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Review Article

Multi-Targeting Approach of αvβ3 Integrin Ligands and their Applications in Diagnostic Field and Cancer Therapy

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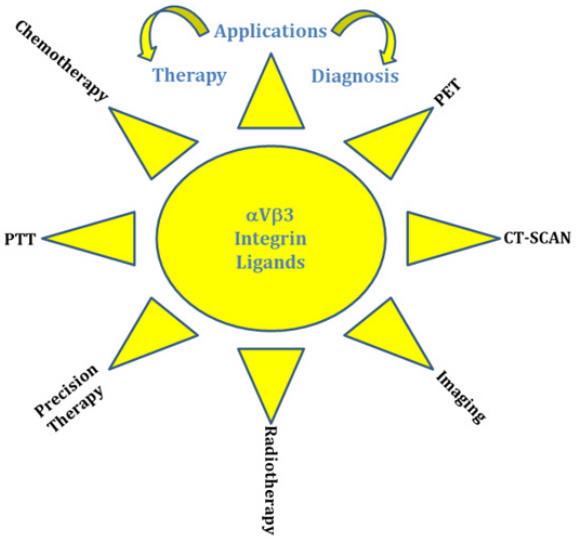
Abstract

Multi-target strategy is an approach where different ligands are conjugated together to give a synergic effect toward cancer cells for applications in diagnosis and cancer therapy. The target most largely in use is the integrin αvβ3 which recognizes the RGD sequence to interact with the proteins of the extracellular matrix. In the first part of this work, we analyzed the usage of RGD mimetics with radiolabeling to enhance the images of positron emission tomography, computer tomography and magnetic resonance imaging. Secondly, the interaction with drugs or with other target peptides to kill in a selective manner the cancer cells without hitting healthy cells.

Keywords: Integrin; αvβ3; Antagonist; Nanomaterials; Drug; Diagnosis; Imaging; Therapy



Abbreviations: ECM, extracellular matrix; CAM, chorioallantoic membrane; SAR, structure- activity relationships; PET, positron emission tomography; CT, computer tomography; NIR, near infrared; HPS, heat protein shock; PTT, photothermal therapy; GRP, gastrin-releasing peptide; BBN, bombesin; FSC, fusarin C; TNBC, triple-negative breast cancer; EGFR, epidermal growth factor receptor; CL, Cerenkoc luminescence; NRP-1, neuropilin-1; MRI, magnetic resonance imaging; CEUS, contrast enhanced ultrasound; PTX, paclitaxel; USPIO, ultra-small super magnetic iron oxide; DOX, doxorubicin; PAMAM, polyamidoamine; GSH, glutathione; RT, radiation therapy; NLS, asparagine leucine serine; EPR, enhanced permeability and retention; IAP, inhibitor apoptosis protein; CD, cyclodextrin; MSN, mesoporous nanoparticle; DBCO, dibenzocyclooctyne; MMAE, monmethyl atristatin E; ING-4, inhibitor of growth-4; IL-24, interleukin-24; Ad, adenovirus.

Introduction

In recent years, binding of different peptide ligands capable of interacting with different cellular targets is of growing interest. One of the major reasons is that bispecific peptide heterodimers can increase the specificity binding with the receptors when compared with binding with mono specific peptide [1]. This "dual targeting" approach gives the possibility to modulate two biological targets with a single system, it can drive to synergistic therapeutic effects and avoids side effects associated with combination therapy (Figure 1).

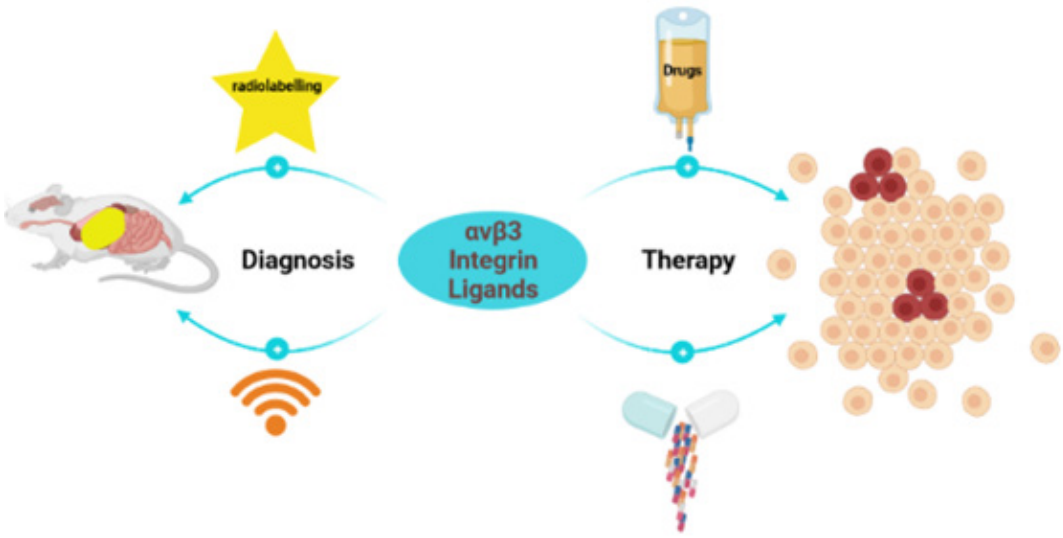


Figure 1: Schematic representation of multi-target approach. Integrin ligands are loaded with radiolabeling, drugs or other peptide targets for diagnostic and therapy studies.

The advantage to use peptides as targeting ligand is a higher metabolic stability and avidity. In the first case, the cyclic structure, the introduction of D-amino acid and modification of the backbone are techniques to enhance the enzymatic instability with greater bioavailability [2]. Secondly, the binding affinity for more receptors induces a synergic effect versus the cancer cells [3].

Integrins are proteins expressed on cell surfaces and extend over the lipid bilayer of cells. They are heterodimer formed to α and β subunits and from their combination are obtained 24 different integrin types involved in different cell behaviours such as proliferation, death, differentiation, and migration so they may perform targets for therapeutic and diagnostic diseases [4]. Integrins are able to mediate the mechanical and chemical signals of extracellular matrix (ECM) through association with the cytoskeleton, which also selects the specific integrin to be involved in the signal pathway due to the differential binding affinity of ECM ligands [5]. This interaction results in integrin clustering, conformational changes and outside signals across the plasma membrane. As a consequence, the activation of focal adhesion complexes and integrin clustering recruits' intracellular proteins that start cascades of signalling events inducing gene expression and cellular behaviour [6].

The sequence Arg-Gly-Asp (RGD), described by Ruoslahty and Pierschbacher in 1986 as minimal sequence to recognize the integrin, was the first sequence motif discovered in fibronectin and in other ECM proteins as vitronectin, laminin, and thrombospondin. The integrin subunits α and β are able to bind RGD sequence at the site for integrin-mediated cell adhesion. RGD sequence was discovered to interact with αvβ3 integrins but also with αvβ1, αvβ5, αvβ6, αvβ8, and αIIbβ3. The specificity of integrin binding to different proteins of ECM depends also by other amino acids that surround the RGD sequence. In this context, the tripeptide has been inserted in the cyclic penta- and hexapeptides containing D-amino acids and only two peptides showed an antagonist behavior and high activity for αvβ3 and αIIbβ3 integrin the c[RGDfV] (IC50 4.9 x 10-8 M) and c[RGDfVG] (IC50 5.0 x 10-8 M). The D-Phe in the sequence makes the cycles able also to suppress tumor-induced angiogenesis in a chick chorio allantoic membrane (CAM) model. From structure-activity relationships (SAR) the Val residue is not fundamental for the activity and can be modified by adding a heterocycle or it can be replaced with another amino acid. This leads to two important molecules, c[RGDfK] and c[RGDf-N-(Me)V] (Figure 2), where the Lys, in the first case, can be used to link with nanomaterials, fluorescent probes or other molecules to create diagnostic and drug delivery systems (see the next sections). For example, the conjugation of integrin ligands with nanomaterials such as zeolite has led to the development of a device capable to accurately recognize cancer cells [7-9]. Instead c[RGDf-N-(Me)V] called cilengitide, showed a higher activity but no selectivity at all for αvβ3 (IC50 6.5 x 10-10 M) (Kessler et al. 1995) (Figure 2).

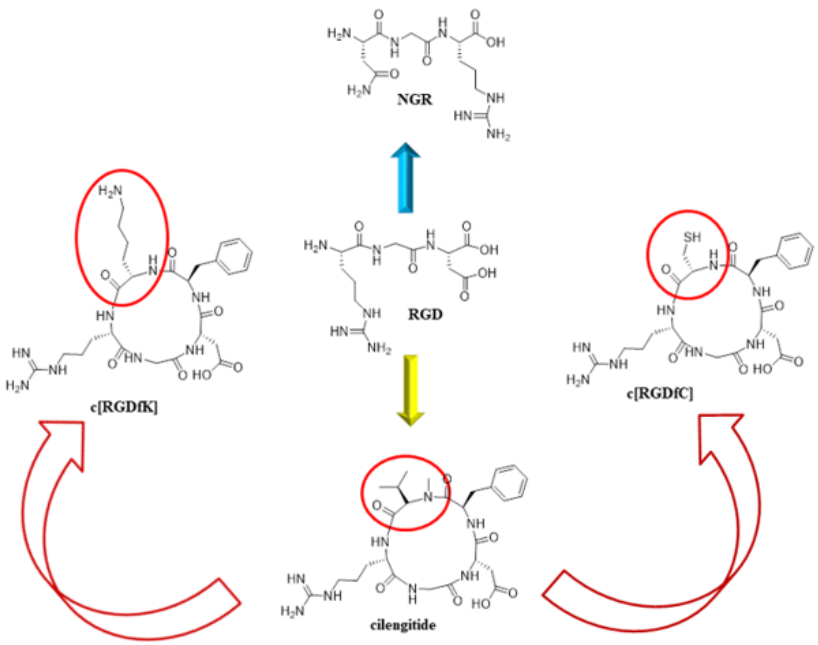


Figure 2: Structure of the αvβ3 integrin ligands.

This review aims to analyze the more innovative applications of the integrin antagonist αvβ3 in a dual-targeting system in diagnostic and therapy fields.

Multi-Target Approach of Integrin αvβ3 in Cancer Diagnosis

One of the most important strategies for tumour diagnosis is the molecular imaging. This technique showed the ability to visualize and measure the function of biological and cellular processes in vivo. Invasive biopsy or surgical procedures were initially used to investigate the presence or the absence of cancer in patients. Molecular imaging has the advantage not to be invasive and gives the possibility to create personalized treatments. The use of nanoparticles capable of complexing with different molecule has proved important in molecular imaging for the following reasons: a) the binding ligand with specific target on the tissue/disease marker, b) the binding or the encapsulation with drug for targeted delivery. For example, a system developed for target delivery to provide the encapsulation in nanoparticles of doxorubicin and the release in high dosage of the drug directly in cancer cells, with pH-responsive mechanism [10,11]. Lastly the possibility to detect contrast agents with different imaging modalities [12], an example being the pyropheophorbide-a (Pyro), a porphyrin used as a photosensitive and a radiolabel chelator, which has been linked with c[RGDfK] and 64Cu. The probes obtained showed high tumor-specific positron emission tomography (PET) and fluorescent imaging in vivo [13].

In this context we analysed different techniques that use integrin as target to improve the selectivity toward cancer cells and can be applied to improve imaging test.

Positron Emission Tomography and Computer Tomography Imaging

Cancer cells often take up more glucose than health cells and Positron Emission Tomography scan (PET-scan), a nuclear scan, takes 3D-pictures and permits to compare the cancer cells with health cells. Computer Tomography-scan (CT-scan) uses computers and rotating X-ray machines to create 3D- images of the organs of the body. The association between near infrared (NIR) fluorescence with CT-scan can diagnosticate the neoplastic and monitor the side effects. A method used to this purpose is to link the W18O49 inorganic nanoparticles with iRGD, with an inhibitor of heat protein shock (HPS) 17AAG and Cy5.5 as fluorescent dye [14]. The final system iRGD-W18O49-17AAG was tested on MKN-45P cells with high levels of integrin αvβ3, and GES-1 gastric epithelial cells with low levels of integrin αvβ3. The flow cytometer displayed an accumulated of iRGD-W18O49-17AAG in MKN-45P cells instead in GES-1 the dual system was no presence. In vivo, an intense fluorescent signal of Cy5.5 was observed in tumour site after 4 hours from the injection of the dual system confirmed the in vitro results. The dual system was used as contrast agent in CT-scan and the results have been compared with iodixanol a common contrast agent. After injection with iRGD-W18O49-17AAG the contour of the tumour tissue can be clear observed, instead with iodixanol this evidence was absent. These results indicated as the dual system can be used in CT-scan to determine the tumour position [14]. The dual system was used also in photothermal therapy (PTT) to kill the selected cancer cells with a specific laser irradiation (see next session).

The functionalization of ultra-small fluorescent core-shell silica nanoparticles called Cornell prime dots (C' dots) with cRGD giving a system used on mouse model of glioblastoma able to partially cross the blood brain barrier. The images were done at 0.5 and 24 hours after intravenous injection of 89ZrcRGD-C' dots in adult brain tumor–bearing mice and compared with the images previously obtained by hematoxylin and eosin that confirmed the presence of the tumour. The data indicated that cRGD-C' dots showed an enhanced accumulation, distribution, and retention in the tumour interstitium with a larger distribution of the system in the malignant brain tumor tissue. The encapsulation also of 124I such as PET labels allowed the system to be used as a multimodal imaging [15].

To obtain early tumor diagnosis by PET imaging, the cRGD was conjugated with bombesin, an amphibian homolog of mammalian gastrin-releasing peptide (GRP). It has been extensively used for the development of molecular probes for the imaging of GRPR after loading with different radionuclides [16,17].

The ligands were linked with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid (NODA-GA) using 64Cu as radiolabel. The binding affinity displayed that NODA-GA-RGD2-PG12-BBN present a highest affinity for bombesin (BBN) receptor (IC50 = 24 nM) and good affinity for αvβ3 receptor (IC50 = 165.3 nM) [18].

In the same year BBN and c[RGDyK] were linked through a glutamate and then labeled with 68Ga. The system was used for PET and CT-scan and it was observed a positive 68Ga-BBN-RGD accumulation in primary and metastases cancer with a significant up take of the dual system compared with 68Ga-BBN [19].

Another dual targeting usage in PET/CT scan is formed by cRGD connected on the Fusarine C (FSC) used like a scaffold and gallium-68 as radionuclide, to obtain a dual-modality imaging agent. Sulfo-Cy7-FSC-RGD showed an IC50 = 1.13 ± 0.22 and 0.81 ± 0.19 nM for metal free and [natGa]. Cell up take was tested on M21 (αvβ3 positive) and M21-L (αvβ3 negative) was 2.45 ± 0.36% and 4.41 ± 1.17% with an internalization receptor-mediated in vitro. RGD-dual system in vivo was high accumulated in tumour tissue after 30 min from the injection. PET images confirm highly specific receptor uptake, high stability and radiolabeling yield [20].

The dynamic microenvironment of hard tumour, an example being triple-negative breast cancer (TNBC) and associated spatiotemporal changes in the expression of the targetable biomarker, is captured by the dual-targeting nanoparticles. The c[RGDfC], where cysteine replace the lysine, and P-selectin have been used to develop a dual ligand system to recognize hard to reveal metastases. The first adhesion of the site metastases is on endothelium, then they pass from P-selectin-dependent cell rolling to interact with the integrin αvβ3. Rolling and tethering of circulating tumour cells to the endothelium and the aggregation between tumour cells, platelets and leukocytes, is mediated by selectins [21]. The conjugation of P-selectin targeting peptide CDAEWVDVS with the cyclopeptide on nanoparticles is specific towards blood vessels associated with metastases at different stages of their development. In this context the radionuclide used was Technetium-99m

(99mTc) and it was incorporated into the nanoparticle using a "shake and bake" radiolabeling method.

The orthotopic 4T1 animal model was used 9 days after tumour inoculation, and to test the accuracy of the 99mTc-dual-ligand-NP to recognize the metastases at an early stage the gamma scintigraphic imaging has been used. After 120 minutes from the administration of the dual-ligands nanoparticles, the scintigraphic imaging showed "hot spots" in the lungs of mice with 4T1 metastasis. The comparison between mice bearing 4T1 metastasis and healthy mice after injection of 99mTc-dual-ligand-NP showed an accumulation of dual-ligands system in metastatic site with a 22% of the injected nanoparticles accumulated in the lungs of mice with early- stage 4T1 metastasis [22]. Starting from these results Peiris, et al. [23] have developed a new multi target system with four different ligands linked on the liposomal nanoparticles. Starting from the previous dual-system with P-selectin and c[RGDfC], in the new system the authors added fibronectin and EGFR targeting. The fibronectin because it is overexpressed on the extracellular matrix in the near perivascular regions of the metastases and the peptide sequence used is CREKA. EGFR because with the integrin □v□3 it is a receptor of the tumour cells, and the sequence used is CYHWYGYTPQNVI. To create this multi ligands system the researchers used liposomes and silica nanoparticles with sizes of 100 nm and Alexa 647 for fluorescence imaging studies and 18F in the silica nanoparticles for PET imaging.

The fluorescence studies for four ligands system were conducted in the same conditions of the dual system (see above). The data were compared with a dual ligand formed by EGFR and integrin αvβ3 because including receptors on the metastasis of the tumour selected and receptors on the remodeled endothelium metastasis. The multi-ligands showed 3-fold higher deposition into lung metastases versus 2-fold higher deposition of the dual system. The 18F-multi target system showed a precise detection of the metastases with a very low dose of the nanoparticles, high sensitivity and specificity [23].

Another peptide ligand used in association with cRGD to obtain a dual-imaging system is GE11 a long peptide with high affinity for EGFR which belongs the receptor of tyrosine kinase, which has a role in the regulation of the cell differentiation, migration and proliferation of normal and cancer cells [24]. The two peptides have been conjugated with 1,4,7-triazacyclononane-N,N′,N″-triacetic acid (NOTA), and Gallium-68 as radionuclide, then the resulting has been compared with the monomer peptides. The results indicated that in vivo the dual-peptide showed an enhanced accumulation in tumour cells [25,26].

A technique that emerged in the recent years plans to exploit Cerenkov luminescence (CL) effect. This effect represents the emission of the radiation of electromagnetic from a material where the molecules are polarized by a charged particle moving through it. The effect was observed when the speed of the particle in the crossed medium is higher than the phase speed of light in the same medium [27]. The usage of this effect has several advantages such us the easy sample preparation, the absence of chemical quenching, the possibility to handle large volume of aqueous solution for counting and it is less expensive [28]. The limitation of this technique is its low signal intensity, but also highly sensitive cameras, sufficiently shielded from background light, and long acquisition times are required. These problems were alleviated transforming weak CL signals in a stronger signal using azide as CL-activated formation of nitrenes to fix a fluorescent probe (Cy7) in the tissue after covalent bound formation. The association with 90Y-DOTA-RGD induced CL-activation and the bound with a chemotherapy drug allowed using CL also in radiotherapy. The accumulation in the tumour cells was observed in presence of azide activated while usage of inactive Cy7 fluoride did not lead CL-dependent accumulation fluorescence within the tumour cells. This data indicated the importance of the azide and RGD for dye accumulation [29].

In 2009 Ruoslahti's group described a new antagonist peptide of integrin αvβ3, called iRGD (internalizing RGD, CRGDK/RGPD/EC) with efficient tissue/cell penetration and high tumour targeting. Proteolytic enzymes cleaved the sequence iRGD after integrin interaction to expose the C-terminal and N-terminal of the half sequence (CRGDK/R) will be able to bind neuropilin-1 (NRP-1) to give the internalization in the tumour targeting [30].

The conjugation with two fluorophores TAMRA and Cy5.5 at two termini of the sequence provided the system TAMRA-iRGD-Cy5.5 used in fluorescence imaging. To evaluate the cell uptake, the probe was tested on U-87 MG cells, and human glioblastoma cell line overexpressing both αvβ3 integrin and NRP-1 receptors. The internalization was confirmed by optical microscopy where it was observed the co-localization of the signals within endosome compartments. in vivo 2 hours post intravenous injection, the signals of the two fluorophores were colocalized in the tumour and this data indicates that the cargo attached at the N- and C- termini of the peptide is delivered systemically in the body [31].

Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) uses high-energy sound waves impossible to hear from the people. The sound waves echo off tissues into the body and the echo creates an image of the tissues or organs. These pictures are named sonogram. Nuclear scan uses small amounts of radiation to create pictures of tissues, bones, and organs inside the body.

To enhance the diagnosis of the hard tumour, dual-modal microbubbles were developed. In this system the ultrasound and microbubbles carry on the drug and gas inert into the lipid layer of the cell. The advantage of this methodology is the release of the gas in defined regions reducing off-target side effect [32]. Paclitaxel (PTX) and RGD were loaded on the lipid nanoparticles and then combined with sulphur hexafluoride (SF6) as internal gas to give the contrast enhanced ultrasound (CEUS) images with ultrasonic targeted microbubble destruction and to improve the sensitivity and the resolution of the ultrasound images. This method increased the cell internalization because it generates pores in the membranes to enable the microbubbles to penetrate in the cell and to release the drug into the cell as necessary. The capability of PTX@RGD-MBs to produce CEUS images was investigated using peristaltic pump in vitro and TNBC subcutaneous tumours in vivo. In the first case PTX@RGD-MBs showed a slow release of the paclitaxel (from 30% to 70% within 2 h) and displayed a large amount of small-dotted echo obtaining effective CEUS images in vitro. The subcutaneous injection of PTX@RGD-MBs showed in vivo a good distribution of the dual system in tumour and excellent CEUS images [33].

Cancer cells showed a high heterogeneity; to increase the specificity and affinity toward the tumour cell, imaging can be achieved with dual probe systems. In this context the tripeptide RGD is conjugated with NGR peptide sequence that binds the aminopeptidase-N involved in the regulation of angiogenesis, tumour growth and metastasis [34]. Both peptides are conjugated on ultra small superparamagnetic iron oxide (USPIO) as negative contrast agents. In cell binding assay, the cell uptake of the dual peptide in HUVECs showed 1.71 and 2.19 greater than a single USPIO peptide. The usage of RGD–NGR-USPIO as contrast agent in MRI was evaluated by xenograft tumour-bearing animal model. 24 hours after the injection of tumour cell it was observed a significant decrease in signal intensity at the tumour site and an increase of the contrast-to-noise ratios in contrast with surrounding tissues when RGD–NGR-USPIO are used to achieve a very clear visualization on MRI, while this is not observed with a single USPIO peptide [35].

Another important dual system was obtained conjugating RGD sequence with and ATWLPPR, a heptapeptide that binds NRP1, and Gd-DTPA as contrast agent on liposome nanoparticles or with Cy5.5 as contrast agent and linked together by click chemistry. This system remained in circulation for long time and showed higher selectivity and specificity for cancer cells compared with single peptide system [36,37].

Multi-Target Approach of Integrin αvβ3 in Cancer Therapy

The traditional therapies are very invasive and involve the surgery to remove the tumor mass (when this is possible), the chemotherapy and radiotherapy alone or in combination. The radiotherapy uses high doses of radiation to kill the cancer cells and shrink tumors. Instead the chemotherapy is a mix of drugs to kill the cancer cells. These are very invasive treatments, in particular the combination of the last two to attack cancer cell, they also affect healthy cells, thus lacking selectivity. During these treatments

the stem cells are completely disrupted and need to transplant to restore blood-forming stem cells. Other treatments developed in recent years are: precision medicine that uses molecules as genes, proteins, and biomarkers to understand the disease and then to develop the appropriate therapy; immunotherapy, in this case the treatment is focused to help the immune system to fight the cancer; hormone therapy is used for breast and prostate cancers, and slows or stops the hormones growth; target therapy, the final goal are the changes in tumor cells responsible to grow, divide and spread using small molecules and monoclonal antibody.

Chemotherapy

The need to prepare new anticancer drugs lacking or reducing side effect is the new challenge for different research fields. One approach is to use a specific ligand to recognize the receptors on the cell membrane over expressed in the tumor cells in association with a classic chemotherapy drug. In this context cRGD peptide ligand and doxorubicin (DOX) was loaded on polyamidoamine dendrimer (PAMAM) modified with PEG by disulfide linker (PSSP). Modifications are request in order to user PMAM, because it showed limitations such as the charge-related toxicity, high clearance by reticuloendothelial system and non-active targeting. The link of PMAM with PEG can prolong the retention time in blood vessel and increase drug accumulation at the tumor site. The final system cRGD-PSSP-DOX was tested in vitro on B16 cancer cells and Huvec cells and in vivo with B16- bearing mouse model. in vitro the release of doxorubicin was pH-dependent. At physiological conditions the release of DOX was negligible, but when the concentration of glutathione (GSH) was increased to 10 mM the release of DOX was increased to 60% in 24 h. Keeping the value of GSH lower than 10 mM the release of DOX was more than 60 % because the pH was decreased from 7.4 to 5.5 with a changed conformation of PMAM from "dense core" at high pH to a "dense shell" at low pH. in vivo studies showed that as the presence of cRGD increases the cellular uptake, as well as the release of DOX by intracellular redox and pH environment with high anti-cancer effect [38]. The association of RGD sequence and DOX was amply used in chemotherapy. In this case, both integrin ligand and the chemotherapy were conjugated on carboxymethyl cellulose polysaccharide. The dual system was tested on MTT assay and the data displayed that the cell viability has been 75-80% for all the cells if compared with free DOX where the cell viability was

reduced in both cells. These results are important to demonstrate the key role of RGD peptide in conjugation with anticancer drugs to achieve the selectivity toward cancer cells [39].

To enhance the activity of the chemotherapies drug it was utilized an octa guanidium sorbitol motif with Arg-8-mer sequence, a cell penetrating peptide, in association with cRGD to recognize the cancer cells. The presence of cathepsin B is important to provide the prodrug activation with the selective release of drug in the specific site. The drug used was an anticancer agent PTX modified to interact with octa guanidium sorbitol. The dual targeting was tested on U-87 glioblastoma cells and the data showed the apoptotic event and the microtubule alteration in these cells [40].

Radiotherapy

Radiation therapy (RT) is a common strategy used in treatment of cancer diseases alone or in combination with chemotherapy or surgery. The X-ray to use in this strategy causes DNA damage that can lead to cellular arrest or cell death.

The conjugation of RGD sequence with nuclear localization peptide Asparagine-Leucine-Serine (NLS) and an aptamer loaded on gold nanoparticles carried out the cancer specific nano radio pharmaceutical, where the aptamer bound both the integrin αvβ3 and vascular endothelial growth factor overexpressed in the tumour. The aim of the final system 177Lu-NP-NLS-RGD-Aptamer is the inhibition of the angiogenesis which is important for the tumour growth. The synergic effect of integrin ligand and NLS increases the effect of the radiotherapy. In particular, when the EA.hy926 cells were exposed with the system, a drastic reduce of the cell adhesion has been observed and in the same time the ability to form vessel tubes in presence of pro-angiogenic stimuli [41].

Photothermal Therapy

Photothermal therapy or PTT is an emerging noninvasive and precise technique widely used in cancer therapy. The aim of this strategy is to use photothermal agents able to penetrate in the cells and destroy them by generating heat by NIR. The advantage of this procedure is very low side effects, temporally and spatially controllable, a good alternative to the traditional therapy for cancer diseases. One of the limits of this method is the poor penetration in tumor cells. A strategy used to solve this problem is to use iRGD peptides with high biocompatibility and specificity for the tumor cells. The HPS can be overstressed by PTT. Some HPS inhibitors are developed to increase the curative effect and reduce the thermo-resistance. In this context a system realized to enhance this technique is iRGD-W18O49-17AAG, where the tripeptide is used to increase the selectivity for cancer cells that with HSP inhibitor 17AAG are linked with W18O49 nanoparticles. These nanoparticles showed high photo-thermal properties, biosafety in vitro and in vivo. The heat shock proteins response was induced with laser irradiation under 808 nm [14].

Precision Therapy

Different strategies are developed to increase the selectivity towards cancer cells. In this perspective the integrin αvβ3 in particular plays a key role. One possible approach in use is the conjugation of the integrin antagonist with gold nanoparticles and octarginine as cell penetrating peptide to cross the BBB. A very important aggregation is the one with legumain to increase the particular size that blocks their backflow to the bloodstream, to narrow their exocytosis by cells and to allow the accumulation in the tumor cells by enhanced permeability and retention (EPR)

effect. This complex system was tested on glioblastoma cells where the association with doxorubicin confirmed the selectivity drug delivery of the system [42].

The large size of the nanoparticles was central to developed poly (ethylene glycol) PEG-liposome with RGD or NGD as a peptide motif and cell penetrating peptide. In this context the authors observed that the nanoparticles with 300 nm of diameter showed a higher accumulation in the cancer cells than the same nanoparticles with 100 nm of size, probably because the large size of the liposomes prevents the extravasation and the recognition of the tumor vessels. On the basis of these results, doxorubicin was encapsulated on the liposomes and the cytotoxicity was evaluated on tumor and endothelial cells. It was observed that the target of the tumor endothelial cells is 3-orders more efficient than tumor cells. These results indicated the possible way to form new anti-angiogenetic chemotherapy for drug resistance cancer [43]. The inhibitor apoptosis protein (IAP) would be likely responsible of the chemo-resistance because IAP induces only the suppression of the intrinsic apoptosis way but not the extrinsic way. This pathway can be inhibited by TRIAL protein with a selective action toward tumor cells sparing the health cells. TRIAL protein showed a short half-life, and this is responsible of the poor usage in the cancer therapy. In this prospective it was developed a dual system formed by RGD-γ-PGA/Moβ-CD-SSPEIpTRAIL, where RGD was used in conjugation with γ -PGA for bound both integrin and gamma-glutamyl transpeptidase [44]. The cyclodextrin (CD) was used as carrier for monensin a potent antibiotic used as potential anticancer drug because it was observed that it sensitizes the cancer cells to TRAIL protein therapy by upregulation of death receptor [45]. The tumour acidic microenvironment causes the detachment of surface coating γ-PGA, which conformation was found to be pH-sensitive. An efficient gene delivery is also favored by fast disassembling of the disulfide crosslinking LMW-PEI, which is redox-sensitive, in the tumour cells.

in vitro assay showed an IC50 = 0.73 μg/mL for γ-PGA/Moβ-CD-SSPEIpTRAIL, instead for γ-PGA/β-CD-SSPEIpTRAIL and Moβ-CD-SSPEIpTRAIL it was 1.6 μg/mL and 2.1 μg/mL, these data confirmed that TRIAL protein and monensin worked in synergy effect. The apoptosis was evaluated on HCT8/ADR cells and γ-PGA/Moβ -CD-SSPEIpTRAIL showed an apoptotic effect up to 93.8% instead for Moβ-CD-SSPEIpTRAIL, with monensin, and γ-PGA/β-CD-SSPEIpTRAIL , without monensin, it was 75.5% and 59.6%, respectively. The conjugation with RGD was tested in vivo on HCT8/ADR tumor- bearing BALB/c nude mice via intravenous injection. The dual system RGD- γ-PGA/ Moβ-CD- SSPEIpTRAIL showed high efficacy 83%, while it was 77% for γ-PGA/ Moβ-CD-SSPEIpTRAIL, data confirmed also by western blotting. The biosafety was tested, and body loss weight not observed, suggesting a biocompatibility biodegradability of the dual system [44].

IAP could be a promising target in the cancer therapy. This approach was used by De Marco et al. 2020 where the inhibition of the IAP was induced by protein SMAC/DIABLO. This is a mitochondrial protein that induces the apoptosis in the cancer cell by inhibiting members of IAP family. The activity was provided by the N-terminal tetrapeptide AVPI and the selectivity was induced by c[RGDfK] integrin ligands. Both the target peptides were conjugated on the silica nanoparticles by click-chemistry. The multi target was tested on A549, U373, and HeLa cancer cells, and it showed a significant toxicity at low concentrations. The internalization was tested after incubation with anti-mouse FITC-conjugated secondary antibody for 1 hour at rt and imagines at

confocal microscopy showed the accumulation of the dual targeting in the cancer cells. The apoptosis was tested on caspase-9 and the double targeting showed an activity 40-fold higher than single peptides.

Drugs based on CIS platinum (Pt IV) are powerful but lack of specificity toward cancer cells and promote side effects. For this reason, the usage of a target ligand like iRGD to increase the specific body distribution and Cy5.5 a fluorophore for imaging is necessary. The final system will be Cy5.5- iRGDC-Pt(IV) where the CIS platinum is bound on the C-termini of the iRGDC motif. CIS platinum is a prodrug and only during the exposition to the tumour environment it reduces CIS platinum from Pt(IV) to Pt(II) which is the active form. The internalization carries out two possible pathways via NRP-1 mediated endocytosis. In the first pathway the proteolytic activity in the extracellular membrane reduces the disulfide bridge of the system Cy5.5-iRGDC-Pt(IV) before internalization, in the second pathway the internalization followed cleavage by proteolytic enzyme of the disulfide bridge in both NRP-1 mediated. After internalization, the complex showed efficacious tumour visualization and growth suppression with minimal systemic toxicity [31].

NRP-1 a co-receptor of VEGFR2, can interact with many integrin in particular with integrin αvβ3 and for this reason it is used with integrin antagonist cRGD in imaging (see above) but also for chemotherapy. In this context the heptapeptide sequence ATWLPPR, ligand of NRP-1, was linked on silica nanoparticles with cRGD and tested on primary endothelial cells. From in vitro assay it was observed that at low concentration of 0.1 nM the dual target is an antagonist of VEGFR2 with high binding efficiency generating an activation of AKT that induce cells to survive, inhibition of caspase 3, and the activation of GSK3β and eNOS. After intravenous injection in vivo the dual target showed a vasodilation of tumour blood vessel [47].

The association of cRGD with peptides used as “gatekeeper” on mesoporous nanoparticles (MSN) is an interesting application. The gatekeeper has the advantage to enhance the biocompatibility, cytotoxicity, and to reduce the biodegradability. The conjugation with MSN and cRGD improves the selectivity towards cancer cells and when the nanoparticles reach the target site the gatekeeper of the MSN releases the entrapped guest molecules in response to stimuli such as pH, temperature, light, enzyme or redox potential.

To enhance the dispersion stability of the MSN, the surface was PEGylated and then functionalized with the cyclic peptides WKCRGDC and KCRGDC used as gatekeeper to obtain the systems PEG-WKCRGDC-SS-Si and PEG-KCRGDC-SS-Si. The efficiency of the entrapped of the DOX was 2.5% for both systems. The biological assay in vitro on A549 cells showed that the release of the DOX was induced by GSH that cleaved the disulfide bond of the gatekeeper and changed the structure from cyclic to linear peptide which is responsible of the release of the drug into the cancer cells and the apoptosis was induced with 3μM of DOX loaded on both systems [48].

In recent years the development of new RGD peptide as inhibitor of integrin αvβ3 with enhanced stability, higher affinity and specificity has represented new perspective in this field. More recently Le's group prepared a dual ligand formed by two moieties of cRGDfK linked by RGD hexapeptide (RGDRGD) or nonapeptide (RGDRGDRGD). Only the dual peptide cRGDfK-RGDRGD showed good activity with IC50=4.2 μM al MTT assay [49]. The conjugation with a dibenzo cyclooctyne (DBCO)-tagged toxin, DBCO-VCPAB-MMAE, where Mono Methyl Atristatin E (MMAE) is a cytotoxic drug with activity on microtubules [50] displayed in vitro no difference in the cytotoxic activity between DBCO-VCPAB-MMAE and cRGDfK-RGDRGD on a negative αvβ3 MCF-7 cells. Instead the cytotoxic activity against a positive αvβ3 SKOV-3 cells was 2-fold higher than DBCO-toxin. This modest anti-tumor activity on both cells of DBCO-VCPAB- MMAE is probably due to the DBCO-modification that reduce its activity [49].

An interesting approach in the cancer therapy is the gene therapy where the targets are molecular defects of the cancer cells. There are three approaches: 1) expressing tumour-suppressor genes that cause the cell-cycle arrest and/or apoptosis; 2) suicide gene therapy where to develop an enzyme able to convert a prodrug in a toxin; 3) selectively replicating viruses, in this case the cancer cells will be susceptible to virus-induced infections [51]. In this context the approach is to express tumor-suppressor genes where inhibitor of growth 4 (ING4) and interleukin-24 (IL-24) are used.

ING4 is able to inhibit the tumor growth through cell cycle alterations, apoptosis, and inhibition of the angiogenesis; instead IL-24 is a secreted cytokine and membrane receptor-mediated tumor growth suppressor, and it is able to induce the apoptosis and the suppressor on the growth tumor in cancer cells only. More important in gene therapy is to develop vectors able to achieve gene expression and to reduce side effects from vectors. To this aim the recombinant adenovirus (Ad) was used because it has the advantage to high transgene expression efficiency, the ability to infect both the dividing and non-dividing cells. The Ad-vector was modified with ING4 and IL-24, and the final complex interacted by electrostatic interaction with Antheraea pernyi silk fibroin that contains an abundant RGD sequence and it was modified with low-molecular-weight PEI. The final complex RGD.Ad-ING4-IL-24 was used to infect human hepatoma carcinoma SMMC-7721 cells and hepatic L-02 cells. The data displayed as ING4, IL-24 and RGD sequence worked in synergic effect with apoptosis of SMMC-7721 cells equal to 19.20% instead the apoptosis induced of the naked Ad was equal to 10.86%. Still it was observed that RGD.Ad-ING4-IL-24 showed higher infection efficiency than naked Ad thus causing increased expression of ING4 and secretion of IL-24 and no toxicity in normal hepatic L-02 cells. The gene therapy has a potential in the clinical treatment of the human liver cancer [52].

Concluding Remarks

In this review, we can view such as the multi-target approach is a strategy that involved the sequence to recognize of the integrin αvβ3, important for the selectivity of the system, and: 1) specific radiolabel to visualize the tumour tissue in technique such us PET, CT-scan or MR-imaging; 2) drugs or other target peptides to induce the death of the cancer cells. This approach is important because adds a piece to the fight against cancer.

Author Contributions

RDM wrote the manuscript, revision and approved the final version and is the senior authorship of the manuscript.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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