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Relationship between the melanin content of a human melanoma cell line and its radiosensitivity and uptake of pimonidazole*

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Summary. The intra-cellular uptake of the weakly basic radiosensitiser pimonidazole (PIMO) was determined as a function of the pigmentation of Na11+ human melanotic melanoma cells in vitro. Two experimental conditions were considered: exponentially growing cells (Exp.) and plateau-phase cells (Pl.). The melanin content of Na11+ cells ranged from 500 μg/g cell weight in exponentially growing cells to 6000 μg/g in heavily pigmented plateau-phase cells. Cells were exposed to PIMO (medium dose, 0.2 mmol/dm^3; 58.2 μg/ml). The intra-cellular concentration ranged from 163 μg/g in Exp. to 900 μg/g in pigmented Pl.; the latter being equivalent to an intra- to extracellular concentration ratio (Ci/Ce) of 17. However, this increase in the cellular uptake of PIMO was not accompanied by an increase in radiosensitising efficiency. In comparison, the Ci/Ce for etanidazole (ETA), a radiosensitiser that is uncharged at physiological pH, remained approximately constant at 1 for all values of melanin contents. Treatment of Na11+ tumours in vivo with [^{3}H]-PIMO resulted in a tumour: blood ratio of about 3 at 30–60 min after administration. However, at 24 h a grain count of label derived from [^{3}H]-PIMO showed that picnotic areas of tumours contained levels that were some 40 times greater than the background value. This high level of label was coincident with areas of highest apparent melanin content. In conclusion, PIMO accumulates in very heavily pigmented melanoma cells present in necrotic zones with picnosis. As these cells are probably non-clonogenic, PIMO is not suitable for use in melanoma radiotherapy.

Introduction

Most rodent and human xenografted tumours contain hypoxic cells [12, 25, 28], and a few clinical studies have suggested that radiotherapy might be improved by the use of agents such as nitroimidazoles that increase the radiosensitivity of these hypoxic cells [6]. The first agents evaluated were metronidazole and misonidazole, but neurotoxicity has limited their use in radiotherapy. A second generation of hypoxic cell sensitisers have been developed, among which pimonidazole (PIMO) is particularly interesting since it is preferentially accumulated by tumour cells in vitro [4]. PIMO also accumulates in rodent tumours as indicated by tumour: blood ratios of >1 [15, 20, 21, 24, 33, 37, 44–46] and, similarly, in human tumour xenografts [21, 35] and in human tumours in patients [1, 8, 26], with the uptake being highest in melanomas as compared with other tumours [1, 5, 8, 20, 21, 26]. The high tumour-cell concentration of PIMO that can be obtained suggests that the radiosensitising effect of PIMO should also be high, and the results obtained using non-melanotic cells growing exponentially in vitro support this hypothesis [30, 40]. In addition, PIMO has generally been found to be effective in non-pigmented rodent tumours in vivo [15, 16, 32, 44]. However, using human tumour xenografts and clinically relevant drug doses, a radiosensitising effect has been detected in rectal adenocarcinoma HRT18 but not in melanoma Na11+, although the accumulation of PIMO in the melanoma was particularly high [21, 35]. We report the results of some studies that may explain the basis for the lack of effect of PIMO on the Na11+ melanoma.

Tumours are very heterogeneous, especially with respect to the cell-proliferation kinetics (exponentially growing cells, plateau-phase cells) and to the presence of necrotic zones. This heterogeneity suggests that the average intra-tumour concentration may not necessarily reflect the concentration of the drug in the clonogenic hypoxic cells, which are the cells essential for tumour radiosensitivity. In vitro radiosensitisation and drug uptake have previously been investigated in exponentially growing cells [30, 38–41]. Therefore, the aim of the present work was, firstly, to measure the uptake of PIMO into exponentially growing and plateau-phase melanotic melanoma cells in vitro and determine whether this might be related to their melanin content and secondly, to determine whether the

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inter- and intra-cellular distribution of [^{3}H]-PIMO in Na11+ tumours might explain the lack of radiosensitisation observed for PIMO in this tumour type.

Materials and methods

Compounds. Etanidazole [ETA, *N*-(2-hydroxyethyl)-2-nitroimidazolyl acetamide; Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA] and pimonidazole {PIMO, α-[(2-nitro-1-imidazolyl)methyl]-1-piperidine-ethanol hydrochloride; Hoffman-La Roche, Switzerland} were dissolved in minimum essential medium (MEM) supplemented with 20 mM HEPES (pH 7.2) at a concentration of 0.2 mmol/dm^3 (42.8 μg/ml for ETA and 58.2 μg/ml for PIMO). [^{3}H]-PIMO [42] dissolved in absolute ethanol had a specific activity of 5.5×10^7 Bq/ml. [^{3}H]-PIMO (10 μl for scintillation or 100 μl for autoradiography) was mixed with non-radioactive PIMO dissolved in Dulbecco's phosphate-buffered saline (PBS). The total amount of PIMO injected i. v. into mice via the retro-orbital sinus in a volume of 0.3 ml was 200 mg/g body weight. Control mice were given PBS alone.

Tumour-cell system. The melanotic melanoma Na11+ originated from a human melanoma. The characteristics and maintenance of this cell line have been described elsewhere [14]. Congenitally athymic nude mice were bred and maintained in a defined flora- and pathogen-free colony. Details of the mouse breeding and tumour production have been published elsewhere [13, 14]. Tumours were obtained by injecting 3×10^6 cells into both flanks of mice that had been whole-body-irradiated with 5 Gy ^{137}Cs γ-rays to increase tumour uptake.

In vitro studies. Plateau-phase cells were obtained 7 days after 5×10^5 cells had been seeded in glass petri dishes (unpublished data). A few experiments were performed using exponentially growing cells obtained 2 days after the cells had been seeded. For the aerobic study, cells were placed in an incubator (37° C, 5% CO_2, 45 min). For hypoxia, open dishes were placed in aluminium chambers and gassed with a humidified mixture of 95% N_2 and 5% CO_2 (<3 ppm O_2) for 45 min. A filtered solution of dithionite-sodium carbonate was placed in the center of the chambers to remove traces of oxygen [18]. After undergoing incubation with PIMO or ETA and/or irradiation, the cells were trypsinised and the surviving fraction was assessed by an in vitro colony assay. Colonies were fixed and stained with crystal violet (0.25%, w/v, in 80% methanol containing 10% formaldehyde).

Cellular uptake and drug concentration in the medium was measured by high-performance liquid chromatography (HPLC) [21, 23]. Briefly, the medium was analysed directly and cells were collected with a rubber policeman and stored at –80° C. On the day of analysis, the cells were sonicated in water and ETA and PIMO were extracted with acetonitrile-water (1 : 1, v/v). The extraction was repeated and the supernatants were pooled and evaporated to dryness. The residue was diluted in eluent B containing an appropriate amount of internal standard (Ro 03-1902), and cellular uptake was analysed using a Varian model 5000 chromatograph equipped with a 5-μm Nucleosil column connected to a Varichrom UV-visible detector at 326 nm. The elution flow rate was 2 ml/min (eluent A, 75% acetonitrile and 25% water; eluent B, 4 mM heptane sulphonic acid, 5 mM dibutylamine and 50 mM glycine adjusted to pH 3); the gradient was: 0 min, 8% eluent A; 10 min, 45% eluent A; 10–11 min, 45%–8% eluent A.

In vivo studies. Tumours were used when they had reached a mean diameter of 9–11 mm. Animals were anaesthetised at various times after drug injection and blood was collected by cardiac puncture into heparinised tubes immediately before tumour excision and frozen in liquid nitrogen. Tumours were removed and immediately frozen in liquid nitrogen. Subsequently, tissue samples were weighed and 100–300 mg was suspended in Optisolve (LKB). Tissues were digested by incubation at 55° C for 16 h, 15 ml liquid scintillation fluid (Optiphase, Hisafe-TM; LKB) was added and samples were counted on a Packard Tricard liquid scintillation counter. A set of variably quenched standards were used for calibration. These were prepared by adding a known activity of tritiated water to a set of eight samples containing between 0 and 0.2 ml whole blood digested as described above. Any very highly coloured samples were bleached by incubation at 60° C for 2 h with benzoyl peroxide [0.4 ml, 5% (w/v) in toluene] prior to the addition of scintillant.

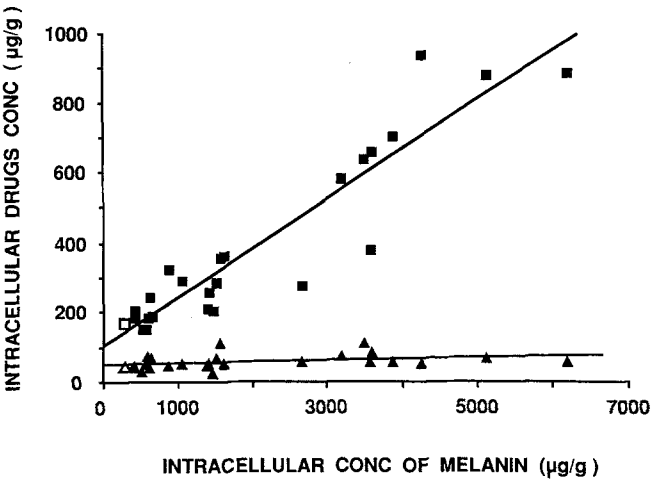


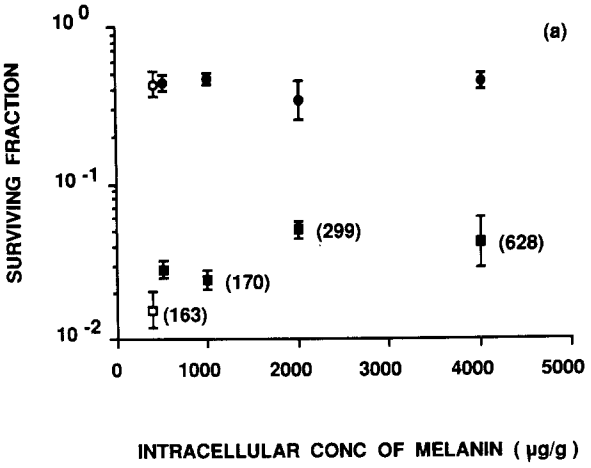
Fig. 1. Intra-cellular concentrations of ETA (▲, △) and PIMO (■, □) as a function of melanin content. *Closed symbols*, plateau-phase cells; *open symbols*, exponentially growing cells

Histology, autoradiography and melanin determination. Tumours were removed at 24 h after [^{3}H]-PIMO administration and were immediately fixed in ethanol: acetic acid (3 : 1, v/v). The tissues were dehydrated and embedded in paraffin and sections (3 μm) were cut and mounted on microscope slides. For autoradiography the slides were dipped in Ilford K2 emulsion and exposed for 2 months. The slides were then developed in Kodak D19b and stained with hemalum and erythrosin. The amount of radioactivity in the tumour was determined by grain counting using an ocular grid. The percentage of cellular areas was identified under a microscope and outlined on photographs of the sections. The areas were weighed and cellular areas were expressed as a percentage of the total areas. Melanin was localized by the method of Fontana-Masson [17].

For melanin determination, the quantitative colourimetric method of Foster et al. [9, 10] as adapted to normal and cultured retinal pigment cells by Whittaker [43] was used, with a few modifications. Cells were sonicated in water, and non-melanic substances that interfered with the assay were removed by three extractions with 5% trichloroacetic acid, two extractions with cold ether-alcohol (1 : 3, v/v) and one extraction with absolute ether. The dried residue extracted from 50–100 mg cells was dissolved in 1 ml 1 *N* KOH and then heated to 100° C for 30 min. It was cooled to room temperature and the optical density was measured at 400 nm (Varian spectrophotometer). A standard curve was constructed using synthetic melanin (Sigma) dissolved in 1 *N* KOH (1–100 μg/ml). Absorbance at 400 nm increased linearly with melanin concentration up to 100 μg/ml.

Results

A preliminary experiment showed that the melanin content was very homogeneous in exponentially growing cells but varied greatly in plateau-phase cells. The melanin content of plateau-phase cells obtained at 7 days after seeding increased by a factor of >6 within 12 h (results not shown). The uptake of PIMO into hypoxic plateau-phase Na11+ cells in vitro was compared with that of ETA. Figure 1 shows that the intra-cellular concentration of PIMO increased linearly (correlation coefficient, 0.9 P:10^{-4}) as a



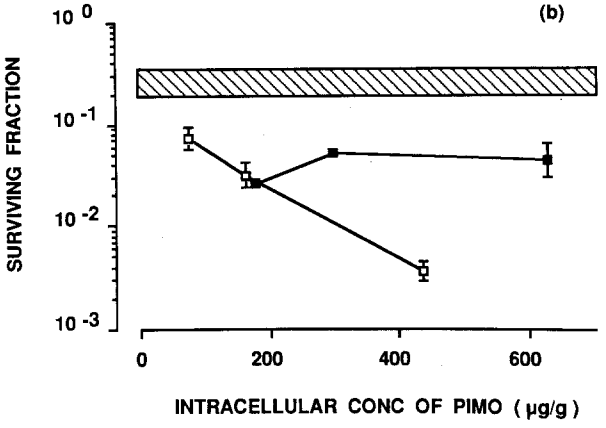


Fig. 2 a, b. Surviving fractions of Na11+ Exp. (*open symbols*) and Na11+ Pl. (*closed symbols*) after the delivery of a radiation dose of 10 Gy under hypoxic conditions in the presence or absence of PIMO. **a** As a function of intra-cellular melanin concentration: (○, ●), in the absence of PIMO; (□, ■), in the presence of PIMO. The intra-cellular PIMO concentration is shown (in µg/g of cells) *in parentheses*. **b** As a function of intra-cellular PIMO concentration. The *shaded area* represents the 95% confidence interval of cell survival in nitrogen without PIMO. Data points represent mean values for 2 experiments; error bars indicate 95% confidence intervals

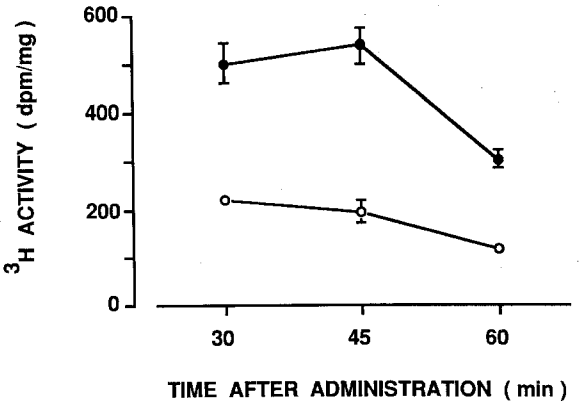


Fig. 3. ^{3}H activity in blood (○) and tumours (●) after the injection of [^{3}H]-PIMO. Data represent mean values ± 95% confidence intervals

function of melanin content. At the lowest melanin content (500 µg/g), the Ci/Ce ratio for PIMO was between 3 and 4, which is very similar to that found for exponentially growing Na11+ cells. However, in plateau-phase cells in which the melanin content increased, the Ci/Ce value also increased to >17. In contrast, the intra-cellular concentration of ETA remained fairly constant, with Ci/Ce values being close to 1.

The radiosensitising effect of 58.2 µg PIMO/ml in hypoxic Na11+ cells was determined as a function of cellular pigmentation. Figure 2 shows that there was no significant change in the radiosensitivity with pigmentation in plateau-phase cells following a radiation dose of 10 Gy in the presence of PIMO (Fig. 2a), although the intra-cellular PIMO concentration measured in the more pigmented cells was at least 3-fold that determined in the less pigmented cells (Fig. 1). In these experiments, no significant change in the plating efficiency was detected as a function of either the pigmentation or the intra-cellular PIMO concentration. In Fig. 2b, these results expressed as intra-cellular concentration (Ci) are compared with those obtained for radiosensitisation of exponentially growing cells (melanin content, <500 µg/g). The extra-cellular concentration (Ce) remained constant (58.2 µg/ml) in plateau-phase cells, whereas it varied from 29.1 to 174.6 µg/g in exponentially growing cells. Clearly, in exponential Na11+ cells, radiosensitisation by PIMO increases as a function of Ci. In contrast, no similar increase in radiosensitisation was observed in plateau-phase cells. Furthermore, the maximal Ci value obtained in exponential cells (437 µg/g) was limited by toxicity, whereas in plateau-phase cells a Ci value of 628 µg/g was achieved with no loss in plating efficiency.

Liquid scintillation studies

[^{3}H]-PIMO radioactivity in the blood and in the tumour is shown in Fig. 3. The radioactivity in the blood decreased with time after administration, whereas that in the tumour increased to a plateau. The tumour/blood ratio was relatively constant (2–3) at between 30 and 60 min.

Histology studies

The cellular zone represented only 51% of the tumour. Figure 4 shows the melanin content, with the highest concentrations of melanin being found in the zones of tumour in which picnotic cells form the largest proportion of cells (Fig. 4b). The radioactivity derived from [^{3}H]-PIMO was not evenly distributed throughout the tumour. The results of grain counts from two experiments are given in Table 1; it is apparent that the necrotic zones containing numerous picnoses were most highly labeled, showing values some 40 times greater than the background level. In contrast, viable zones of tumour and necrotic regions containing no identifiable cell fragments or picnotic cells showed substantially lower grain counts. Only one-fifth of the total radioactivity was located in cellular zones. Although the

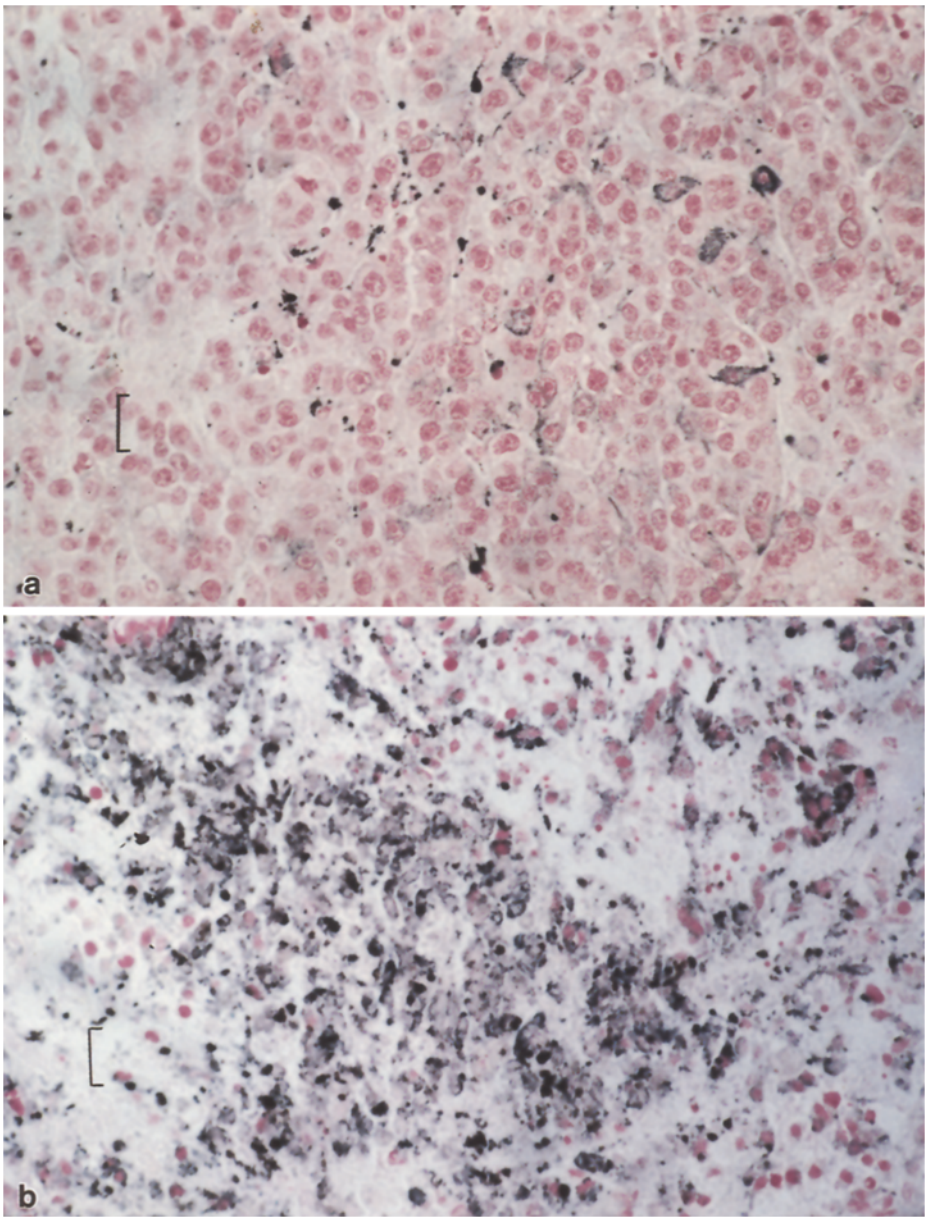


Fig. 4 a, b. Histological sections of Na11+ tumours showing melanin **a** in the cellular zones and **b** in necrotic zones where picnotic cells are found. Bar = 40 µm

Table 1. Distribution of radioactivity derived from [^{3}H]-PIMO in tumours and percentage of total radioactivity

	Tumour 1			Tumour 2		
	Cellular zone (CZ)[a]	Necrotic zone		Cellular zone (CZ)[a]	Necrotic zone	
		With picnotic cells and cell fragments (NZP)[b]	Without any tumour-cell material (NZ)		With picnotic cells and cell fragments (NZP)[b]	Without any tumour-cell material (NZ)
Mean number of grains (MNG)	156 (20)	621 (86)	209 (21)	140 (7)	533 (30)	81 (13)
% Total area (TA)	52	35	13	44	43	13
% Total radioactivity[c]	25	67	8	20	76	4

Numbers in parentheses represent standard errors; 8–40 fields were studied per zone (background value = 15)

[a] See Fig. 4 a for the histology of the cellular zone

[b] See Fig. 4 b for the histology of the necrotic zone with picnotic cells and cell fragments

[c] % Total radioactivity: $MNG_{CZ} \times$ % $TA_{CZ}+MNG_{NZP} \times$ % $TA_{NZP}+MNG_{NZ} \times$ % $TA_{NZ} = 100\%$

melanin content and the radioactivity derived from [^{3}H]-PIMO were not identified on the same slide, it is clear that the highest activity and the highest staining of melanin were found in the same zones.

Discussion

It has been suggested that weak bases can concentrate in melanin-containing cells [3, 20–22, 31, 35, 36]. The intracellular concentrations of weak bases such as PIMO [4], RSU 1069 and RSU 1164 [37] are increased at elevated extra-cellular pH, and their much higher uptake into melanotic as compared with non-melanotic cells led to the suggestion that the intra-cellular pH of melanotic melanoma cells is lower than the pH of non-pigmented cells. However, other mechanisms are likely to be involved in the concentration of weak bases in melanotic melanomas. The present in vitro results show that the pigmentation is higher in plateau-phase cells relative to exponentially growing Na11+ cells; furthermore, when the cells reach the plateau phase, a continuous and rapid increase in pigmentation is observed. For plateau-phase melanotic cells, the results clearly show that the intra-cellular concentration of PIMO depends strongly on the intra-cellular concentration of melanin: the higher the melanin content, the higher the PIMO concentration (Fig. 1). PIMO had no greater radiosensitising effect on these cells than it had on exponentially growing cells, despite the observation that the intra-cellular concentration of PIMO was higher and the plating efficiency was not modified. An association between PIMO and melanin could explain these results, especially if the melanin is remote from the DNA [34], thus spatially preventing PIMO from exerting its radiosensitising effect.

The data for melanoma transplanted into nude mice show that at a short time after its administration, PIMO accumulates in tumours (Fig. 3). Histological data derived from tumours excised at 24 h after the administration of [^{3}H]-PIMO show the presence of label to be coincident with areas of high melanin content, which would be consistent with the in vitro results. It is unlikely that this localisation would be a consequence of passive sequestration onto or association with melanin; rather, it is probably due to selective hypoxia-induced binding in these areas. In non-melanotic tumours it has been claimed that [^{14}C]-misonidazole [2, 11] and PIMO [19, 29] accumulate in viable hypoxic tumour cells, whereas other investigators have reported that uptake is lower in necrotic tumours [7, 27] or that there is no link between accumulation and necrosis [21, 26]. On the basis of our results, it is difficult to determine the influence of the necrotic areas on PIMO accumulation, as these regions were also those in which the melanin content was the highest. It must be emphasised that although it was not possible to evaluate the melanin content in the necrotic zones, these dying cells probably contained much more melanin than did the plateau-phase cells we studied; indeed, the plateau obtained in the present studies is not a perfect one, since 11% of the cells remained in the S phase (unpublished results).

On the basis of the above-mentioned observations, it is possible to provide an explanation for our previous results, which indicated an inability of PIMO to sensitise Na11+ tumours [35]. The accumulation of PIMO in the tumour (liquid scintillation studies) may well have resulted from the accumulation of PIMO in necrotic zones containing picnotic cells of very high melanin content. Its accumulation in these regions cannot influence radiosensitivity; only the PIMO in areas of clonogenic hypoxic cells is important.

In conclusion, the present results obtained both in vitro and in vivo indicate that PIMO accumulates in very heavily pigmented melanoma cells. The in vivo results also show that the accumulation of label derived from PIMO is higher in the necrotic zones with picnosis than in the cellular areas. The latter findings could explain why the intra-cellular accumulation of PIMO is not linked with a radiosensitising effect in melanotic melanoma. To explain the lack of efficiency of PIMO in vitro, a mechanism such as the localisation of PIMO far from the DNA close to the melanin content or a very low sub-cellular pH must be supposed. As far as melanotic melanomas are concerned, PIMO is probably not the compound to be used in clinical radiotherapy.

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