5.4 Biological activity of Compound 31 on human cancer cells

5.4.1 Introductory remarks

The data previously obtained on endothelial cells strongly suggested to test Compound 31 on cancer cells with the same biological assays, in order to determine its putative activity as integrin antagonist in cancer settings. To this end, Compound 31 was tested on a panel of human cancer cell lines in adhesion assays, and this screening pointed out that multiforme glioblastoma cells were the most responsive to the treatment. These cells were then further tested in wound healing assays in order to investigate the putative *in vitro* anti-migratory activity of Compound 31 on this cell line. Adhesion and wound healing assays were performed as previously described (see **paragraphs 4.3.3** and **4.3.4**, respectively).

Finally, Compound 31 was tested for its ability to drive actin remodeling as well. Indeed, integrins cluster in focal contacts and focal adhesions and mediate the cross-talk between ECM and actin cytoskeleton (see **paragraph 2.1.1**). This is the reason why it was intriguing to understand if the treatment with an integrin antagonist might be able to impair the onset of such structures, with a particular focus on actin assembly itself.

5.4.2 Compound 31 impairs cell adhesion to either vitronectin or fibronectin in human cancer cells

ECV-304, T98G, PC-3, Caki-1, H460 and MDA-MB-231 cells were allowed to adhere to immobilized vitronectin or fibronectin, in the presence of increasing concentrations of the compound.

Cell line	IC_{50} vitronectin (μM)	IC_{50} fibronectin (μM)
ECV-304	14.1 ± 1.6	25.0 ± 1.5
T98G	3.2 ± 0.7	1.5 ± 0.3
PC-3	7.8 ± 0.4	8.0 ± 1.2
Caki-1	12.2 ± 2.6	5.2 ± 1.0
H460	29.1 ± 8.0	77.0 ± 14.6
MDA-MB-231	21.8 ± 5.4	9.4 ± 2.6

Table XII. *Effect of Compound 31 on human cancer cells adhesion to vitronectin or fibronectin. Each data point was performed in triplicate, in two independent experiments.*

As outlined in **Table XII**, Compound 31 significantly inhibited cell adhesion to either vitronectin of fibronectin in all cell lines in a similar micromolar range, except for multiforme glioblastoma, renal clear cell carcinoma and breast adenocarcinoma cells. These cell lines displayed a two fold stronger inhibition on fibronectin in respect of vitronectin. Given that vitronectin is the ligand for integrins αvβ3 and αvβ5, whereas fibronectin is bound predominantly by integrin α5β1 and (to a lesser extent) αvβ3, the adhesion assay strongly suggested that Compound 31 was able to target all the integrins of interest (namely αvβ3, αvβ5 and α5β1). However, the stronger inhibition observed on T98G, Caki-1 and MDA-MB-231 cells plated on fibronectin was probably

due to the inhibitory effect Compound 31 exerted on integrin α5β1. This molecule was in fact expressed in higher amounts in respect of integrin αvβ3 and αvβ5 in the previously cited cell lines (see paragraph **5.1.2**).
Prostate cancer cells deserved a different emphasis, because this cell line was really efficiently inhibited by Compound 31, even if there was not an appreciable difference between vitronectin or fibronectin incubated cells. As underlined before, PC-3 cells derive from a bone metastasis and bone is known to generally express good amounts of integrin αvβ3, which plays pivotal roles in bone resorption and remodeling. Matching this evidence with adhesion assay results and with the high surface expression of integrin α5β1 on PC-3 cells, this type of metastatic prostate cancer cells could be in the future another valuable model to study the role of cyclic RGD compounds in a context different from cancer, namely bone resorption and bone biology.
Overall, multiforme glioblastoma was considered the best responsive cell line because of its very low IC_{50} values in both conditions. The good responsiveness of multiforme glioblastoma cells was not surprising. As underlined in the Introduction, this tumour is currently treated in Clinical Trials with standard chemotherapeutic agents (such as temozolomide) in association with the reference cyclic RGD compound Cilengitide® [19]. Even if the data presented in this work regard mainly an *in vitro* ideal situation, it is however encouraging to observe such a good anti-adhesive activity of Compound 31 on this kind of cerebral neoplasia, in the light of the clinical results gained so far by Cilengitide®. As a reference for cancer and because of the experimental evidences highlighted before, multiforme glioblastoma cells were then chosen as a model.
Figure 20 outlines the graphical analysis of the adhesion assays performed on the cell panel.

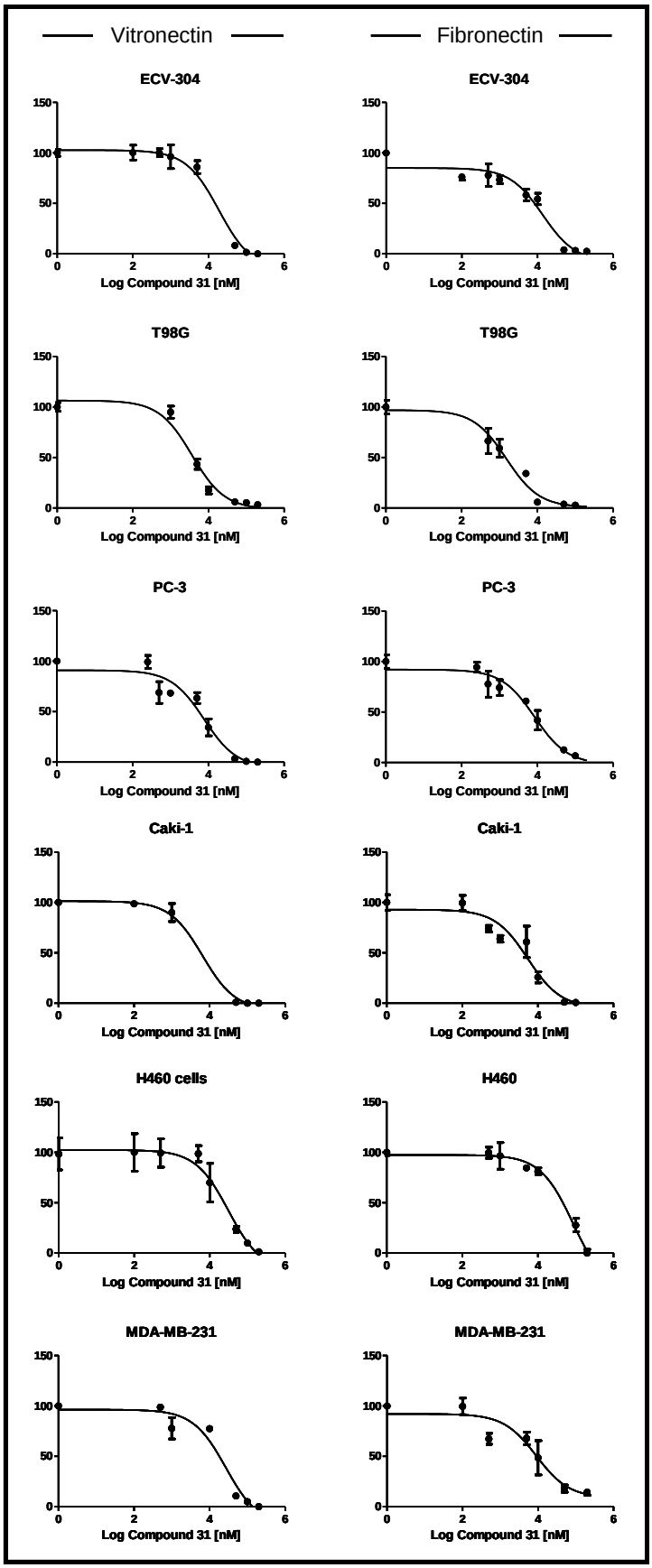

Figure 20. *Graphical analysis of adhesion assays performed with Compound 31 on the cancer cell panel. Cells were plated on vitronectin or fibronectin.*

5.4.3 Compound 31 displays *in vitro* anti-migratory activity on multiforme glioblastoma cells

The putative bi-dimensional anti-migratory activity of Compound 31 was measured by means of wound healing assay on T98G cells. Multiforme glioblastoma cells displayed a two-fold adhesive inhibition when Compound 31 was administered in the presence of fibronectin as a substrate. T98G healing potential was then determined on either vitronectin or fibronectin coated wells, in order to understand if the migratory behavior of these cells in response to the cyclic RGD peptide could have been affected by the presence of different extracellular substrates. Overall, the presence of different substrates could be really important in the putative idea to administer cyclic RGD peptides *in vivo*, where there is a much more intensive and complex cross-talk between cells and ECM proteins.

Wound healing assays were performed with the cell line and substrate specific IC_{20} of Compound 31, namely 0.9 µM for vitronectin and 0.3 µM for fibronectin. This values were graphically extrapolated by the IC_{50} values measured by means of adhesion assays.

As shown in **Figure 21**, Compound 31 significantly slowed the healing of the wounded area in T98G cells, whereas control wells healed properly. In addition, it was noteworthy that multiforme glioblastoma cells plated on fibronectin healed less than if plated on vitronectin. This was in line with the results previously obtained by means of adhesion assay, and confirmed that the higher expression of integrin α5β1 on T98G cells rendered them more responsive to the cyclic RGD peptide.

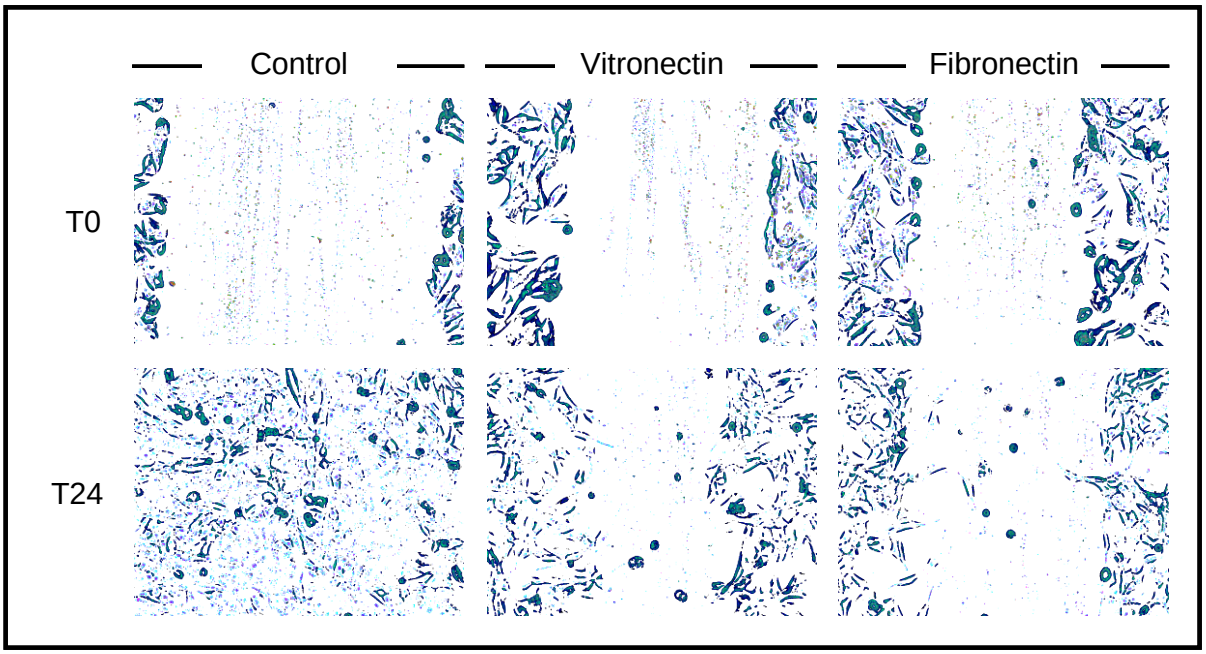

Figure 21. *Wound healing assay on T98G cells. The IC_{20} of Compound 31 was incubated on T98G cells plated on vitronectin or fibronectin for 24 hours, in order to determine its anti-migratory activity. T0: starting point; T24: 24 hours incubation. Original magnification: 10x.*

5.4.4 Compound 31 drives actin remodelling in multiforme glioblastoma cells

Because of their responsiveness in both adhesion and wound healing assays, multiforme glioblastoma cells were further treated with Compound 31 in order to determine if the cyclic RGD peptidomimetic might functionally affect actin cytoskeleton assembly as well. In particular, Compound 31 was tested at a concentration of 1 µM or 2 µM because those values corresponded to IC_{20} and IC_{30}, respectively, and no cytotoxic effect was desired. Multiforme glioblastoma cells were cultured and treated on vitronectin or fibronectin in order to understand if the presence of a different substrate might be relevant in such treatment. Compound 31 was incubated for 3 hours at 37 °C, and actin cytoskeleton was stained according to the experimental protocol presented in **paragraph 4.3.5**. Cells were firstly analyzed with fluorescence microscopy and secondly through confocal microscopy. The latter approach allowed me to better analyze the tri-dimensional structure of the actin cytoskeleton and the putative effect of Compound 31 on the stability of focal adhesion complexes.

As shown by the fluorescence microscopy analysis (**Figure 22**), the actin cytoskeleton of un-treated cells retained its structure, with elongated filaments well distributed all over the cell shape. The treatment with 2 µM of Compound 31 was able to strongly impair actin cytoskeleton structure in both conditions, with a particular focus on cells plated on fibronectin. The same was true for cells treated with 1 µM of Compound 31 as well, even if the overall effect of the Compound was less evident and few actin filaments were still present (in particular in cells plated on vitronectin). These evidences suggested that when integrins αvβ3, αvβ5 and α5β1 were involved at the same time, as occured when cells were plated on fibronectin, the effect on actin cytoskeleton assembly was more pronounced. Indeed, when only integrins αvβ3 and αvβ5 were targeted, as occurred when cells were plated on vitronectin, the actin cytoskeleton structure was less affected. This ultimately supported the real effectiveness of Compound 31 in the inhibition of integrin α5β1.

The confocal microscopy analysis basically confirmed what previously observed. The cytoskeleton of treated cells displayed a completely subverted structure of actin filaments. In addition, the confocal images depicting focal adhesions strongly suggested that the treatment with Compound 31 (in particular with a concentration of 2 µM) was able to disrupt those structure, thus ultimately compromising cell shape (**Figure 23**).

On the whole, Compound 31 was demonstrated to drive actin cytoskeleton remodelling in multiforme glioblastoma cells.

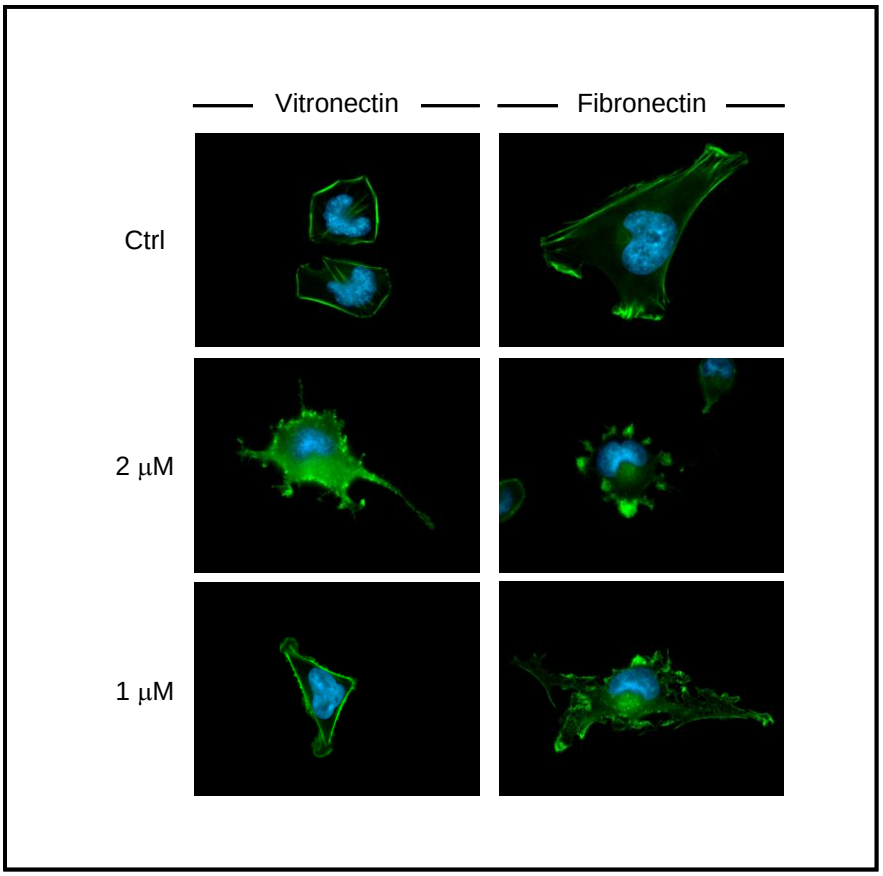

Figure 22. *Actin remodelling test: fluorescence microscopy analysis. Compound 31 was able to drive actin remodelling in multiforme glioblastoma cells plated on vitronectin (left column) or fibronectin (right column). Cells were incubated 3 hours at 37°C with treatment medium alone (first row), Compound 31 at 2 μM (second row) or 1 μM (third row). Original magnification: 63x.*