DESIGN AND SYNTHESIS OF $\alpha_v\beta_3$ AND $\alpha_5\beta_1$ INTEGRIN LIGANDS AS DELIVERY SYSTEMS FOR FLUORESCENT MOLECULES

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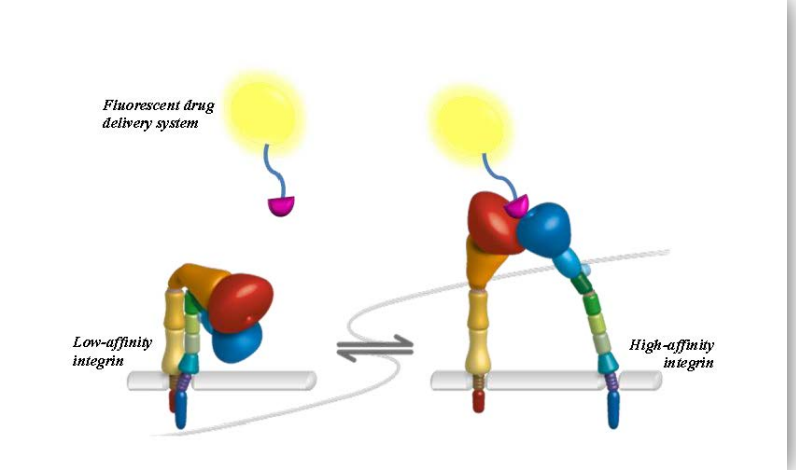


The design, synthesis and biological evaluation of integrin ligands is an important goal for their potential application as diagnostic and therapeutic tools in cancer research. We have synthesized isoxazoline-containing peptidomimetics, that showed great affinity towards $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. These receptors are involved in tumor angiogenesis and the obtained ligands may be thus conjugated to fluorescent molecules to provide potential diagnostic tools

Progettazione e sintesi di ligandi di integrine $\alpha_v\beta_3$ e $\alpha_5\beta_1$ come sistemi di rilascio per molecole fluorescenti
La progettazione, la sintesi e la valutazione biologica di ligandi di integrine rappresenta un valido obiettivo nella ricerca sul cancro per la loro potenziale applicazione terapeutica e diagnostica. Il nostro gruppo ha sintetizzato peptidomimetici contenenti isossazoline che hanno mostrato grande affinità verso integrine $\alpha_v\beta_3$ e $\alpha_5\beta_1$, i due recettori coinvioti nell'angiogenensi tumorale. Questi ligandi possono essere coniugati a molecole fluorescenti al fine di ottenere dei potenziali strumenti diagnostici.

The development of novel tools for the early detection, diagnosis, and therapy of cancer is one of the most important goal in medicinal chemistry. Integrins are a large family of heterodimeric transmembrane glycoproteins involved in several cellular activities as adhesion, differentiation, proliferation and cellular migration. Alterations or aberrations in integrin-mediated cell adhesion have been connected with the pathogenesis of several diseases such as atherosclerosis, osteoporosis, cancer and a variety of inflammatory disorders and for these reasons integrins are an attractive target for the development of therapeutic agents[1]. Among the different classes of receptors, the $\alpha_V\beta_3$ has been deeply investigated, as it is involved in tumor proliferation and metastasis through the formation of new blood vessels. It is known that $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins bind the extracellular matrix (ECM) proteins through a specific recognition motif: the RGD tripeptide sequence (Arg-Gly-Asp). Using this recognition triad, the receptors are able to bind a wide number of ECM components like fibronectin, fibrinogen, vitronectin and osteopontin.

The $\alpha_V\beta_3$ integrin is present at low levels on healthy tissues, whereas it is overexpressed in certain pathologies such as metastatic melanoma, late-stage glioblastoma, and breast and prostate tumors. It is also preferentially expressed on cancer blood vessels, mediating angiogenesis initiated by basic fibroblast growth factor (bFGF) or tumor necrosis factor-α (TNF- α). On the other hand, the $\alpha_5\beta_1$ integrin has been recognized as proangiogenic receptor, as antagonists to its extracellular matrix partner fibronectin are able to block growth-factor- and tumor-

induced angiogenesis. Moreover, during its migration in vitro or angiogenesis in vivo, this integrin may regulate the function of integrin $\alpha_V\beta_3$. Simultaneous blockade of $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrins was reported to inhibit bFGF-induced angiogenesis, and evidence of cross-talk between the two receptors has been provided[2]. Therefore, the identification of synthetic ligands that are able to engage with both these cell-surface receptors may give access to imaging biomarkers for early detection of cancer and assessment of therapy response, as well as in the engineering of cell-targeted anticancer agents.

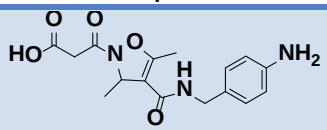
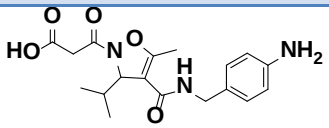
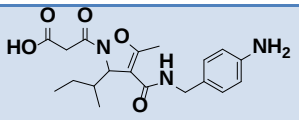
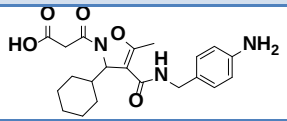
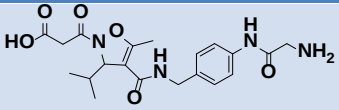
For this reason, we thought that a single antagonist mimicking the RGD sequence, could mediate the activity of both types of integrin and then block the same pathway of angiogenesis. Docking studies have shown that isoxazoline-containing peptidomimetics, already synthesized by our group[3], may assume a conformation suitable to bind these receptors and the excellent affinity has been confirmed by the values obtained in cell adhesion inhibition assays and in ERK phosphorylation assays.

Starting from these bases, new isoxazolines were synthesized which, while retaining the attributes that make them good structural ligands of integrin receptors, possess a further moiety to conjugate to fragments ending with fluorescent residues. The compounds thus obtained should be used as effective diagnostic tools. The ability to highlight only cancer cells, leaving untouched the cells of normal tissues, should allow a greater efficiency of the contrast medium. The increased sensitivity of the method should lead to the use of lower doses of the diagnostic and to the detection of cancer in a very early stage[4].

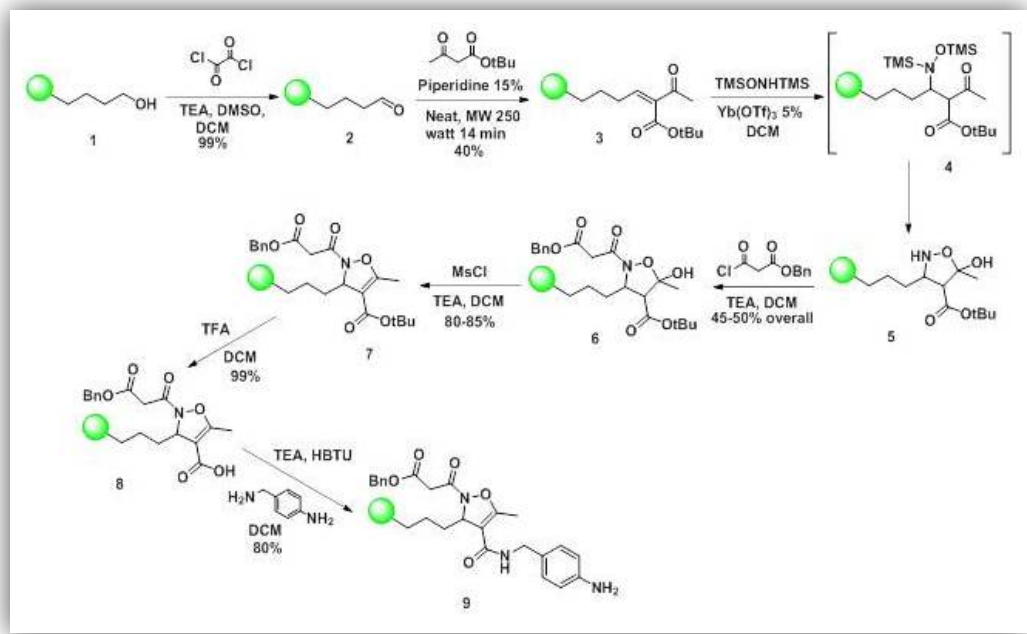
Synthesis of isoxazolines

Willing to find a good scaffold for integrin ligands, our choice has fallen on isoxazoline because its isoxazolidine precursor may be considered as a masked β-amino acid, as a 1,3 amino alcohol equivalent, or as a furanose ring mimetic in the preparation of bioactive compounds. Indeed, several transcriptional activators contain the isoxazolinic nucleus as conformational constrain element.. Aware of that, we developed a small library of integrin ligands that present the isoxazoline core as scaffold and opportune appendages inserted to mimic the RGD sequence (Tab. 1). The synthesized molecules showed a good activity against $\alpha_V\beta_3$ and $\alpha_5\beta_1$ and this results encouraged us to continue to work on this class of compounds.

Tab.1
Cell adhesion inhibition mediated by integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ in the presence of isoxazoline

Entry	Compound	IC_{50}(nM) $\alpha_v\beta_3$	IC50(nM) $\alpha_5\beta_1$
1		32±3	12±4
2		8.8±0.6	1.05±0.3
3		360±70	1,320±80
4		20±6	1,030±50
5		15,500±900	>100,000

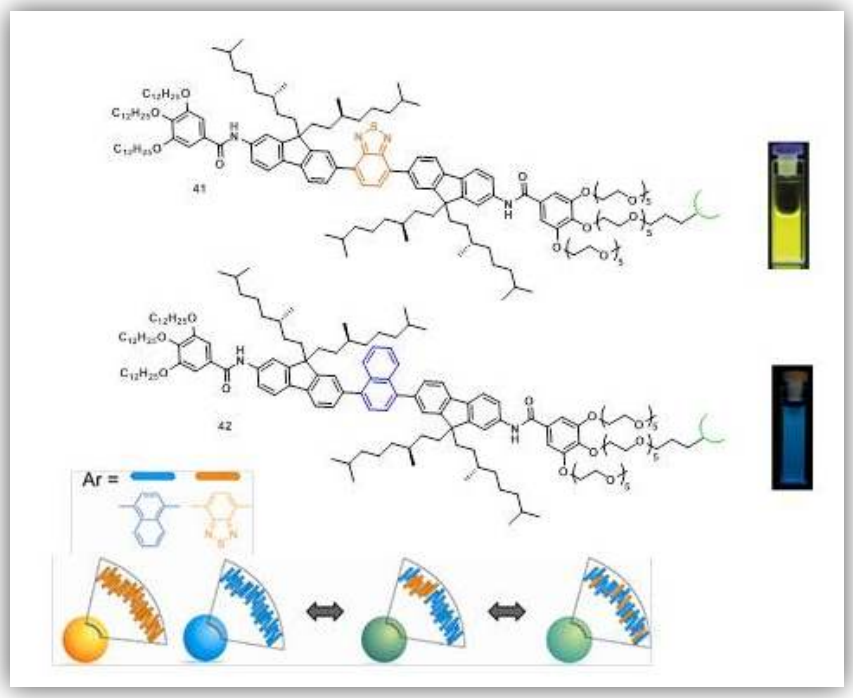
Starting from the obtained results, we synthesized new ligands able to link a fluorescent molecule in order to obtain potential drug delivery systems to be employed as diagnostic tools.



The candidate molecules used to conjugate the fluorescent compound present in their structure new functionalities, as amines, multiple bonds or carboxylates, able to react with complementary chemical moieties, in order to conjugate the two synthons.

Scheme 1
Synthesis of functionalized isoxazoline ligands

To synthesize this kind of isoxazolinic ligand, we prepared the aldehyde **2** which presents the opportune functionality, by Swern oxidation on alcohol **1** using oxalyl chloride (99% yield), and then subjected it to Knoevenagel reaction with *t*-butyl acetoacetate in presence of a catalytic amount of piperidine under MW irradiation, following the procedure reported in our previous work. From this reaction we obtained the corresponding alkylidene acetoacetate **3** in good to modest yield depending on the nature of the side chain. The α,β-unsaturated compound obtained was subjected to Michael addition with TMSONHTMS and ytterbium triflate as catalyst to obtain the intermediate **4** that spontaneously provided isoxazolidine **5** (Scheme 1).
In some cases, difficulties in the isolation of **5** were observed, since several intermediates resulting from hemiacetalic nature of isoxazolidine are present and for this reason we performed the subsequent reaction using the crude product. After treatment with benzyl malonyl chloride and TEA, **6** was obtained in 45÷50% yield after two steps. Using mesyl chloride and TEA in DCM isoxazoline **7** was finally obtained with good yield (80÷85%). The *t*-butylester hydrolysis, carried out with TFA in DCM, provided the corresponding acid **8** in quantitative yield and the final coupling with p-aminobenzylamine performed with HBTU and TEA in dichloromethane allowed us to obtain the desired product **9** in 80% yield (Scheme 1).



Synthesis of fluorescent molecule
Concerning the fluorescent compound to link to the latter, a class of amphiphilic molecules developed in Prof. Brunsveld's laboratory in Eindhoven (NL) was chosen (Fig. 1)[5].
As shown in Fig. 1, these molecules consist of different segments: a bicyclic aromatic central core, two fluorenyl entities which are respectively connected to different gallic acid derivatives.

Fig. 1
Fluorescent molecules synthesized and relative nanoparticles formation

The chains linked to the gallic acid portions, have different nature: one is hydrophobic, since formed by a lipophilic saturated chain, and the other is hydrophilic, because formed by a polyethylene glycol chain.
The specific affinity towards different solvents of these two portions allowed to obtain amphiphilic nanoparticles. The presence of the fluorenyl moieties and the bicyclic aromatic central core provides excellent detection systems and, in addition, by changing the byciclic aromatic central core, it is possible to obtain different colors upon UV irradiation. It is also interesting to note that a terminal chemical function is present on the hydrophilic terminal, allowing the connection with the integrin ligand that presents a complementary side chain.

Conclusions

Aiming to apply our expertise in the preparation of small libraries of integrin ligands to the synthesis of diagnostic tools for cancer detection, we have revisited the synthesis of isoxazoline-containing RGD mimetics by modifying the central core structure. We report herein our preliminary results in the introduction of side chains that may be linked to fluorescent molecules through complementary functional groups and the synthesis of the fluorescent fragments.

REFERENCES

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