The integrin antagonist cilengitide increases the antitumor activity of temozolomide against malignant melanoma

LUCIO TENTORI[1], ANNALISA SUSANNA DORIO[1], ALESSIA MUZI[1], PEDRO MIGUEL LACAL[2], FEDERICA RUFFINI[2], PIERLUIGI NAVARRA[3] and GRAZIA GRAZIANI[1]

[1]Department of Neuroscience, University of Rome 'Tor Vergata';
[2]Laboratory of Molecular Oncology, 'Istituto Dermopatico dell'Immacolata' (IDI-IRCCS);
[3]Department of Pharmacology, Catholic University Medical School, Rome, Italy

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Abstract. Inhibitors of α_v integrins have been developed as anti-angiogenic agents for cancer therapy and, among them, cyclic RGD-containing pentapeptides, such as cilengitide, are the most commonly used integrin antagonists. In this study, cilengitide was tested in combination with the methylating agent temozolomide (TMZ), a well-tolerated anticancer drug with favourable pharmacokinetic properties currently used for the therapy of metastatic melanoma. To this end, the influence of cilengitide and TMZ on malignant melanoma growth and endothelial cell proliferation were investigated, using *in vitro* and *in vivo* models. The results indicated that cilengitide and TMZ exerted synergistic antiproliferative effects against melanoma and endothelial cells *in vitro* and induced a statistically significant reduction of *in vivo* melanoma growth with respect to treatment with the methylating agent only. In conclusion, this study proposes the use of cilengitide in combination with TMZ for the treatment of metastatic melanoma, thereby opening novel perspectives for the use of integrin inhibitors to enhance the efficacy of chemotherapy.

Introduction

Integrins, a family of cell adhesion molecules, mediate the interactions between cells or between cells and the extracellular matrix (ECM). They are heterodimeric cell surface glycoproteins composed of non-covalently associated transmembrane subunits, α and ß chains, which link the cytoskeleton to ECM components. In mammals, 18 different α and eight different ß subunits associate, giving rise to >20 receptors, capable of recognizing one or more ligands (1).

It is well established that integrin signaling plays an important role in tumor angiogenesis and metastasis. In particular, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are highly expressed on activated endothelial cells, modulating cell migration and survival during angiogenesis, and in tumor cells, facilitating cell invasion and movement across blood vessels due to their ability to recruit and activate matrix metallo-proteases (2). In contrast to $\alpha_v\beta_5$ which solely binds to vitronectin, $\alpha_v\beta_3$ interacts with various matrix ligands. In particular, $\alpha_v\beta_3$ binds to the Arginine-Glycine-Aspartic acid (RGD)-sequence present in vitronectin, tenascin, thrombo-spondin, fibronectin and fibrinogen (3).

Inhibitors of α_v integrins have been developed as anti-angiogenic agents for cancer therapy and cyclic RGD-containing pentapeptides are the most commonly used integrin antagonists (4,5). Among these, a compound which recently entered clinical trials is cilengitide (EMD121974, cycloL-Arg-Gly-L-Asp-D-Phe-N[-Methyl]L-Val), a cyclic RGD containing peptide which binds $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins with nanomolar affinity (3). Cilengitide selectively inhibits the $\alpha_v\beta_3$ integrin binding to vitronectin with an IC_{50} value of 0.58-1 nM and also antagonizes the $\alpha_v\beta_5$ integrin binding to vitronectin with a high affinity (IC_{50} 37-140 nM) (6,7). Preclinical studies indicated that cilengitide blocks tumor growth by inhibiting tumor angiogenesis. However, the compound may have antitumor effects that are independent of tumor vascularization (8-11). In phase I studies, the drug did not show any dose-limiting toxicity in solid tumors (7) and glioma (12,13). Several phase II clinical trials have been performed for the treatment of a variety of cancer types including prostatic (14,15), pancreatic (16), melanoma (17) and glioblastoma (18,19).

Among the different solid tumors, malignant melanoma expresses α_v integrins, that activate and control signaling pathways which enable tumor cells to switch from stationary to a migratory and invasive phenotype (20). The treatment of metastatic melanoma is still unsatisfactory, with a 5-year survival of treated patients ranging from 3 to 14%. The

Correspondence to: Dr Grazia Graziani, Department of Neuroscience, University of Rome 'Tor Vergata', Via Montpellier 1, 00133 Rome, Italy
E-mail: graziani@uniroma2.it

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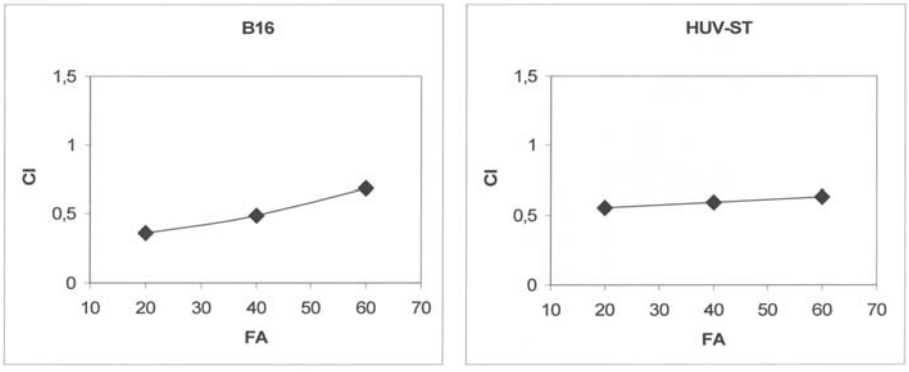

Figure 1. Synergistic effect of the TMZ and cilengitide combination in melanoma or endothelial cells. The chemosensitivity of melanoma (B16) or endothelial cells (HUV-ST) to TMZ or cilengitide as single agents, or in combination, was assessed by colony-formation assay. Cells were treated with TMZ + cilengitide at fixed molar ratios (1, 0.5, 0.2 and 0.1 times the IC_{50} concentration for each drug). Colony-forming ability was evaluated after 10-14 days. A combination index (CI) fraction-affected (Fa) plots of interactions between TMZ and cilengitide were generated by computer analysis using Calcusyn software Version 2.0. CI indicates a synergistic (CI<1), additive (CI=1) or antagonistic effect (CI>1).

methylating agent dacarbazine (DTIC), is historically the reference drug for the treatment of malignant melanoma and recently its congener temozolomide (TMZ) has in part replaced DTIC due to a number of favourable properties. TMZ is a lipophilic compound with a high oral bio-availability, penetration of the blood-brain barrier and mild to moderate adverse effects (21). Although response rates were comparable, TMZ has been shown to improve progression-free survival compared to DTIC, to allow a better quality of life and to reduce the incidence of CNS relapse in patients affected by metastatic melanoma (22,23). To enhance efficacy and counteract tumor resistance, TMZ is currently used in combination therapy with other chemotherapeutic agents and clinical trials, recently completed or still underway, are evaluating the efficacy of TMZ treatment in combination with inhibitors of DNA repair (24-26). In the present study, we demonstrated that cilengitide is capable of increasing the anti-melanoma activity of TMZ with no increase of systemic toxicity.

Materials and methods

Cell lines. The murine melanoma cell line B16 of C57BL/6 ($H\text{-}2^b/H\text{-}2^b$) origin (ATCC, Manassas, VA) was cultured in RPMI-1640 containing 10% fetal calf serum (Sigma-Aldrich, Milan, Italy), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), at 37˚C in a 5% CO_2 humidified atmosphere.

The immortalized human endothelial cell line HUV-ST was generated as previously described (27). Cells were maintained in culture in endothelial growth factor medium (EGM-2, Clonetics, BioWhittaker Inc, Walkersville, MD) and supplemented with 0.4 mg/ml geneticin and 5 μg/ml puromycin.

Colony-formation assay. Cells were seeded in triplicate into 6-well plates ($2x10^2$/well) and, after overnight incubation, were treated with TMZ (Schering-Plough, Kenilworth, NJ, USA, 15-500 μM) or cilengitide (Merck, KGaA, Darmstadt, Germany, 0.01-10 μM). Cells were cultured to allow colony formation and, after 10-14 days, colonies were fixed and stained with rhodamine B basic violet 10 (Sigma-Aldrich). Only colonies comprising >50 cells were scored as survival colonies. Chemosensitivity was evaluated in terms of IC_{50}, i.e. the concentration of the drug capable of inhibiting the colony-forming ability by 50%.

To evaluate whether the combination of TMZ + cilengitide was synergic, cells were exposed to TMZ or cilengitide alone or in combination at fixed equipotent ratios (corresponding to 1, 0.5, 0.2 and 0.1 times the IC_{50} for each drug). The dose-effect curves were analyzed by the median-effect method of Chou and Talalay using the Calcusyn software (Biosoft, Cambridge, UK). The combination index (CI) indicates a quantitative measure of the degree of drug interaction in terms of synergistic (CI<1), additive (CI=1) or antagonistic effect (CI>1). Graphs were generated, plotting CI as a function of the fraction of cells affected (Fa) by the dose of the drugs.

Cell adhesion assays. Solid support was prepared by coating immunological 96-multiwell plates with 10 μg/ml vitronectin (Sigma-Aldrich) in PBS for 18 h and blocking with 3% BSA in PBS for 2 h. B16 cells were plated in serum-free medium supplemented with 0.1% BSA at a concentration of $4x10^4$ cells/well. After incubation at 37˚C for 45 min, the non-adherent cells were washed out with PBS, whereas the attached cells were fixed with 3% formaldehyde and stained with 0.5% crystal violet. The attachment efficiency was determined by a quantitative dye extraction and the spectrophotometric measurement of the absorbance at 595 nm.

In vivo studies on antitumor activity. Drug toxicity was evaluated by treating intact mice (5/group) with the compounds under study, used as single agents or in combination. Body weight was measured three times weekly and survival was recorded for 3 weeks after the last treatment. Toxicity was assessed on the basis of apparent drug-related deaths (within 7 days after the last treatment) and net body weight loss [i.e., (initial weight - lowest weight)/initial weight x 100%].

B16 cells ($2.5x10^5$) were inoculated intra-muscle (i.m.) in seven week-old male C57BL/6 (8/group, Charles River, Calco, Italy). Tumors were measured with a caliper and the treatment was started when the nodules reached 200-250 mm^3.

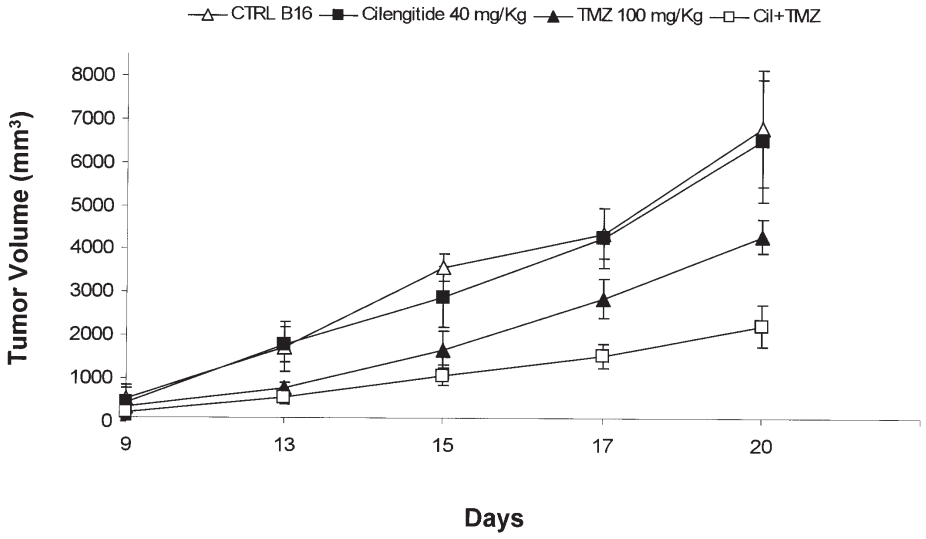

Figure 2. Cilengitide increases the antitumor activity of TMZ against B16 melanoma. Treatment (TMZ 100 mg/kg/day/i.p. for 5 days or cilengitide 30 mg/kg/day/i.p. for 5 days) started on day 6, when the volume of tumor nodules reached 200-250 mm^3. Symbols represent the means of tumor nodule volumes determined in 8 animals for each group every 2-4 days. Bars: ± SD. Statistical analysis of tumor growth was performed by one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple comparisons. Differences between TMZ + cilengitide versus control or cilengitide groups were statistically significant starting from day 13 onward ($P<0.05$); differences between TMZ + cilengitide versus TMZ were statistically significant starting from day 17 onward ($P<0.05$); differences between TMZ versus control or cilengitide groups were statistically significant starting from day 13 onward ($P<0.05$).

Tumor growth was monitored by measuring tumor nodules every 2-4 days for 3 weeks.

TMZ (Shering Plough) was dissolved in dimethylsulfoxide, diluted in saline (5 mg/ml) and administered intraperitoneally (i.p.) at 100 mg/kg/dx5d. Cilengitide (Merck KgaA) was dissolved in sterile physiological saline (5 mg/ml) and administered i.p. at 40 mg/kg/dx5d, a dose corresponding to ~1/2 of LD_{10} (7). Control mice were always treated with vehicles. The results of the *in vivo* tumor growth were analyzed by one-way analysis of variance (ANOVA) for multiple comparisons followed by a Bonferroni test (Primer of Biostatistics Statistical Software Program, McGraw-Hill Medical, USA). A P-value of <0.05 was considered significant.

All procedures involving animals and their care were performed in compliance with the national and international guidelines (European Economy Community Council Directive 86/109, OLJ318, Dec. 1, 1987).

Results

Cilengitide increases the antiproliferative effect of TMZ against melanoma and endothelial cells. B16 melanoma cells were exposed to 0.01-10 μM cilengitide and an analysis of the long-term survival of the drug-treated cells, by colony-formation assay, indicated that the drug induced a dose-dependent reduction of cell growth with an IC_{50} of 0.2±0.07 μM, while the cilengitide IC_{50} for B16 adhesion to vitronectin was 8±1.5 μM.

To evaluate the influence of cilengitide on endothelial cell growth, an immortalized human endothelial cell line generated in our laboratory from HUVEC was used. This cell line (HUV-ST) possesses a stabilized telomere length and an increased proliferation rate with respect to parental cells, but it is not tumorigenic and displays all major endothelial phenotypic markers (27). Notably, HUV-ST cells overexpress the tumor endothelial marker TEM-1, which is regarded as the most differentially expressed molecule in tumor-derived endothelium versus normal-derived endothelium. Therefore, the immortalized human endothelial line HUV-ST is a suitable model for studying the efficacy of anti-neovascular therapy and mimicking proliferating neovascular endothelial cells associated to the tumor mass. An analysis of cell growth showed that cilengitide reduced the colony-forming ability of endothelial cells with an IC_{50} of 6.7±1.2 μM, a concentration which approximately corresponds to the IC_{50} for HUVEC attachment on vitronectin (10).

The TMZ IC_{50} of B16 and HUV-ST cells were 115±12 and 77±7 μM, respectively, according to previous studies (27,28). Cells were then treated with TMZ and cilengitide in combination at fixed equipotent ratios selected on the basis of the IC_{50} values of each cell line, as described in Materials and methods. Fig. 1 shows the results of the median effect analysis using Calcusyn software. The CI values indicate that the combination of TMZ + cilengitide was synergistic in the two cell lines.

Systemic administration of cilengitide increases the antitumor activity of TMZ against melanoma. The antitumor activity of the drug combination (cilengitide 40 mg/kg/day/i.p. + TMZ 100 mg/kg/day/i.p., for 5 days), was tested in B16 melanoma growing i.m. in C57/BL6 mice and compared to the effects induced by TMZ or cilengitide, when used as single agents. The treatment produced animal body weight loss <10% of the original weight and all mice recovered the initial body weight one week after treatment. The results of multiple comparisons between groups using ANOVA analysis of variance show that, starting from day 17 onward, cilengitide significantly enhanced ($P<0.05$) the antitumor effect of TMZ, while the treatment with cilengitide, used as a single agent, did not affect tumor growth (Fig. 2).

Discussion

In the present study we demonstrated for the first time that the administration of the α_vß-integrin antagonist cilengitide enhances the antitumor activity of TMZ against malignant melanoma. In particular, the combination of cilengitide and TMZ showed synergistic antiproliferative effects against melanoma and endothelial cells *in vitro* and induced a statistically significant reduction of *in vivo* melanoma growth with respect to treatment with the methylating agent alone.

The rationale of combining cilengitide with TMZ relies on their common activity against melanoma. While the mechanism underlying the antitumor activity of cilengitide primarily relies on the inhibition of tumor angiogenesis through the disruption of the integrin-ligand interaction in endothelial cells, TMZ methylates DNA generating a wide spectrum of base adducts mainly represented by N7-methylguanine, N3-methyladenine and O^6-methylguanine (29). Despite being produced in low amounts, O^6-methylguanine is generally considered the main cytotoxic lesion produced by TMZ. If not repaired by O^6-alkylguanine DNA alkyltransferase (AGT), O^6-methylguanine inappropriately pairs with thymine and the resultant mismatches trigger the intervention of the mismatch repair system (MR), which removes the improperly paired pyrimidine to re-insert it again opposite the O^6-methylguanine. Repeated cycles of MR-mediated excision/re-synthesis eventually provoke DNA nicks with apoptosis induction and growth arrest. Unfortunately, resistance to TMZ occurs quite frequently and is due to high AGT levels or to the functional defects of MR, soliciting a therapeutic approach that may counteract the emergence of drug resistance possibly without worsening the advantageous toxicological profile of the methylating agent (30). In this regard, it should be noted that the administration of cilengitide in combination with TMZ did not increase systemic toxicity.

The interest in the use of integrin inhibitors for cancer therapy has lately been revived by recent phase I or II clinical studies in which cilengitide has been safely used as a single agent or in combination with chemotherapy (12,16). Protracted treatment with cilengitide induced partial or complete responses in patients affected by recurrent malignant glioma (13). The lack of the *in vivo* effect of cilengitide as a single agent observed in the melanoma model used for this study is likely due to the limited time frame of the drug administration (i.e. a single 5-day cycle), which has been adopted to mimic the schedule currently approved for the use of TMZ in the clinic.

The enhancing effect of the TMZ antitumor activity induced by a short course of cilengitide observed in the *in vivo* preclinical model is likely due to the well-recognized anti-angiogenic effect of cilengitide (31,32). Besides its direct effect on tumor cells, TMZ has been shown to possess anti-angiogenic properties, especially when used in low doses (33). A possible modulation of the AGT activity by pretreatment with cilengitide cannot be invoked to explain the synergistic effect of the combination TMZ + cilengitide since the B16 and HUV-ST cell lines possess an almost undetectable basal activity of the repair enzyme (27,34).

In conclusion, this study suggests the use of cilengitide in combination with TMZ for the treatment of metastatic melanoma, thereby opening novel perspectives for the use of integrin inhibitors not only in an adjuvant setting to prevent tumor spreading but also to enhance the efficacy of chemotherapy.

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