL-60 cells. HL-60 cells were seeded at 6.8×10^4 cells/ml in 96-well round bottomed microtitre plates and grown for 48 h prior to increasing concentrations of Ant 4,4 and left for 48 h. Results shown are the mean ± SEM ($n = 12$) with six replicates per experiment. Data were analysed statistically using one-way ANOVA with Dunnett's post test (***$p < 0.001$)

Results

The polyamine–anthracene conjugate Ant-4,4 was found to be cytotoxic to the HL-60 human promyelogenous leukaemia cell line in a dose-dependent manner, with a significant cytotoxic response being observed above 5 μM concentration ($p < 0.001$: Fig. 1). After 48 h exposure the IC_{50}, value, at which 50 % of growth inhibition was observed, was determined to be 8.6 ± 2.1 μM from the MTT data. However, previous observations in our laboratory have shown that the MTT assay overestimates the IC_{50} value of drugs by 10–30 % (data not shown). Therefore, a concentration of 5 μM Ant-4,4 ($\approx IC_{30}$) rather than 8.6 μM was used in all further experiments. At this concentration Ant-4,4 inhibited HL-60 cellular growth in a time-dependent manner. Inhibition of growth was observed by significant decreases in both cell number and protein content 24 h after the addition of the drug (Fig. 2 i, ii). Despite the observed cytotoxic response after the addition of the drug, the cellular viability remained high (>90 %: Fig. 2 iii), a feature common to apoptotic, rather than necrotic, cell death (Fraser et al. 2002). These results are in agreement with the previous observations for the anthracene–putrescine conjugate (Palmer et al. 2009).

The growth-inhibitory effects of Ant-4,4 were further evaluated by examining the cell-cycle distribution through FACS analysis. The DNA of untreated and drug-treated cells was thus stained with propidium iodide and the percentage of cells in G_1, S and G_2 phase of the cell cycle was determined (Table 1). After 6 h of the addition of Ant-4,4 (Table 1 i), the population of cells in S phase was shown to be decreased significantly ($p < 0.01$) by around 10 % in comparison with untreated cells. After 24 (Table 1ii) and 48 h (Table 1iii) exposure to the conjugate, a slightly higher difference was observed, as the population of drug-treated cells in the S phase of the cell cycle was depleted by 13 % approximately ($p < 0.01$ after 24 h and $p < 0.05$ after 48 h exposure) in comparison with the controls. The observed decreases in S phase population of drug-treated cells were accompanied by a significant increase in sub G_1 populations at the same time points, as drug-treated cells distribution was 5 % (6 h treatment, $p < 0.05$), 10 % (24 h treatment, $p < 0.01$) and 6 % (48 h treatment, $p < 0.05$) higher than the controls.

Furthermore, the growth-inhibitory effects induced by Ant-4,4 were accompanied by changes in the polyamine intracellular content (Fig. 3). The total polyamine pool of HL-60 cells decreased significantly ($p < 0.001$) by around 49 % at 24 h, 68 % at 48 h, 80 % at 72 h and 76 % at 96 h after the addition of Ant-4,4 (Fig. 3 i). This was mainly due to a significant decrease ($p < 0.001$) in intracellular spermidine and spermine. Spermidine intracellular pool was depleted by around 44 % at 24 h, 70 % at 48 h, 89 % at 72 h and 88 % at 96 h after the addition of Ant-4,4 (Fig. 3 ii), whereas spermine was depleted by 64 % at 24 and 48 h, 86 % at 72 h and 85 % at 96 h after treatment (Fig. 3 iii).

Although suggesting promising anti-tumour activity towards HL-60 cells, it was of interest to assess whether Ant-4,4 effects were still present in the absence of the drug. Therefore, HL-60 cells were treated with 5 μM concentrations of Ant-4,4 for 48 h and the drug removed from cells thoroughly. After 48 h exposure, Ant-4,4 effectively inhibited HL-60 growth by around 31 % ($p < 0.05$) in comparison with untreated cells. The decrease in viable cell number was approximately what was expected for 5 μM concentrations of Ant-4,4, therefore enabling evaluation of cellular recovery.

After removing the drug ($t = 0$ h), the recovery capability of HL-60 cells to drug treatment was evaluated by different parameters. In terms of cellular growth, the results

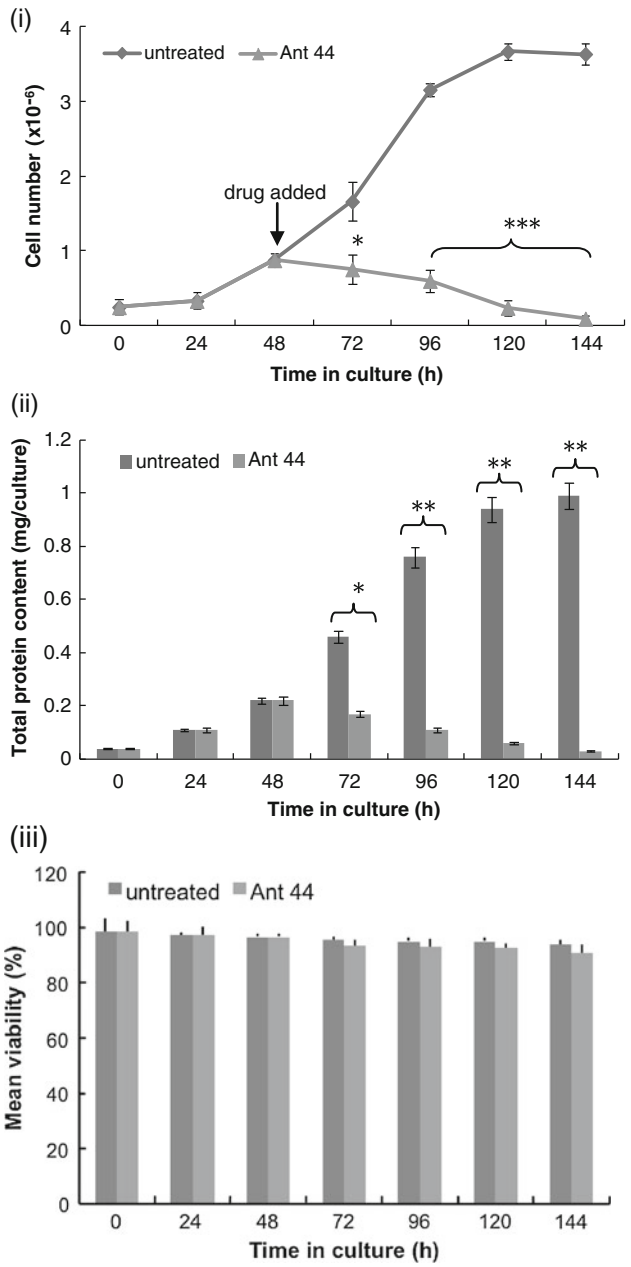


Fig. 2 Effect of Ant-4,4 on HL-60 cells over time. HL-60 cells were seeded at 6.8×10^4 cells/ml in 3.5 cm plates and grown for 48 h. Cells were then treated with vehicle (VC) or Ant-4,4. After 48 h growth, cells were treated with vehicle or Ant-4,4. Duplicate plates were harvested at 0 h and then every 24 h. Cell number (**i**) and viability (**iii**) were determined through trypan blue exclusion and protein content (**ii**) was determined by the Lowry assay after extraction with NaOH. Results are shown as mean ± SEM ($n = 10$) with two replicates per experiment. Statistical analysis was by two-way ANOVA with Bonferroni post-test (*$p < 0.05$; ***$p < 0.001$)

obtained suggest that Ant-4,4-treated cells are able to recover from treatment in the absence of the drug, as the viable cell number increases over time (Fig. 4 i). However, whereas untreated cells grow exponentially as expected, Ant-4,4-treated cells have been shown to grow to a slightly smaller extent with significant differences in viable cell number being observed at 56 ($p < 0.05$), 72 ($p < 0.001$) and 96 h ($p < 0.001$) in comparison with the controls. These results were further supported by total protein content, which also increased over time in the absence of the drug (Fig. 4 ii). Such an increase was seen to a smaller extent for Ant-4,4-treated cells than for the untreated cells, with significant differences being observed at 72 ($p < 0.01$) and 96 h ($p < 0.001$). To evaluate this effect into more detail, the generation time, the time taken for the cell population to double after the removal of Ant-4,4 was calculated (Table 2). During the first 48 h, the time taken for drug-treated cells to double their population was approximately two times higher than the time taken for untreated cells, with significant differences being shown at 32 ($p < 0.01$) and 48 h ($p < 0.05$). After 48 h of Ant-4,4 removal, however, the generation time of drug-treated cells decreased gradually to near values to those of the untreated ones, suggesting that HL-60 cells are slowly recovering from treatment.

Bearing in mind that Ant-4,4 depletes the total polyamine content after 48 h exposure (Fig. 3), it was of interest to assess if cells were able to overcome this effect over time. Indeed, after drug removal Ant-4,4-treated cells have shown an increase in the total polyamine content over time (Fig. 5 i). However, the total intracellular polyamine content of drug-treated cells increased at a smaller rate than the one observed for the controls, being significantly different after 32 h ($p < 0.05$) and 56 h ($p < 0.001$) of Ant-4,4 removal.

The changes in total polyamine content over time were made up predominantly by changes in spermine content, rather than changes in spermidine. Whereas it is clear that spermine content increased over time for both untreated and drug-treated cells (Fig. 5 iii), rather small fluctuations were observed for spermidine (Fig. 5 ii). However, the intracellular content of both spermidine and spermine was consistently lower for the Ant-4,4-treated cells than for the controls, with significant differences being observed at 56 h for both spermidine ($p < 0.05$) and spermine ($p < 0.01$).

The depletion of polyamine intracellular content observed for the controls at 72 and 96 h after drug removal is consistent with the previous published observations (Palmer et al. 2009; Fraser et al. 2002), as polyamine intracellular concentrations decrease with increasing time in culture.

Ant-4,4 growth-inhibitory effects on HL-60 cells were observed previously to have concomitant alterations in cell-cycle distribution, namely by arresting G_1/S phase transition (Table 1). For this reason, we assessed whether these effects were maintained after drug removal. After

Table 1 Cell-cycle distribution after exposure to Ant-4,4 (%)

	Sub G_1	G_1	S	G_2/M
(i) Cell-cycle distribution after 6 h exposure to Ant-4,4 (%)				
Untreated	1.03 ± 0.13	59.55 ± 1.58	21.28 ± 2.41	16.22 ± 1.19
Ant 44	6.09 ± 0.46*	58.01 ± 3.95	11.45 ± 1.27**	22.54 ± 3.33
(ii) Cell-cycle distribution after 24 h exposure to Ant-4,4 (%)				
Untreated	2.10 ± 0.23	50.6 ± 2.45	21.65 ± 2.11	25.65 ± 1.19
Ant 44	12.17 ± 1.10**	52.62 ± 1.99	8.05 ± 0.78**	27.16 ± 0.98
(iii) Cell-cycle distribution after 48 h exposure to Ant-4,4 (%)				
Untreated	2.19 ± 0.15	47.99 ± 1.18	25.65 ± 2.15	26.17 ± 1.01
Ant 44	8.10 ± 1.12*	53.08 ± 2.11	13.01 ± 0.23*	25.81 ± 1.18

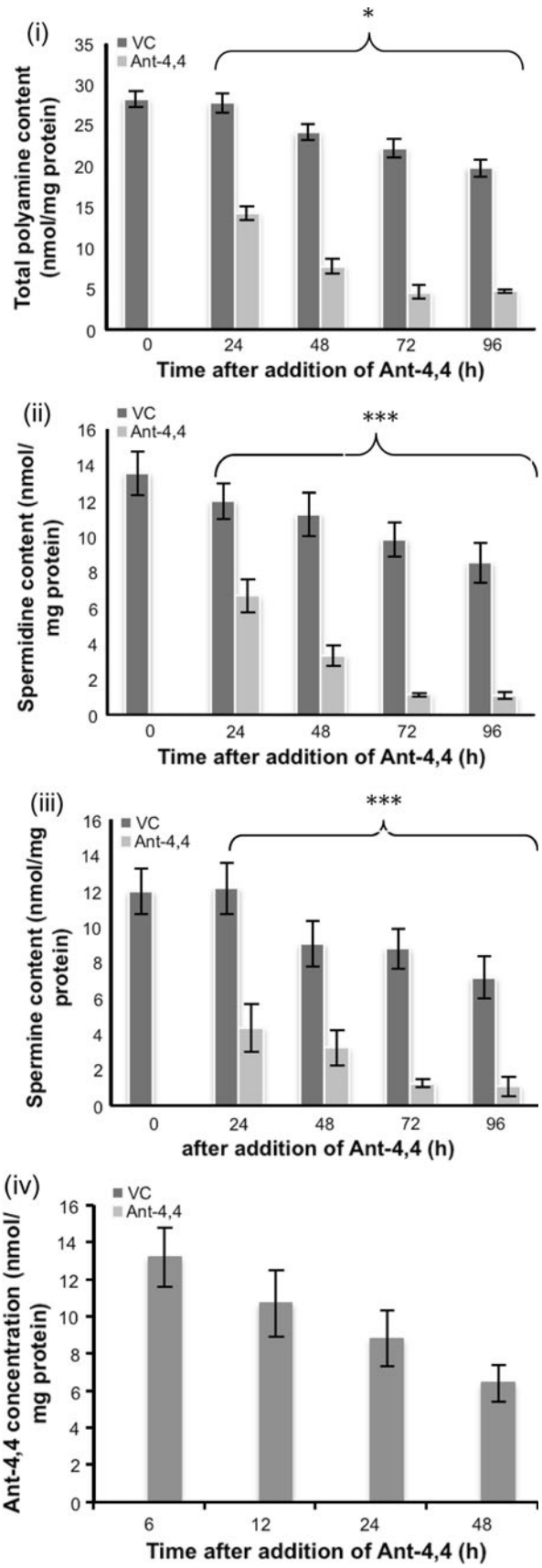
HL-60 cells were seeded at 6.8×10^4 cells/ml and grown in 5 cm plates for 48 h. Cells were then left untreated (VC) or exposed to 5 μM Ant-4,4. Plates were harvested at 6 (i), 24 (ii) and 48 h (iii) after treatment. DNA was stained with propidium iodide (PI) and samples analysed through flow cytometry. Data were analysed through the Dean–Jett–Fox (DJF) mathematical model and adjusted as population of cells (%) at each phase. Data shown are the mean ± SEM ($n = 6$) with duplicates for each experiment. Statistical analysis was done using ANOVA with Bonferroni post-test (* $p < 0.05$, ** $p < 0.01$)

48 h exposure to Ant-4,4 ($t = 0$ h), an increase in G_1 population by around 10 % with a concomitant decrease in S phase population was observed on drug-treated cells (Table 3), thus corroborating Ant-4,4 inhibitory effects on G_1–S phase transition. However, those effects were observed to disappear gradually over time after removing Ant-4,4. Up to 96 h after drug removal, the G_1 phase population of drug-treated cells decreased gradually to near values to those of the untreated ones. In addition, both treated and untreated cells were observed to have a similar cell-cycle profile 72 h after drug removal, therefore suggesting that HL-60 successfully recover from Ant-4,4 inhibitory effects over time.

It was previously established in our laboratory, either by morphological or biochemical determinations, that after 48 h treatment Ant-4,4 induces cell death on HL-60 cells through the activation of apoptosis (data not shown). To evaluate the remaining incidence of apoptotic cells after the removal of the drug, the nuclear chromatin of the cells was stained with DAPI. Untreated cells were shown as having uniform staining of the chromatin, regular shape and regular size of the nuclear envelope (Fig. 6a, e), therefore suggesting no apoptotic signals. On the other hand, after 48 h exposure to Ant-4,4, HL-60 cells were shown as being shrunken with the formation of highly stained condensed chromatin (Fig. 6j, n), which is in agreement with the previous data obtained in our laboratory. This typical apoptotic morphology was less clear 24 h after the removal of the drug (Fig. 6k, o) when comparing to the controls (Fig. 6c, g), while at 48 h after Ant-4,4 removal (Fig. 6l, p) no apoptotic traces at all were observed, as cells reacquired their normal shape. The same absence in apoptotic cells was also evident after 72 h removal (data not shown). Untreated cells got smaller with increasing time in culture, as it is evident when comparing them before exposure to the drug and 48 h after removing vehicle control (Fig. 6d, h). However, although morphologically different they were not considered as being apoptotic cells since no typical signs were observed.

Discussion

A number of polyamine conjugates have been synthesised over the last few years as potential anti-proliferative drugs mainly due to the lack of tumour specificity of many chemotherapeutic agents in current clinical use. To date, however, the reports available on these potential tumour-selective drugs are focused in their ability to interact with the PTS, with only a few being focused on their effects on cancer cells. To elucidate their mechanisms of action and biological activity, the cytotoxicity of a number of polyamine–anthracence conjugates have been established in our group on several tumours including non-small lung cancer (A549), human erythromyeloblastoid leukaemia (K562), breast cancer cells lines (MDA), among others (data not shown). Regardless of the cell line used, they all have responded in the same micromolar range, with Ant-4,4, in particular, showing great promise. In this study, we established the dose-dependent cytotoxic effect of Ant-4,4 on HL-60 cells, which was seen to be comparable with the few studies performed on different tumour models. These include L1210 murine leukaemia cells (Wang et al. 2003b), Chinese hamster ovary cells (CHO) (Wang et al. 2003c), B16 murine melanoma cells as well as human cervical cancer cells (HeLa) (Xie et al. 2007a). Furthermore, we have shown that a small concentration of Ant-4,4 induces significant decreases in both HL-60 viable cell number and protein content. Indeed, Ant-4,4 effects were seen to be significantly more potent than Ant-4 under the same



◀ **Fig. 3** Effect of Ant-4,4 on total polyamine (**i**), spermidine (**ii**) and spermine (**iii**) content. Ant-4,4 concentration after the addition of the drug (iv). HL-60 cells were seeded at 6.8×10^4 cells/ml and grown in 3.5 cm plates for 48 h. Cells were then treated with vehicle (VC) or 5 μM Ant-4,4. Plates were harvested at 0 h and then every 24 h after treatment. The polyamine fraction was extracted in $HClO_4$ and stored at −20 °C until its measurement. Samples were then dansylated and the protein content determined by HPLC. Results are shown as mean ± SEM ($n = 10$) with two replicates per experiment. Statistical analysis was by two-way ANOVA with Bonferroni post-test (***$p < 0.01$)

conditions (Palmer et al. 2009). This data corroborate previous studies suggesting the homospermidine moiety as being an ideal backbone for selective delivery (Wang et al. 2003b). In addition, it also demonstrates the importance of establishing structure–activity relationships for the development of effective anti-proliferative agents, as small changes in polyamine backbone resulted in substantial differences in their anti-tumour activity. On the other hand, HL-60 growth inhibition in response to Ant-4,4 treatment was accompanied by a decrease in the intracellular polyamine content, an effect also seen with Ant-4 (Palmer et al. 2009). Interference with polyamine metabolism once Ant-4,4 is administrated to cells is an unexpected finding. The principle behind these molecules is to enhance selective delivery of cytotoxic drugs, rather than deplete polyamine intracellular pools in an analogue-like manner (Wallace and Fraser 2003; Wallace and Niiranen 2007). The reason why these drugs are interfering with polyamine metabolism is therefore not yet understood. One could speculate that feedback mechanisms are being activated by the polyamine backbone to compensate for the uptake. In this regard, there are a few studies with benzyl–homospermidine conjugates reporting the liberation of the appended polyamine from the drug cargo once within cells (Xie et al. 2010). From our data, however, we were not able to visualise homospermidine alone by HPLC and therefore suggest that the conjugate is not metabolised to any significant extent. Nevertheless, we have shown that after Ant-4,4 treatment spermidine was depleted in a greater extent than spermine. Given that the former is absolutely required for eIF-5A maturation (Park 2006), a ubiquitous protein essential for cell growth and protein synthesis, our data suggests that the polyamine conjugates may have an additional mode of toxicity. Interestingly, spermidine depletion resulted in a concomitant arrest in G1–S phase transition of the cell cycle. Since it is established that eIF-5A is associated with cell proliferation in the G1–S stage of the cell cycle, this data suggests that the polyamine backbone could indeed be contributing to the biological activity of the conjugate, rather than only improving anthracene uptake.

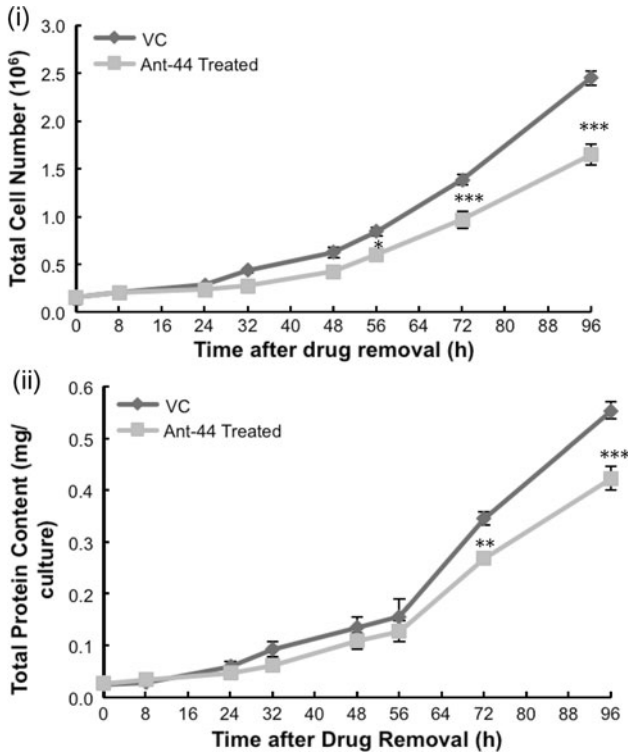


Fig. 4 HL-60 cellular growth after removal of Ant-4,4 ($t = 0$ h). Cell number (**i**) and total protein content (**ii**) was determined. HL-60 cells were seeded at 1.5×10^6 cells/ml on T75 cm^2 culture flasks and grown for 48 h. Cells were then treated with vehicle or exposed to 5 μM Ant-4,4 for a further 48 h. After treatment, the cells were washed twice to remove the drug. Cells were then seeded at 6.8×10^4 cells/ml on 3.5 cm plates and grown up to 96 h. Plates were harvested at each time point and viable cell number was determined by Trypan Blue exclusion assay, whereas the protein fraction was extracted in 0.3 M NaOH and further analysed through Lowry assay. Results are shown as mean ± SEM ($n = 4$, with two replicates per experiment). Analysis was by two-way ANOVA with Bonferroni post-test (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$)

Taken together, these results demonstrated the potential that these conjugates have as paradigm for selective delivery of an established cytotoxic agent—namely, the 4,4

Table 2 Generation time of HL-60 cells after removal of Ant-4,4

Time (h)	Control	Ant-4,4
0	–	–
24	25.4 ± 1.8	41.6 ± 8.4
32	20.6 ± 0.7	40.5 ± 2.6**
48	24.0 ± 1.4	42.5 ± 2.8*
56	22.5 ± 1.2	30.1 ± 2.6
72	22.4 ± 1.1	28.7 ± 2.8
96	23.8 ± 0.8	28.3 ± 1.6

Generation time (h) of HL-60 cells after removal of Ant-4,4. Generation time was measured as viable cell number at each time point compared to viable cell number after removal of Ant-4,4 ($t = 0$ h). The results are shown as mean ± SEM ($n = 4$) with two replicates per experiment. Statistical analysis was by unpaired t test (* $p < 0.05$; ** $p < 0.01$)

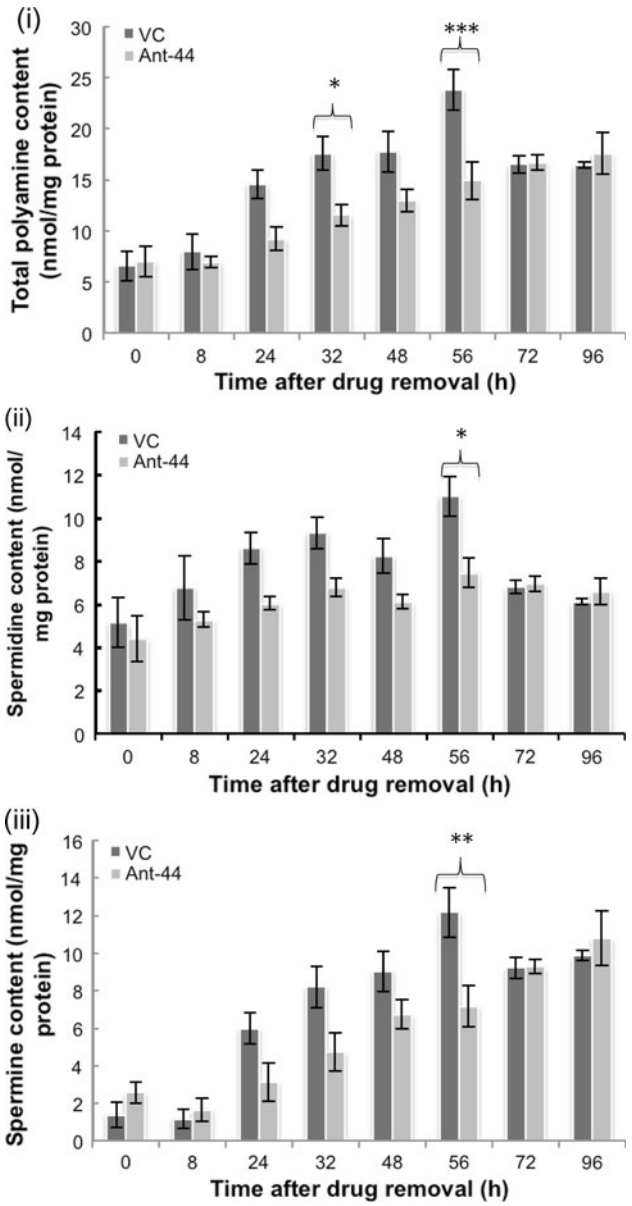


Fig. 5 Total polyamine (**i**), spermidine (**ii**) and spermine content after removal of Ant-4,4. HL-60 cells were seeded at 1.5×10^6 cells/ml on T75 cm^2 culture flasks and grown for 48 h. Cells were then exposed to 5 μM Ant-4,4 for a further 48 h. After treatment, the cells were washed twice to remove the drug. Cells were then seeded at 6.8×10^4 cells/ml on 3.5 cm plates and grown up to 96 h. Plates were harvested at each time point with the polyamine fraction being extracted in 0.2 M $HClO_4$ and stored at −20 °C until its measurement. Samples were dansylated and the polyamine content determined by HPLC. Results are shown as mean ± SEM ($n = 3$, with two replicates per experiment). Analysis was by two-way ANOVA with Bonferroni post-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

homospermidine backbone is an ideal vector to target tumours more effectively. Despite extensive research, to date no evidence has yet been provided regarding whether the potential anti-tumour effects exerted by the polyamine conjugates were long lasting or, on the contrary, if tumour

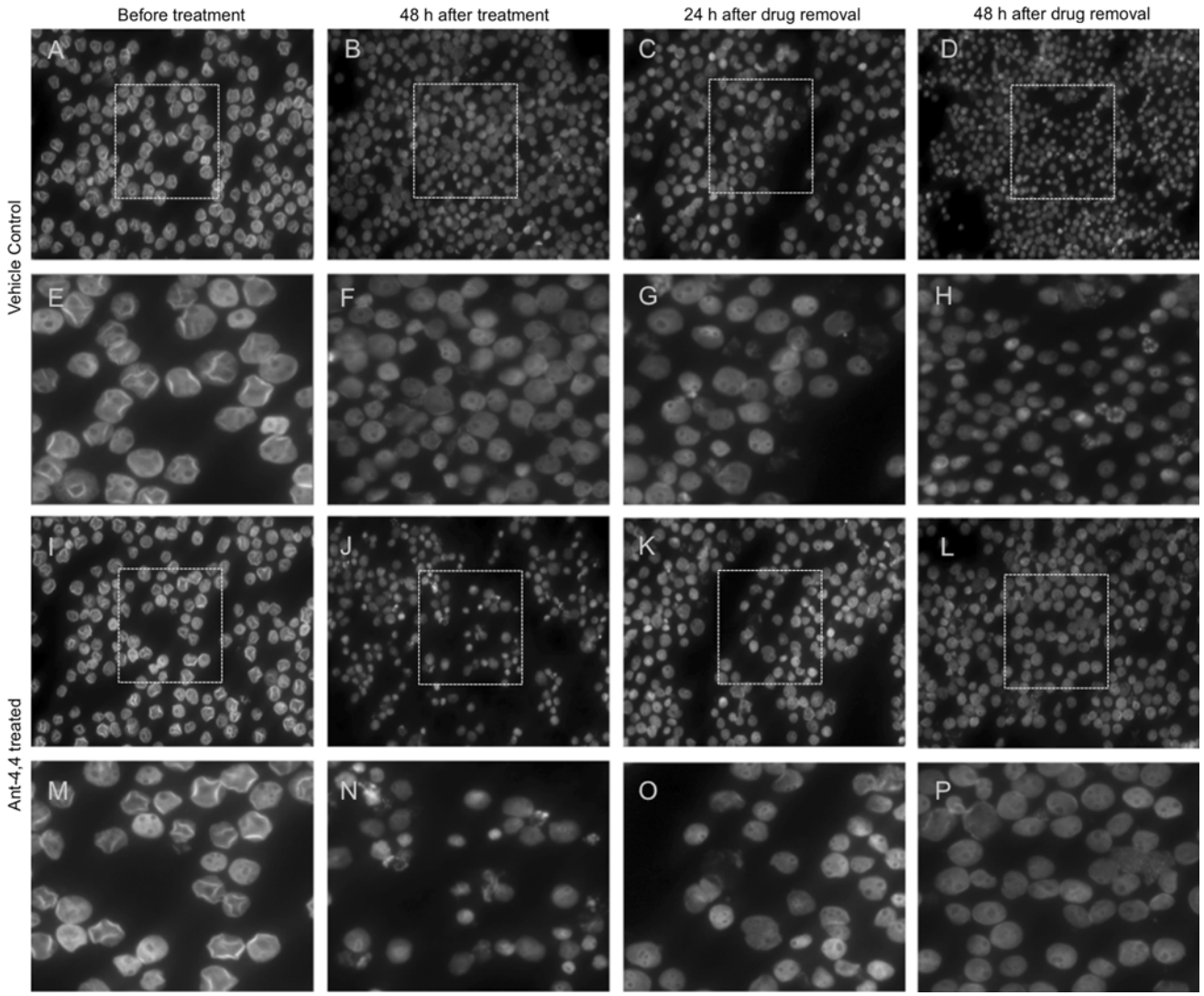


Fig. 6 Morphological determination of cell death of untreated (**a–d**) and Ant-4,4-treated HL-60 cells (**i–l**) before treatment; after 48 h exposure; and 24 and 48 h after the removal of Ant-4,4. HL-60 cells were seeded at 6.8×10^4 cells/ml on 10 cm plates and grown for 48 h. Cells were then exposed to 5 μM Ant-4,4 for a further 48 h. After treatment, the cells were washed twice to remove the drug. At each time point, cells were harvested and washed in cold PBS to remove medium. Cytospins of cells were stained with Vectashield DAPI mounting medium and apoptosis evaluated by microscopy. All images were taken with 20× amplification. Images shown from **e–h** and **m–p** are magnifications of the region depicted in **a–d** and **i–l**, respectively

growth inhibition was followed by relapse in the long term. Together with a selective delivery of chemotherapeutic agents, this question is of utmost importance in cancer therapy since it is also highly desirable to have agents inducing irreversible harmful effects on their targets. Therefore, to address whether HL-60 cells were able to overcome drug treatment the recovery of these tumour cells was evaluated after treatment.

After removing Ant-4,4 thoroughly we have shown for the first time that HL-60 cells are able to recover from Ant-4,4 treatment. The results also suggest that drug-induced cytotoxicity was being exerted in the first hours but gradually disappeared over time. Once the drug was removed, an increase in total cell number as well as protein content was observed over time up to 96 h. These changes were accompanied by increases in polyamine content, showing the positive correlation between their intracellular content and the rate of cell growth. As a comparator, we exposed cells in an identical manner to etoposide and found that these cells showed no recovery whatsoever (data not shown).

Since one of the main characteristics of cancer cells is that their regulation of the cell cycle is defective, it is important to evaluate cell-cycle kinetics after drug

Table 3 Summary of HL-60 cell-cycle distribution after Ant-4,4 removal

Time after removal (h)	Control (% of cells)			Ant-4,4 (% of cells)		
	G_1	S	G_2/M	G_1	S	G_2/M
0	51.5	32.9	14.8	61.8	28.0	8.6
24	28.3	57.5	12.4	42.2	49.5	6.2
48	36.9	54.5	7.5	42.7	42.2	10.9
72	33.9	47.7	16.6	37	46.1	12.6
96	44.8	38	11.5	32.4	49.9	12.3

HL-60 cells were seeded at 6.8×10^4 cells/ml and grown in 10 cm plates for 48 h. Cells were then left untreated (VC) or exposed to 5 μM Ant-4,4 for a further 48 h. After treatment the cells were washed twice to remove the drug. Cells were then seeded at 6.8×10^4 cells/ml on 10 cm plates. These were harvested every 24 h up to 96 h after drug removal. DNA was stained with propidium iodide (PI) and samples analysed through flow cytometry. Data were analysed through the Dean–Jett–Fox (DJF) mathematical model. Results are shown as mean population of cells (%) at each phase of the cell cycle ($n = 1$, 2 replicates per sample)

treatment. We have provided evidence suggesting that Ant-4,4 induces a decrease in G_1–S phase transition of HL-60 cells. This data is in agreement with the previous reports showing that Ant-4,4 also activates cell-cycle perturbations at the G_1–S phase transition in melanoma B16 (Xie et al. 2007a) and Hepatoma BEL-7402 cell lines (Xie et al. 2007b). However, in this study we have also shown that these cell-cycle perturbations resolved after drug removal, therefore suggesting that at least in HL-60 cells such effects are readily reversible over time. The way in which Ant-4,4 may interfere with the different opposing forces and checkpoints that participate in the regulation of the cell cycle remains to be defined.

Beyond Ant-4,4 activity in decreasing polyamine intracellular concentrations and inhibiting tumour growth whilst under drug treatment, anti-tumour effects were also established through the activation of apoptotic cell-death mechanisms. These results are in agreement with others obtained from the literature, where it was shown that Ant-4,4 induced apoptosis not only in the treatment of HL-60 cells (Xie et al. 2008) but also in human hepatoma BEL-7402 (Xie et al. 2007b) and in B16 melanoma cells (Xie et al. 2007a). Indeed, Ant-4,4, has been shown to cause a loss in mitochondrial membrane potential and subsequent release of cytochrome *c* in HL-60 cells, therefore indicating apoptosis to be occurring via the mitochondrial pathway (Xie et al. 2008). This evidence is further substantiated by studies with B16 melanoma cells, as Ant-4,4 induced the accumulation of reactive oxygen species (ROS) and subsequent oxidative stress by means of APAO activity upregulation (Xie et al. 2009). Previous studies undertaken in our laboratory with the putrescine–anthracene conjugate, Ant-4 give further strength to this hypothesis, as this drug also induced oxidative stress in HL-60 cells (Palmer et al. 2009). Here, we have confirmed through morphological determinations that Ant-4,4 induced apoptotic cell death in HL-60 cells. However, for the first time we have shown that this apoptotic stimuli induced by Ant-4,4 treatment were no longer present after the removal of the drug, with cells recovering their natural shape over time.

In summary, the overall aim of this study was to investigate the potential of Ant-4,4 as an anti-proliferative agent, therefore evaluating further the use of polyamine backbones for selective drug delivery. Particularly, we were interested in whether the anti-tumour effects of Ant-4,4 were long lasting. For the first time, we have shown that despite being an efficient way of delivering anthracene to cancer cells more effectively, the Ant-4,4 conjugate does not exert irreversible harmful effects on HL-60 cells. However, this does not interfere with the validation of polyamine conjugates as a promising anti-cancer approach, as it must be taken into account that the drug part in the conjugate is a key factor in determining its total properties. Therefore, the work conducted in our laboratory with polyamine–anthracene conjugates in combination with the work from others will allow establishing the best polyamine vector as well as the best drug to be delivered selectively to cancer cells. So far, the idea of using polyamines as vectors for selective drug delivery of known cytotoxic drugs shows great promise, and it is our belief that in future it will lead to the development of a novel and selective anti-cancer therapy.

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Conflict of interest There is no conflict of interest with regard to this work.

References

Chari RV (2008) Targeted cancer therapy: conferring specificity to cytotoxic drugs. Acc Chem Res 41:98–107. doi:10.1021/ar700108g

Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improvised sensitivity and reliability. J Immunol Methods 89:271–277

Fraser AV, Woster PM, Wallace HM (2002) Induction of apoptosis in human leukaemic cells by IPENSpm, a novel polyamine analogue and anti-metabolite. Biochem J 367:307–312. doi:10.1042/BJ20020156

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 65:55–63

Palmer AJ, Wallace HM (2010) The polyamine transport system as a target for anticancer drug development. Amino Acids 38:415–422. doi:10.1007/s00726-009-0400-2

Palmer AJ, Ghani RA, Kaur N, Phanstiel O, Wallace HM (2009) A putrescine–anthracene conjugate: a paradigm for selective drug delivery. Biochem J 424:431–438. doi:10.1042/BJ20090815

Park MH (2006) The post-translational synthesis of a polyamine-derived amino acid, hypusine, in the eukaryotic translation initiation factor 5A (eIF5A). J Biochem 139:161–169. doi:10.1093/jb/mvj034

Phanstiel O IV, Kaur N, Delcros JG (2007) Structure-activity investigations of polyamine–anthracene conjugates and their uptake via the polyamine transporter. Amino Acids 33:305–313. doi:10.1007/s00726-007-0527-y

Seiler N, Sarhan S, Grauffel C, Jones R, Knodgen B, Moulinoux J-P (1990) Endogenous and exogenous polyamines in support of tumor growth. Cancer Res 50:5077–5083

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244–258

Wallace HM, Fraser AV (2003) Polyamine analogues as anticancer drugs. Biochem Soc Trans 31:393–396

Wallace HM, Niiranen K (2007) Polyamine analogues—an update. Amino Acids 33:261–265. doi:10.1007/s00726-007-0534-z

Wallace HM, Fraser AV, Hughes A (2003) A perspective of polyamine metabolism. Biochem J 376:1–14. doi:10.1042/BJ20031327

Walters JD, Wojcik MS (1994) Polyamine transport in human promyelocytic leukemia cells and polymorphonuclear leukocytes. Leuk Res 18:703–708. doi:10.1016/0145-2126(94)90070-1

Wang C, Delcros JG, Biggerstaff J, Phanstiel O IV (2003a) Synthesis and biological evaluation of N^1-(Antracen-9-ylmethyl)triamines as molecular recognition elements for the polyamine transporter. J Med Chem 46:2663–2671. doi:10.1021/jm030028w

Wang C, Delcros JG, Biggerstaff J, Phanstiel O IV (2003b) Molecular requirements for targeting the polyamine transport system. Synthesis and biological evaluation of polyamine–anthracene conjugates. J Med Chem 46:2672–2682. doi:10.1021/jm020598g

Wang C, Delcros JG, Cannon L, Konate F, Biggerstaff J, Gardner RA, Phanstiel O IV (2003c) Defining the molecular requirements for the selective delivery of polyamine conjugates into cells containing active polyamine transporters. J Med Chem 46:5129–5138. doi:10.1021/jm030223a

Xie S, Cheng P, Ma Y, Zhao J, Chehtane M, Khaled AR, Phanstiel O IV, Wang C (2007a) Synthesis and bioevaluation of *N*-(arylalkyl)-homospermidine conjugates. Bioorg Med Chem Lett 17:4471–4475. doi:10.1016/j.bmcl.2007.06.009

Xie SQ, Cheng PF, Wang MW, Liu GC, Ma YF, Zhao J, Wang CJ (2007b) A novel homospermidine conjugate inhibits growth and induces apoptosis in human hepatoma cells. Acta Pharmacol Sin 28:1827–1834. doi:10.1111/j.1745-7254.2007.00639.x

Xie SQ, Liu GC, Ma YF, Cheng PF, Wu YL, Wang MW, Ji BS, Zhao J, Wang CJ (2008) Synergistic antitumor effects of anthracenylmethyl homospermidine and alpha-difluoromethylornithine on promyelocytic leukemia HL-60 cells. Toxicol In Vitro 22:352–358. doi:10.1016/j.tiv.2007.09.017

Xie SQ, Wang JH, Ma HX, Cheng PF, Zhao J, Wang CJ (2009) Polyamine transporter recognition and antitumour effects of anthracenylmethyl homospermidine. Toxicology 263:127–133. doi:10.1016/j.tox.2009.07.001

Xie S, Wang J, Zhang Y, Wang C (2010) Antitumour conjugates with polyamine vectors and their molecular mechanisms. Expert Opin Deliv 7(9):1049–1061. doi:10.1517/17425247.2010.504205