Invest New Drugs (2009) 27:412–418
DOI 10.1007/s10637-008-9188-2

PRECLINICAL STUDIES

Artemisinin reduces human melanoma cell migration by down-regulating αVβ3 integrin and reducing metalloproteinase 2 production

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Received: 29 July 2008 / Accepted: 30 September 2008 / Published online: 28 October 2008

Summary Artemisinin and its derivatives are well known antimalarial drugs, particularly useful after resistance to traditional antimalarial pharmaceuticals has started to occur in *Plasmodium falciparum*. In recent years, anticancer activity of artemisinin has been reported both in vitro and in vivo. Artemisinin has inhibitory effects on cancer cell growth and anti-angiogenetic activity. In the present investigation, we analyzed the inhibitory effects of artemisinin on migratory ability of melanoma cell lines (A375P and A375M, low and medium metastatic properties, respectively). We demonstrate that artemisinin induces cell growth arrest in A375M, and affects A375P cells viability with cytotoxic and growth inhibitory effects, while it was not effective in contrasting proliferation of other tumor cell lines (MCF7 and MKN). In addition, artemisinin affected the migratory ability of A375M cells by reducing metalloproteinase 2 (MMP-2) production and down-regulating αvβ3 integrin expression. These findings introduce a potential of artemisinin as a chemotherapeutic agent in melanoma treatment.

Keywords Artemisinin · Melanoma · Integrin · Metalloproteinase · Migration

Introduction

Melanoma is the most aggressive form of skin cancer, well-known for its resistance to current therapeutic modalities and tendency to induce metastasis [1]. Several studies have showed that an altered expression of different molecular markers correlated with melanoma metastatic phenotype such as integrin β3 subunit occurs during melanoma progression [2]. Furthermore, it appears that the invasiveness and the metastatic phenotype of melanoma depend on the stromal response. In fact, a persistent inflammation, fibroblast activation, the release of proteinases for matrix remodelling, and induction of angiogenesis can be observed during melanoma progression [3].

Artemisinin is a sesquiterpenoid compound extracted from the leaves of wormweed (*Artemisia annua)*, that has been used in traditional Chinese medicine for several centuries. At present, artemisinin and its derivatives are used in human medicine for the treatment of malaria [4]. These molecules are well tolerated by patients and are more and more employed upon occurrence of resistance to the traditional antimalarial agents [5]. Previous studies have shown that artemisinin, apart from its antimalarial activity, also possesses antitumor, anti-angiogenic, and pro-apoptotic effects [6–8]. Consequently, in recent years the hypothesis

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has been advanced that artemisinin and its derivatives can be used as anticancer drugs [9]. Several studies revealed that artemisinin inhibits the growth of many transformed cell lines and has a selective cytotoxic effect. In fact, artemisinin proved to be more toxic to cancer than normal cells, and the artemisinin-tagged holotransferrin showed enhanced potency and selectivity in killing human leukaemia cells [10, 11]. Probably, the cytotoxic effect is due to the high concentration of transferrin receptors in cancer cells that determines an increased iron influx compared to the normal cells [12]. Finally, artemisinin presents an anti-angiogenic activity [13]. Angiogenesis, occurring through the proliferation and migration of endothelial cells, is very important for tumor development. The treatment with artesunate, another artemisinin derivative, inhibits the growth of Kaposi's sarcoma cells, a highly angiogenic multifocal tumor. Furthermore, a correlation exists between the cell growth inhibition and the induction of apoptosis [14]. As for in vivo models, dihydroartemisinin was found to inhibit the growth of rat-implanted fibrosarcoma tumors [15] and cell migration in murine lymphatic endothelial cells [16], while artemisinin itself induced preventive effects on breast cancer development in rats treated with a carcinogenic agent [9].

Considering the importance to contrast melanoma progression by avoiding metastatic cell spread at lymph nodes, we investigated the effect of artemisinin on human melanoma A375M cells, a metastatic derivative of the parental A375P cell line displaying a medium metastatic behaviour.

Materials and methods

Cell culture and treatments

A375P and A375M are human melanoma cell lines with low and medium metastatic properties, respectively [17]. MCF-7 is an human breast cancer cell line, and MKN is a human gastric adenocarcinoma cell line. Cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2. A preliminary dose–response curve was performed to determine the concentration and the time by which artemisinin (Sigma-Aldrich Co., St. Louis, MO) produced significant effects on cell morphology and growth inhibition. Artemisinin was administered to cell cultures as ethanolic solution (1 mM). 3×10^5 cells were plated in 6-multiwell plates (35 mm diameter) with 2 ml DMEM and treated with increasing artemisinin concentrations (25, 100 and 150 μM) for 24, 48 and 72 h. Control cultures received absolute ethanol. Morphological features of A375P and A375M, cells were defined by phase-contrast microscopy (Olympus CDK40) at 20× magnification.

MTT cell proliferation assay

Cells (2×10^3) treated or not with artemisinin were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 μl DMEM, at 37°C and 5% CO_2. After 24 h, 10 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) labeling reagent (Roche Diagnostics, Basel, Switzerland; final concentration 0.5 mg/ml) were added to each well. After 4 h, 100 μl of the solubilization solution (10% SDS in 0.01 M HCl) were added, and the cultures were incubated overnight. The spectrophotometric absorbance was measured using a microplate ELISA reader (Biorad) at 600 nm wavelenght.

Cell migration assay

Cell migration assays were performed under serum-free conditions using Boyden chambers and 8 mm pore size polyvinylpyrrolidone-free polycarbonate filters coated with 5 μg/ml gelatin (Sigma-Aldrich), forming a barrier that is disrupted as cells produce proteinases. A375M cells were treated or not with artemisinin 100 and 150 μM for 72 h, harvested by mild trypsinization, and counted. Viable cells were evaluated by trypan blue exclusion test. A375M cells were allowed to migrate toward the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma-Aldrich) (100 nM for 3 h at 37°C, 5% CO_2) introduced in the lower compartment of the chamber. At the end of the assay, cells that migrated on the lower surface of the filter were fixed in ethanol, stained with haematoxylin, and counted in ten randomly-selected microscopic fields (100×) per filter. Each assay was carried out in triplicate and repeated five times. The data are reported as percentage of the basal random migration toward FMLP in the absence of the artemisinin.

RT-PCR (reverse transcription-polymerase chain reaction) analysis

Total RNA, isolated by the High Pure RNA Isolation Kit (Roche Diagnostics) from A375M cells before and after the treatment with artemisinin 100 and 150 μM for 72 h, was transcribed by reverse transcriptase (Expand Reverse Transcriptase, Roche Diagnostics) at 42°C for 45 min according to the manufacturer's instructions. Two microliters of complementary DNA (cDNA) were amplified in a reaction mixture containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTP, and 2.5 units of Taq DNA polymerase (Roche Diagnostics) in a final volume of 25 μl. For the co-amplification conditions the

PCR was carried out in the presence of 0.5 μM sense and antisense αv, β3 and MMP-2 primers, and 0.05 μM sense and antisense GAPDH or β-actin primers. Conditions and size of the products are reported in Table 1. The reaction was carried out in a DNA thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). β-actin RT-PCR was performed on mRNA extracted at 24 h to confirm that mRNAs were suitable for RT-PCR analysis. The PCR products were analyzed by electrophoresis on 1.8% agarose gel in TBE. Densitometric analysis of ethidium bromide-stained agarose gel was carried out by NIH image V1.6 software. The ratio between the yields of each amplified product and of the co-amplified internal control allowed a relative estimate of mRNA levels in the sample analyzed. The internal control was an housekeeping gene whose PCR product was not overlapping with the gene analyzed.

Protein extraction and western blotting analysis

A375M cells were treated with 100 and 150 μM for 72 h. The cells were scraped with 1 ml PBS and the cell pellet was homogenized with 300 μl ice-cold buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton, 1.5 mM $MgCl_2$, 5 mM EGTA) supplemented with 20 mM sodium pyrophosphate, 40 μg/ml aprotinin, 4 mM PMSF, 10 mM sodium orthovanadate, 25 mM NaF. Total extracts were cleared by centrifugation for 30 min at 4°C at 10,000 rpm and assayed for the protein content by Bradford's method. Fifty micrograms of protein from each cell lysates were separated by a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitro-cellulose membranes; the filters were stained with 10% Ponceau S solution for 2 min to verify equal loading and transfer efficiency. Blots were blocked overnight with 5% non fat dry milk, then incubated with MMP-2 rabbit polyclonal antibody (Santa Cruz, CA), anti-αv rabbit polyclonal antibody (Chemicon, Temecula, CA) and β3 integrin mouse polyclonal antibody (Santa Cruz), 1 μg/ml in TBS (150 mM NaCl, 20 mM Tris–HCl pH 8) for 2 h at room temperature. After washing with 0.1% Tween-20 PBS, the filter was incubated with 1:2,500 peroxidase-conjugated anti-rabbit immunoglobulins (Santa Cruz) and 1:2,500 anti-mouse immunoglobulin (Santa Cruz) for 1 h at 22°C. It was extensively washed and finally analyzed using the Enhanced ChemiLuminescence system (ECL-Amersham, Little Chalfont, United Kingdom). Protein loading was checked by reprobing the membranes with α-tubulin, in order to show that protein levels were not changed.

Statistical analysis

Each experiment was performed five times. The results are expressed as mean±standard deviation (SD). ANOVA was performed for each experiment (e.g. MTT proliferation assay, RT-PCR analysis). The P value was generally evaluated between 0.01 and 0.05, confirming the statistical significance of results.

Results

Artemisinin inhibits the growth of melanoma cell lines

Several studies have previously shown that artemisinin inhibits the growth of different transformed cell lines [11, 12]. In the present study artemisinin displayed a different effect on the tumor cell lines tested. Cell morphology was not modified after treatments with artemisinin at all the concentrations, and neither necrotic nor apoptotic phenomena were evident in MCF7 and MKN cells (data not shown). In contrast, at the dose of 150 μM artemisinin A375P cells showed a rounded outline and picnotic nuclei, that are typical features of cell death (Fig. 1d), associated to a marked growth inhibition (data not shown). Interestingly, 100 and 150 μM artemisinin induced a strong growth inhibition in A375M cells treated for 48 h (Fig. 2), in the absence of changes in cell morphology (Fig. 1c). The effect was even stronger after

Table 1 Human primers sense and antisense sequences and expected PCR products (bp)

Gene	Sense and antisense sequences	Conditions	Size of product
αV	5′-TAAAGGCAGATGGCAAAGGAGT-3′ 5′-CAGTGGAATGGAAACGATGAGC-3′	30 cycles at 94°C for 30 s 64°C for 40 s, 72°C for 40 s	510
β3	5′-CCTTTGATTGTGACTGTGCC-3′ 5′-GCAGACACATTGACCACAGAGG-3′	30 cycles at 95°C for 30 s 58°C for 30 s, 72°C for 40 s	235
MMP2	5′-TGACGGTAAGGACGGACTC-3′ 5′-TGGAAGCGGATTGGAAACT-3′	32 cycles at 95°C for 60 s 57°C for 60 s, 72°C for 60 s	342
GAPDH	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′		306
β-actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′		661

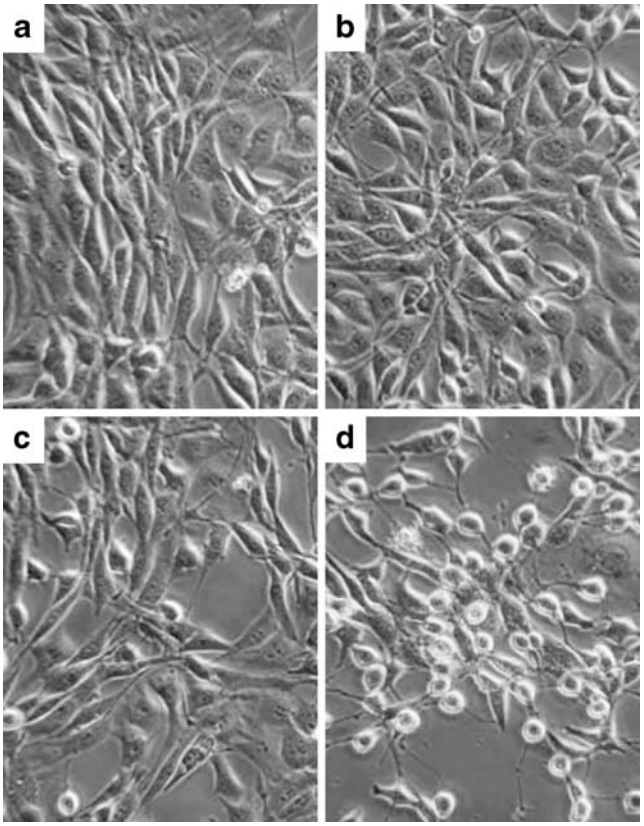

Fig. 1 Effect of OMF treatment on cell morphology of A375M cells. **a** Untreated-A375M cells; **b** untreated-A375P cells; **c** 150 μM artemisinin treated A375M cells for 72 h. **d** 150 μM artemisinin treated A375P cells for 72 h. Magnification: ×20

72 h. As the best effect in term of growth inhibition was observed when A375M cells were treated with 100 and 150 μM artemisinin for 72 h, we chose these experimental conditions and A375M cell line for further investigations.

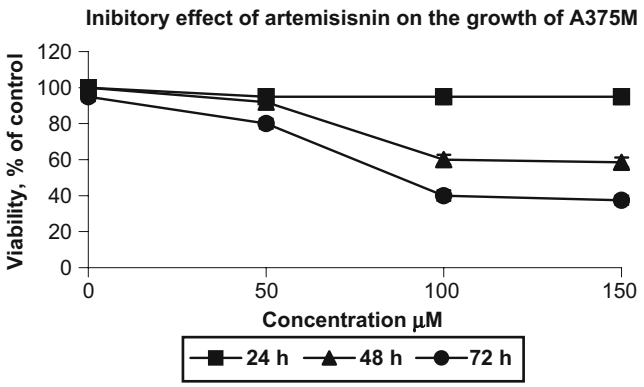

Fig. 2 Effect of artemisinin treatment on cell proliferation (MTT assay). A375M cells were treated with various concentrations of artemisinin for 24, 48, and 72 h. The data are presented as the mean± SD of results of five independent experiments ($P<0.05$)

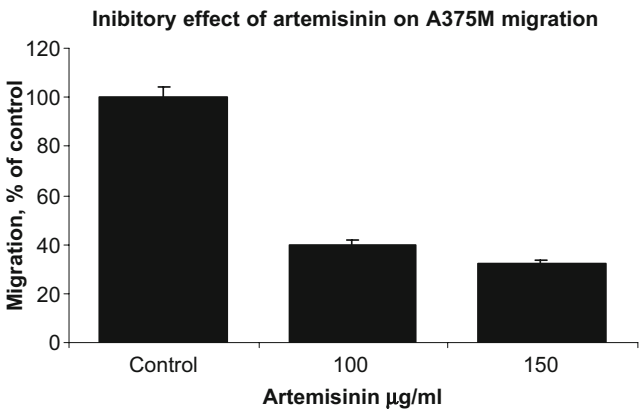

Fig. 3 Effect of artemisinin treatment on chemotactic migration of A375M cells. Control and artemisinin treated cells were left to migrate in presence of FMLP. The data are reported as percentage of the basal random migration in the presence of the chemoattractant. The results are reported as the mean value of five different experiments

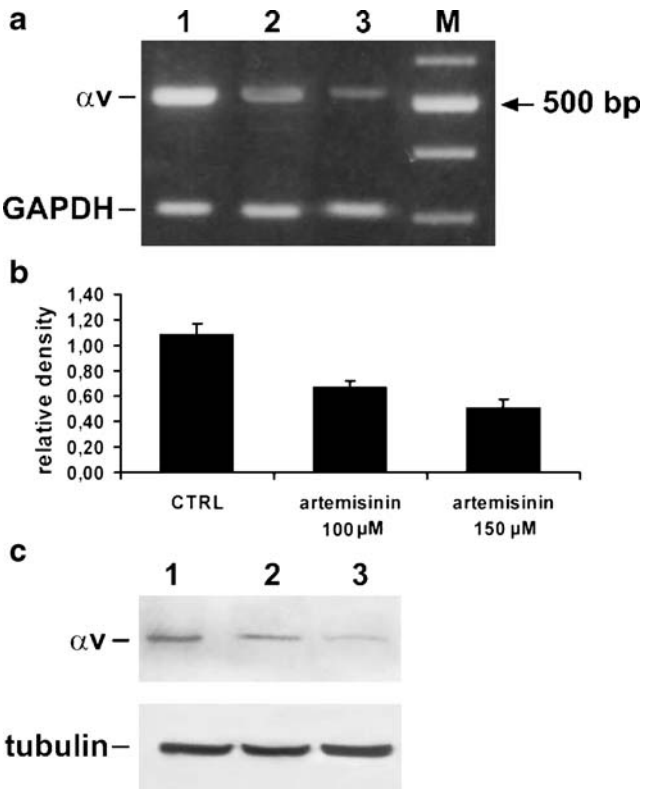

Fig. 4 RT-PCR analysis using specific primers for αV mRNA expression (**a**) and Western blot analysis (**c**). **a** and **c** *lane 1* untreated-A375M cells, *lane 2* A375M cells treated with 100 μM artemisinin for 72 h, *lane 3* A375M cells treated with 150 μM artemisinin for 72 h. *M* 100 bp ladder MW-marker (Roche Diagnostics). **b** The quantitative measurements of the band intensities relatives to RT-PCR analysis. The data shown are representative of five different experiments ±SD ($P<0.05$)

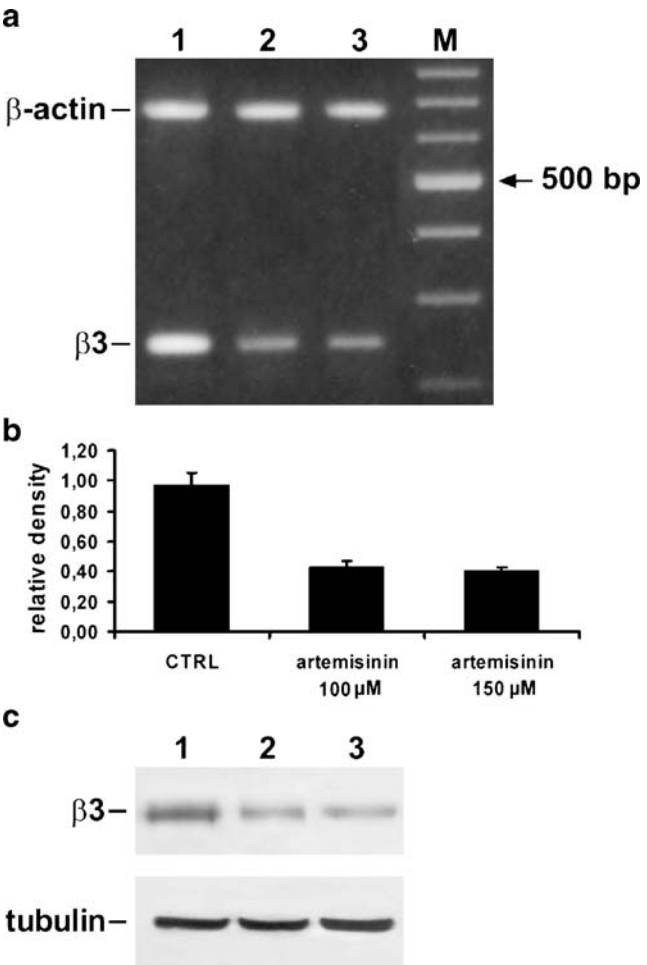

Fig. 5 RT-PCR analysis using specific primers for β3 mRNA expression (**a**) and Western blot analysis (**c**). **a** and **c** *lane 1* untreated-A375M cells, *lane 2* A375M cells treated with 100 μM artemisinin for 72 h, *lane 3* A375M cells treated with 150 μM artemisinin for 72 h. *M* 100 bp ladder MW-marker (Roche Diagnostics). **b** The quantitative measurements of the band intensities relatives to RT-PCR analysis. The data shown are representative of five different experiments ±SD ($P<0.05$)

Effect of artemisinin on the chemotactic migration of A375M cells

To investigate if cell growth inhibition was also associated to a reduced invasive ability, we performed a chemotaxis assay. As shown in Fig. 3, untreated A375M cells were induced to migrate from the upper to the lower compartment of the Boyden chamber through the gelatine-coated filter, by using the chemoattractant FMPL. In contrast, the chemotactic response of A375M cells in the presence of FMPL was inhibited when cells were treated with 100 and 150 μM artemisinin for 72 h. Cell migration inhibition in comparison with untreated cells was about 60% and 68% respectively

Effect of artemisinin on αvβ3 integrins and matrix metalloproteinase (MMP-2)

To investigate the mechanism by which A375M motility was inhibited, we analyzed the mRNA levels of αv and β3 integrin subunits and MMP-2 by RT-PCR and Western blot analysis. As shown in Figs. 4 and 5 artemisinin induces a strong down-regulation of αv and β3 integrin subunit, respectively. In particular, when A375M cells were treated with 100 and 150 μM artemisinin for 72 h about 40% and 60% αv decrease is observed, respectively, whereas the same percentage of decrease (60%) is observed when β3 expression was evaluated at both concentrations. We also demonstrated that the treatment is effective in reducing MMP-2 mRNA expression. MMPs are a family of structurally related zinc-dependent neutral endopeptidases that are involved in the extracellular matrix degradation

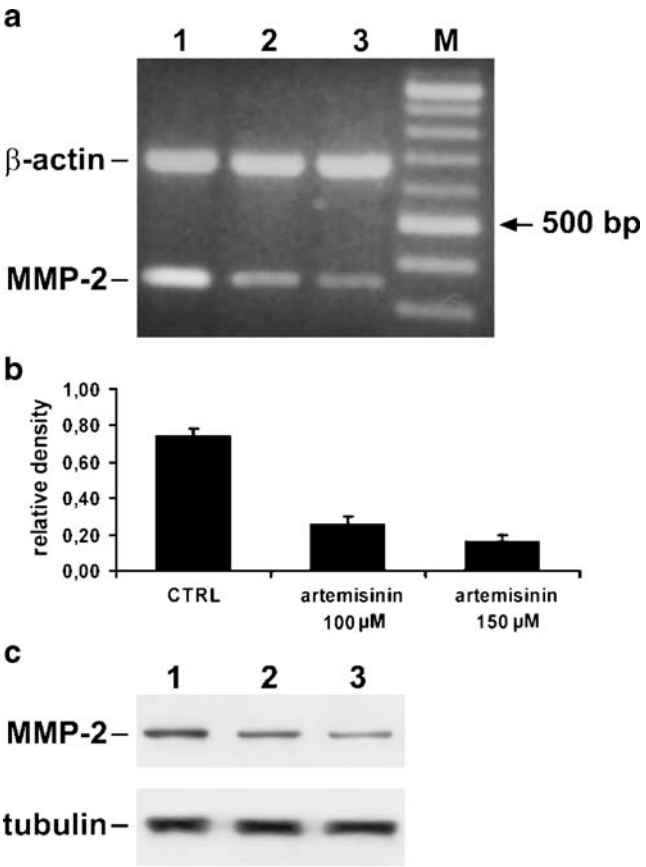

Fig. 6 RT-PCR analysis using specific primers for MMP2 mRNA expression (**a**) and Western blot analysis (**c**). **a** and **c** *lane 1* untreated-A375M cells, *lane 2* A375M cells treated with 100 μM artemisinin for 72 h, *lane 3* A375M cells treated with 150 μM artemisinin for 72 h. *M* 100 bp ladder MW-marker (Roche Diagnostics). **b** The quantitative measurements of the band intensities relatives to RT-PCR analysis. The data shown are representative of five different experiments ±SD ($P<0.05$)

required for migration [18]. These proteins have been shown to be involved in cancer metastasis. We studied by RT-PCR and Western blot analysis whether artemisinin affects the in vitro secretion of MMP-2 by A375M cells. MMP-2 production clearly decreased after 72 hours of treatment in A375M cells at both concentrations as demonstrated by RT-PCR and Western blot, with a stronger effect at the higher concentration (Fig. 6).

Discussion

In the present investigation we have demonstrated that artemisinin may induce cell growth arrest in a human metastatic melanoma cell line, A375M, while it was not effective in contrasting proliferation of MCF7 and MKN cells, that for this reason were not considered in the next stage of the study. In contrast, artemisinin seems to affect A375P cells vitality with cytotoxic and growth inhibitory effects. Considering the cytotoxic effects observed, the latter cell line was also not considered for the migration assay. The effect on A375M cells was clearly evident after 72 h at the concentration of 100 μM, whereas the highest concentration (150 μM) did not induce a stronger cytotoxic effect; in addition a strong inhibition of the migratory ability of artemisinin-treated cells was also demonstrated by the chemotactic assays. At the same experimental conditions the molecule showed the ability to influence cell migration by affecting integrin αvβ3 and down-regulating the MMP-2 production, two biomolecular effects that have been implicated in many steps of tumor dissemination [19, 20].

The integrins are a large family of receptors that attach cells to the extracellular matrix (ECM), organize their cytoskeleton and cooperate with receptor protein kinases to regulate the fate of the cell [21]. They are composed of non-covalently associated α and β subunits that heterodimerize to produce more than 20 different receptors for the ECM proteins; αv integrins are a major subfamily with restricted tissue/cell distribution [22]. Integrins function in cell-to-cell and cell-to-matrix adhesive interactions and transducer signals from the ECM to the cell interior and vice versa. Hence, the integrins mediate the ECM influence on cell growth and differentiation [23]. Besides mediating cell attachment and spread, thus facilitating cell migration and ligand-receptor internalization, they play a functional role in angiogenesis and metastasis and promote tumor cell survival [24]. Increased expression of αvβ3 integrins appear to be positively correlated with increased malignancy in melanomas [25]. Consequently, the ability of artemisinin to significantly affect A375M cell migration, by down-regulating αvβ3 integrin and reducing MMP-2 production, is of great interest. αv integrins are frequently associated to MMPs [20], and both play an essential role in tumor angiogenesis, cell motility, metastasis and tissue remodeling and repair [19]. Evidence is provided that MMP-2 can be localized in a proteolytically active form on the surface of invasive melanoma cells, based on its ability to bind directly αv/β3 integrin. Expression of αv/β3 on cultured melanoma cells enabled their binding to MMP-2 in a proteolytically active form, facilitating cell-mediated collagen degradation [26, 27]. These data reinforce the importance of the results obtained in our study. Since melanoma is notoriously resistant to current therapeutic modalities, and known to possibly evolve in metastasis, the ability to control its onset at the early stages and to avoid cell spreading at the lymph nodes is of primary importance [1]. Therefore, the ability of artemisinin to contrast cell growth and migration represent an important step in the development of new chemotherapeutic strategies for treating melanoma, corroborated by the fact that this molecule is already known as a well tolerated pharmaceutical.

Acknowledgments This study was supported by grants from Legge 5 (2006), Regione Campania.

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