## 3. AIM OF THE WORK

Because of the ubiquitous expression of integrins and of their pivotal role in pathologic conditions such as cancer and tumor-associated angiogenesis, a lot of effort has been recently made in the development of integrin antagonists.

Our group is particularly focused on the generation of conformationally constrained cyclic RGD peptidomimetics selectively driven to RGD-binding integrins (namely integrins αvβ3, αvβ5 and α5β1), in order to develop new putative cancer antagonists and imaging vehicles for cancer diagnosis. My attention was mainly focused on integrin αvβ3 because it is probably the best-known and ubiquitously expressed receptor, in this context. Given these evidences and that I could took advantage of a panel of human cell lines (endothelial and epithelial cancer cells) and of a small library of cyclic RGD peptidomimetics and their derivatives, the aim of this work was to:

- characterize the panel of human cell lines for the expression of the markers of interest, namely integrins αvβ3, αvβ5 and α5β1;
- screen selected compounds among those comprised in the small library for their binding affinity for integrin αvβ3 receptor, in order to choose the most promising one;
- test that compound on the cell panel in order to confirm its anti-adhesive and anti-migratory activity, having a look on its possible effects on the organization of the actin cytoskeleton as well;
- test a couple of fluorescein-conjugated cyclic RGD derivatives of the progenitor compound on a human cell panel for their ability to tailor the imaging vehicle (e.g. fluorescein) into the cell by means of integrin αvβ3-dependent internalization and, ultimately, for their effectiveness in staining the cells;
- test gold nanoparticles functionalized with the most active cyclic RGD derivative and fluorescein on selected cancer cell lines. This approach should take advantage of the selectivity of the cyclic RGD compound together with the low susceptibility to photobleaching ensured by gold nanoparticles themselves, in order to allow the development of new potent imaging vehicles for cancer diagnosis or therapy.

# 4. MATERIALS AND METHODS

## *4.1 Cell biology*

### 4.1.1 General procedures

All manipulations involving cell culture were performed in a sterile environment provided by a laminar flow sterile hood. All liquid reagents were either purchased sterile or filtered through a 0.22 µm filter and stored in sterile autoclaved containers. All reagents were purchased form Sigma-Aldrich (unless otherwise indicated). Routine culturing was performed at 37 °C in humidified incubators in the presence of 5% $CO_2$. Centrifugation of cells was routinely performed at 1200 rpm for 5 minutes.

### 4.1.2 Culture of adherent cell lines

Adherent tumour cell lines were grown as monolayers in plastic tissue culture flask (BD Biosciences). Human Umbilical Vein Endothelial Cells (HUVECs, Promocell Gmbh, Heidelberg, Germany) were grown in M199 medium. HUVECs were grown in culture medium supplemented with 20% v/v FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL porcine heparin and 50 ng/mL ECGF. Late-Endothelial Progenitor Cells (EPCs) (kindly provided by Prof. M.L. Villa) were obtained and cultured as previously described (Della Bella et al. 2008). ECV-304 (kindly provided by Prof. M.L. Villa), T98G (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy), PC-3 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy), Caki-1 (ATCC), H460 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy) and MDA-MB-231 (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy) were grown in RPMI-1640 medium supplemented with 10% v/v FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. Cells were cultured until reaching 90% confluence and were then sub-cultured. Sub-culturing was performed using trypsin (0.05% w/v)/5 mM EDTA to detach the cells, which were immediately pelleted by centrifugation. Pelleted cells were plated at a density of $1.2x10^4$ cells/$cm^2$. Precise cell counts were obtained when needed using a Burker's chamber visualized through an inverted microscope. All details about cell lines used in this work are outlined in **Table V**.

### 4.1.3 Storage and recovery of eukaryotic cells from liquid nitrogen

Harvested cells were pelleted by centrifugation and resuspended at approximately $2x10^6$ cells/mL in FBS containing 10% v/v DMSO, the presence of which prevented the formation of ice crystals. Aliquots of 1 mL were transferred to 1.5 mL cryotubes and stored O/N at -80 °C. Frozen cells were transferred the following day to liquid nitrogen tanks (-196 °C).

Recovery of cells from storage was performed by rapidly thawing in a 37 °C $H_20$ bath. Thawed cells were washed in 10 mL fresh and pre-warmed medium, pelleted by centrifugation and then transferred to the appropriate culturing conditions for the cell type of interest.

| Cell line | Description | Reference |
|---|---|---|
| HUVEC | Primary vascular endothelial cells | Promocell Gmbh |
| EPC | Endothelial Progenitor Cells | Prof. M.L. Villa (University of Milan, Italy) |
| ECV-304 | Bladder carcinoma | Prof. M.L. Villa (University of Milan, Italy) |
| T98G | Multiforme glioblastoma | Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy) (BS TCL 63) |
| PC-3 | Prostate carcinoma | Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy) (BS TCL 175) |
| Caki-1 | Renal clear cell carcinoma | ATCC (HTB-46) |
| H460 | Human non-small cell lung carcinoma | Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy) (BS TCL 197) |
| MDA-MB-231 | Breast adenocarcinoma | Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia (Brescia, Italy) (BS TCL 223) |

***Table V.*** *Adherent cell lines used.*

## *4.2 Cell free procedures*

### 4.2.1 Human Vitronectin biotinylation

Biotinylated human vitronectin was necessary for solid-phase receptor-binding assays.

Human vitronectin was purchased by Molecular Innovations (Peary Court, MI, USA). Vitronectin biotinylation was performed through EZ-Link® Micro Sulfo-NHS-Biotinylation Kit (Pierce, Rockford, IL, USA), according to manufacturer's instructions. Briefly, $6.6x10^{-5}$ mmoles of biotin were incubated with 100 µg vitronectin (Duotech) for 2 hours at 4 °C. The final solution was eluted through a wash in desalting column, for 2 minutes at 1000 rcf. Biotinylated vitronectin was quantified by means of EZ™ Biotin Quantitation Kit (Pierce, Rockford, IL, USA), according to manufacturer's instructions.

### 4.2.2 Solid-phase receptor-binding assay

Solid-phase receptor-binding assay was performed in order to determine the binding affinity of test compounds to integrin αvβ3 and αvβ5.

Purified αvβ3 and αvβ5 receptors (Chemicon International, Inc., Temecula, CA, USA) were diluted to 0.5 µg/mL in coating buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MnCl_2$, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. An aliquot of diluted receptors (100 µL/well) was added to 96-well microtiter plates (NUNC MW

96F Medisorp Straight) and incubated O/N at 4 °C. The plates were then incubated with blocking solution (coating buffer supplemented with 1% w/v BSA) for an additional 2 hours at RT to block nonspecific binding, followed by 3 hours incubation at RT with various concentrations ($10^{-5}$ – $10^{-12}$ M) of test compounds in the presence of biotinylated vitronectin (1 µg/mL). Biotinylation was performed as previously described. After washing, the plates were incubated for 1 hour at RT with biotinylated streptavidin-peroxidase complex (Amersham Biosciences, Uppsala, Sweden) followed by 30 minutes incubation with 100 µL Substrate Reagent Solution (R&D Systems, Minneapolis, MN, USA) before stopping the reaction with the addition of 50 µL of 1N $H_2SO_4$. Absorbance at 415 nM was read in the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.). Each data point represented the average of triplicate wells. Data analysis was carried out by nonlinear regression analysis with GraphPad Prism Software®.

## *4.3 Biological assays*

### 4.3.1 Immunofluorescence on adherent cells

Immunofluorescence was used to detect the expression of integrins αvβ3, αvβ5 and α5β1 on the surface of cells of interest. For each sample, cells were plated at a density of $1.6x10^4$ cells/$cm^2$ on glass slides alone or previously coated with 10 µg/mL of either vitronectin or fibronectin.

For integrin αvβ3 staining, cells were washed in PBS and fixed in 4% w/v PAF (Fluka) for 10 minutes at RT, in the dark. Cells were then washed and permeabilized with 0.1% v/v Triton-X100 (Fluka) in PBS for 10 minutes, at RT. For integrin αvβ5 and α5β1 staining, cells were washed in PBS and fixed in ice cold methanol for 3 minutes at -20 °C. After fixation, blocking was performed in PBS supplemented with 5% w/v BSA for 1 hour at RT. Cells were then incubated with primary antibody diluted in PBS supplemented with 5% w/v BSA, O/N at 4 °C. Cells were then extensively washed in PBS supplemented with 5% w/v BSA and incubated with the secondary antibody diluted in PBS supplemented with 5% w/v BSA, for 1 hour at RT in the dark. Nuclei were counterstained for 5 minutes at RT with 0.25 µg/mL DAPI diluted in PBS. Cells were finally mounted with Vectashield® (VectorLabs-DBA, Italy) mounting medium and visualized with Zeiss Axio Observer A1 Microscope (Carl Zeiss Inc., Chester, VA, USA). All fluorescence microscopy scans were acquired with Zeiss AxioCam MRm (Carl Zeiss Inc., Chester, VA, USA).

### 4.3.2 Flow cytometry

Flow cytometry screening was performed in collaboration with Prof. M.L. Villa's Immunology Laboratory (Dr. E. Colombo and Dr. S. Della Bella), University of Milan.

Flow cytometry was used to detect the expression of integrins αvβ3, αvβ5 and α5β1 on the surface of human cells of interest. Cells were detached and harvested as previously described. The entire protocol was performed at 4 °C in order to avoid the internalization of the antibodies by un-fixed cells. For each sample, approximately $10^5$ cells were harvested, washed twice in ice-cold PBS and incubated with the primary antibody diluted in PBS supplemented with 1% w/v BSA for 30 minutes. After two washes in PBS supplemented with 1% w/v BSA, cells were incubated with the secondary antibody diluted in PBS + 1% w/v BSA for 30 minutes. Cells were washed twice in PBS + 1% w/v BSA and finally fixed in 1% w/v PAF. Isotype-matched irrelevant mAbs were used as negative controls. Data were acquired on a FACSCanto flow

cytometer (Becton-Dickinson) and analyzed using FACSDiva software. Cells were electronically gated according to light scatter properties to exclude cell debris. Results were quantified as MFI.

### 4.3.3 Adhesion assay

Adhesion assay is a cell biology test widely used to determine the ability of chemical compounds to compete with the adhesion of a cell line to its physiological ligand/s [41]. The output of the adhesion assay is represented by $IC_{50}$ values, namely the concentration of test compounds able to give the 50% inhibition of cell adhesion. In this work adhesion assay-derived $IC_{50}$ graphical values have been used to extrapolate the cell line and substrate specific $IC_{20}$ values, necessary to measure the anti-migratory activity of test compounds by means of wound healing assay.

Briefly, round bottomed 96-well plates were coated with vitronectin or fibronectin at 10 µg/mL in PBS, O/N at 37 °C. Before starting the adhesion assay, wells were incubated with cell specific culture medium supplemented with 1% w/v BSA, for 30 minutes at 37 °C. During this incubation, $7.8x10^4$ cells/$cm^2$/well were harvested by trypsinisation and resuspended in culture medium alone (negative control) or containing increasing concentrations of the compound, namely: 100 nM, 250 nM, 500 nM, 1 µM, 5 µM, 10 µM, 50 µM, 100 µM and 200 µM. Then, 50 µL of cell/compound suspension were seeded in each well and allowed to adhere for 2 hours at 37 °C. Nonadherent cells were removed with PBS. The remaining adherent cells were stained with a 0.5 w/v Crystal Violet solution, for 10 minutes at RT. Plates were finally rinsed with $H_20$ and dried O/N at RT. Stained cells were solubilized with 10% w/v SDS and quantified on a microtiter plate reader at a wavelength of 595 nM. Experiments were carried out in triplicate. Results are expressed as $IC_{50} \pm SD$, calculated using GraphPad Prism 5 Software®.

### 4.3.4 Wound healing assay

Wound healing assay is a cell biology test used to observe how cells migrate in response to a mechanical scratch (directly performed on the dish by the operator with a p200 pipette tip), in order to determine the anti-migratory activity of test compounds. Briefly, after the scratch cells are incubated with the $IC_{20}$ of test compounds for an experimental time ranging from 24 to 48 hours, in dependence on the cell type. $IC_{20}$ is graphically extrapolated by $IC_{50}$ values and is used in order to avoid the toxic effects often given by the treatment with the $IC_{50}$ of test compounds.

Round bottomed 24-well plates were coated with vitronectin or fibronectin at 10 µg/mL in PBS, O/N at 37 °C. $1.2x10^4$ cells/$cm^2$/well were harvested by trypsinization, seeded and allowed to adhere until a nearly confluent state. Adherent cells were then scratched with a p200 pipette tip and washed three times with culture medium. The scratch was immediately photographed with a Zeiss Axio Observer A1 Inverted Microscope, equipped with a Zeiss AxioCam MRm. Subsequently, cells were incubated for 24 hours with the $IC_{20}$ of test compound, as graphically extrapolated by the reference $IC_{50}$ values determined by means of adhesion assay. Test compound was diluted in culture medium supplemented with 2% v/v FBS. Control cells were maintained in culture medium supplemented with 2% v/v FBS. After treatment, cells were washed once in culture medium and the scratch was photographed as previously described. This allowed to measure and compare the width of the scratches before and after the treatment. Each point was done in triplicate, in two separate experiments. The best representative images were chosen.

### 4.3.5 Actin remodeling test after Compound 31 treatment

Cells ($1.6x10^4/cm^2$) were seeded and allowed to adhere O/N on 10 µg/mL vitronectin or fibronectin coated glass slides in complete medium supplemented with 10% v/v FBS. Cells were then treated with different concentrations of Compound 31 (namely 2 µM and 1 µM) diluted in complete medium supplemented with 0,1% v/v FBS, for 3 hours at 37 °C. After the incubation, cells were estensively washed in PBS and fixed in 4% PAF, for 10 minutes at RT. Cells were then subjected to actin stainining with phalloidin (Oregon Green® 488) at 4 U/mL diluted in PBS supplemented with 5% w/v BSA, for 1 hour at RT, in the dark. After an extensive washing, nuclei were counterstained with DAPI, as previously described. Cells were finally mounted and visualized as previously described or with a Leica TCSNT Confocal Microscope (Leica Mikrosysteme Vertrieb GmbH, Germany).

### 4.3.6 Treatment of cells with fluorescein-conjugated compounds and cyclic RGD functionalized gold nanoparticles and analysis

Cells ($1.6x10^4/cm^2$) were seeded and allowed to adhere O/N in complete medium on glass slides. Cells were treated 4 hours at 37 °C with selected compounds diluted in fresh medium supplemented with 0.1% v/v FBS. After the incubation, cells were extensively washed in PBS and fixed in 4% PAF, for 10 minutes at RT. Nuclei were then counterstained with DAPI, as previously described. Cells were finally mounted and visualized as described in the immunofluorescence protocol.

### 4.3.7 Antibodies

**Tables VI** and **VII** resume the antibodies used in this study to perform immunofluorescence and flow cytometry experiments.

| Specificity | Clone | Isotype | Species | Working concentration | Supplier (reference) |
|---|---|---|---|---|---|
| Integrin αvβ3 | LM609 | Ms $IgG_1$ | Human | 10 µg/mL (IF, FACS) | Brooks et al., 1994 |
| Integrin αvβ5 | P1F6 | Ms $IgG_1$ | Human | 10 µg/mL (IF, FACS) | Wayner et al., 1991 |
| CD49e (Integrin α5) | NKI-SAM-1 | Ms $IgG_{2b}$ | Human | 10 µg/mL (IF, FACS) | te Velde et al., 1988 |

***Table VI.*** *Primary antibodies.*

| Specificity | Species | Conjugation | Working concentration | Supplier (reference) |
|---|---|---|---|---|
| Mouse | Donkey | Cyanine 3 | 3.75 µg/mL (IF) | Jackson Immunoresearch laboratories Inc. (UK) |
| Mouse | Donkey | Allophycocyanin | 0.5 mg/ml (FACS) | Jackson Immunoresearch Laboratories Inc. (UK) |

***Table VII.*** *Secondary antibodies.*