The effect of pentamidine on melanoma *ex vivo*

Jason Smith, Benjamin J. Stewart, Sharon Glaysher, Katharine Peregrin, Louise A. Knight, David J. Weber and Ian A. Cree

Pentamidine is a small molecule inhibitor of the Ca^{2+}-binding protein S100B and disrupts the S100B–p53 protein–protein interaction; this is thought to restore wild-type p53 tumour suppressor function in melanoma. Additional anticancer effects may be the result of inhibition of regenerating liver family phosphatases. In this study, we have used a standardized ATP-tumour chemosensitivity assay to investigate the effect of pentamidine on cells derived from 18 skin melanoma samples and one uveal melanoma sample. The cells were tested at six concentrations from which the IC_{50} and IC_{90} were calculated. To allow comparison between samples, an $index_{sum}$ was calculated based on the percentage of tumour growth inhibition at each concentration. Of the skin melanoma samples tested, 78% exhibited an $index_{sum}$ less than 300 indicating strong inhibition. The median $index_{sum}$ of 237 also indicates considerable activity against these samples. The median IC_{90} (30.2 μmol/l) may be clinically achievable in a proportion of patients. The uveal melanoma sample exhibited an $index_{sum}$ of 333 indicating moderate inhibition, and 86% inhibition at test drug concentration (37.96 μmol/l). These results show that pentamidine has activity against melanoma, and support the prospect of its development for therapeutic use. *Anti-Cancer Drugs* 21:181–185 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Translational Oncology Research Centre, Pathology Centre, Queen Alexandra Hospital, Portsmouth, UK

Correspondence to Ian A. Cree, PhD, Translational Oncology Research Centre, Queen Alexandra Hospital, Portsmouth, PO6 3LY, UK
Tel: +44 23 92 286378; fax: +44 23 92 286379; e-mail: ian.cree@porthosp.nhs.uk

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Introduction

1,5-Bis(4-amidinophenoxy)pentane (pentamidine) is an aromatic diamidine, pharmacologically active as an antiprotozoal agent. It is used in the treatment and prevention of *Pneumocystis carinii* pneumonia, particularly in patients with HIV infection, and in the treatment of trypanosomiasis and visceral leishmaniasis. Pentamidine has recently been highlighted as a potential anticancer drug, particularly in the context of melanoma where it is thought to inhibit the S100B–p53 protein–protein interaction [1,2].

S100B is a highly conserved 21.5-kDa homodimer belonging to the S100 family of EF-hand Ca^{2+}-binding proteins, structurally related to other Ca^{2+}-binding EF hand motif superfamily proteins such as calmodulin and troponin C [3,4]. S100B interacts with p53 at its C terminus in a Ca^{2+}-dependent manner and binds through hydrophobic and salt bridge interactions [2]. In addition to this interaction, the C terminus of p53 has been shown to be a substrate of protein kinase C [5,6]. These findings serve to link p53 activity to calcium signaling. It is thought that S100B inhibits the transcriptional activity of p53 by disrupting tetramerization and inhibiting phosphorylation of the C terminus by protein kinase C [7,8]. p53 has long been recognized as a vital transcriptional activator of many genes involved in apoptosis and cell-cycle control; stabilization and activation of this protein (by tetramerization and modifications such as C-terminus phosphorylation) halts inappropriate growth and cell cycling, a tumour suppressor function [9].

Pentamidine has been identified as a molecule that binds to the p53 binding site on S100B, and the structure of pentamidine bound to S100B has been characterized at atomic resolution by nuclear magnetic resonance and X-ray crystallography [2,10–12]. Pentamidine may therefore act to prevent S100B–p53 binding and prevent loss of tetramerization (stabilization) and C-terminus phosphorylation caused by this protein–protein interaction.

High levels of S100B are associated with melanoma and are commonly used in diagnosis by immunohistochemistry [7,13,14]. Lin *et al.* [1] have shown a direct relationship between levels of p53 and S100B protein in six melanoma cell lines (LOX-IM, UACC-62, SK-MEL-5, UACC-2571, C8146A and Malme-3M) with a wild-type p53 genotype, where a high S100B level is directly related to a low level of p53, and a low level of S100B is directly related to a high level of p53 as measured by western blot [1]. Furthermore, these authors develop a physiological theory of S100B, suggesting that p53 binds the S100B promoter at levels above which are required for most p53 transcriptional targets, and the generation of S100B acts as a negative feedback on p53, in a functionally similar manner to hdm2 [1,7].

There is some evidence that pentamidine may also exert its anticancer effects by acting as an inhibitor of

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regenerating liver (PRL) family phosphatases whose biological functions are poorly understood, but which are overexpressed in many cancers [15]. Pathak *et al.* [16] report that pentamidine inhibits all three PRLs *in vitro* and exhibits an inhibitory effect against WM9 human melanoma cell line xenografts in nude mice [15]. Wang *et al.* [17] found high levels of PRL-1 expression in five of six melanoma cell lines studied by quantitative real-time PCR.

However, all of the in-vitro and xenograft data are derived from cell lines, which are highly passaged and adapted to the cell culture environment, resulting in high growth rates and greater sensitivity to chemotherapeutic agents [18,19]. The use of tumour-derived cells or low passage number cell lines can offset this disadvantage, as we have previously shown in ovarian cancer [18] and in melanoma (Fernando *et al.* unpublished). We therefore took the opportunity to study the activity of pentamidine against human tumour-derived melanoma cells *in vitro* using the ATP-tumour chemosensitivity assay (ATP-TCA) [20].

Materials and methods

Tumours

A total of 18 metastatic skin melanoma samples (10 males, 8 females) and one uveal melanoma sample (female) were tested in the study. These samples were obtained at debulking surgery for regional lymph node metastasis. The individual tumours are described in Table 1. Only one patient had received previous chemotherapy (temozolomide). In each case, only tumour material not required for diagnosis was used in the ATP-TCA. Individual patient consent was obtained for all samples and permission for tissue use was granted by the local ethics committee.

ATP-tumour chemosensitivity assay

The ATP-TCA assay was performed as described earlier [20,21]. Cells were obtained by enzymatic dissociation of solid tumour tissue by collagenase (Sigma C8051, Poole, Dorset, UK). These cells were diluted in serum-free complete assay medium (available from DCS Innovative Diagnostik Systeme, Hamburg, Germany) and plated in 96-well polypropylene plates (Corning Life Sciences, High Wycombe, UK) at 20 000 cells per well.

Pentamidine was added in triplicate to wells at serial dilutions of 200, 100, 50, 25, 12.5, 6.25% test drug concentration (TDC). For pentamidine, the TDC was set at 37.96 μmol/l (22.5 μg/ml) based on previous in-vitro experiments defining the inhibition of S100B–p53 interaction [2]. Each plate included two controls: a medium only row (MO) which contained no drug, and a row to which a maximum inhibitor (MI: available from DCS Innovative Diagnostik Systeme) was added, killing all cells present.

Cells were incubated for 6 days at 37°C in 5% CO_2. After the incubation period, cells were lysed by the addition of a cell extraction reagent (available from DCS Innovative Diagnostik Systeme). An aliquot of lysate (0.05 ml) from each well was added to the corresponding well of a white 96-well microplate (Thermo Life Sciences, Basingstoke, UK), to which 0.05 ml luciferin–luciferase counting reagent [D-luciferin and recombinant luciferase (R&D Systems, Abingdon, UK)] was then added. The light output corresponding to the level of ATP present was measured using a luminometer (MPLX, Berthold Diagnostic Systems, Hamburg, Germany). The light output data were transferred to a spreadsheet and the percentage inhibition at each concentration was calculated using the equation: $1 - (\text{test} - \text{MI})/(\text{MO} - \text{MI})$.

Table 1 Data for individual patient samples including the site of primary tumour and lymph node metastasis, IC_{50}, IC_{90} and $index_{sum}$

Sample type	Age (years)	Sex	Primary tumour site	Lymph node metastasis site	IC_{50} (μmol/l)	IC_{90} (μmol/l)	$Index_{sum}$
Uveal melanoma	37	F	Left ciliary body	NA	22.776	50.11	333
Skin melanoma	49	M	Right thigh	Right thigh	27.0	63.4	366
	36	F	Left groin	Left groin	14.0	22.8	393
	49	F	Back	Back	8.73	32.3	235
	64	M	Left leg	Left leg	6.83	11.8	192
	33	F	Unknown	Unknown	17.1	35.7	274
	27	M	Unknown	Groin	21.3	35.3	266
	63	M	Left leg	Left Leg	4.56	16.3	138
	63	F	Unknown	Left Inguinal	16.7	40.6	310
	72	F	Unknown	Unknown	3.80	22.0	140
	81	M	Unknown	Groin	3.80	11.0	126
	45	M	Unknown	Left axilla	4.56	17.5	156
	61	F	Unknown	Right groin	9.11	34.5	239
	60	M	Unknown	Left axilla	15.9	55.4	306
	75	M	Scalp	Right neck	9.11	28.1	233
	73	M	Right leg	Right groin	14.8	34.5	275
	85	F	Fingertip	Left axilla	4.18	14.0	141
	66	F	Lateral malleolus	Left groin	3.80	14.0	123
	65	M	Chest	Left axilla	14.0	35.7	277
Median ($n=18$)					9.11	30.2	237

F, female; M, male.

To compare results between tumours, a simple logarithmic sum index of cell survival ($index_{sum}$) was calculated by adding the percentage inhibition at each of the six concentrations used and subtracting this from 600: index = 600 – sum ($\%inhibition_{6.25,\ 12.5 \ldots 200}$). Total inhibition across all concentrations produces an $index_{sum}$ of 0, whereas no inhibition produces an $index_{sum}$ of 600.

Pentamidine

The pentamidine (pentamidine isothionate salt) used in the ATP-TCA assay was sourced from Sigma Aldrich (PO547, Poole, Dorset, UK). The drug was dissolved in dimethyl sulfoxide at 0.18 g/µl, and stock aliquots stored at −20°C. The stock was diluted in complete assay medium for testing in the ATP-TCA at six concentrations ranging from 6.25% TDC (2.37 µmol/l) to 200% TDC (75.92 µmol/l). The highest percentage of dimethyl sulfoxide in the assay was 0.05%.

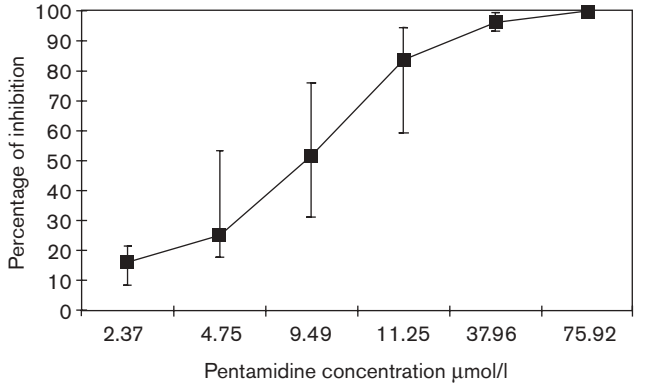
Data analysis

The data for the 19 samples were input to an Access database (Microsoft, Reading, Berkshire, UK) and transferred to Excel (Microsoft) for further analysis. The median percentage inhibition and interquartile range was calculated at each percentage TDC. The percentage TDC was converted to micromoles per litre concentrations and a concentration–response curve was plotted using the natural logarithmic scale produced by serial drug dilution. The IC_{50} and IC_{90} for pentamidine in each tumour sample were calculated by the trapezoidal rule.

Results

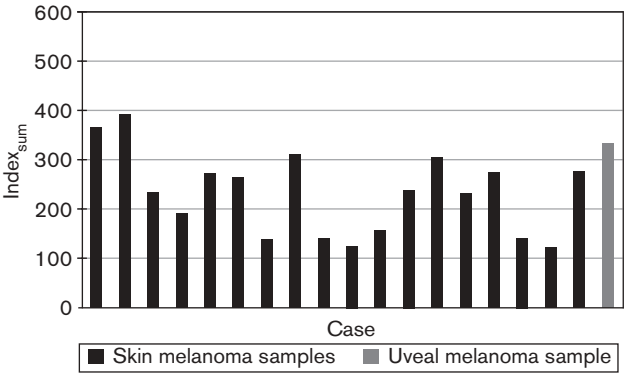
Pentamidine exhibited strong inhibition in all melanoma tumour samples tested, though with some heterogeneity between samples and less activity than reported in cell lines. There was increasing inhibition with increasing drug concentration (Fig. 1). In all, 50% of the skin melanoma samples tested exhibited greater than 95% inhibition at 100% TDC (37.96 µmol/l), whereas *22%* (four of 18) exhibited greater than 95% inhibition at 50% TDC (11.25 µmol/l). Pentamidine exhibited increasing inhibition of the uveal melanoma sample at increasing concentrations, and 86% inhibition at 100% TDC (37.96 µmol/l). There was heterogeneity of response (Fig. 2). Heterogeneity of response to single agents has been observed previously in melanoma [22], and was expected in this study. An $index_{sum}$ less than 300 corresponding to 50% inhibition across the range of concentrations tested is a useful way to compare samples, and 78% (14 of 18) of the skin melanoma samples exhibited an $index_{sum}$ of less than 300 indicating strong inhibition (Fig. 2). The median $index_{sum}$ of 237 and the median IC_{90} of 79.5% TDC (30.2 µmol/l) also show a strong response in skin melanoma samples. However, a considerable range of IC_{90} values (11.0–55.4 µmol/l) was observed (Fig. 3). The uveal melanoma sample exhibited an $index_{sum}$ of 333, indicating moderate inhibition (Fig. 2) and an IC_{90} of 50.11 µmol/l (Fig. 3).

Fig. 1



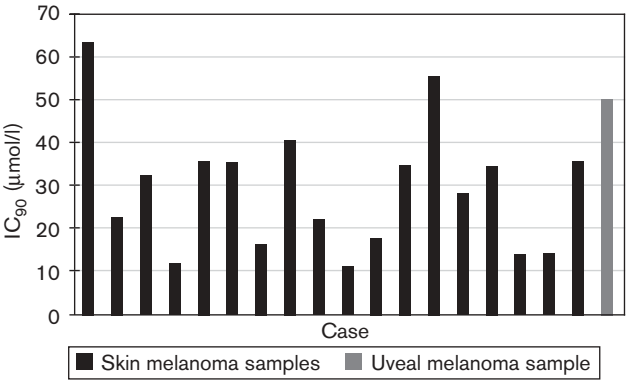
Median percentage inhibition at increasing pentamidine concentrations, with interquartile range, in skin melanoma samples ($n = 18$).

Fig. 2



$Index_{sum}$ of individual samples.

Fig. 3



IC_{90} for individual samples.

Discussion

Several studies have linked pentamidine to anticancer effects in melanoma [1,2,7,12,16]. However, these effects have been observed in cell lines whose characteristics are not directly comparable with the original tissue [18]. This study investigated the effects of pentamidine on tumour-derived melanoma cells in the ATP-TCA, and as a result is likely to give a more accurate indication of the potential effect of pentamidine on melanoma *in vivo* than can be achieved with cell lines. Despite heterogeneity of response, the median $index_{sum}$ remained low (< 300), and at high concentrations (100% TDC) the interquartile range of inhibition was low (Fig. 2), showing a decreased heterogeneity of response at higher concentrations and considerable pentamidine activity at the concentrations measured.

The results show pentamidine to be active against melanoma over a range of concentrations, which are probably just clinically attainable. It has been shown that in patients with leishmaniasis, a peak plasma concentration (C_{max}) on day 7 of 751 nmol/l is achievable (area under the curve: 6738 nmol/h/l), based on a dose of 3–9 mg/kg/day given by intravenous infusion over 4 h once daily [23]. Others have shown higher values within 8 h of administration. In trypanosomiasis patients, given a 2 h intravenous infusion of 3.0–4.8 mg/kg, C_{max} was noted to be 713–2461 nmol/l (median 923 nmol/l) [24]. Other studies show similar figures for both C_{max} and area under the curve [25,26]. Metabolism is by cytochrome P450 and excretion is mainly through the kidney [24]. This compares with our data showing IC_{90} values of 11.0–55.4 μmol/l, suggesting that the concentrations observed to be active could perhaps be achieved in patients. However, little protein is present in our culture medium, and pentamidine is 70% protein bound in plasma. Much depends on the degree to which pentamidine is taken up by tumour tissue, and this is unknown.

There may be several mechanisms by which pentamidine exerts its anticancer effects; the binding of pentamidine to S100B has been rigorously established [1,2,12] and it is likely that pentamidine restores wild-type p53 tumour suppressor function. A second potential mechanism, inhibition of PRL family phosphatases, may halt cell-cycle progression; PRL-1 has been shown to be required for normal cell-cycle progression [17], and Lee *et al.* [27] report that when used in conjunction with chlorpromazine, pentamidine has a synergistic effect in halting mitosis in tumours. Both mechanisms are likely to play a role in tumour suppression, and further mechanistic studies must be conducted to conclude which is the most important, and which can be best targeted. Both of these targets are involved in cell-cycle progression; however, given the extremely important role of p53 as an upstream regulator of the cell cycle, this target is likely to be the most significant.

This study shows pentamidine to be active *in vitro* against tumour-derived melanoma cells and supports the prospect of its future therapeutic use in patients with metastatic melanoma, though the concentration required is probably only just clinically achievable. Its frequent and serious side effects, particularly renal and pancreatic damage, are a concern [23,28,29], but are no worse and probably better than many anticancer agents or combinations in use for melanoma. They may be ameliorated by careful scheduling [30]. A phase II trial is being conducted investigating the effect of pentamidine against melanoma with wild-type p53 and detectable S100B in human participants with relapsed or refractory melanoma (*www.clinicaltrials.gov* Identifier: NCT00729807).

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