Induction of apoptosis in human leukaemic cells by IPENSpm, a novel polyamine analogue and anti-metabolite

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INTRODUCTION

The naturally occurring polyamines, putrescine, spermidine and spermine, are known to be present in increased concentrations in tumour cells with, for example, breast and colon cancers containing more than four times the amount found in normal cells [1,2]. As a result, many studies have targeted polyamine metabolism in cancer cells as a potential site for chemotherapeutic intervention [3]. Three different approaches to chemotherapy have been used. Firstly, prevention of polyamine synthesis through inhibition or regulation of individual biosynthetic enzymes has been found to lower intracellular polyamine concentrations below those values required for cellular growth [4,5]. This method has had limited success since it rarely depletes all three intracellular polyamines. Secondly, polyamine transport inhibitors can prevent uptake of exogenous polyamines by blocking membrane transporters and so, either alone or in combination with single enzyme inhibitors, can limit cell growth [6]. This method shows some improvement over a single enzyme inhibition but still does not fully deplete intracellular polyamine content. Finally, an increasingly popular and successful method is to manipulate natural intracellular polyamine concentrations using polyamine analogues [7,8]. These analogues are synthesized artificially, and may be either symmetrically or unsymmetrically alkyl-substituted molecules (for a review see [9]). The major polyamine analogues synthesized thus far have been analogues of spermidine or spermine [10,11]. The principle of analogue theory is that these molecules will be readily taken up by growing cells, using the polyamine transporter system, and once intracellular, will cause depletion of natural polyamine pools through feedback inhibition of biosynthesis and, coincidentally, increased catabolism and export of natural polyamines. For example, unsymmetrically substituted spermine analogues, CHENSpm [N1-(cycloheptylmethyl)-N5,N11-ethyl-4,8-diazauaundecane] and CPENSpm [N3,N7-(cyclopentylmethyl)-N9,N11-ethyl-4,8-diazauaundecane] have both been shown to decrease intracellular polyamine concentrations in different cell types. In HL-60 cells, CHENSpm and CPENSpm both caused a decrease in intracellular polyamine content to below 50% of control values over 24 h [12]. However, in DU145 prostate cancer cells, although CPENSpm was found to be effective in depleting intracellular spermidine and spermine pools over 24 h, this was not the case for CHENSpm, despite its intracellular accumulation [13].

In an attempt to classify the actions of these inhibitors the analogues have been divided into two groups, polyamine mimetics and polyamine anti-metabolites [14]. Our definition of a polyamine mimic is a polyamine analogue that enters the cell and mimics the function of natural polyamines. Polyamine mimetics generally do not cause depletion of intracellular polyamine pools. The other group of polyamine inhibitors, the anti-metabolites, deplete intracellular polyamine concentrations and trigger increased catabolism in addition to down-regulation of biosynthesis [14,15]. Such anti-proliferative analogues may be useful anti-cancer agents.

The aim of this study was to characterize a more recently developed, novel, unsymmetrically substituted spermine analogue, IPENSpm [(S)-N1,N7-(2-methyl-1-butyl)-N9,N11-ethyl-4,8-diazauaundecane] [16] in HL-60 human leukaemic cells, which have been shown previously to respond to other polyamine analogues [12]. Of particular interest was the mechanism by which this compound induced cell death and altered polyamine metabolism.

MATERIALS AND METHODS

Materials

RPMI 1640 growth medium and penicillin and streptomycin were from Life Technologies (Paisley, Scotland, U.K.). All cell culture plastics were from Nunc (Nalge Nunc International, Roskilde, Denmark). Foetal bovine serum was from PAA Laboratories (Yeovil, Somerset, U.K.). Trypan Blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldextrazolium bromide (MTT), EDTA, EGTA, L-cysteine, CHAPS, 1,10-phenanthroline, PMSF,
Humidified atmosphere flushed with 5% CO\textsubscript{2}. For experiments, cells were seeded at 6.8 × 10\textsuperscript{3} cells/ml and grown for 48 h prior to treatment.

### Cell-culture methods

HL-60 (human promyelogenous leukaemic) cells were grown in suspension in RPMI\textsubscript{1640} medium (supplemented with 10% foetal bovine serum) containing 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were grown at 37 °C in a humidified atmosphere flushed with 5% CO\textsubscript{2}. Due to the susceptibility of HL-60 cells to differentiate spontaneously beyond the promyelocytic stage with time in culture, cells were routinely passaged every 2–3 days and reseeded at a ratio of 1:6. For experiments, cells were seeded at 6.8 × 10\textsuperscript{3} cells/ml and grown for 48 h prior to treatment.

### Measurement of cytotoxicity

Cytotoxicity was measured using MTT assay as modified by the method of Denizot and Lang [17], from the original by Mossman [18]. In summary, HL-60 cells were plated on to 96-well plates and left to grow for 48 h, after which time cells were treated with analogue. At the appropriate time, MTT was added to the plates at a concentration of 5 mg/ml as a sterile solution dissolved in RPMI\textsubscript{1640}. Plates were then left for 3–4 h in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C. Metabolism of MTT by actively respiring cells produces an insoluble formazan salt that can be dissolved using 100 µl DMSO. Absorbance values were measured at wavelengths of 540 and 690 nm.

### IPENSpm activity on cell growth

HL-60 cell growth was determined by use of the Trypan Blue exclusion assay. This technique involved mixing cells with Trypan Blue, then counting the cells using a Neubauer haemocytometer to determine cell number. The cell viability was also determined using this method, since Trypan Blue penetrates non-viable cells, staining them blue. A modification of the method described by Lowry et al. [19] was used to determine total cellular protein. Samples were quantified against standards, prepared from a stock solution of 300 µg/ml BSA, to provide a range of standards ranging from 0 to 250 µg/ml BSA.

### Determination of polyamines

Polyamine data were obtained by perchloric acid extraction of intracellular polyamines from cells [20]. Samples were stored at −20 °C until analysis by HPLC. The method of HPLC was used that of pre-column dansylation [21]. This allowed quantification of IPENSpm as well as polyamines. Samples were dansylated at 25 °C overnight, extracted in toluene, blown to dryness in a nitrogen stream, then resuspended in acetonitrile. Samples were analysed by reverse-phase HPLC on a Hichrom RPB 5 µm column using a gradient of 100% acetonitrile to 40:60 acetonitrile/water [22].

### Detection of apoptosis

The technique used to identify the type of cell death involved staining of chromosomal material in cells that had been fixed in 0.4% formaldehyde. Cells were cytopsion on to glass slides and stained with Hoechst no. 33342 to detect apoptosis, followed by propidium iodide to detect necrosis. Hoechst no. 33342 is a small molecule capable of diffusion through the cell membrane and so will stain all chromosomal material, whereas propidium iodide is a larger molecule and can only enter cells where the cell membrane has been compromised. Apoptotic and necrotic cells were identified morphologically using fluorescence microscopy. Percentage apoptosis and necrosis was calculated by counting 100 cells five times from non-overlapping views.

### Measurement of caspase activity

The caspase-3-like activity measured in response to IPENSpm treatment was a direct result of the cleavage of a fluorogenic substrate, Ac-DEVD-AMC (where AMC is aminomethyl coumarin). Cell lysates were prepared by resuspending cells in 200 µl of lysis buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 10 mM EGTA, 0.5% CHAPS, 1 mM PMSF and 0.1 mM 1,10-phenanthroline]. After 10 min on ice in lysis buffer, cell were sonicated to induce cell lysis. Cell supernatant (50 µl) was added to 1.5 ml of assay buffer [50 mM Tris (pH 7.4), 1 mM EDTA, 10 mM EGTA, 0.5% CHAPS and 5 mM L-cysteine]. Substrate was then added and fluorescence measured immediately. Cleavage of this substrate generated fluorescence that was measured 1 h after the addition of substrate at excitation and emission wavelengths of 380 and 460 nm respectively.

### Determination of cell-cycle profile

Cell-cycle analysis and percentage apoptosis was determined by flow cytometry. Cells were resuspended in 1% paraformaldehyde (w/v) and left on ice for 20 min to induce membrane permeabilization, after which cells were suspended in DNA prep stain (kit number PN 6607055) containing propidium iodide (Coulter, Miami, FL, U.S.A.) and analysed by flow cytometry using a Coulter Flow Cytometer.

### Measurement of polyamine metabolic enzyme activities

Assays for ornithine decarboxylase (ODC) and spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT) were carried out on cell supernatant fractions. For ODC, incubation with [\textsuperscript{14}C]ornithine allowed determination of the level of enzyme activity by measurement of the amount of [\textsuperscript{14}C]CO\textsubscript{2} generated from cleavage of [\textsuperscript{14}C]ornithine [23]. SSAT activity was measured by quantification of the amount of [\textsuperscript{3}H]acetyl-spermidine generated from metabolism of [\textsuperscript{3}H]acetyl-CoA [24].

### Determination of polyamine export

Polyamine export was quantified by the method of Wallace and Mackarel [25]. Briefly, administration of [\textsuperscript{3}H]putrescine (0.5 µCi/ml) for 36 h was undertaken to allow radiolabelling of intracellular polyamine pools, since putrescine is a precursor for both spermidine and spermine. After an equilibrium period of 12 h and several washes, plates were harvested every 24 h and polyamines isolated using perchloric acid extraction. Samples of cell supernatant and medium were taken for analysis on a Packard liquid scintillation counter.

Analysis of intra- and extra-cellular polyamines was determined by TLC. Samples were dansylated as before but reconstituted in 100 µl of toluene. 20 µl was spotted on to a TLC plate and developed in 3:2 cyclohexane/ethyl acetate. The plate was dried and radioactivity in individual polyamines determined using a Packard liquid scintillation counter.
Statistical analysis

Statistical analysis was performed where appropriate using ANOVA with Dunnett’s post test. All data shown are means ± S.E.M. with each experiment performed a minimum of three times with replicates.

RESULTS

Cytotoxicity of IPENSpm was tested over a range of concentrations and incubation times (Figure 1). IPENSpm was inhibitory to growth at all concentrations tested and showed a dose- and time-dependence. With exposures of 48 h or longer, the inhibition was statistically significant at concentrations above 2.5 μM (P < 0.01). The IC_{50} value, at which 50% inhibition of cell growth was observed, was calculated to be 2.5 μM at 48 h exposure, and this concentration was used in all subsequent experiments. Similar inhibitory effects were seen on cell number (Figure 2) and protein content (results not shown), with cytotoxic effects being observed after 24 h exposure to IPENSpm. Despite the low cell number, cell viability remained high (> 90%) in all treatments, a feature common to apoptotic rather than necrotic cell death.

After a cytotoxic concentration of IPENSpm had been established, the type of cell death induced by this concentration of drug was determined. Hoechst no. 33342 staining of the cellular chromatin showed there to be a significant induction (4-fold) of apoptosis in the cells treated with IPENSpm compared with controls (Table 1). This result compares favourably with an equimolar dose of etoposide, a known inducer of apoptosis in these cell lines [26]. Counterstaining with propidium iodide showed there to be minimal necrosis as a result of IPENSpm administration (Table 1), the level being similar to that of controls. In an attempt to ascertain the mechanism of IPENSpm-induced apoptosis, flow cytometry was used to quantify the cell-cycle distribution and apoptosis of cells treated with IPENSpm for 48 h (Figure 3). The percentage of cells in G_{0}/G_{1} phase was elevated, while those in both S and G_{2}/M phases were decreased by IPENSpm compared with the control profile. In control cells the percentage apoptosis was 15.8 ± 4.4% (n = 4), while in IPENSpm-treated cells it was 32.7 ± 5.8% (n = 6).

Induction of apoptosis frequently involves the activation of caspase cascades within cells [28]. The activity of caspase-3-like enzymes was measured using a fluorogenic substrate. Results showed a 2.8-fold increase after 48 h of 2.5 μM IPENSpm treatment compared with controls (2470 compared with 897 units of activity/μg of protein respectively). Etoposide, a classic inducer of apoptosis, was used as a positive control (10 μM for 4 h) and produced an 8-fold increase in activity (4753 units of activity/μg of protein) of caspase-3-like enzymes over control values.

Previously, treatment of cancer cell lines with polyamine analogues has been shown to cause up-regulation of SSAT, an

Table 1 Type of cell death induced by IPENSpm in HL-60 cells

<table>
<thead>
<tr>
<th>Cell distribution</th>
<th>Treatment</th>
<th>Percentage of total cell number</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>exposure time (h)</td>
<td>24</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>Viable</td>
<td></td>
<td>92.6</td>
</tr>
<tr>
<td>Apoptotic</td>
<td></td>
<td>6.2</td>
</tr>
<tr>
<td>Necrotic</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>IPENSpm</td>
<td></td>
<td>84.2</td>
</tr>
<tr>
<td>Viable</td>
<td></td>
<td>15.2</td>
</tr>
<tr>
<td>Apoptotic</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Necrotic</td>
<td></td>
<td>12.4</td>
</tr>
</tbody>
</table>

‡ P < 0.01 when compared with untreated controls.

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HL-60 cells were seeded on 10 cm plates at $6.8 \times 10^4$ cells/ml and grown in RFC10 for 48 h prior to addition of 2.5 $\mu$M IPENSpm. After 48 h, harvested cells were fixed on ice in 1% (v/v) parafomaldehyde, then resuspended in DNA prep stain containing propidium iodide to allow analysis by flow cytometry. Results are the average of six separate experiments. $^*P < 0.01$ when compared with untreated control (ANOVA with Dunnett's post test).

Table 2 Effect of IPENSpm on polyamine metabolic enzymes

Cells were seeded on 10 cm plates at $6.8 \times 10^4$ cells/ml and after 48 h growth exposed to 2.5 $\mu$M treatments for 48 h. For the ODC assay, cells were washed, resuspended in assay buffer then sonicated to rupture the cells. The supernatant was then isolated and used to measure ODC activity by the amount of CO2 produced by cleavage of [14C]ornithine. For the SSAT assay, again cells were washed and, after sonication, the cell supernatant was isolated. The activity of SSAT was measured by formation of [3H]acetyl-spermine from [3H]acetyl-CoA. Results shown are means ± S.E.M., $n = 3$, with two replicates per experiment. Results were analysed by ANOVA with Dunnett's post test ($^*P < 0.01$).

<table>
<thead>
<tr>
<th>Enzyme activity (pmol/min per mg of protein)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODC</td>
<td>SSAT</td>
</tr>
<tr>
<td>Control</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>IPENSpm</td>
<td>6.3 ± 1.4*</td>
</tr>
<tr>
<td>Etoposide</td>
<td>5.1 ± 1.5*</td>
</tr>
</tbody>
</table>

Table 3 Polyamine data and intracellular IPENSpm concentration

Cells were seeded on 5 cm plates at $6.8 \times 10^4$ cells/ml, grown for 48 h, and then treated with 2.5 $\mu$M IPENSpm. Plates were harvested at 24 h intervals from 24–72 h after IPENSpm addition. Cells from harvested plates were washed before acid extraction of polyamines on ice using 0.2 M HClO4. The acid fraction was stored at $-20$ °C until analysis by HPLC. Results are shown for each polyamine expressed in nmol/mg of protein $n = 3$ ± S.E.M. Results were analysed by ANOVA with Dunnett's post test. $^*P < 0.01$ when compared to control data.

<table>
<thead>
<tr>
<th>Polyamine content (nmol/mg of protein)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exposure time (h) ... 24 48 72</td>
</tr>
<tr>
<td>Control</td>
<td>Putrescine</td>
</tr>
<tr>
<td></td>
<td>Spermidine</td>
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<tr>
<td></td>
<td>Spermine</td>
</tr>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>IPENSpm</td>
<td>Putrescine</td>
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<tr>
<td></td>
<td>Spermidine</td>
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<td></td>
<td>Spermine</td>
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<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>IPENSpm</td>
<td>Putrescine</td>
</tr>
</tbody>
</table>

The cytotoxicity of IPENSpm was tested in the presence of transport inhibitors denoted ORI 1090 and ORI 1202 (Figure 4).
shown to be the case for IPENSpm by the use of transport inhibitors, ORI 1202 and ORI 1090, which both inhibited polyamine accumulation (not shown) and the cytotoxic effect of the drug (Figure 4). The polyamine transport inhibitors used, when tested independently, showed no toxicity in HL-60 cells up to 125 \( \mu \)M, the maximum concentration used (results not shown). The inhibition of IPENSpm toxicity was more pronounced with ORI 1202 than ORI 1090, but the efficacy of both compounds indicates the involvement of the polyamine transport system. Further experiments have revealed ORI 1202 to be consistently more effective than ORI 1090 at inhibiting polyamine and polyamine analogue uptake in human cancer cell lines (results not shown).

Depletion of polyamines occurred despite the increase in ODC and the relatively small change in SSAT (Table 2). IPENSpm clearly does not superinduce SSAT in the way CPENSpm does, but it did cause a significant up-regulation compared with controls, and an increase consistent with the induction seen in NCI H157 non-small-cell lung carcinoma cells in response to IPENSpm treatment [16]. Thus polyamine depletion may be related more to the increased export observed (Figure 5). A similar effect was observed recently in hybrid transgenic mice overexpressing ODC and SSAT, where increased export was observed despite only small increases in SSAT activity [32]. The difference in distribution of radiolabel between the intra- and extra-cellular polyamines argues for a specific transport system out of the cell. Although IPENSpm and etoposide increased the total amount of polyamine exported, the distribution remained the same as the control, again arguing for a regulated export process as suggested previously [33,34].

The role of ODC in apoptosis is controversial, with both increases and decreases being observed in response to cytotoxic agents [35,36]. In HL-60 cells, etoposide was found to produce a transient increase in ODC activity at 2–6 h before the onset of etoposide-induced apoptosis. However, by 24 h ODC was markedly inhibited (H. M. Wallace and G. S. Lindsay, unpublished work). On the other hand, apoptosis induced by withdrawal of interleukin-3 from mouse T cells results in inhibition of ODC activity [23]. The same authors have shown ODC to be a transcriptional target of c-Myc, and contribute to c-Myc-induced apoptosis [37]. Thus it may be that a transient up-regulation of ODC is a response to the initiation of apoptosis, while inhibition of ODC is required later in the process. The involvement of c-Myc in the up-regulation of ODC [38] is interesting, as HL-60 cells are known to over express c-Myc [39].

The type of cell death induced by IPENSpm in HL-60 cells was shown to be apoptosis through a caspase-3-dependent pathway (Table 1). Apoptosis is a form of cell suicide that is particularly favourable for chemotherapeutic agents due to its altruistic nature [40]. The induction of apoptosis in HL-60 cells by IPENSpm was linked to an altered cell-cycle profile. This may be associated with an effect on tubulin polymerization that has been suggested previously to cause cell-cycle arrest and apoptosis [16]. The alteration in the cell-cycle profile, although different from previous data for IPENSpm [16], can be explained by cell-type differences in response to specific polyamine analogues. For example, the cell-cycle profile for CPENSpm in NCI H157 is unchanged from control cells, despite its apparent toxicity [16,29, 41]. The spermine analogue CPENSpm shows cytotoxicity on NCI H157 non-small lung carcinoma cells as well as MCF-7 and MDA-MB-468 breast cancer cells, yet DNA fragmentation is only observed in the breast cancer cells. These results clearly show that polyamine analogues have cell-type specific effects.

In summary, in this study IPENSpm appears to be acting as a polyamine anti-metabolite in HL-60 cells. Toxicity is associated with intracellular IPENSpm accumulation via the polyamine transporter and depletion of polyamine content through in-

**DISCUSSION**

The cytotoxic effect seen with IPENSpm alone (Figure 1) was prevented in the presence of a polyamine transport inhibitor.

Export of polyamines from the cell contributes significantly to the regulation of intracellular polyamine content [31]. Control cells exported approx. 14%, labelled polyamines over 72 h. In the presence of IPENSpm, the amount of polyamines exported increased and reached 42% by 72 h. By comparison, etoposide caused 85% export of polyamines over the same time period (Figure 5). Analysis of intra- and extra-cellular radioactivity showed that spermine and spermidine were the major intracellular radiolabelled polyamines, accounting for more than 70% of the total (control 73%; IPENSpm 78%; etoposide 78%). The pattern of exported polyamines was, however, different, with acetyl-polyamines and putrescine increasing at the expense of spermine (control, 51%; IPENSpm, 40%; etoposide, 37%).

The polyamine analogue IPENSpm was found to be cytotoxic in HL-60 human leukaemic cells. All the observed responses to IPENSpm were found to be comparable with the effects of the topoisomerase II inhibitor etoposide [26]. Closer inspection showed IPENSpm-induced G1, cell-cycle arrest and caspase-dependent apoptosis. Concomitant with this cell death were decreases in intracellular polyamine pools and increases in polyamine catabolism and export. Taken together, our results suggest that IPENSpm is acting as a cytotoxic polyamine anti-metabolite.

The theory of polyamine analogues is that they are taken up by cells and replace the natural polyamines, as well as decreasing polyamine synthesis and increasing polyamine catabolism and export. This results in the cell being unable to divide and instead leads to cell death. The theory assumes polyamine analogues to be taken up by the polyamine transporter system. This was shown to be the case for IPENSpm by the use of transport inhibitors, ORI 1202 and ORI 1090, which both inhibited polyamine uptake by the polyamine transporter system. This was suggested previously to cause cell-cycle arrest and apoptosis [16]. The alteration in the cell-cycle profile, although different from previous data for IPENSpm [16], can be explained by cell-type differences in response to specific polyamine analogues. For example, the cell-cycle profile for CPENSpm in NCI H157 is unchanged from control cells, despite its apparent toxicity [16,29, 41]. The spermine analogue CPENSpm shows cytotoxicity on NCI H157 non-small lung carcinoma cells as well as MCF-7 and MDA-MB-468 breast cancer cells, yet DNA fragmentation is only observed in the breast cancer cells. These results clearly show that polyamine analogues have cell-type specific effects.

In summary, in this study IPENSpm appears to be acting as a polyamine anti-metabolite in HL-60 cells. Toxicity is associated with intracellular IPENSpm accumulation via the polyamine transporter and depletion of polyamine content through in-

**Figure 5 Polyamine efflux in HL-60 cells in response to IPENSpm treatment**

Cells were seeded in 5 cm plates at 6.8 \( \times \) 10^4 cells/ml, and grown in RFC, for 36 h in the presence of [3H]putrescine. Cells were washed and grown on for another 12 h, then washed again. After addition of fresh medium, cells were treated with the appropriate drug, control; ▲ IPENSpm, △ 2.5 \( \mu \)M etoposide; Plates were harvested at 24 hours after drug addition. Cells from harvested plates were washed with complete PBS before acid extraction of the polyamines by 0.2 M HClO4 on ice for 30 min. Polyamines were also isolated from cell medium by 0.2 M HClO4 extraction. 50 \( \mu \)l of cell lysate and 450 \( \mu \)l of medium were taken for analysis on a Packard liquid scintillation counter. The remainder was stored at –20 °C until analysis by TLC. Values are shown as percentage of polyamine exported into the medium expressed as a percentage of the total polyamine content, \( \sigma = 3 \pm S.E.M. \) with 2 replicates per treatment. Results were analysed by ANOVA with Dunnett's post test. *P < 0.01 when compared with control.
creased polyamine catabolism and export. IPENSpm induces classical apoptotic cell death through a caspase-3-like-dependent mechanism and G2 arrest. IPENSpm, therefore, shows promising anti-proliferative activity in human cells and may be a useful agent in the fight against cancer.

We thank Reitha S. Weeks and MediQuest for their generous gift of the transport inhibitors, ORI 1090 and ORI 1202, and for the helpful comments on the manuscript. Thanks also to the European Social Fund and Gramian Universities Hospitals Trust for their financial support of this work.

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