5.2 Cell-free screening of test compounds

5.2.1 Introductory remarks

Solid-phase receptor-binding assay is a simple and precise method to analyze protein-protein interactions. Moreover, this test is well established for the study of interactions between ECM proteins and integrins [41]. In this thesis, solid-phase receptor-binding assay was performed in order to quantify the binding affinity of test compounds for integrins αvβ3 and αvβ5. Unfortunately, a binding affinity test for integrin α5β1 was not performed because of the lack of a suitable commercial isolated receptor.

5.2.2 Compounds 31, 13 and 15 show a good binding affinity for integrin αvβ3

Compound 31 was selected in a library of cyclic RGD-containing functionalized azabicycloalkane peptides designed to be carriers for therapeutic and diagnostic purposes. All RGD derivatives in the library were in fact screened for their conformational properties by spectroscopic and computational methods, comprising docking studies with the X-ray crystal structure of the extracellular domain of integrin αvβ3. Compound 31 was tested in solid-phase receptor-binding assay and displayed a good binding affinity for both αvβ3 and αvβ5, with IC_{50} values of 53.7 ± 17.3 nM and 205 ± 33.5 nM, respectively [36].

In addition, the small molecule was functionalized with a fluorescein probe, which is approved for human use in diagnostic techniques [15] and has already been tested in a fluorescent polarization assay. The latter is a protocol widely used for high-throughput screening in the drug discovery process [51]. Compound 31 was therefore conjugated with a fluorescein moiety (as described in **paragraph 2.2.3**), thus obtaining Compound 13 and Compound 15. Because of the higher affinity of Compound 31 for integrin αvβ3, Compounds 13 and 15 were tested in solid-phase receptor-binding assay against integrin αvβ3 only. Both Compounds were demonstrated to bind integrin αvβ3, even if the IC_{50} values were higher than those of Compound 31 (namely: 142 ± 6 nM for Compound 13, 207 ± 85 nM for Compound 15). This could be probably due to the steric hindrance given by the fluorescein moiety [2].

Results of solid-phase receptor-binding assays performed on Compounds 31, 13, and 15 are outlined in **Table IX**.

Compound	IC_{50} (nM) ± SD for αvβ3	IC_{50} (nM) ± SD for αvβ5
31	53.7 ± 17.3	205 ± 33.5
13	142.0 ± 6.0	n.p.
15	207 ± 85	n.p.

Table IX. *Results of solid-phase receptor-binding assays performed on Compounds 31, 13 and 15. N.p.: not performed.*

5.3 The biological activity of Compound 31 on human endothelial cells suggests its possible use as an anti-angiogenic compound

5.3.1 Introductory remarks

The good binding affinity of Compound 31 for integrin αvβ3 receptor suggested to further investigate its biological activity. The first screening was performed on endothelial cells, namely EPCs and HUVECs. Apart from literature data highlighting the expression of integrin αvβ3 by vascular endothelial cells, the previously performed surface antigen characterization (see **paragraph 5.1**) confirmed that both cell lines expressed good and comparable amounts of integrin αvβ3 and very high amounts of the other putative target of Compound 31, namely integrin α5β1. Endothelial progenitor cells were screened because no data about the putative effects of antagonist compounds were available on such cell model. In addition, it was interesting to understand if and how compounds such as cyclic RGD peptidomimetics could be theoretically applied to endothelial cells with stem-like characteristics. HUVECs were used during the screening as well because this cell line is considered the gold standard for vascular biology tests.

The biological activity (namely, its putative anti-angiogenic and anti-migratory activities) of Compound 31 on endothelial cells was investigated by means of adhesion and wound healing assays. Adhesion and wound healing assays were performed as previously described (see **paragraphs 4.3.3** and **4.3.4**, respectively).

5.3.2 Compound 31 impairs endothelial cells adhesion to either vitronectin or fibronectin

Adhesion assay provides a good method to understand the ability of a compound to compete against a physiological ligand in an *in vitro* system such as cell culture. As was previously underlined (see **Table I**), in the case of the integrins studied in this work the physiological ligands are represented by vitronectin (integrins αvβ3 and αvβ5) and fibronectin (integrins αvβ3 and α5β1).

Compound 31 was tested in adhesion assays on EPCs and HUVECs. Namely, EPCs and HUVECs 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)
EPCs	10.6 ± 4.7	21.1 ± 2.2
HUVECs	29.5 ± 1.1	26.2 ± 1.4

Table X. *Effect of Compound 31 on EPC and HUVEC cells adhesion to vitronectin or fibronectin. Each data point was performed in triplicate, in two independent experiments.*

As outlined in **Table X**, Compound 31 effectively inhibited cell adhesion to either vitronectin or fibronectin in both cell lines. Endothelial progenitor cells are particularly frail and tricky to maintain *in vitro*, thus rendering difficult to obtain a good experimental and biological reproducibility. However, endothelial progenitor cells adhesion was two-fold more efficiently impaired by Compound 31 when cells were cultured on vitronectin. This was surprising, because surface expression data indicated that EPCs expressed four fold integrin α5β1 in respect of integrin αvβ3. A stronger inhibition of these cells adhesion on fibronectin was thus expected. Given that vitronectin is the physiological ligand for integrins αvβ3 and αvβ5, it is possible that the presence

of vitronectin might stimulate endothelial progenitor cells to express higher amounts of integrin αvβ3 and αvβ5 *in vitro*. Moreover, if this is true it is reasonable that in EPCs vitronectin might stimulate integrins αvβ3 and αvβ5 expression more efficiently than fibronectin in respect to integrin α5β1. Finally, it is important to remember that Compound 31 was selectively designed to target integrins αvβ3 and αvβ5, even if the absence of a cell free test for integrin α5β1 did not allow to determine if the Compound might target integrin α5β1 too. In addition, the good IC_{50} values displayed by HUVECs suggested that Compound 31 might have a role on endothelial cells and, consequently, in vascular biology. It is in fact known that fibronectin, vitronectin and the related integrin receptors play crucial and specific roles during angiogenic events. In particular, the fibronectin-α5β1 complex is mainly involved in developmental angiogenesis, whereas vitronectin-αvβ3 and vitronectin-αvβ5 complexes are mainly related to post-natal tumor angiogenesis.
The graphical analysis of the adhesion assays described above is shown in **Figure 17**.

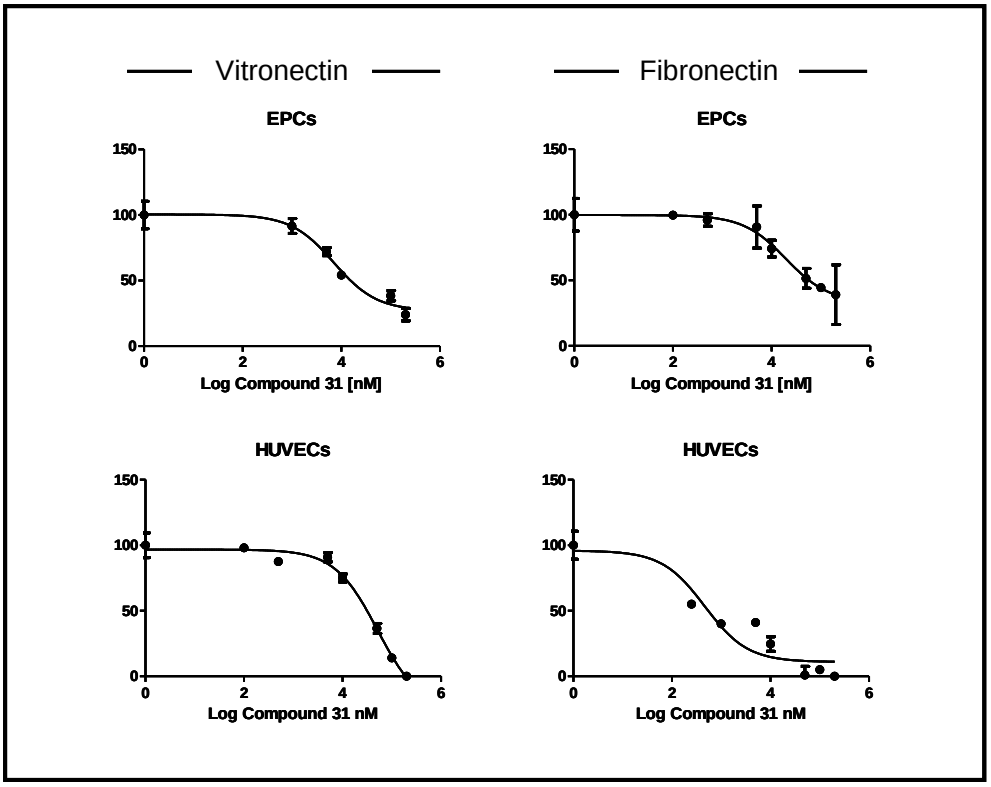

Figure 17. *Graphical analysis of adhesion assays performed with Compound 31 on EPCs and HUVECs plated on vitronectin or fibronectin.*

Overall, it was amazing that Compound 31 was able to inhibit cell adhesion also in endothelial progenitor cells. It was then mandatory to further investigate whether Compound 31 might exert some other biological effects (e.g. anti-migratory activity) on both endothelial progenitor and primary vascular cells.

5.3.3 Compound 31 displays *in vitro* anti-migratory activity on endothelial cells

Because Compound 31 inhibited the adhesive activity of HUVECs on vitronectin or fibronectin in a similar micromolar range, HUVECs were exposed to the wound assay only on vitronectin coated wells. Vitronectin was chosen as a substrate for primary endothelial cells because probably Compound 31 was mainly able to bind integrin αvβ3 and αvβ5 on this cell line. EPCs were exposed to the wound assay on both vitronectin and

fibronectin because there was a two-fold difference in adhesive inhibition between the substrates, as was underlined above (see paragraph **5.3.2**).

Wound healing assays were performed with the cell line and substrate specific IC_{20} of Compound 31, relating to the IC_{50} values measured by means of adhesion assays. IC_{20} values are outlined in **Table XI**.

Cell line	IC_{20} vitronectin (μM)	IC_{20} fibronectin (μM)
HUVECs	10.0	n.p.
EPCs	3.0	8.0

Table XI. *Cell line and substrate specific IC_{20} of Compound 31. IC_{20} values were graphically extrapolated by adhesion assays IC_{50} values.*

As shown by **Figure 18** and **Figure 19**, Compound 31 significantly slowed the healing of the wounded area in both EPCs and HUVECs, whereas control wells healed properly. In particular, no gross discrepancy was observed in EPCs wounded on vitronectin or fibronectin, indicating that Compound 31 retained the same anti-migratory activity on both substrates. This seemed to suggest that the overall anti-migratory activity of Compound 31 was not affected by the presence of different extracellular ligands, in contrast with what observed during the adhesion assay.

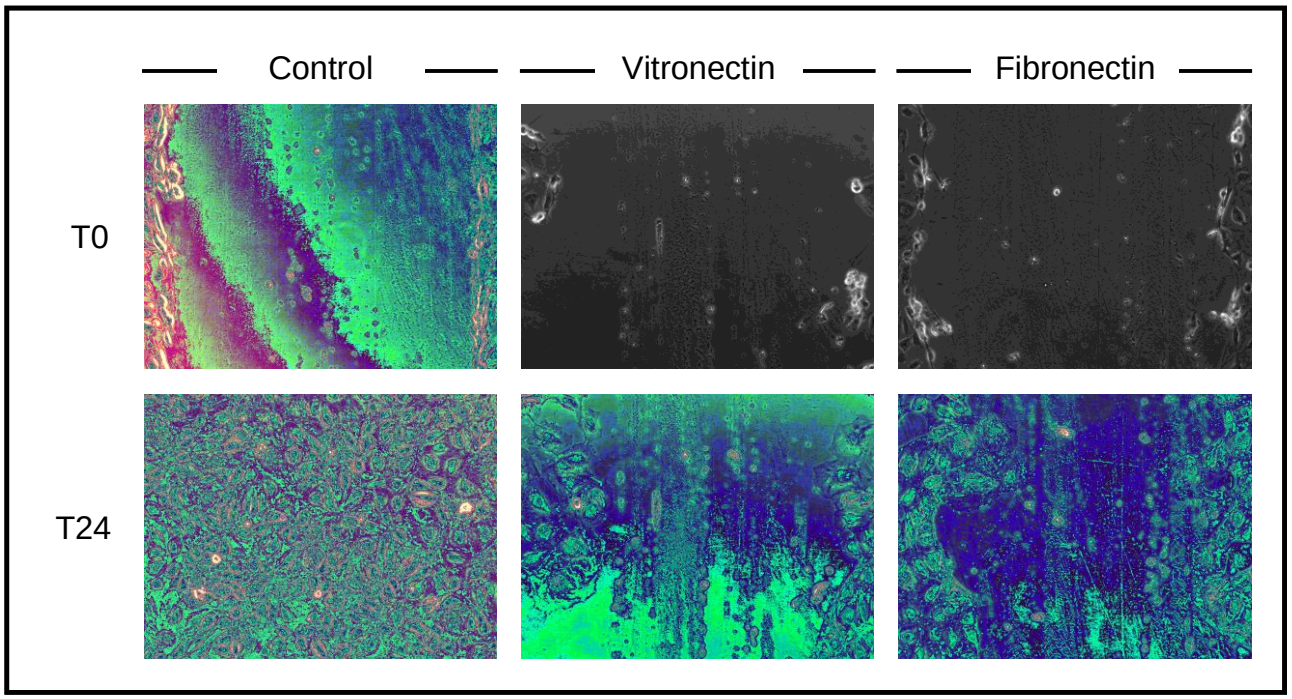

Figure 18. *Wound healing assay on EPCs. The IC_{20} of Compound 31 was incubated on EPCs plated on vitronectin or fibronectin for 24 hours, in order to determine its anti-migratory activity. T0: starting point; T24: 24 hours incubation. Original magnification: 10x.*

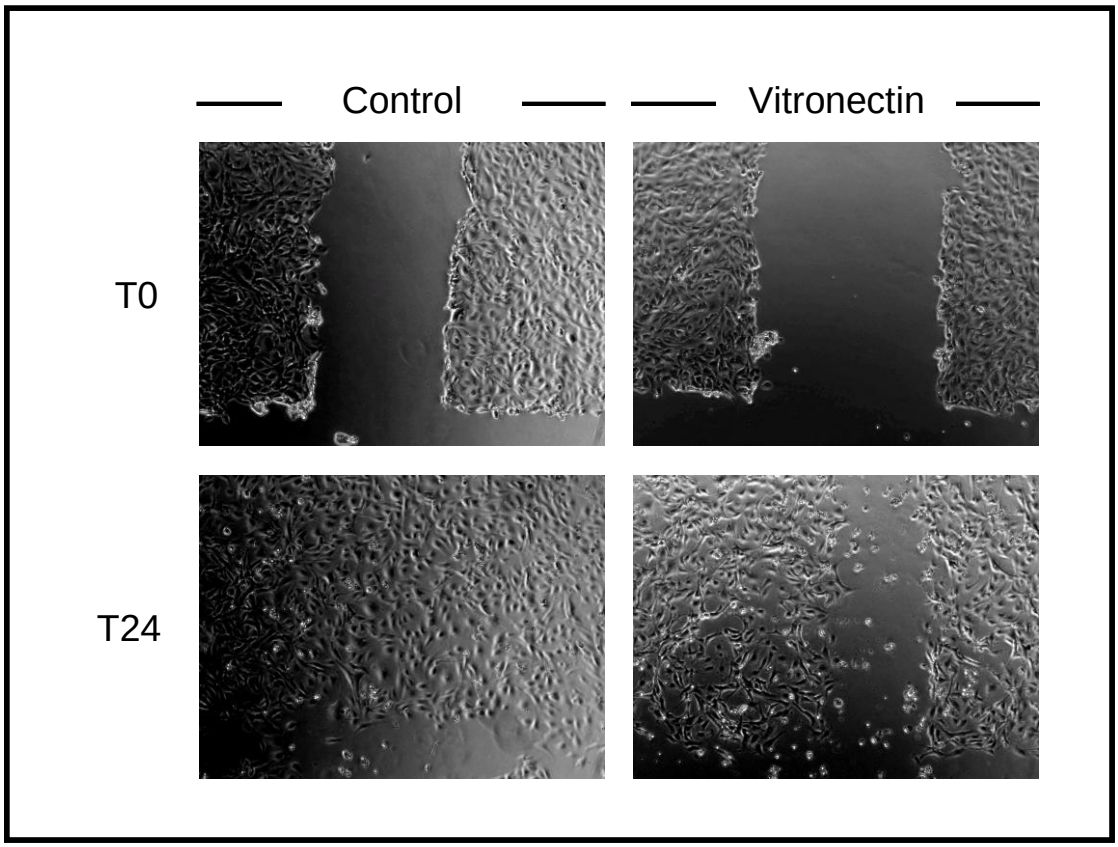

Figure 19. *Wound healing assay on HUVECs. The IC_{20} of Compound 31 was incubated on HUVECs plated on vitronectin or fibronectin for 24 hours, in order to determine its anti-migratory activity. T0: starting point; T24: 24 hours incubation. Original magnification: 10x.*