# Immunophenotypical markers, ultrastructure and chemosensitivity profile of metastatic melanoma cells

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## Abstract

The survival of patients affected by cutaneous melanoma has improved dramatically in the last 10 years, because of earlier diagnosis. Despite this, the therapeutic results obtained in metastatic melanoma (MM) are very disappointing due to its poor responsiveness to cytotoxic agents. In this type of solid tumor, tumor chemosensitivity assays have been suggested to be an important tool to predict clinical responsiveness to therapy. Metastatic melanoma cells (MMCs) were obtained from subcutaneous melanoma metastases of five patients and cultured for several consecutive passages. An immunofluorescence and an electron microscopic study were performed in order to establish the ultrastructural and physiopathological features of MMCs. A sulphorodamine-B test was used to measure in vitro sensitivity of MMCs to temozolomide, cisplatin, vindesine, taxol and interpheron alpha-2a. Following a 72 h exposure, maximum activity was obtained with vindesine (median inhibitory concentration, $IC_{50}$, 0.23 nM) and taxol (median $IC_{50}$ 0.31 nM). Cisplatin median $IC_{50}$ values were higher (4.6 $\mu$M) than taxol and vindesine, but still in the range of clinically achievable plasma concentrations. Temozolomide inhibited cell proliferation only at very high concentrations (median $IC_{50}$ 228 $\mu$M). No significant cell growth inhibitory effects ($\leq$25%) were observed with interferon alpha-2a concentrations up to 8000 IU/ml. MMCs expressed progression markers typical of cutaneous metastatic melanoma and showed poor sensitivity in vitro to most anticancer drugs tested, including temozolomide. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords*: Melanoma immunophenotypical markers; Ultrastructure; In vitro chemosensitivity test; Sulphorhodamine B assay

## 1. Introduction

The survival of patients with cutaneous melanoma has improved dramatically in the last 10 years, especially due to the increasingly earlier diagnosis [1]. Metastatic melanoma (MM), conversely, represents a very difficult challenge for medical oncologists having a median survival of only 7 months and an overall 5-year survival <10% [2,3], despite the several immunochemotherapy approaches attempted. These poor results are due to the particularly low chemosensitivity of MM.

The basic defects which underlie chemoresistance in human solid tumors are related to altered expression of specific drug resistance genes [4] or of oncogenes (e.g. p53, bcl2) [5–7] also involved in the development of generalized drug resistance. For instance, the development of resistance to temozolomide in melanoma cells is often accompanied by an increase in $O^6$-methylguanine-DNA methyltransferase activity and by reduction of DNA mismatch repair proteins [8], the latter also being a main mechanism

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involved in resistance to platinum compounds [9]. The acquisition of an altered oncogenic phenotype (e.g. mutated p53, loss of p16 gene expression) which occurs frequently in primary cutaneous melanoma [10–12] may also be the basis for melanoma cells to become resistant to cytotoxic drugs.

According to many reported clinical trials, the chemosensitivity of MM to currently used drugs is heterogeneous [13]. This offers a possible explanation for the observed clinical response, often unpredictable and overall poor. Chemosensitivity assays have been recently proposed as an important tool to predict tumor responsiveness to clinical treatment [14–16].

In this study, the cytotoxicity of the drugs usually used in the clinical treatment of MM was assessed in vitro by a sulphorodamine-B colorimetric assay [17] against metastatic melanoma cells (MMCs) from patients. MMCs isolated from subcutaneous melanoma metastases and cultured for several consecutive passages, were also studied in immunofluorescence and at electron microscopy.

## 2. Material and methods

### 2.1. Patient characteristics

Cutaneous melanoma metastases were taken from five patients whose major clinical patterns are summarized in Table 1. Three patients had not received previous drug treatment (nos. 1, 2 and 4), while two patients (nos. 3 and 5) were administered interferon alpha-2a (10 millions s.c. three times weekly). Following biopsy, all five patients received medical treatment for their advanced disease. Patient no. 1 received two courses of temozolomide, (250 mg/$m^2$ i.v. daily for 5 consecutive days every 1 month) but progressed and died after 4 months due to distant metastases. Patient no. 2 was given interferon alpha-2a (10 millions s.c. three times weekly) and this treatment is still ongoing 16 months later. The patient is still experiencing a partial response. The brain metastasis of this last patient was treated with standard whole brain radiotherapy.

Patient no. 3 received combined chemotherapy (one course of the CVD regimen, i.e. a combination of cisplatin, vindesine, dacarbazine) but died due to disease progression after 1.5 months. Patient no. 4

underwent six courses of dacarbazine treatment, experiencing stable disease, thereafter new metastases in transit appeared and interferon alpha-2a was administered (for 3 more months) but disease progressed and the patient died 8 months after therapy stop. Patient no. 5 was given a combination of dacarbazine and interferon alpha-2a for 8 months (disease stabilization was obtained) and then two courses of CVD, but he died 3 months after the latter treatment.

### 2.2. Melanoma specimens and cell culture

The metastatic skin samples were taken from subcutaneous metastases of five melanoma patients. Each skin metastasis was deprived of fat, washed many times in phosphate buffered saline (PBS) and antibiotics and cut into many tiny pieces. The first enzymatic digestion was performed with protease (Boeheringer/Mannheim, Mannheim, Germany) for 4 h at 37°C in RPMI 1640 medium (Calbiochem, Parmstad, Germany) with 1% penicillin/streptomycin (Sigma-Aldrich, Milan, Italy). The medium with the cells was then centrifuged and the supernatant was discarded. A second enzymatic digestion was then performed with collagenase (Sigma) for 3 h at 37°C in 5% $CO_2$ humidified atmosphere. The enzymatic action was stopped with a combination of Tris Base, NaCl and EDTA. The cells were then put in RPMI 1640 medium with 10% fetal calf serum (FCS) (Sigma), 5% penicillin/streptomycin (Sigma) and 5% glutamine (Sigma) and cultured for numerous consecutive passages (at least 20 passages for each patient).

### 2.3. Assessment of immunophenotype

MMCs were grown on chamber slides (Nunc International, Roskilde, Denmark), were rehydrated, blocked with 5% FCS, and incubated with primary monoclonal antibodies against S-100 (Sigma), HMB-45 (Dako, Milan, Italy), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sera Lab, Heidelberg, Germany), c-kit (Novo Castra, Segrate, Milan, Italy). This incubation was protracted for 90 min at 37°C and was followed by a fluorescein-isothiocyanate-conjugated goat anti-mouse antibody (Sigma). The slides were mounted with Gel/Mount (Biomeda, Augst, Switzerland) for 1 h at 37°C and observed under a Zeiss Axioskop microscope

Table 1
Clinical and immunophenotypic characteristics of the patients[a]

| Patient no. | Age/sex | Stage | Metastatic site (s) | Pre-treatment | Post-treatment | Resp | Survival (months) | S-100 | HMB-45 | GM-CSF | c-kit |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 34/M | IV | Skin<br>Surrenal gland<br>Brain | None | Temozolomide | P | 4 | + | + | − | − |
| 2 | 70/F | III-B | Skin<br>Brain | None | IFN-α2a | PR | 16+ | + | + | − | + |
| 3 | 51/M | IV | Lung<br>Lymphonodes<br>(multiple sites) | IFN-α2a | CVD regimen | P | 1.5 | + | + | + | + |
| 4 | 72/M | III-B | Skin<br>Liver<br>Lymphonodes<br>Brain | None | 1 DTIC<br><br>2 IFN-α2a | S<br><br>P | 17 | + | + | + | − |
| 5 | 79/M | IV | Skin<br>Lung<br>Lymphonodes | IFN-α2a | 1 DTIC + IFN-α2a<br><br>2 CVD regimen | S<br><br>P | 13 | + | + | + | |

[a] CVD, cisplatin, vindesine and dacarbazine; P, progression; PR, partial response; and S, stable disease.

equipped for epifluorescence. The working dilutions for the monoclonal antibodies were the following: prediluted for S-100, 1:30 for HMB-45, 1:60 for GM-CSF, 1:200 for c-kit.

## 2.4. Morphology

For electron microscopy analysis, samples were collected at the very beginning of all cultures, after five passage and after ten passages in vitro. This procedure has already been standardized in our laboratory [18]. At each time point, MMCs grown in Petri culture dishes (Costar, Cambridge, MA) were washed with PBS, trypsinized and then fixed with a mixture of 2% formaldehyde and 2.5% glutaraldehyde in 0.1 mmol/l cacodylate buffer, pH 7.4, at room temperature for 10 min; the MMCs were then osmicated and embedded in Epon. Ultrathin sections were stained with uranyl acetate followed by bismuth tartrate and lead citrate and observed in a Siemens 102 electron microscope, at 80 kV.

## 2.5. Drugs

Temozolomide, cisplatin, vindesine, taxol and interferon alpha-2a were obtained from commercial sources. Stock solutions of temozolomide were prepared in RPMI 1640. Stock solution of temozolomide was freshly prepared in dimethylsulphoxide (0.66% in RPMI); serial dilutions of all the drugs for incubation were prepared in RPMI 1640 complete medium.

## 2.6. Cell growth inhibition studies

MMCs were seeded in 96-well microplates at a cell density of $5 \times 10^3$ cells/ml and allowed to grow for 24 h before treatment. In vitro cytotoxic single agent activity was evaluated following incubation of cells for 72 h to: temozolomide ($5 \times 10^{-8}$–$5 \times 10^{-4}$ M), cisplatin ($1 \times 10^{-8}$–$5 \times 10^{-5}$ M), vindesine ($5 \times 10^{-13}$–$5 \times 10^{-9}$ M), taxol ($5 \times 10^{-11}$–$5 \times 10^{-7}$ M) and interferon alpha-2a (125–8000 IU/ml). The drug concentrations tested were in the range of those achievable in the plasma following administration of the usual therapeutic drug doses [19–23]. At the end of treatment cells were fixed with trichloroacetic acid (50% aqueous solution) at 4°C for 1 h, air-dried and stained for 30 min with sulphorodamine

B (SRB) 0.4% in 1% acetic acid at room temperature. Then SRB was removed and protein-bound dye was extracted with 10 mM unbuffered Tris base (pH 10) for 15 min in a gyratory shaker. The optical density was read in a computer-interfaced, 96-well microtiter plate reader at 540 nm (Model Benchmark, Biorad).

Chemosensitivity of MMCs was expressed as $IC_{50}$ corresponding to the concentration of drug producing a 50% inhibition of cell proliferation as compared to untreated cell control.

## 2.7. Statistical analysis

Cell growth inhibitory effects produced by the different drugs on MMCs were analyzed by Student's t-test.

## 3. Results

### 3.1. Ultrastructure data

All MMCs expressed the S-100 (Fig. 1a and Table 1) and HMB-45 antigens (Fig. 1b and Table 1) which are both melanocyte markers; MMCs obtained from three patients were GM-CSF positive (Fig. 1d and Table 1); MMCs obtained from one patient were c-kit positive (Fig. 1c and Table 1); MMCs from the fourth patient were GM-CSF and c-kit positive and MMC of the fifth patient expressed neither GM-CSF nor c-kit. This phenotype was evaluated at each passage in vitro for S-100 and HMB-45, and at each third passage for the antigens GM-CSF and c-kit. The MMCs were cultured for 20 passages and the immunophenotype was always the same.

While the expression of c-kit by MMCs is indicative of a biological influence by stem cell factor (SCF) on MMCs and is variously expressed by MMCs, GM-CSF is always up-regulated in late melanoma metastases. By electron microscopy, MMCs showed the typical morphology of well differentiated melanocytes, which were characterized by dendritic morphology, were rich in organelles such as mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus (Fig. 2) and, above all, there were melanosomes in different stages of maturation in their cytoplasm (Fig. 3). The cells connected to each other without specialized junctions such as
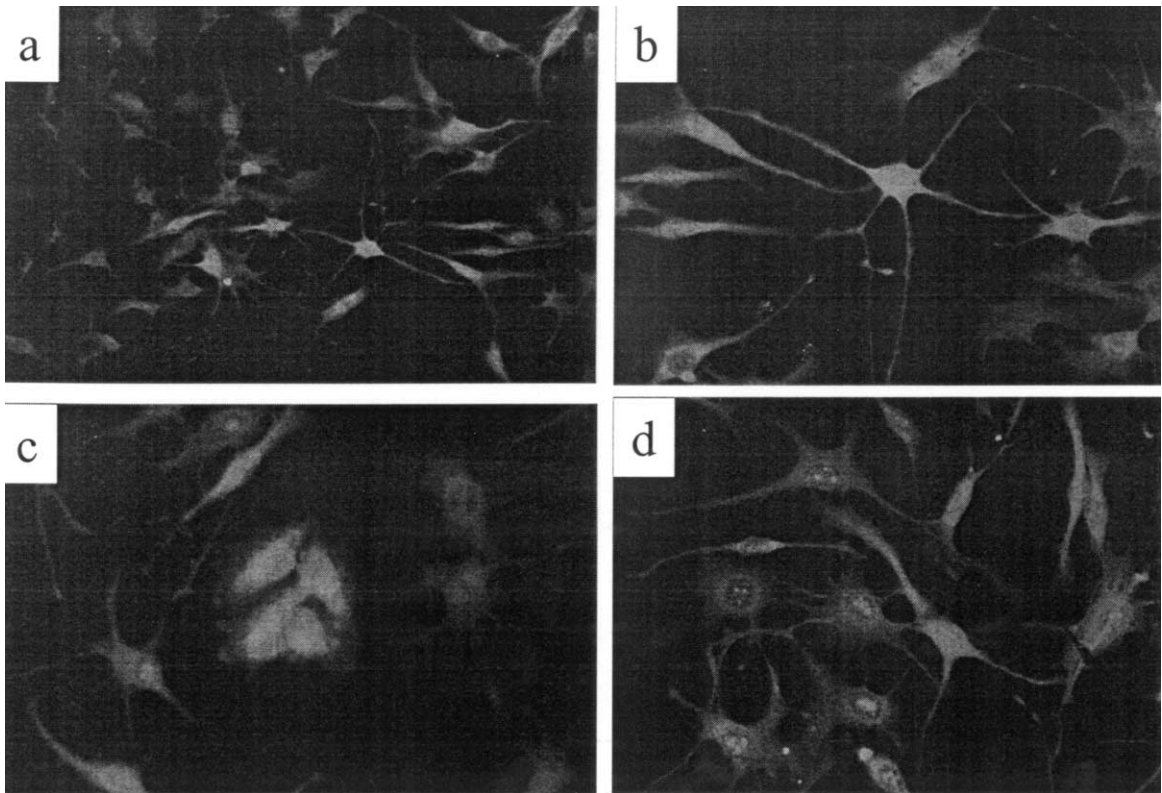
Fig. 1. Indirect immunofluorescence: MMC$_s$ in culture, expression of melanocyte immunophenotypical markers: (a) S-100; (b) HMB-45; (c) cells positive to c-kit; and (d) to GM-CSF. (Magnification: ×200).

desmosomes. All the cells showed no signs of apoptosis, neither at cytoplasmic and nuclear membranes nor at organelles, as shown in all figures.

## 3.2. Effects of drugs on cell growth

Concentration-response curves and IC$_{50}$ values concerning in vitro cytotoxicity tests are reported in Fig. 4 and Table 2. The most in vitro active drugs were taxol and vindesine, with IC$_{50}$ values ranging from 0.079 to 0.4 nM and from 0.12 to 0.27 nM, respectively, in the five cases tested (median IC$_{50}$ values 0.31 and 0.23 nM, respectively).

MMCs were less sensitive to cisplatin with IC$_{50}$ values ranging from 3.3 and 4.7 μM (median value 3.8 μM). In all five cases temozolomide IC$_{50}$ values higher than 200 μM have been obtained (median value 228 μM). No significant inhibitory effects on cell growth ($\geq 25\%$) were noted using interferon alpha-2a concentrations up to 8000 IU/ml.

Thus the in vitro potency of the drugs tested could be graded as follows: vindesine = taxol > cisplatin > temozolomide > interferon alpha-2a. The differences in cytotoxicity observed among the different drugs were statistically significant ($P < 0.05$).

Interestingly, the drug sensitivity patterns of MMCs did not vary in relationship to expression of tumor progression markers tested (GM-CSF, c-kit).

Despite the limited number of cases a correlation between in vitro cytotoxicity data and clinical response of patients treated with chemotherapy was attempted. Disparate clinical results were obtained in patients who were administered interferon alpha-2a after biopsy (i.e. a partial response in patient no. 2, progression in patient no. 4 who received single agent interferon alpha-2a as first-and second-line treatment for their metastatic disease, respectively; stable disease in
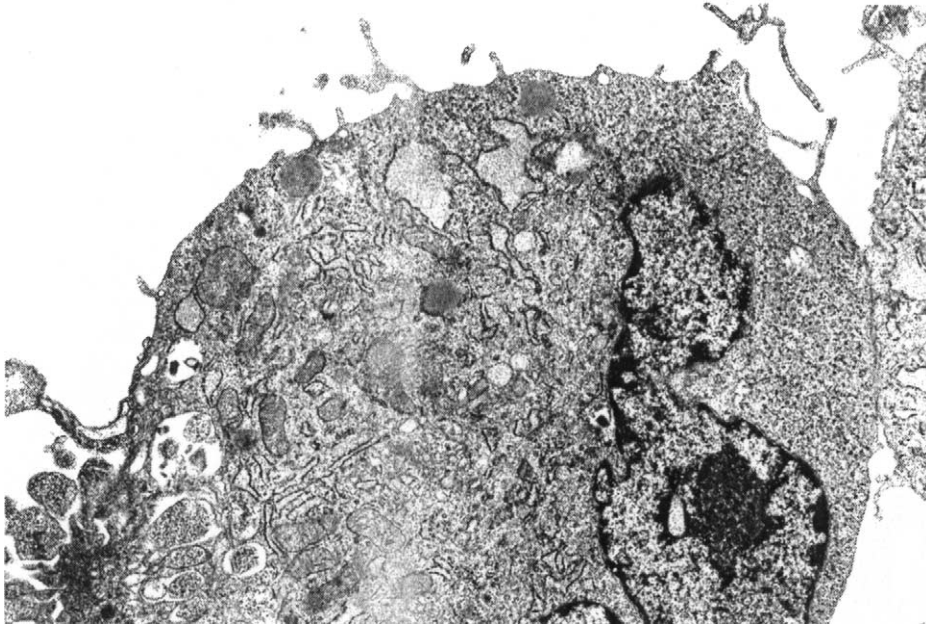
Fig. 2. Electron microscopy: a melanocyte characterized by dendritic morphology; its cytoplasm appeared rich in organelles, especially mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus. (Magnification: ×15 000).
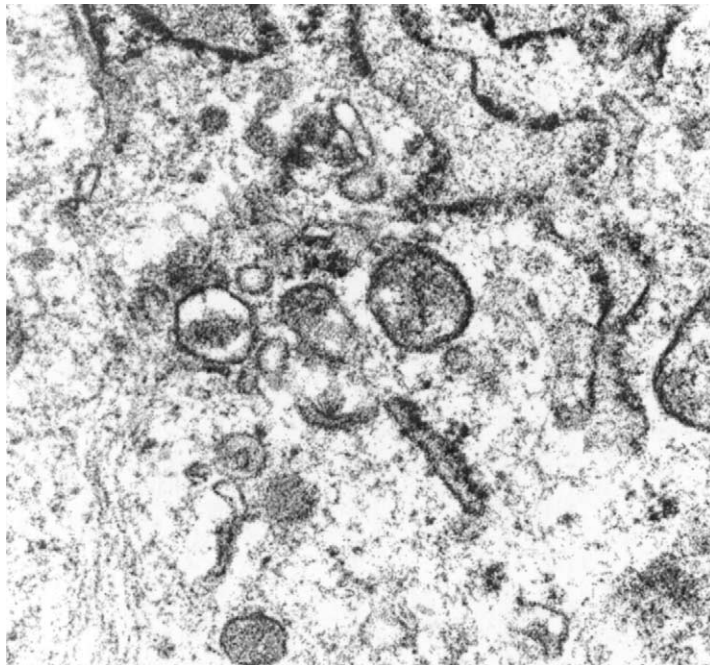


Fig. 3. Electron microscopy: melanosomes in various degree of differentiation were present in the cytoplasm of MMCs. (Magnification: ×75 000).
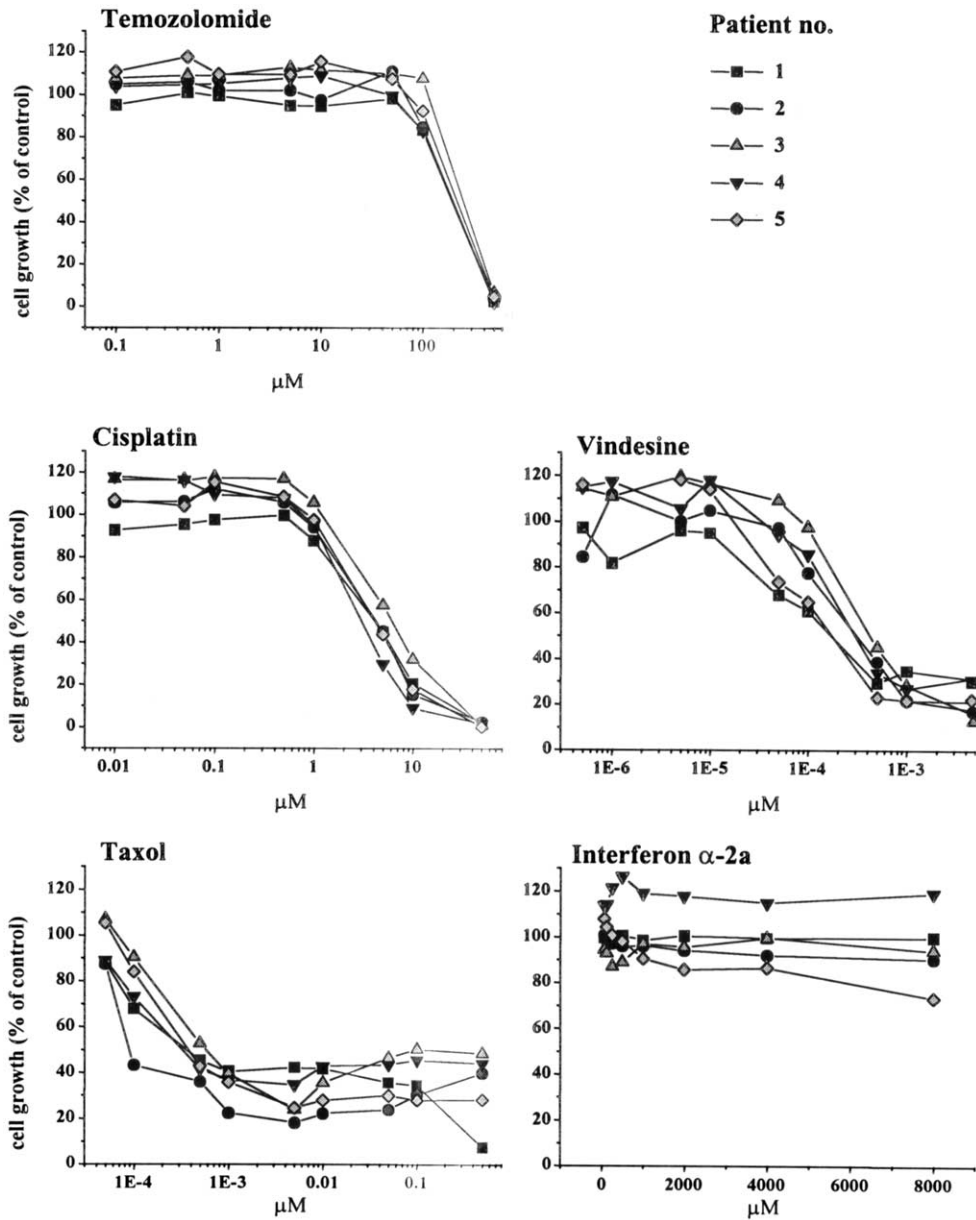
Fig. 4. Effect of drugs on metastatic melanoma cell growth. Cells were exposed to drugs for 72 h. Each value represents the mean of eight wells of a representative experiment repeated at least twice.

patient no. 5 who was treated with a combination of interferon alpha-2a and dacarbazine after tumor biopsy).

Following temozolomide or dacarbazine treatment clinical responses also varied. In two cases (patients nos. 4 and 5) first-line treatment with dacarbazine alone or dacarbazine plus interferon-alpha-2a induced disease stabilization, while in two other cases (patients nos. 3 and 5) a dacarbazine based regimen (CVD) did not hamper disease progression.

Table 2
Cytotoxicity data following continuous drug exposure (72 h) of metastatic melanoma cell cultures[a]

| Drugs | IC$_{50}$ ($\mu$M) Patient no. | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | Median |
| Temozolomide | 228 | 227.5 | 245 | 220 | 246.5 | 228 |
| Cisplatin | 3.0 ± 0.5 | 3.3 ± 0.4 | 3.8 | 4.6 | 4.7 | 3.8 |
| Vindesine | 0.00012 | 0.00027 | 0.00027 | 0.00023 | 0.00021 | 0.00023 |
| Taxol | 0.00034 ± 0.0001 | 0.000079 ± 0.00001 | 0.00031 | 0.00026 | 0.0004 | 0.00031 |
| Interferon-$\alpha$-2a (UI) | >8000 | >8000 | >8000 | >8000 | >8000 | nd |

[a] Data are reported as IC$_{50}$ ($\mu$M) values expressed as mean ± SE of at least three determinations or mean of two determinations.

Temozolomide was used in only one patient (patient no. 1) but failed to control disease progression. The clinical use of cisplatin and vindesine was limited, in our case series, to two patients, within the CVD regimen, (patients nos. 3 and 5) and was unsuccessful. None of the patients received taxol therapy. Agreement between in vitro drug sensitivity/resistance data and clinical response to the seven treatments performed was present in only two cases while discrepancies were observed in the other five cases.

## 4. Discussion

The immunophenotypical and ultrastructural data proved that all the cell cultures obtained with this isolation tecnique were melanocytes. All five cell cultures expressed the antigens S-100 and HM-B45, immunophenotypical markers of melanocytes [24]. Melanocytes obtained from two patients were c-kit positive; c-kit is the natural ligand of SCF, a cytokine which controls human melanocyte homeostasis, promoting hyperplasia and functional activation on melanocytes in vivo [25] and growth inhibitory effects on c-kit expressing melanoma cells [26]; a downregulation of c-kit has been reported in the later stages of melanoma that might permit escape from c-kit growth control [27] or allow melanoma cells to evade immunosurveillance by reducing the expression of differentiation antigens [28].

Three MMCs were positive for GM-CSF. The expression of this cytokine and other growth factors is correlated with tumor progression and is usually increased in later stages of melanoma [29,30].

The ultrastructural data proved that all the cells were well differentiated melanocytes as shown by the presence of smooth and rough endoplasmic reticulum, Golgi apparatus, the richness in mitochondria and above all by the presence of many melanine granules in various stages of maturation within the cytoplasm of MMCs. None of the melanocytes was in apoptosis, as demonstrated by the integrity of cytoplasmic and nuclear membranes, as well as that of mitochondria and by the total absence of apoptotic bodies.

In regard to the chemosensitivity assay data, the concentration-response curves for each single drug were similar in all cell cultures tested. Although a slight variability in cytotoxic effects was observed at low drug concentrations for each single melanoma cell culture, the IC$_{50}$ values were in fact very similar.

Differences in in vitro cytotoxicity induced by the different drugs used were however remarkable. The in vitro cyotoxic activity of taxol and vindesine was very high (IC$_{50}$ values in the nanomolar range), mainly below the peak concentrations obtainable in the plasma with clinical administration of these drugs [23,19].

Considerable in vitro cytotoxic effects were also obtained with cisplatin at median IC$_{50}$ in the range of clinically achievable plasma concentrations [22].

On the contrary a high degree of in vitro resistance to temozolomide and interferon alpha-2a was observed.

In our experience disparate clinical results were obtained in patients receiving interferon alpha-2a after biopsy, with poor correlation to in vitro results.

To explain the lack of in vitro activity by interferon alpha-2a, it should be remembered that this drug has only minor direct cytotoxic effects on MMCs but possesses diverse immunomodulatory effects on

tumoral cells which cannot be fully reproduced in vitro. In particular, interferon is much more effective on cells of the microenviroment than on MMCs [31,32]; its antiproliferative and immunomediated effects are obtained through enhanced natural-killer cell activity or upregulation of tumor antigens and/or human leukocyte antigen classes I and II antigens [32].

Temozolomide is a novel oral alkylating agent which, at variance with dacarbazine, does not need hepatic cytochrome p450-mediated metabolic activation to generate an active drug such as imidazole-4-carboxamide [20]. It is thus an important tool for detecting possible in vitro activity of triazenes.

While the efficacy of temozolomide in metastatic melanoma has been widely demonstrated in clinical trials [33], high levels of in vitro resistance were observed in our case series. Raymond and coworkers [34] reported, however, 33% in vitro activity of temozolomide in melanomas as detected by a soft agar tumor cloning system.

The clinical use of cisplatin and vindesine (within the CVD regimen) was unsuccessful, leading to disease progression. Also these results are at odds with the in vitro chemosensitivity data that showed generalized activity of cisplatin (of moderate degree) and of vindesine (of very high degree).

Our data arise from a limited case-series and it is not possible to draw a clear-cut conclusion about the in vitro-studies and the clinicopathological relationship; further research on a more extended number of observations will be necessary. They raise, however, some doubts about the possible clinical predictability of in vitro tumor chemosensitivity assay-results for cutaneous metastatic melanoma. The tecnique that we have used (sulphorodamine B assay) appears to be sensitive and accurate for detecting cytotoxic drug effects in long term primary cultures of MMCs. Other techniques such as those based on soft agar cloning [35] or measurement of intracellular adenosine-5$'$-triphosphate content by chemoluminescence [16,36–37] have obtained positive correlation between in vitro sensitivity data and clinical responsiveness, allowing speculation about the use of tumor chemosensitivity assays to tailor patient therapy. Our results appear to suggest caution in promoting assay-directed chemotherapy for cutaneous melanoma patients, as pointed out also by other authors [38].

## Acknowledgements

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