Selzer, J Cancer Sci Ther 2018, 10:10
DOI: 10.4172/1948-5956.1000561

Journal of Cancer Science & Therapy

Research Article Open Access

Comparative Analysis of Cilengitide with a Novel Integrin Antagonist AV-38/398 in 2D/3D Melanoma Cultures

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Rec date: July 23, 2018; **Acc date:** October 12, 2018; **Pub date:** October 15, 2018

Abstract

Objective: Integrin receptors are differentially expressed between normal and malignant cells and are therefore potential targets for inhibitors. One of the most thoroughly investigated integrin antagonists is the αvβ3/αvβ5 antagonist Cilengitide, which has been studied in several clinical trials. We investigated a novel integrin antagonist designated AV-38/398 and compared its biological effects with those of Cilengitide.

Methods: We assessed drug sensitivity towards AV-38/398 and Cilengitide in a panel of melanoma cell lines from different disease stages. Anti-migrative and anti-invasive effects were studied using scratch assays and a modified Boyden chamber assay with matrigel coated inserts. Spheroid growth under various concentrations of AV-38/398 or Cilengitide was monitored and spheroid viability was assessed by immunofluorescent staining and cell viability ELISA. Anti-invasive effects were studied by embedding melanoma spheroids in an invasion matrix.

Results: Differences in efficacy and the mode of action between AV-38/398 and Cilengitide were found. In 2D cultures, both compounds displayed anti-cancer effects within a comparable low micromolar range (<10 μM). At concentrations above their respective IC50 values (for anti-proliferative effects), AV-38/398, but not Cilengitide, induced additional cytotoxic effects. AV-38/398 dose-dependently inhibited spheroid growth, invasion, and survival under 3D-culture conditions. Cilengitide did not affect growth, invasion or survival in 3D spheroid cultures at concentrations up to 25 μM, which are far above its published concentrations necessary for binding to its cognate receptor. In fact, Cilengitide had no effect on spheroid growth at concentrations up to 5 mM in 3D cultures.

Conclusion: Cilengitide, in contrast to AV-38/398, is not effective under 3D-culture conditions. AV-38/398, or structurally related compounds, may be promising candidates for preclinical development.

Keywords: Melanoma; Integrins; AV-38/398; Cilengitide; Integrin antagonists: 3D spheroid culture

Introduction

Melanoma is an aggressive type of cancer arising from melanocytes of the skin or eye [1]. Due to its highly metastatic nature, melanomas are responsible for most skin cancer-related death [1-4]. Apart from genetic predispositions (Familial melanoma) and frequently occurring mutations (BRAF, NRAS, KIT, CDKN2A, and PTEN) [5,6], melanoma cells depict an altered integrin expression profile, which may contribute to its malignant characteristics.

Integrins are a family of heterodimeric cell surface receptors that mediate several biological processes such as cell attachment, motility, proliferation, and survival [7–12]. Each cell type has its specific integrin expression pattern that depends on its natural tissue-environment. Integrin-mediated survival signaling may be regarded as a mechanism assuring that normal cells are only able to survive in their physiological tissue environment [13–15]. Tumor cells often show altered integrin expression patterns, which promotes tumor invasion and survival in different tissues [10,16–20]. In melanoma, integrins α3β1 [21,22], α4β1 [22,23], α6β1 [24], α7β1 [25,26] and αVβ3 are frequently found to be dysregulated compared to normal melanocytes (reviewed in [11,27]). Particularly the upregulation of integrin αVβ3 expression is thought to be associated with malignant progression [17,28,29]. Currently, several integrin targeting drugs are in clinical development for application in various types of diseases and also some for cancer (reviewed in [30,31]). However, no integrin antagonist has been approved in the treatment of cancer so far. The majority of integrin targeting drugs for application in cancer aim at inhibiting the function of RGD (Arg-Gly-Asp)-binding integrins [14,19,31,32]. Especially integrin αVβ3 and αVβ5 seem to be favorable drug targets, as these integrins are not only upregulated in tumor cells, but also in the tumor neovasculature [19,33,34].

One of the most promising and well-investigated integrin antagonists is Cilengitide, the primary targets of which are αVβ3 and αVβ5 [35,36]. Cilengitide has been investigated in over 30 clinical trials for various types of cancer, such as pancreatic, head and neck, lung cancer, as well as in melanoma and glioblastoma [37–44]. However, it recently failed in phase III studies, as it did not show a significant improvement over current standard treatment concept [40,45].

Novel antagonists targeting integrin αVβ3 have been developed, amongst others the compound which is being investigated in this study, designated AV-38/398 [46,47]. Preliminary data by others as well as our group showed a binding affinity of AV-38/398 for integrin αVβ3 at 240 nM and favorable drug-like properties [46–49]. *In vitro* data showed significant cytotoxic effects in normal endothelial cells and

cancer cell lines of different histology with IC50-values typically in the nano-to low micromolar range [46,47]. As was recently reported by our group (Christenheit), AV-38/398 is effective against proliferating primary human umbilical vein endothelial cells (HUVEC), which express αVβ3 integrin, whereas AV-38/398 had significantly less effects on HT-29 cells, which do not express αVβ3 [47].

In HUVECs, AV-38/398 induced cellular detachment and triggered cell death by anoikis. The drug also exhibited anti-migratory and anti-angiogenic effects *in vitro* and *in vivo* [50]. These features, together with the reported anti-proliferative effects in 2D cell cultures, make it an attractive candidate for further evaluation.

Based on these promising results, we decided to test AV-38/398 in melanoma cells and to compare its mode of action with Cilengitide. In the present study, we placed particular emphasis on a comparative analysis of the effects on growth, survival, migration, and invasion, in 2D as well as in 3D culture systems, as these may better represent the *in vivo* situation.

Materials and Methods

Cell lines and reagents

Human melanoma cell lines A375 (ATCC) and 518A2 were cultured in DMEM [51]. SK-Mel-28 (provided by Dr. Walter Berger, Medical University of Vienna) and WM-115 (ATCC) were cultured in MEM. WM-35 cells (obtained from Wistar Institute) were cultured in RPMI 1640. All media were supplemented with 10% FCS and 1% Pen-Strep. Cell culture reagents for melanoma cell lines were purchased from Gibco.

The structure of the compound AV-38/398 (4-({(Z)-5-[(Z)-3-Ethoxy-4-hydroxybenzylidene]-3-methyl-4-oxo-1,3-thiazolidin-2-ylidene} amino) benzoic acid dimethyl-formamide monosolvate) has been described by our group [52] and was synthesized by ChemCon GmbH (Freiburg i.Br., Germany). A stock solution of AV-38/398 (25 mM) was prepared in DMSO (Sigma) and stored at -80°C. Cilengitide (EMD121974) was kindly provided by Dr. Gerhard Prager (Medical University of Vienna).

Cytotoxicity assays and measurement of cell proliferation kinetics

Cell lines were plated in 24 well plates and treated the next day with the indicated drug concentrations. After 72 h, adherent cells were harvested, and viable cell numbers were determined on a ViCell XR cell counter (Beckman Coulter). IC50 values were determined for each cell line. For determination of cell proliferation kinetics, A375 and SK-Mel-28 cell lines were cultured using a similar setup. Cells were plated and treated the next day with indicated drug concentrations. After 24, 48 and 72 h total cell populations were harvested, and viable cell numbers were assessed.

Flow cytometric analysis of integrin αVβ3 and αVβ5 expression

Subconfluent cultures of melanoma cell lines were harvested after gentle detachment with Accutase™ solution (BD Biosciences) and kept on ice during the further steps to inhibit internalization of integrins during the staining procedure. Staining was performed according to the manufacturer's protocol for FITC-integrin αVβ3 (CD51/61) antibody (BD Biosciences). Briefly, cells were centrifuged for 5min at 500g (4°C) and cell pellets were washed in cold FACS-buffer (PBS with Ca2+ and Mg2+, 2% FCS, 0.1% NaN_3). For each cell line, 1 x 10^6 cells were incubated with FITC-labelled integrin αVβ3 antibodies (BD Biosciences), αVβ5 (Millipore) or isotype control (BD Biosciences) diluted in FACS-buffer for 30 min at 4°C. Cells were washed twice in PBS and analyzed by FACS (FACSCalibur™, Becton Dickinson) using Cell Quest Pro software (Becton Dickinson).

Scratch assay

SK-Mel-28 and 518A2 cells were cultured in 6-well plates, grown to confluence and scratches with Eppendorf pipet tips (200 µl) were made. Cell debris was gently washed away, and adherent cells were overplayed with medium containing the indicated drug concentrations. Photographs were taken at 0 and after 24 hrs with an Olympus PEN Lite E-PL5 camera mounted on a Nikon PH-2 inverse microscope (40x magnification). Cell migration into the cell-free scratch was measured using TScratch software (ETH Zurich).

Invasion assay

Invasion capability after drug treatment was analyzed using Matrigel-coated inserts from Corning. Assays were performed according to manufacturer's protocol. Inserts were rehydrated for 2h before SK-Mel-28 or 518A2 cells were seeded on top in medium with 1% FCS and the indicated drug concentrations. The insert was placed into a 24 well plate containing standard medium (10% FCS) to attract cell migration initiating from the upper part. After 24hr, cells from the upper chamber were removed with a cotton swab and the invaded cells on the lower side of the filter were simultaneously fixed and stained with 0.5% Crystal violet solution (Sigma). Photos were taken (Olympus PEN Lite E-PL5 camera+Nikon PH-2 inverse microscope; 100x magnification, 10 images/filter) and the number of invaded cells was counted using Image J software (National Institutes of Health).

Spheroid culture and 3D growth kinetics

Spheroids were cultured from 518A2, WM-115 and WM-35 cell lines using U-shaped ultra-low attachment plates (Corning). Defined cell numbers were seeded per well and incubated for three days until they formed tight 3D aggregates. For analysis of drug-dependent growth kinetics, spheroids with a diameter of approximately 200µm were treated with increasing drug concentrations. After 72 h, 50% of the medium was replaced with fresh medium (without drug), followed by further equivalent changes every 3-4 days, thereby continuously diluting the concentration of the drug. Photos were taken every 2-3 days (40x magnification, Olympus PEN Lite E-PL5 camera+Nikon PH-2 inverse microscope) from at least five spheroids per dose point over a period of 17 days. The spheroid size was measured using Image J software.

Spheroid viability assay

Spheroids were treated as described above. On day 17, melanoma spheroids (518A2) were stained with LIVE/DEAD™ cell viability assay kit (Molecular Probes™) according to the manufacturer's protocol. Spheroids were incubated with 2 µM calcein AM and 4µM ethidium homodimer-1 for 30 min before immunofluorescence imaging (Zeiss LSM700 laser scanning microscope). For quantification of viability, CellTiter-Glo® luminescent cell viability assay (Promega) was used according to the manufacturer's protocol. On day three, spheroids treated with AV-38/398 were mixed with CellTiter-Glo® reagent (ratio

1:1), lysed and incubated for another 25 min at room temperature. Luminescence was measured on a Hidex Sense plate reader.

Spheroid invasion

Spheroid invasion was analyzed with the Cultrex® 3D spheroid cell invasion assay. The assay was conducted according to the manufacturer's protocol. 518A2 and WM-115 spheroids (diameter of ~300 µm) were embedded in invasion matrix. After solidification, gels were overlayed with equal amounts of media with or without drug and incubated for three days. Photos were taken at the beginning and after three days treatment (Olympus PEN Lite E-PL5 camera+Nikon PH-2 inverse microscope; 40x magnification). The size of the invaded area was analyzed using Image J software. Additionally, the viability of 518A2 spheroids was analyzed using LIVE/DEAD™ cell viability assay kit as previously described.

Colony assay

For assessment of clonogenic survival in 2D monolayer cultures, 518A2 cells were seeded in 6 cm dishes (500-2000 cells/dish, in triplicates). After attachment, the medium was carefully removed and indicated drug concentrations were applied. For 3D cultures, spheroids were treated as previously described with indicated drug concentrations. After 3 days spheroids were collected, disintegrated with Accutase™ solution (BD Biosciences), and re-seeded as described. Dishes were incubated for two weeks until assessable colonies were visible. Colonies were fixed and stained with 0.5% Crystal violet solution. Colonies (>50 cells/colony) were counted manually.

Data analysis and statistics

Data analysis if not otherwise indicated was performed with GraphPad Prism 5.0. For analysis of statistical significance, two-way analysis of variance (ANOVA) was performed. All experiments were repeated at least two times.

Results

AV-38/398 exerts anti-cancer activity in the low micromolar range

To obtain a better understanding of the anti-cancer activity of the new integrin antagonist, AV-38/398 was tested together with the integrin inhibitor Cilengitide in a panel of commonly used melanoma cell lines from different disease stages. SK-Mel-28, 518A2 and A375 were derived from metastatic melanoma, WM-115 cells from vertical growth phase (VGP) melanoma, and WM-35 cell line from a radial growth phase (RGP) melanoma. All melanoma cell lines assessed showed surface expression of both integrin αVβ3 and αVβ5 albeit at different levels (Figures 1A and 1B).

Dose-response curves after 72 h drug treatment were generated for both compounds (Figures 2A and 2B) and half-maximal growth inhibitory concentrations (IC50-values) were determined (Table 1).

Both AV-38/398 and Cilengitide exerted anti-cancer activity within a low micromolar range. IC50-values (AV-38/398) for the melanoma lines tested ranged between 1.6-3.4 µM, except for 518A2 cells, which had a higher IC50 (8.7 µM). Cilengitide, at a molar level, had comparable activity to AV-38/398 in all cell lines investigated, with IC50-values ranging from 0.5 to 9.1 µM.

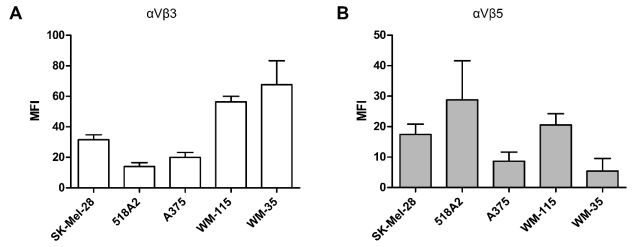

Figure 1: Integrin αVβ3 and αVβ5 expression in melanoma cell lines. Cell surface expression of target integrins (**A**) αVβ3 and (**B**) αVβ5 in the melanoma cell line panel. Bars represent mean fluorescent intensities (MFI) of respective integrin heterodimers (± SD) of at least 3 independent experiments.

Variable	AV-38/398	Cilengitide
	IC50 [µM]	IC50 [µM]
SK-Mel-28	1.67 ± 0.38	1.87 ± 0.36
518A2	8.74 ± 0.96	1.16 ± 0.50
A375	3.37 ± 0.57	9.13 ± 1.94
WM-115	1.89 ± 0.25	0.63 ± 0.46
WM-35	2.42 ± 0.25	0.48 ± 0.05

Table 1: AV-38/398 and Cilengitide exert anti-cancer activity in the low micromolar range. Mean IC50-values (± S.D.) for AV-38/398 and Cilengitide calculated from respective dose-response curves from at least 3 independent experiments are shown.

Regarding the association of drug sensitivity (IC50 values) and target integrin expression, we observed a tendency towards a correlation between sensitivity against AV-38/398 and target integrin expression levels. For example, melanoma cell lines 518A2 and A375, which expressed comparably low integrin αVβ3 levels, were more resistant towards the drug than WM-35 and WM-115 cells, which expressed higher levels of αVβ3. However, in the analyzed melanoma panel of cell lines, the correlation coefficient for AV-38/398 sensitivity and integrin αVβ3 expression was either weak or not significant ($r=-0.6$; $p=0.35$), as it also was the case for Cilengitide and integrin αVβ3 expression ($r=-0.7$; $p=0.233$) or integrin αVβ5 ($r=0.1$; $p=0.95$).

For a better understanding of the anti-cancer effect, drug-dependent proliferation kinetics were assessed in A375 and SK-Mel-28 melanoma cells from the total cell population (attached and detached cells, Figures 2C-2F). Both drugs induced dose-dependent growth inhibition. AV-38/398 inhibited cell proliferation at lower concentrations, whereas, at higher concentrations (A375>9 µM, SK-Mel-28>3 µM), a decline in viable cell numbers was observed. This observation suggests a direct cytotoxic activity of the drug at higher concentrations. Cilengitide, in contrast, did not show any cytotoxic effect, as the number of viable cells was not reduced even at concentrations up to 25 µM.

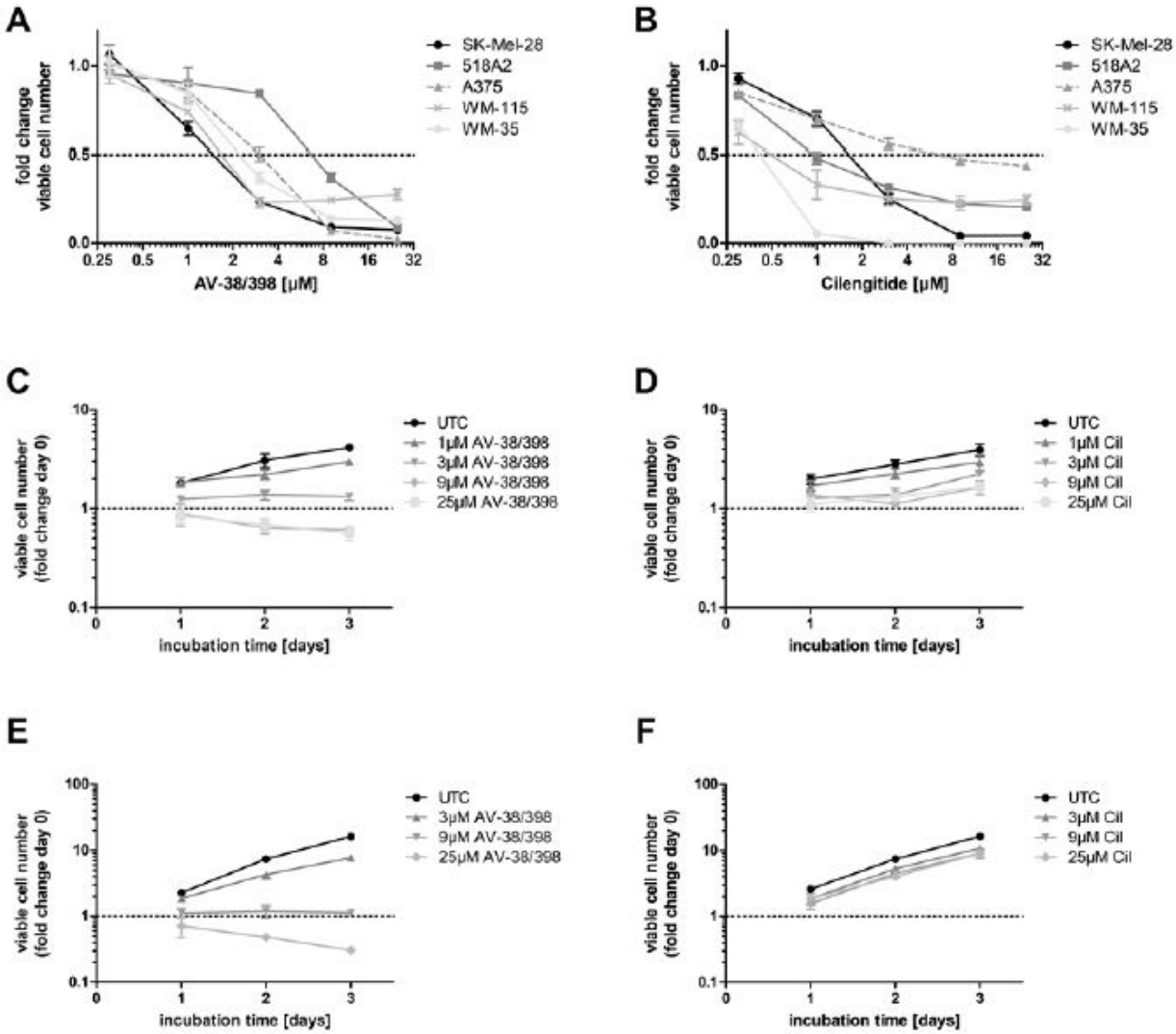

Figure 2: AV-38/398 and Cilengitide exert anti-cancer activity and dose-dependently inhibit melanoma growth. (A+B) Dose-response curves for melanoma cell lines. Cells were treated for 72h at the indicated concentrations of (**A**) AV-38/398 or (**B**) Cilengitide and viable cell numbers of adherent cells were determined. The dotted line (Figure A + represents half-maximal inhibitory effect. (**C-F**) Proliferation kinetics of (C+D) SK-Mel-28 and (E+F) A375 melanoma cells for AV-38/398 and Cilengitide (Cil). Cells were treated with indicated drug concentrations and number of viable cells from total cell populations was measured every day. Dotted lines (Figures 2C-2F) correspond to the cell numbers at the time of start of treatment.

Melanoma migration and invasion is inhibited by AV-38/398

Next, we investigated the migration-inhibiting activity of AV-38/398 in SK-Mel-28 and 518A2 cells using the scratch assay. We found a dose-dependent inhibition of cell migration in the low micromolar range (Figures 3A and 3B). Even in 518A2 melanoma cells that were least sensitive to AV-38/398 (IC50=9.3 µM), concentrations below the respective IC50 were able to reduce cellular migration by more than 50%. The effects of Cilengitide on the two assessed cell lines - within the same concentration range – were even more pronounced.

Next we investigated the anti-invasive effects of the drugs. The invasion potential was determined using a Matrigel-coated transwell filter assay (Figures 3C and 3D), in which we found a dose-dependent inhibition of cell invasion under AV-38/398 treatment in both cell lines. Cilengitide inhibited invasion at comparable drug concentrations more effectively than AV-38/398.

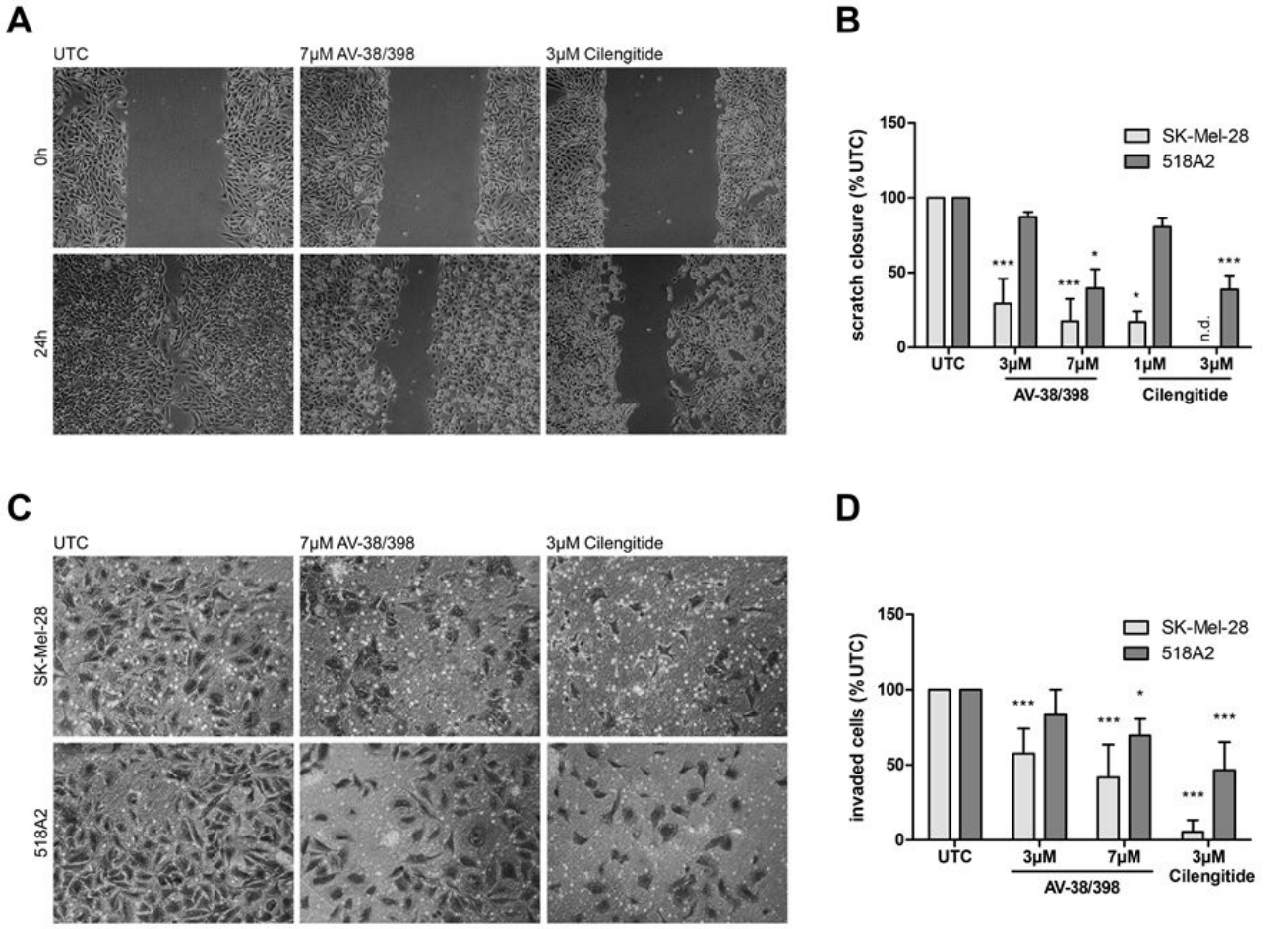

Figure 3: Melanoma cell migration and invasion is inhibited by AV-38/398 and Cilengitide. (A+B) Scratch assay. Figure (**A**) depicts 518A2 melanoma cells at 0 and 24 h (40x magnification). (**B**) Quantification of cell migration. Bars represent mean percentages of gap closure (± SD) after 24 h relative to untreated controls (UTC) from at least 3 independent experiments. * p<0.05; *** p<0.001 (Two-way ANOVA). n.d. not determined. (C+D) Invasion assay of 518A2 and SK-Mel-28 melanoma cells. After 24 h, cells which invaded through the matrigel layer were counted. (**C**) Representative photographs of crystal violet stained invading cells (100x magnification). (**D**) Quantification of invaded cells. Bars represent mean percentage of invaded cells (± SD) normalized to untreated controls (UTC) from 3 independent experiments. * p<0.05; *** p<0.001 (Two-way ANOVA).

AV-38/398, but not Cilengitide, inhibits melanoma spheroid growth

Figure 4 shows the results of a series of growth experiments with 518A2 spheroids treated with AV-38/398 or with Cilengitide. AV-38/398 treatment (25 μM) led to a structural disintegration of the spheroids that was visible on day 7. At that time point, approximately 50% of the spheroids disintegrated, whereas non-disintegrated spheroids, after discontinuing treatment with the drug, were capable of regrowth at day 11. Spheroids treated with 25 μM Cilengitide did not show any evidence of growth inhibition, neither in 518A2 cells nor WM-115 or WM-35 melanoma cell lines (data not shown). In 518A2 melanoma spheroids, Cilengitide did not affect proliferation at a concentration of up to 5 mM (Figure 5).

Analysis with live/dead immunofluorescence staining of the spheroids at day 17 after treatment showed that in AV-38/398 treated spheroids only the cells in the center were viable, whereas the surrounding, visibly disintegrated cells, were dead (Figures 6A and 6B). In contrast, Cilengitide had no effect on spheroid size or spheroid viability.

Further studies were conducted with AV-38/398 and showed that this drug led to dose-dependent growth inhibition (Figure 6C) and had a disintegrating and cytotoxic effect on 518A2 spheroids. At concentrations corresponding to the IC50 under 2D conditions, spheroid growth was significantly inhibited by AV-38/398 while at higher concentrations spheroids started to disintegrate. Similar dose-dependent growth-inhibiting effects could also be observed in WM-115 and WM-35 melanoma spheroids treated with AV-38/398 (Figures 6A and 6B).

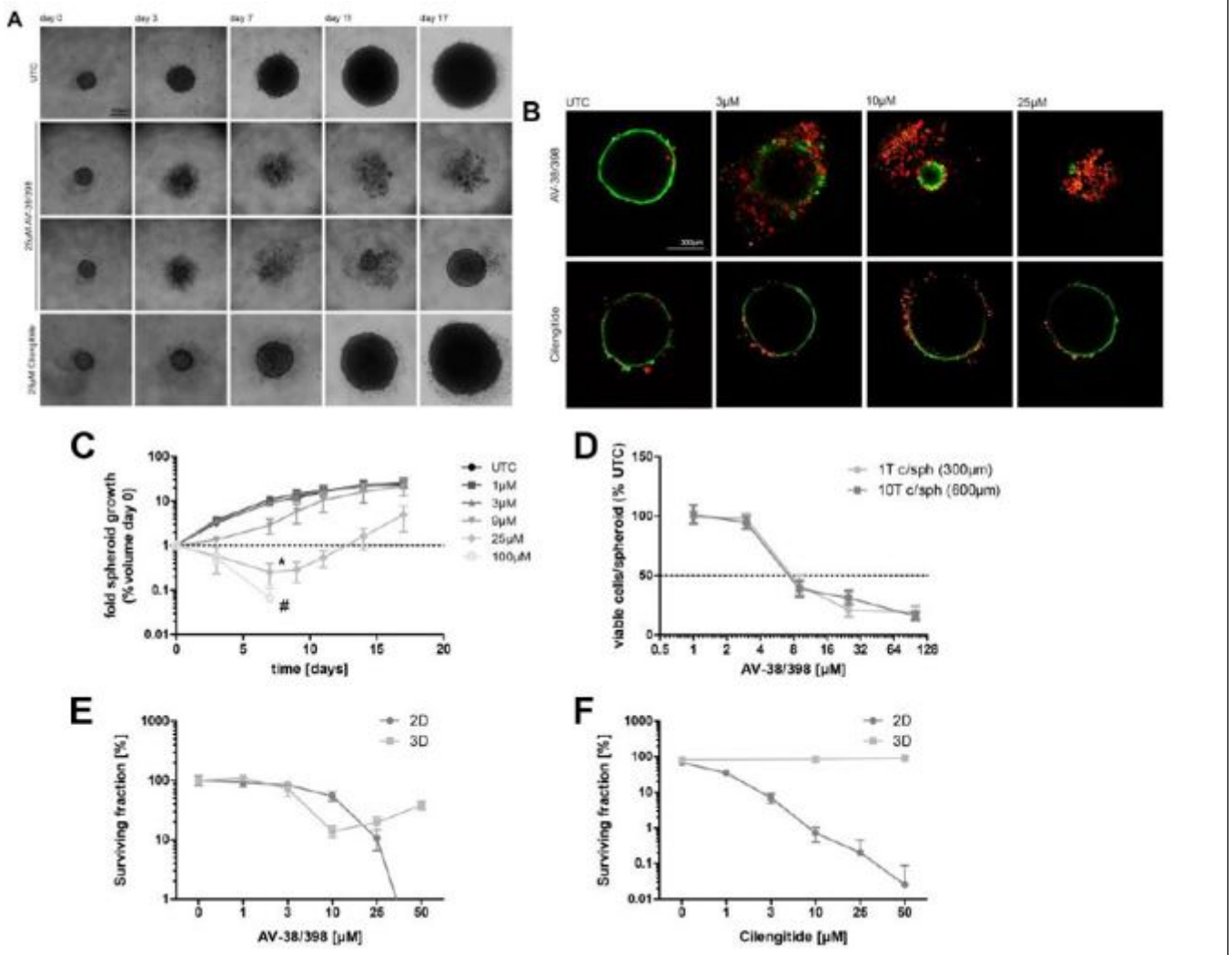

Figure 4: AV-38/398 inhibits melanoma spheroid growth and also has cytotoxic effects in 3D cultures. (**A**) 518A2 melanoma (~300 µm diameter) spheroids were treated with 25 µM AV-38/398 or with Cilengitide and growth was monitored over 17 days. Representative photographs (40x magnification) are shown. (**B**) Immunofluorescence viability staining of 518A2 spheroids treated with various concentrations of AV-38/398 or Cilengitide at day 17. Viable cells are labeled in green (calcein AM) and dead cells in red (ethidium homodimer-1). Representative images are shown. (**C**) Spheroid growth curves of 518A2 melanoma treated with AV-38/398. Data represent mean fold-increase in spheroid volume (± SD) relative to day 0. Disintegration of spheroids is observed at concentrations >25 µM. * Approximately 50% of the spheroids show signs of disintegration, the remaining spheroids show evidence for re-growth. # all spheroids completely disintegrated without re-growth. (**D**) Spheroid viability after 3 days of AV-38/398 treatment in small (~300 µm) and large (~600 µm) 518A2 melanoma spheroids. Percentage of viable cells per spheroid was assessed using the "Cell Titer Glo"-assay. Graphs represent mean luminescence intensity per spheroid (± SD) relative to untreated control (UTC). Data points were assessed in triplicates in 3 independent experiments. (E+F) Colony formation assay comparing dose-dependent survival in 2D *versus* 3D cultures of 518A2 melanoma cells. (**E**) AV-38/398 treated cells depict comparable sensitivity in 2D and 3D cultures, whereas (**F**) Cilengitide is effective in 2D, but not in spheroid cultures. Data represent mean percentages of surviving cells (± SD) relative to untreated controls (UTC).

Next, we investigated the proportion of viable cells per spheroid (at 72 hr) following treatment with different concentrations of AV-38/398. Viability was assessed in spheroids of different size to investigate the potential of spheroid size-dependent (for example diffusion-dependent) effects. In small and large spheroids a similar decrease in the percentage of viable cells after 3 days was observed (IC50 ~7.5 µM). For additional data in WM115 and WM-35 cells see Figures 4C and 4D. Only in WM-35 cells on day three, a size-dependent effect was observed.

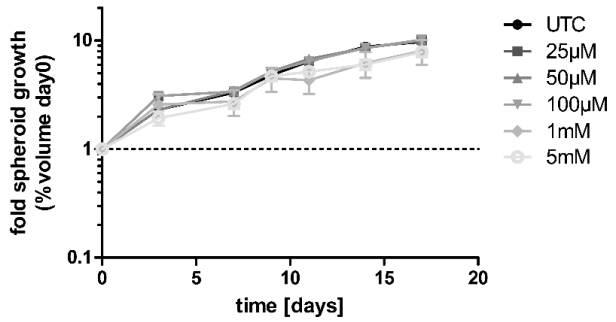

Figure 5: Dose escalation studies of Cilengitide in 3D melanoma spheroids. Growth curves of 518A2 melanoma spheroids treated with increasing doses (25 µM-5 mM) of Cilengitide. Data represent mean fold increase in spheroid volume (± SD) relative to day 0.

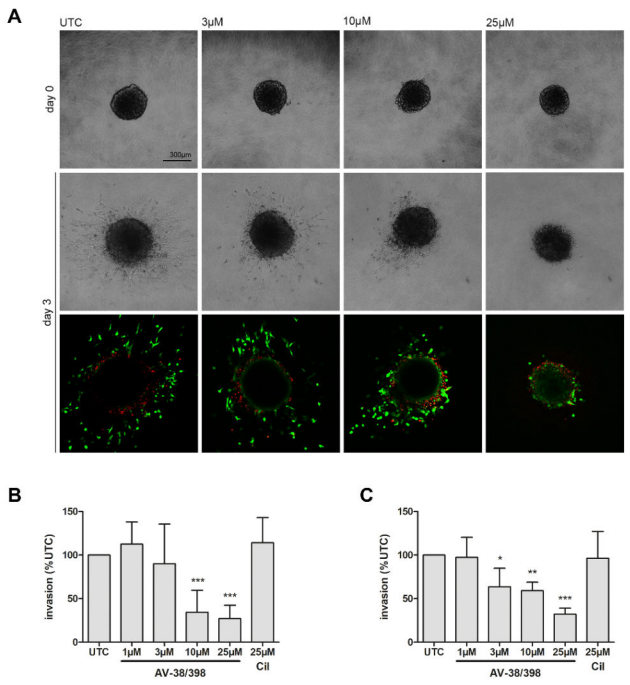

Figure 6: AV-38/398 inhibits spheroid invasion. (**A**) Spheroid invasion of AV-38/398 treated 518A2 melanoma spheroids into extracellular matrix (ECM) gels. B right field and Immunofluorescence (live/dead staining; viable cells in green, dead cells in red) images at day 0 and 3. (B+C) Quantification of spheroid invasion of AV-38/398 (AV) or Cilengitide (Cil) treated 518A2 (**B**) or WM-115 (**C**) spheroids. Bars represent mean percentage of invaded area (± SD) relative to untreated control areas (UTC). * p<0.05; ** p<0.01; *** p<0.001 (Two-way ANOVA).

Additionally, the cytotoxic effects of AV-38/398 and Cilengitide were comparatively evaluated in colony formation assays with 518A2 melanoma cells. The effects of AV-38/398 on survival were comparable in 2D and 3D models.

At a concentration of 10 µM (AV-38/398), a stronger effect on clonogenic survival of 518A2 spheroids was observed compared with 2D cultures (surviving fraction 13.9% *vs.* 54.6%). Cilengitide had no effect on clonogenic survival of 518A2 spheroids at concentrations of up to 50 µM.

Spheroid invasion is inhibited by AV-38/398, but not by Cilengitide

To investigate invasion capabilities in the presence of AV-38/398, we embedded spheroids into extracellular matrix gels and analyzed cell invasion. At the IC50 (10 µM), 518A2 melanoma spheroids invasion was significantly reduced; the surface of the invaded area was reduced by more than 50% (Figures 6A and 6B). We performed in parallel live/dead staining assays of embedded spheroids after three days of treatment with AV-38/398 (Figure 6B).

Similar spheroid invasion inhibition effects of AV-38/398 were observed in WM-115 cells (Figure 7). Invasion of WM-115 cells was inhibited at 3 µM, which is in agreement with our data regarding spheroid growth inhibition. However, no inhibitory effect on invasion by Cilengitide was observed at concentrations up to 25 µM in both cell lines tested.

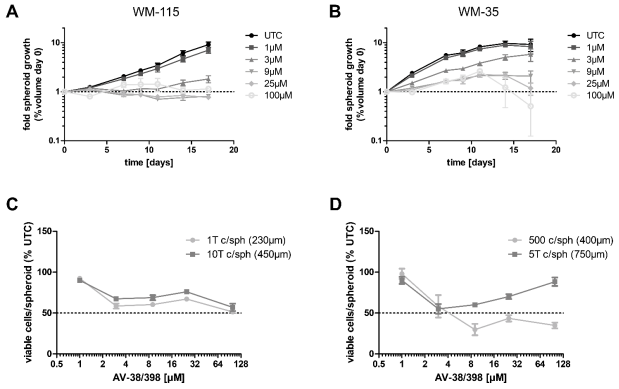

Figure 7: Inhibition of melanoma growth by AV-38/398 in WM-115 and WM-35 spheroids. (A+B) Growth curves of (**A**) WM-115 and (**B**) WM-35 spheroids treated with increasing concentrations of AV-38/398. Data represent mean fold increase in spheroid volume (± SD) relative to day 0. (C+D) Spheroid viability measurements after 3 days of AV-38/398 treatment in small and bigger sized (**C**) WM-115 and (**D**) WM-35 melanoma spheroids. Amount of viable cells per spheroid was assessed using the "Cell Titer Glo"-assay. Graphs represent mean luminescence intensity per spheroid (± SD) relative to untreated controls (UTC). Data points were assessed in triplicates in 3 independent experiments.

Discussion

We investigated in this study the anti-cancer and migration/invasion inhibiting capability of the well-described integrin antagonist Cilengitide and compared the results with the effects of a new, less well-investigated small molecule integrin antagonist. The anti-cancer activity of this compound has been previously determined in a panel of cancer cell lines and normal human endothelial cells [46,47]. Respective IC50 values of AV-38/398 were in a comparable range to

those evaluated for the comparator drug Cilengitide, an integrin antagonist which has already been tested in clinical trials for various cancer types [33]. Antiproliferative effects of AV-38/398 were not significantly correlated with the levels of αVβ3 expression. However, melanoma cell lines 518A2 and A375 with low target integrin αVβ3 expression were also less sensitive towards AV-38/398 treatment as compared to the other cell lines investigated. Our group (Christenheit) recently reported that HT-29 cells, which do not express integrin αVβ3, but αVβ5, are not sensitive towards AV-38/398, but towards Cilengitide treatment [47]. We observed a dose-dependent reduction of viable cell numbers by AV-38/398. At higher concentrations, AV-38/398, but not Cilengitide, induced a decline in cell numbers below those of untreated controls, suggesting additional direct cytotoxic effects of the drug.

Cell migration, as well as invasion, was inhibited by both AV-38/398 and Cilengitide. In scratch assays, AV-38/398 inhibited migration by more than 50% at concentrations near to its respective IC50 for inhibition of proliferation.

The effects of both compounds were also investigated under 3D culture conditions. Spheroid growth assays showed dose-dependent growth inhibiting and cytotoxic effects of AV-38/398 in the low micromolar range. Spheroid disintegration (518A2 and WM-35 cells) was observed at concentrations higher than the respective IC50 for inhibiting proliferation in 2D cultures. In spheroid viability assays, similar IC50 values for AV-38/398 were observed as under 2D culture conditions. 3D colony formation assays showed pronounced cytotoxic effects of AV-38/398 in 518A2 spheroids. However, in 518A2 spheroids cultures treated with concentrations up to 25 μM AV-38/398, approximately 50% of the disintegrated spheroids were able to regrow. In contrast, treatment with Cilengitide even up to 5 mM concentrations did not induce a significant growth inhibitory effect in spheroids. Also, in colony formation assays, no reduction in the number of viable cells by Cilengitide was observed at concentrations up to 50 μM. In invasion experiments under 3D culture conditions, we observed dose-dependent effects on invasion of the spheroid into extracellular matrix gels by AV-38/398, but not by Cilengitide.

Ideally, the clinically prescribed dose of a medicament should be as low as possible, and the mode of its action of a targeted compound should be primarily determined by the number and type of the occupied specific receptors. In the case of AV-38/398, there is evidence that the observed *in vitro* biological effects are mediated by its cognate receptor. This notion is based on the fact that the IC50 of AV-38/398 for growth inhibition was determined to be 0,25 μM in primary normal human endothelial cells [47], and between 1 to 10 μM in tumor cell lines of different histology [46,47]. In contrast to Cilengitide, the effective concentration range of AV-38/398 therefore corresponds well with its published binding affinity [46,47]. By comparison, Cilengitide has a sub-nanomolar antagonistic binding affinity to the αvβ3 receptor and affinities in the lower nanomolar range for its related integrins αvβ5 and α5β1. The corresponding binding data for Cilengitide have been published by several groups (IC50 for αvβ3 (0.61 nM) and αvβ5 (8.4 nM)) [33,53]. However, the concentrations of Cilengitide necessary to inhibit proliferation and survival in 2D cultures were significantly higher (in the micromolar range). Assuming that the postulated mode of action of a compound is mediated by the receptor against which the compound has been designed, its receptor binding constant and the IC50 for the biological effect of interest would be expected to be in a similar concentration range. While this is definitively the case for our comparator compound AV-38/398, the affinity of Cilengitide and the IC50 for growth inhibition, differs by a factor of least 1000. Cilengitide may therefore affect the function of additional subtypes of integrins at the concentrations used and may have off-target effects.

Conclusion

In summary, we could show that the novel integrin antagonist AV-38/398 exhibits promising anti-cancer effects in melanoma cells. The drug demonstrated strong anti-cancer effects and inhibited migration and invasion in the low micromolar range, both in 2D and more complex 3D spheroid models. The IC50 for inhibition of proliferation and the other desired effects correspond well with the reported binding affinity of AV-38/398 [46]. Regarding the results obtained for Cilengitide, besides this significant difference in the published binding affinity and IC50 values, our results show that Cilengitide is not effective in potentially clinically more relevant 3D culture models even at high concentrations. AV-38/398 showed to be more effective than Cilengitide, which was developed until clinical phase III, where it recently failed.

In conclusion, the results of our study provide a rationale for considering AV-38/398 or compounds of similar structure for further preclinical development.

Acknowledgements

The authors thank Prof. Walter Berger for providing melanoma cell lines and R. Hoffmann for technical assistance. Images were taken in cooperation with the Core Facility Imaging of the Medical University of Vienna, which is hereby gratefully acknowledged.

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