

Induction of arginosuccinate synthetase (ASS) expression affects the antiproliferative activity of arginine deiminase (ADI) in melanoma cells

ANTONELLA MANCA¹, MARIA CRISTINA SINI¹, FRANCESCO IZZO², PAOLO A. ASCIERTO²,
 FABIANA TATANGELO², GERARDO BOTTI², GIUSY GENTILCORE², MARILENA CAPONE²,
 NICOLA MOZZILLO², CARLA ROZZO¹, ANTONIO COSSU³, FRANCESCO TANDA⁴ and GIUSEPPE PALMIERI¹

¹Institute of Biomolecular Chemistry (ICB), National Research Council (CNR), Traversa La Crucca, 07100 Sassari;
²National Cancer Institute-Fondazione Pascale, Via Mariano Semmola, 80131 Naples; ³Pathology Service, Hospital-University Health Unit (AOU), Via Matteotti 58; ⁴Institute of Pathology, University of Sassari, Via Matteotti 48, 07100 Sassari, Italy

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Abstract. Arginine deiminase (ADI), an arginine-degrading enzyme, has been used in the treatment of tumours sensitive to arginine deprivation, such as malignant melanoma (MM) and hepatocellular carcinoma (HCC). Endogenous production of arginine is mainly dependent on activity of ornithine transcarbamylase (OTC) and arginosuccinate synthetase (ASS) enzymes. We evaluated the effect of ADI treatment on OTC and ASS expression in a series of melanoma cell lines. Twenty-five primary melanoma cell lines and normal fibroblasts as controls underwent cell proliferation assays and Western blot analyses in the presence or absence of ADI. Tissue sections from primary MMs (N=20) and HCCs (N=20) were investigated by immunohistochemistry for ASS expression. Overall, 21/25 (84%) MM cell lines presented a cell growth inhibition by ADI treatment; none of them presented constitutive detectable levels of the ASS protein. However, 7/21 (33%) ADI-sensitive melanoma cell lines presented markedly increased expression levels of the ASS protein following ADI treatment, with a significantly higher IC₅₀ median value. Growth was not inhibited and the IC₅₀ was not reached among the remaining 4/25 (16%) MM cell lines; all of them showed constitutive ASS expression. The OTC protein was found expressed in all melanoma cell lines before and after the ADI treatment. Lack of ASS immunostaining was observed in all analyzed *in vivo* specimens. Our findings suggest that response to ADI treatment in melanoma is significantly correlated with the ability of cells to express ASS either constitutively at basal

level (inducing drug resistance) or after the treatment (reducing sensitivity to ADI).

Introduction

Arginine is involved in the synthesis of a wide range of proteins, production of creatine and nitric oxide, and several metabolic pathways (1). In addition to dietary intake and muscle degradation, a fundamental source of arginine is its endogenous synthesis in somatic cells ubiquitously (this is the reason why arginine is considered as a semi-essential amino acid in adult humans) (1,2). In particular, the ornithine transcarbamylase (OTC) catalyzes the synthesis of citrulline, which, in turn, is converted to arginine by arginosuccinate synthetase (ASS) and arginosuccinate lyase (ASL) (3,4). However, the reactions induced by OTC and ASS represent the rate-limiting step in the recycling of citrulline to arginine; among them, ASS is considered as a key enzyme in the synthesis of arginine at somatic levels in humans (3,4).

Malignant melanoma (MM) and hepatocellular carcinoma (HCC) have been demonstrated to be dependent on exogenous arginine for growth because of the lack of capacity of their neoplastic cells to synthesize arginine (5,6). Depletion of arginine could be achieved by inducing the arginase, which converts arginine to ornithine and urea in mammals (7,8); another enzyme able to deplete the arginine is represented by the arginine deiminase (ADI), which catalyzes, in microbes but not in mammals, the hydrolysis of arginine into citrulline and ammonia (9-11). Recently, the use of ADI conjugated to polyethylene glycol (ADI-PEG) has been introduced into the therapy of both MM and HCC, aimed to achieve an antiproliferative effect through depletion of arginine in cancer cells of such patients (6,12,13). As previously demonstrated by our group, the ADI-PEG treatment of patients with unresectable HCC or advanced MM indeed resulted in measurable antitumour response (14,15), showing also to be effective in lowering plasma concentration of arginine as well as improving liver function in HCC patients with chronic hepatitis C virus infection (16).

Correspondence to: Dr Giuseppe Palmieri, Institute of Biomolecular Chemistry (ICB)-National Research Council (CNR), Traversa La Crucca 3 - Balduina Li Punti, 07100 Sassari (SS), Italy
 E-mail: gpalmieri@yahoo.com

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Finally, resistance to ADI treatment in several cancer cell lines seems to correlate with the rate of activity of the enzymes (mainly, ASS) involved in converting arginine from citrulline (17). In other words, the antitumour activity of ADI-mediated arginine depletion may be strongly dependent on the efficiency by which ASS can regenerate arginine from citrulline at cellular level (probably, the impact of this mechanism on effectiveness of the ADI-PEG therapy is much higher than the auxotrophic requirement of arginine) (6,11,17). In the present study, we evaluated the effect of ADI treatment on OTC and ASS expression in a representative series of primary melanoma cell lines, by evaluating the capability of the ADI antiproliferative activity to induce the ASS expression.

Materials and methods

Cell lines and culture conditions. Melanoma cell lines were obtained from the Institute Dermopatico dell' Immacolata (IDI) of Rome and from the National Cancer Institute of Naples. They were established as primary short-term cell cultures starting from tumour samples of donors patients with documented diagnosis of melanoma, after obtaining their informed consent, as previously reported (18). Short-term cultures of normal human fibroblasts from healthy donors named BJ (CRL-2522) were purchased from the American Type Culture Collection (ATCC) and served as controls. All cells were grown either in Dulbecco's minimal essential (DMEM; Sigma, St. Louis, MO, USA) or in RPMI (Invitrogen, Carlsbad, CA, USA) media, supplemented with 10% FBS and penicillin-streptomycin (100 IU/50 µg/ml), as previously described (19,20).

Cell proliferation assay. Cells were plated in triplicate in 96-well plates, at a density of $3-5 \times 10^3$ per well, in complete medium; after 24 h, medium was replaced with fresh medium only (controls) or medium containing different concentrations of ADI (0-2.5 mU/ml). The percentage of cell proliferation was estimated on day 6 by the colorimetric assay as in Kueng *et al* (21), modified as follows: cells were fixed for 20 min at room temperature with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet in 20% methanol for 20 min, washed in PBS, solubilized with 10% acetic acid and read at 595 nm in a microplate reader (SpectraFluor Plus, Tecan, Austria).

Western blot analysis. Cells were plated in two T75 tissue culture flasks (6×10^5 cells/flask) in complete medium; at days 1, 3 and 5, cells in one flask were treated with medium containing ADI at a concentration required for 50% growth inhibition (IC_{50}), whereas those in the second flasks received the simple replacement of medium (control). At day 6, cells were harvested, using cell-scraper and cold PBS, and then lysed with Cell Extraction Buffer (BioSource International, Camarillo, CA, USA) plus protease inhibitor cocktail (Sigma). For each cell lysate, protein concentration was determined by the QuantiPro BCA Assay Kit (Sigma). Proteins (50 µg per lane) were electrophoresed on sodium dodecyl sulphate (SDS) 12% polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). For immunoblotting, membranes were then

incubated with monoclonal antibodies against ASS (1:2500; BD, Franklin Lakes, NJ, USA) and OTC (1:1000, Santa Cruz Biotech, Santa Cruz, CA, USA). Detection was achieved by HRP-conjugated anti-mouse (1:10.000; Chemicon, Billerica, MA, USA) for anti-ASS antibody or HRP-conjugated anti-rabbit (Santa Cruz Biotech, 1:10.000) for anti-OTC antibody, and enhanced chemiluminescent kit (ECL Advance, GE Healthcare), according to the manufacturer's instructions; expression was normalized to internal controls, using a mouse antibody against GAPDH (Santa Cruz Biotech).

Immunohistochemistry (IHC). For IHC analysis, 4-µm sections from formalin-fixed, paraffin-embedded tissues from 10 primary melanomas and 20 hepatocellular carcinomas were immunostained using anti-ASS monoclonal antibody (BD).

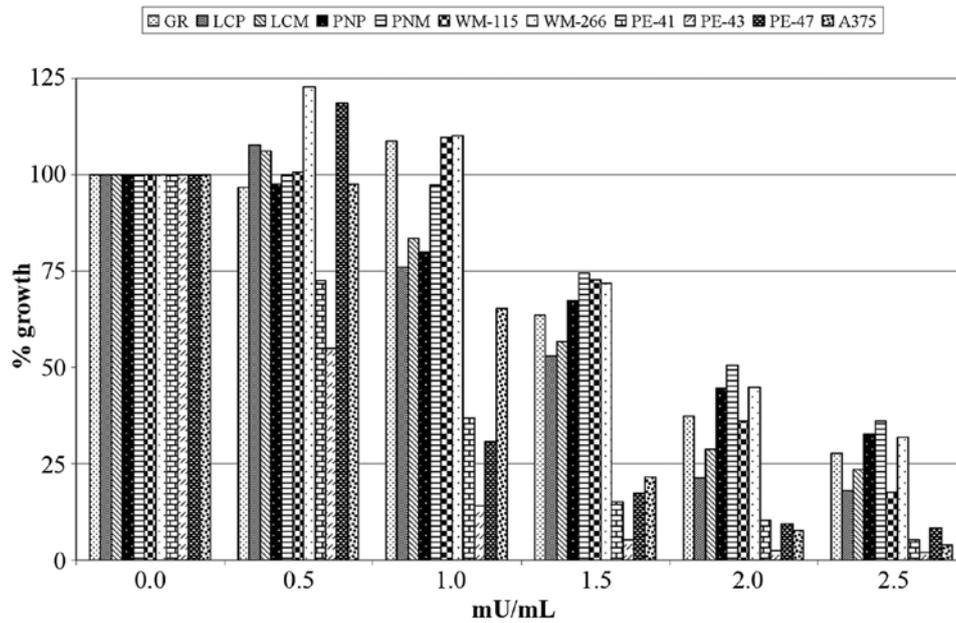
Protocol for immunostaining was previously described by our group (22). Briefly, tissue sections were deparaffinized in xylol and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. After incubation with the monoclonal antibody anti-ASS (dilution, 1:50), the bound antibody was visualized using a biotinylated secondary antibody, avidin-biotin peroxidase complex (Biogenex-Menarini), and the 3-amino-9-ethylcarbazole (DakoCytomation). Nuclear counterstaining was performed with hematoxylin (DakoCytomation). Slides were viewed using a BX61 Olympus Microscope supplied with DP 50 camera and Viewfinder Lite 1.0 Version (Pixera Corporation) image analysis system. Labelling intensity was expressed as percentage of stained area; subcellular staining was evaluated with a x10, x20 and x40 objectives, by two independent observers.

Results

Growth inhibition in ADI-treated melanoma cell lines. To assess the antiproliferative activity of ADI in melanoma, growth assays were performed on 25 MM cell lines established as short-term cultures from primary and metastatic tumours of melanoma patients, using five different concentrations of the drug: 0.5, 1.0, 1.5, 2.0 and 2.5 mU/ml (in addition to the negative control represented by the medium alone). A significant suppression in cell proliferation was observed in 21/25 (84%) MM cell lines treated with ADI in a dose-dependent manner; the median IC_{50} value in this large subset was 1.3 mU/ml (range, 0.4-2.0). Fig. 1 shows the cell growth inhibition of all ADI-sensitive MM cell lines, after five days of treatment with increasing concentrations of ADI. In the remaining 4 (16%) melanoma cell lines, growth was not inhibited and the IC_{50} was not reached, even after a remarkable increase of the ADI concentrations into the medium (up to 100 mU/ml) (Fig. 2). As expected, proliferation of the fibroblasts as control was not inhibited by the ADI treatment (Fig. 2). A summary of all the IC_{50} values obtained in our series of MM cell lines is reported in Table I.

Expression of ASS and OTC proteins in ADI-treated melanoma cell lines. Expression analysis revealed that none of the 21 ADI-responsive cell lines presented detectable levels of the ASS protein before the ADI treatment; conversely, the

A



B

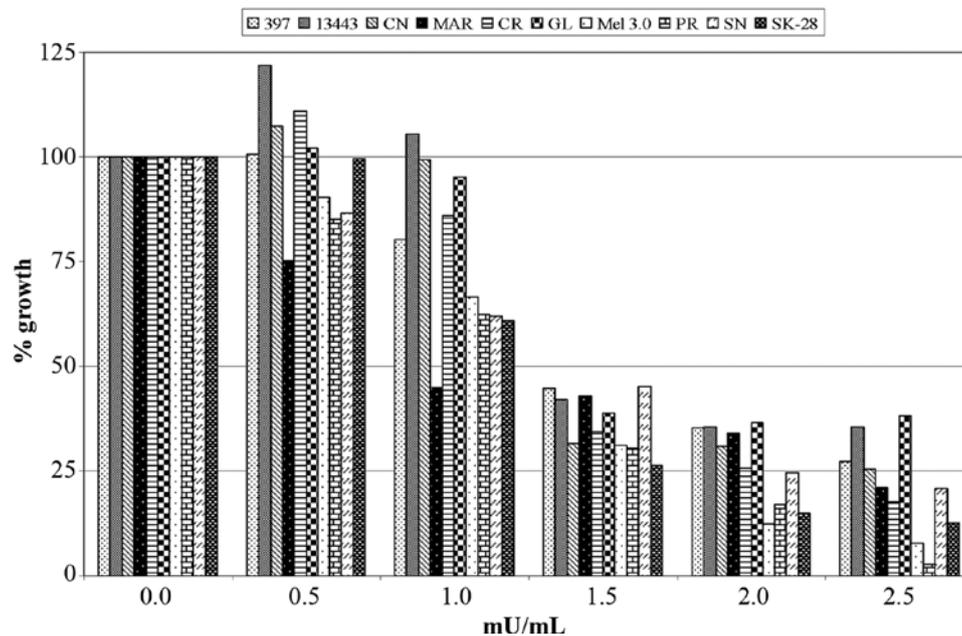


Figure 1. Growth diagrams of ADI-sensitive melanoma cell lines. Human MM cell lines were cultured in presence of RPMI medium alone (negative control) and increasing concentration of ADI for 6 days. Results are expressed as percent of cell growth and represent the average of triplicate cultures performed twice. (A), paired cell lines derived from primary melanomas and MM metastases of the same patients are included.

four melanoma cell lines and the normal fibroblasts, which did not reach an IC_{50} value, showed a constitutive ASS expression in both treated and untreated cells (Table I). In Fig. 3, representative examples of the ASS expression as detected by Western blot hybridization are shown. After treatment with ADI at concentrations corresponding to the IC_{50} values, 7/21 (33%) melanoma cell lines presented a remarkable increase of the expression levels of the ASS protein; six of them were from paired primary and secondary lesions of three patients (Table I). In this subset, the median value of

IC_{50} was 1.8 mU/ml (range, 1.3-2.0) and the average of IC_{50} values was 1.82 mU/ml (Table I). The remaining 14/21 (67%) ADI-responsive cell lines maintained a negative ASS expression after treatment; among them, three cell lines were from paired secondary lesions of the same patient (Table I). In this subset, median value of IC_{50} was 1.2 mU/ml (range, 0.4-1.4) and the average of IC_{50} values was 1.12 mU/ml (Table I). Considering the relationship between the IC_{50} values and the ASS expression status before and after the treatment with ADI (negative/negative, negative/positive and positive/positive), the

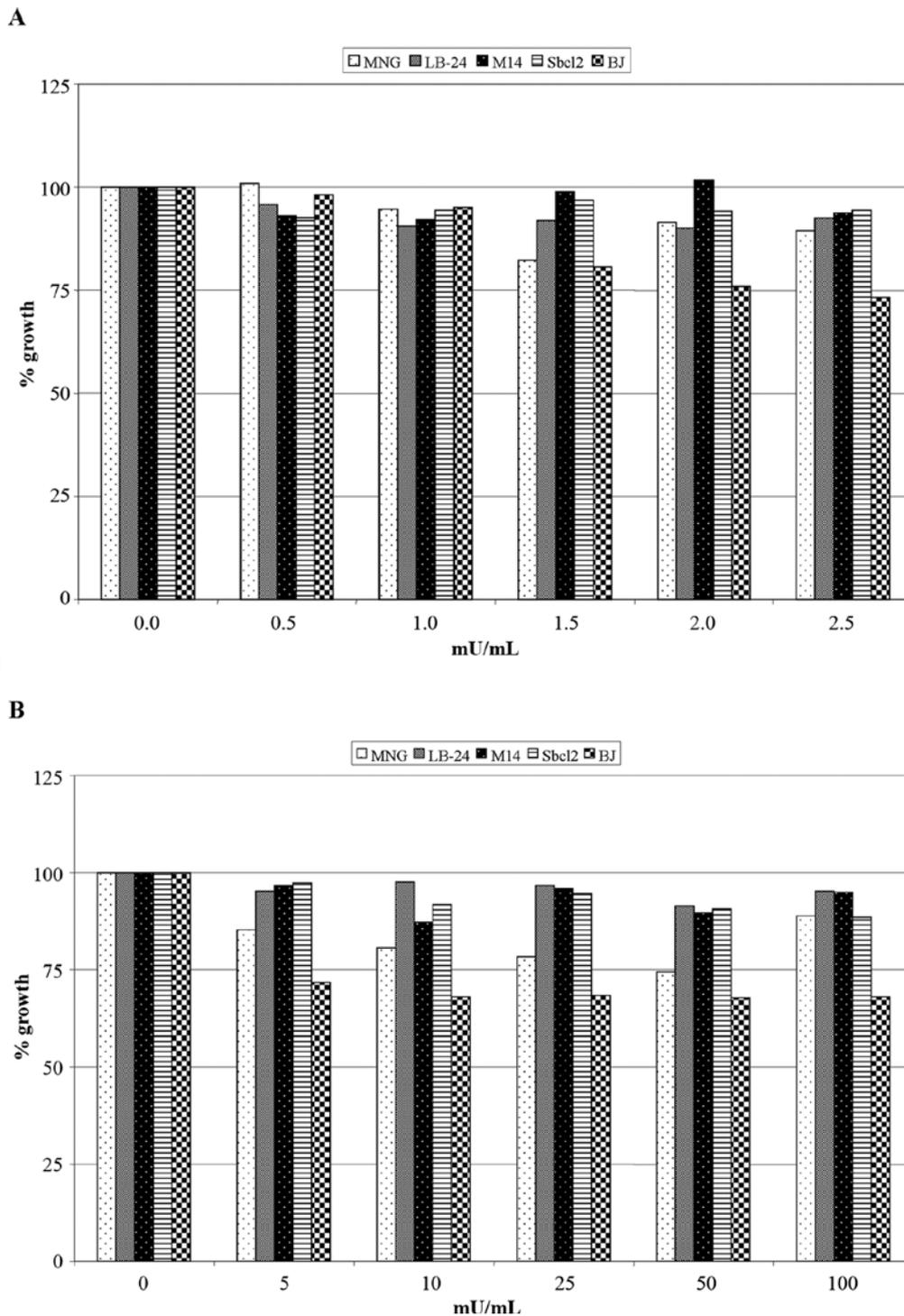


Figure 2. Growth diagrams of ADI-resistant melanoma cell lines. Human MM cell lines were cultured in presence of RPMI medium alone (negative control) and increasing concentrations, (A), as for Fig. 1; (B), up to 100 mU/ml of ADI for 6 days. Results are expressed as percent of cell growth and represent the average of triplicate cultures performed twice.

most drug-sensitive cell lines were those with no detectable expression of ASS in any phase of the treatment; conversely, the MM cell lines with induction of the ASS expression presented a reduced degree of sensitivity to ADI. As expected, the cell lines with constitutive ASS expression, which was not modified by the treatment, were clearly resistant to ADI, even at higher concentrations of the drug. Statistical analysis performed using the IC_{50} values and the ASS expression status as continuous variables indicated that the linear trend

of such an association was significant ($p=0.023$; not shown). Interestingly, the ADI-sensitive cell lines derived from paired primary and metastatic lesions of the same MM patients presented identical responses for ASS expression (presence vs. absence of ASS induction after treatment) as well as similar IC_{50} values when treated with ADI (Table I). Finally, no association was inferred considering the anatomical site of melanoma tissues from which MM cell lines were established (data not shown).

Table I. Comparison between IC₅₀ and ASS expression, before and after ADI treatment.

Cell line	Origin	IC ₅₀ mU/ml	ASS untreated	ASS treated
01 GR-Mel	Primary MM	1,80	-	+
02a LCP-Mel	Primary MM	1,60	-	+
02b LCM-Mel	MM lymph node	1,65	-	+
03a PNP-Mel	Primary MM	1,95	-	+
03b PNM-Mel	MM lymph node	2,00	-	+
04a WM-115	Primary MM	1,80	-	+
04b WM-266	Skin nodule	1,95	-	+
05a PE-Mel-41	Skin nodule	0,80	-	-
05b PE-Mel-43	Lung nodule	0,40	-	-
05c PE-Mel-47	Skin nodule	0,65	-	-
06 A375	Unknown	1,20	-	-
07 397-Mel	Skin nodule	1,40	-	-
08 13443-Mel	MM lymph node	1,40	-	-
09 CN-Mel	MM lymph node	1,35	-	-
10 MAR-Mel	MM lymph node	0,90	-	-
11 CR-Mel	MM lymph node	1,30	-	-
12 GL-Mel	MM lymph node	1,40	-	-
13 Mel 3.0	Unknown	1,20	-	-
14 PR-Mel	Skin nodule	1,20	-	-
15 SN-Mel	Skin nodule	1,35	-	-
16 SK-Mel-28	Unknown	1,15	-	-
17 MNG-Mel	Primary MM	Not reached	+	+
18 LB-24-Mel	Skin nodule	Not reached	+	+
19 M14-RECI	Skin nodule	Not reached	+	+
20 Sbc12	Primary MM	Not reached	+	+
21 BJ	Fibroblasts	Not reached	+	+

Cell lines from paired primary and metastatic MM lesion of the same patients appear in bold.

Since OTC activity has been demonstrated to affect the sensitivity to ADI treatment, the cell lysates from the same series of MM cells were also analyzed for OTC expression by Western blot hybridization. A constitutive expression of the OTC protein was found in all 25 melanoma cell lines; no change in OTC expression was observed after the ADI treatment (Fig. 3). Therefore, OTC did not play any role in determining the responsiveness to ADI treatment in our series.

Immunohistochemical analysis of in vivo tumour tissue. To evaluate the ASS expression among *in vivo* tumour tissues of MM patients from our population (in other words, patients with the same geographical origin of those from whom cell lines were derived), immunohistochemistry was carried out with anti-ASS antibody on sections from the archival tissues of 20 primary melanomas. Moreover, 20 primary hepatocellular carcinomas were included into the IHC analysis, since this tumour is the other main malignancy tightly depending on endogenous ASS activity. A negative immunostaining for ASS protein expression was observed in all analyzed tissues.

In Fig. 4, representative IHC results for MM and HCC cases are provided (including the analysis of a normal kidney tissue section as positive control).

Discussion

The recent introduction of novel anticancer molecules into the clinical practice for the treatment of advanced melanoma [i.e., new drugs targeting signal-transduction pathways or achieving a CTLA-4 blockade (23,24)] further supports the idea that is time for a more appropriate selection of patients to be addressed to the various innovative therapies. Nevertheless, a better clarification of any cause-effect relationship between the new antitumour drugs and their molecular targets will improve the characterization of the subsets of patients who would be expected to be more or less likely to respond to such specific therapeutic interventions.

Recently, a reasonable antitumour activity has been reported by our group for a novel arginine-degrading enzyme-based drug, pegylated arginine deiminase (ADI-PEG), as a treatment

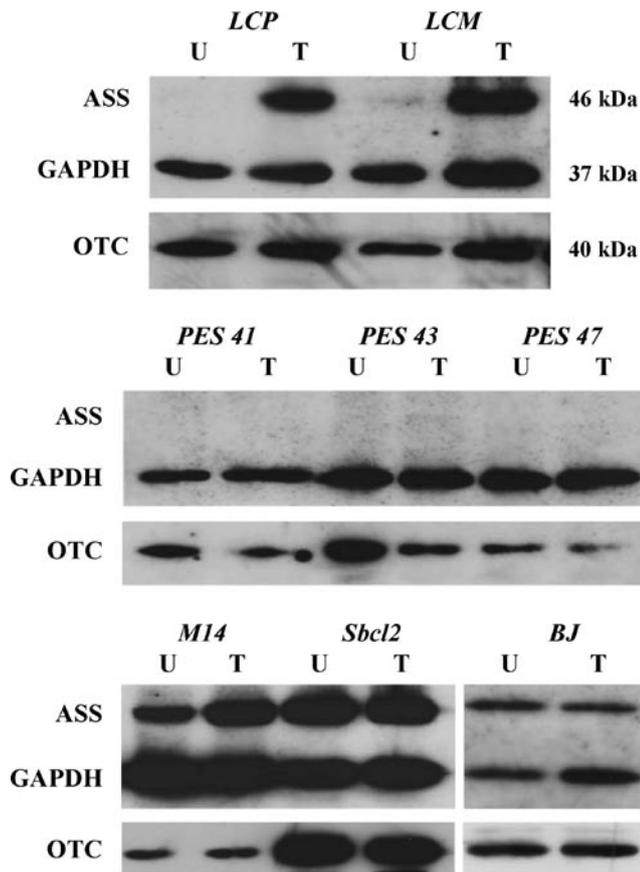


Figure 3. Representative Western blot analysis for ASS and OTC expression in MM cell lines. U, untreated; T, treated.

for melanoma and hepatocellular carcinoma (both tumours are auxotrophic for arginine) (14,15,25). Since the production of endogenous arginine is mainly the result of sequential actions of intracellular OTC and ASS enzymes, an inability to express either enzyme would be expected to result in arginine auxotrophy and sensitivity to ADI-induced arginine deprivation (3,5,26). In this study, we further examined the relationship between the intracellular expression levels of the ASS and OTC proteins and the ADI treatment in a representative series of primary melanoma cell lines (n=25; most of them were established as primary short-term cell cultures from tumour samples of donors patients).

A limited fraction (4/25; 16%) of MM cell lines presented high levels of constitutive ASS expression; as expected, no growth inhibition was achieved when treatment with ADI was performed in such a subset. On the other hand, vast majority (21/25; 84%) of MM cell lines from our series did not produce detectable amount of constitutive ASS protein and were responsive to ADI-driven inhibition of proliferation. However, one third (7/21; 33%) of these ADI-sensitive cells presented an induction of the ASS expression after the drug treatment; in this group, the IC_{50} was obtained using higher concentrations of ADI (Table I). Our findings seem to provide further details on the relationship between cell growth and ADI treatment. Consistently with previous reports (6-7,27-29), lack of expression of ASS markedly predicts sensitivity to ADI treatment; in other words, ADI only inhibits the growth of ASS-deficient cancer cells. As a confirmation of this, all cells presenting

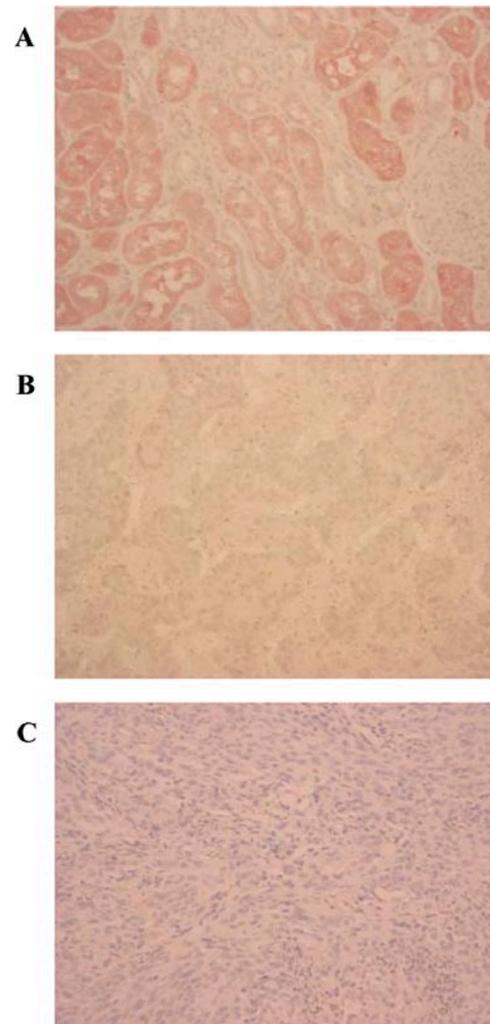


Figure 4. Immunohistochemistry for ASS in *in vivo* tissue sections. Typical examples of IHC analysis for ASS protein on paraffin-embedded tissues: (A) normal kidney as positive control; (B) primary hepatocellular carcinoma; (C) primary melanoma.

constitutive ASS expression were indeed resistant to ADI treatment. In our series, cells showing the capability of inducing the expression of ASS after ADI treatment presented an increased resistance to ADI; in particular, the ADI-driven inhibition of proliferation was significantly affected in a consistent fraction (11/25; 44%) of melanoma cell lines. Conversely, no role for OTC protein on sensitivity to ADI was observed, since expression of such an enzyme was present either before or after the ADI treatment in all cell lines. This latter finding is further evidence of the difference in arginine auxotrophy between human melanomas and hepatocellular carcinomas; indeed, most of the HCC cells seems to be quite unable to express OTC (which may thus act as a key enzyme into the metabolic pathway producing intracellular levels of arginine in this type of tumour) (7,8).

All *in vivo* melanoma and hepatocellular carcinoma tissue samples from our series were negative for ASS expression, as detected by IHC analysis. Unfortunately, only a small fraction of tissues (5 melanomas and 6 hepatocellular carcinomas) from patients previously evaluated for clinical response to ADI treatment [as documented in previous reports (14,15)]

were included in the present study; among them, a partial response was observed in 1/5 (20%) MM patients and 1/6 (17%) HCC patients. Although the amount of such cases is very limited, it is noticeable that the absence of ASS expression in primary tumours did not act as a full predictor of the clinical responsiveness to ADI treatment in our series [putting together the analyzed patients, all eleven cases presented lack of tissue staining for ASS protein whereas only two of them (18%) were found to achieve an objective clinical response to ADI therapy]. In light of our *in vitro* findings, one could speculate that additional clues for a better assessment of the patients' responsiveness to the ADI treatment may be provided by IHC-based evaluations of the ASS expression levels on tissue sections from biopsies obtained after the beginning of the therapy (though, this implies the development of more complex trials, with several ethical aspects to be satisfied). In our case, the absence of available tissue biopsies after ADI treatment within the same series of patients did not allow to fully assess whether the low prevalence of clinical responses in such patients could in some way correlate with a putative induction of the ASS protein expression at somatic level.

Finally, it appears that targeting a single component within the multiple signaling pathways involved in the development and progression of human cancers is unlikely to yield significant antitumour responses. Therefore, further clarification of the main mechanisms involved in controlling the cancer cell growth could permit to select the subsets of patients who would be expected more likely to respond to any specific targeted treatment. In general, our efforts should be focused on avoiding that target-oriented therapies in patients carrying specific molecular alterations achieve different clinical outcomes due to the coexistence of additional alterations in alternative pathways. Correlation between *in vitro* and *in vivo* studies represents the way to evaluate the role of such novel therapies against cancer.

In conclusion, sensitivity to ADI treatment in melanoma seems to be significantly correlated with the capability of cells to express ASS either constitutively at basal level (inducing maximal resistance to the drug) or after the treatment (increasing resistance to ADI).

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