6. DISCUSSION

Integrins are heterodimeric cell surface adhesion receptors evolutionary old and playing very important roles in both physiological and pathological processes, such as neo-angiogenesis and cancer biology. Integrins act mainly as molecular bridges between the cell and the extracellular matrix. The same extracellular ligands bound by integrins are able to activate the heterodimer, so that integrins adopt an active conformation able to achieve the activation of specific signalling pathways. Integrins recognize specific short peptide sequences on their ligands, but the same ligand may be bound by different integrins because of their redundancy. In this way the cell is always able to respond to a diverse array of extracellular stimuli [4].

Integrins do not have intrinsic kinase activity, and this is the reason why activated integrins generally need to associate with molecular partners such as adaptor proteins from the cytoplasm. This association gives rise to the adhesion complexes which, if activated together with growth factor and cytokine receptors, promote cell spreading, migration, survival and proliferation. In addition, the stabilization of adhesion complexes in adherent cells avoids the onset of apoptosis. The ability of integrins to induce cell survival and proliferation may obviously be applied to physiological processes (such as cell motility during development) but also to pathological phenomena (such as cancer spreading and metastasis) [19].

A lot of interest was gained by Arg-Gly-Asp (RGD) recognizing integrins, with a particular focus on integrins αvβ3, αvβ5 and α5β1. On the whole, αv integrins have a pivotal role in endothelial cell growth, survival and migration during angiogenesis and lymphangiogenesis. Undoubtedly, the importance of αv integrins in vascular biology is related to both physiological angiogenesis (e.g. wound healing) and pathologic neo-angiogenesis (e.g. during inflammation or cancer spreading and metastasis). In addition, the aforementioned integrins, and in particular integrins αvβ3 and α5β1, have been demonstrated to promote cancer cell migration, survival and invasion in different organs given to their ability to degrade basement membrane by the interaction with metalloproteases. This kind of activity is carried on during the epithelial-mesenchymal transition as well. All these evidences drove a lot of scientific efforts in the development of integrin targeting agents, in order to inhibit the activity of both tumor cells and tumor-associated host cells. Several integrin targeting methods have been then studied and a wide array of integrin antagonists was rendered available so far [19].

Integrin antagonists comprise function blocking antibodies, synthetic peptides and peptidomimetics. The development of cyclic RGD peptidomimetics which could selectively inhibit integrins αvβ3, αvβ5 and α5β1 in angiogenesis and cancer settings is particularly interesting. In particular, the conformationally constrained cyclic structure of our molecules and its chemical modifications allow the improvement of the binding affinity and bioavailability. This is the reason why peptidomimetic compounds generally display a powerful activity and are less subjected to enxzymatic degradation [25].

Belvisi et al. previously reported the synthesis of a small library of cyclic RGD peptidomimetics functionalized with heteroalkyl side chains ending with a hydroxyl group. This group can be easily applied in the conjugation of various chemical entities for applications in medical diagnosis and therapy [6, 36]. The final aim of this work was then to identify putative cyclic RGD peptidomimetics able to provide anti-angiogenic and anti-neoplastic activity, together with the possibility to use them as imaging vehicles for cancer diagnosis.

The first part of this work was dedicated to the characterization of a panel of human cell lines and to the investigation of the binding affinity of the most promising cyclic-RGD compound, namely Compound 31, for integrins αvβ3 and αvβ5 only (given the absence of a commercial available integrin α5β1 isolated receptor). Because the heteroalkyl side chain of Compound 31 allowed its conjugation with fluorescein through two different linkers, two derivative compounds were generated, namely Compounds 13 and 15, ideally dedicated to imaging applications. The binding affinity of such Compounds was investigated as well.

The panel of human cell lines screened for the expression of our markers of interest (namely integrins αvβ3, αvβ5 and α5β1) comprised endothelial progenitor cells and primary endothelial cells; bladder, glioblastoma multiforme, prostate, renal, non-small cell lung and breast cancer cells. Flow cytometry and immunofluorescence helped me to quantitatively and qualitatively determine the expression of each marker on the cells. These analyses pointed out that progenitor and primary endothelial cells expressed higher amounts of integrin α5β1 in respect of integrin αvβ3, according to literature data underlying that integrin α5β1 is mainly involved in physiological angiogenesis. On the other side, glioblastoma multiforme cells and renal clear cell carcinoma cells were identified as the highly expressing integrins α5β1 and αvβ3, with a two-fold expression of integrin α5β1 in respect of that of integrin αvβ3. Bladder cancer cells expressed approximately the same amounts of all receptors. Prostate cancer and non-small cell lung cancer cells expressed high levels of integrin α5β1, and breast adenocarcinoma cells displayed the most remarkable discrepancy between a high expression of integrin α5β1 and a low expression of integrin αvβ3. Integrin αvβ5 was very faintly expressed in all cell lines.

In the meantime, Compound 31 was selected among the small library of cyclic RGD peptidomimetics by means of computational analysis (docking studies with the X-ray structure). Compound 31 was tested in solid-phase receptor-binding assay for its affinity for integrins αvβ3 and αvβ5, underlying its higher specificity for integrin αvβ3. For this reason Compounds 13 and 15 were tested in the same assay against integrin αvβ3 only, confirming that the fluorescein-conjugated cyclic RGD compounds were able to efficiently bind the receptor as well (even if with less affinity than that of Compound 31).

Later on, the good binding affinity of Compound 31 for integrin αvβ3 suggested to further test its biological activity on the panel of cell lines, with a particular focus on its putative anti-adhesive and anti-migratory properties. To this reason, adhesion and wound healing assays were chosen as preferred protocols. Adhesion assay provides a good method to understand the ability of a compound to compete against a physiological ligand. Wound healing assay allows the investigation of the possible anti-migratory activity of a compound in a bi-dimensional setting.

Progenitor and primary endothelial cells were investigated first. Compound 31 inhibited cell adhesion to either vitronectin or fibronectin in both cell lines. In particular, endothelial progenitor cells adhesion was two-fold more efficiently impaired by Compound 31 when cells were cultured on vitronectin. Overall, the good results obtained with Compound 31 on both endothelial cell lines suggested that it might really have a role in vascular biology and during angiogenic events. The putative anti-migratory activity of Compound 31 on progenitor and primary endothelial cells was further investigated, underlying that the Compound significantly

slowed the healing of the wounded area in both cell lines, independently upon the substrate. This indicated that Compound 31 was effective in the inhibition of endothelial cell migration as well.

Then, the analysis of the anti-adhesive and anti-migratory activity of Compound 31 was extended to the cancer cell panel as well. The screening underlined that multiforme glioblastoma cells were the most responsive to the treatment, with a two-fold stronger inhibition on fibronectin in respect of vitronectin. This was in line with the expression data of integrins αvβ3 and α5β1 determined by flow cytometry. Moreover, the good responsiveness of multiforme glioblastoma cells was not surprising, given that this cerebral tumor is currently treated in Clinical Trials with standard chemotherapeutic drugs in association with the reference cyclic RGD compound Cilengitide[19]. This encouraging data prompted us to further investigate the anti-migratory activity of Compound 31 on multiforme glioblastoma cells. Wound healing assays performed on these cells highlighted that Compound 31 slowed the healing of the wounded area, and that cells plated on fibronectin healed less than those plated on vitronectin. This evidence confirmed what previously observed by means of adhesion assay and stressed that Compound 31 might have a role as an inhibitor of cell adhesion and migration in cancer biology too.

As underlined before, integrins usually associate with cytoskeletal proteins so that they act as molecular bridges between the extracellular matrix and the actin cytoskeleton. To further investigate whether the treatment with Compound 31 might have a functional effect on the assembly of actin cytoskeleton, Compound 31 was tested again on multiforme glioblastoma cells and the actin cytoskeleton was stained after the treatment. The actin cytoskeleton of un-treated cells retained its structure. On the other side, the treatment with Compound 31 impaired actin cytoskeleton structure in a very pronounced way. In addition, confocal microscopy revealed that Compound 31 was able to completely impair the onset of focal adhesions, thus consequently compromising cell shape as well. These results underlined that the treatment with the cyclic RGD peptidomimetic was effectively able to drive actin cytoskeleton remodelling impairing its structure and, ultimately, affecting cell physiology.

After the cell biology screening with Compound 31, the biological activity of its derivatives was investigated as well. To this end, Compounds 13 and 15 were tested on a panel of human cells in order to determine their ability to stain cells. The cyclic RGD scaffold of such molecules should have had the property to drive them toward an integrin αvβ3-dependent internalization, so that cells highly expressing integrin αvβ3 should have been able to internalize Compounds 13 and 15. The cell panel comprised primary endothelial cells and bladder, multiforme glioblastoma, prostate, renal and breast adenocarcinoma cells. Overall, each Compound displayed a fluorescence distributed both at the cell surface and in putative cytosolic vesicles, suggesting that both Compounds were internalized upon binding with integrin αvβ3. The most encouraging results were observed in endothelial, multiforme glioblastoma and prostate cancer cells. Notably, breast adenocarcinoma cells subjected to the treatment with Compounds 13 or 15 did not show any sign of fluorescence. Given the high expression of integrin α5β1 and the low amounts of integrin αvβ3 on these cells as highlighted by flow cytometry and immunofluorescence, these data indicated that both functionalized cyclic RGD peptidomimetics were highly specific for integrin αvβ3 [2].

Finally, an opportunely modified Compound 31 was recently conjugated on the surface of gold nanoparticles together with fluorescein, giving rise to Compound DA425. Gold nanoparticles are considered the ideal candidate for optical imaging because of their low susceptibility to photobleaching, and may be functionalized with several fluorochromes and cyclic RGD peptidomimetics.

Compound 425 was tested on prostate cancer cells because of their good responsiveness to Compound 13 and 15 administration. Breast adenocarcinoma cells were used as a negative control given the results of flow cytometry mentioned above. Compound DA425 positively stained prostate cancer cells in a very peculiar way, whereas the control Compound DA426 (gold nanoparticles coated with fluorescein alone) gave no signal at all. In addition the confocal analysis of cells stained with Compound DA425 revealed that the signal provided by fluorescein and that given by gold were perfectly merged and localized near the focal contacts. This evidences finally suggested that the internalization of Compound DA425 took place in the membrane zones where integrin αvβ3 is mainly expressed, the cell adhesive contacts. The control compound DA426 did not stain prostate cancer cells at all. Notably, breast adenocarcinoma cells did not show any sign of fluorescence neither in the case of Compound DA425 nor of Compound DA426 treatment, suggesting that Compound DA425 staining was specific for integrin αvβ3 over-expressing cells (because of the coating of gold nanoparticles with the cyclic RGD peptidomimetic).

In summary, during this work the biological activity of some integrin αvβ3-targeting compounds, namely a cyclic RGD peptidomimetic and its functionalized derivatives, was investigated in vascular and cancer biology settings. The progenitor compound displayed a good *in vitro* anti-adhesive and anti-migratory activity on human endothelial and epithelial cancer cells, with a particular focus on endothelial progenitor cells and multiforme glioblastoma cells. In addition, the compound was demonstrated to be able to drive actin cytoskeleton remodelling on glioblastoma multiforme cells, thus impairing the structure of focal adhesions, cell shape and cell physiology. Fluorescein-conjugated derivatives of the progenitor compound, selectively driven toward integrin αvβ3, positively stained a panel of human cell lines ranging from primary endothelial cells to cancer epithelial cells, with the most encouraging results observed on endothelial, glioblastoma multiforme and prostate cancer cells. Finally, the last developed chemical entities, namely gold nanoparticles conjugated in turn with fluorescein and cyclic RGD, were tested on cancer cells for their imaging properties. The analysis revealed that functionalized gold nanoparticles positively and specifically stained cells given their internalization mainly near the focal contacts, where integrin αvβ3 is normally expressed.

It could be now interesting to understand and deepen the putative molecular and signalling mechanisms promoted by the treatment of the cells with cyclic RGD peptidomimetics. A particular effort should be made in defining if and how such compounds might impair the interaction between integrins and growth factor or cytokines receptors, in order to plan a putative combination therapy with already known cancer inhibitors (e.g. cytokines or growth factor antagonists.) In fact, it is not reasonable to think about an exclusive treatment with cyclic RGD peptidomimetics alone. Integrins are widely expressed targets, so the side effects of their inhibition might probably overcome the desired results. This is one of the reasons why cyclic RGD scaffolds could be used mainly as carriers to deliver already known therapeutic agents or imaging vehicles to integrin over-expressing cells. For what regards imaging, the work presented here is the result of an

exhaustive study suggesting that even low concentrations of the cyclic RGD peptidomimetic should be able to deliver the imaging vehicles into target cells. However, Reynolds and colleagues recently published a paper blaming that the proangiogenic effects of low concentrations of RGD-mimetic integrin inhibitors could compromise their efficacy as anticancer agents or imaging vehicles, thus displaying major implications for the use of such compounds in humans [44]. Apart from the good results observed *in vitro* and presented here, it could be then mandatory to test these molecules directly *in vivo*. This approach should help to understand if and how the concentration of the cyclic-RGD scaffold (namely, the 'Trojan horse') might affect the overall activity of the imaging compound when it is administered in a complex setting, such as tissue and organ microenvironment.

7. CONCLUSIONS

Thanks to the experimental work presented in this Thesis it was possible to:

- characterize a panel of human endothelial and cancer cells for their expression of markers of interest for this work, namely integrin αvβ3, αvβ5, and α5β1. That kind of characterization has never been reported in the literature;
- choose and screen several cyclic RGD peptidomimetics selectively driven toward integrin targets, and comprising a progenitor cyclic RGD compound and its functionalized derivatives. A particular focus was pointed on integrin αvβ3 targeting, given that this integrin is in fact already considered as a molecular target in known anti-angiogenic and anti-cancer therapies;
- test the progenitor compound on the human cell panel, thus determining its anti-adhesive and anti-migratory activity in respect of both endothelial and cancer cells. In addition, it has been demonstrated that the compound was able to drive the remodelling of the actin cytoskeleton, providing its disassembly and altering the morphology of the cells;
- test on a human cell panel the fluorescein-conjugated cyclic RGD derivatives of the progenitor compound as imaging agents. The RGD scaffold allowed to have a specific selectivity for integrin αvβ3, so that the compounds were efficiently tailored and internalized into the cell. Thus, both fluorescein-conjugated compounds positively stained endothelial and cancer cells, with a particularly good staining on primary endothelial cells, glioblastoma multiforme and prostate cancer cells. No signs of cytotoxicity were observed;
- test on prostate and breast cancer cells the most recently developed chemical entities displaying a RGD scaffold as 'Trojan horses', namely gold nanoparticles functionalized with fluorescein and the cyclic RGD. Such molecules were not toxic and were able to positively stain in a very peculiar way integrin αvβ3 expressing prostate cancer cells, but not breast cancer cells which expressed lower amounts of the receptor. It was reasonable to think that the cyclic RGD moiety was able to drive the gold nanoparticles to integrin αvβ3 clusters present near the focal contacts, promoting the internalization of the compound and, ultimately, the staining of the cells.

In conclusion, the results obtained during this work by means of basically cell biology tests could pave the way for a further *in vivo* evaluation of cyclic RGD peptides and their functionalized derivatives in several scenarios, such as physiologic and disease-associated angiogenesis, cancer treatment and diagnosis.