Small Molecule Integrin Antagonists in Cancer Therapy

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Abstract: Integrins are a large family of dimeric receptors composed by α and β subunits that, once bound to extracellular matrix (ECM) proteins, regulate a variety of cellular processes such as cell motility, migration, and proliferation. The integrins transduce signals from inside-out and outside-in the cell, thus representing the cellular link to the external environment. For these properties, integrin activation has been involved in pathological processes like tumor growth and metastasis formation. Recent advances in the elucidation of the crystallographic structures of the αvβ3 and αIIβ3 integrins are promoting studies focused to the search of small molecule antagonists that can block the integrin binding to ECM and inhibit the biological effects exerted by these receptors. In this review we will focus on small molecule antagonists of αvβ3 and αvβ5 integrin as tools for cancer therapy while other integrins will only be briefly mentioned. Cilengitide (cyclic peptidic αvβ3 and αvβ5 antagonist) is currently in clinical trials for anti cancer therapy. Combination of integrin αvβ3 antagonists and other traditional therapeutic approaches may represent a future strategy to inhibit tumor growth and metastasis spreading.

Key Words: Integrin antagonists, RGD-peptides, metastasis, anoikis.

INTRODUCTION

A fundamental aspect related to cancer therapy is the control of tumor cells proliferative and aggressive behaviour. Different strategies have been tested but the main problem encountered by the most recent therapeutic approaches is represented by tumor heterogeneity. Tumors greatly vary in their cell composition and genotype and even tumors belonging to the same hystological classification may bear different mutations. This heterogeneity makes the identification of new pathways and promising therapeutic targets difficult and slow. At present, the cellular growth control is generally achieved by traditional chemotherapeutic agents but new strategies supporting the classical antiproliferative approach, like antiangiogenic agents, are explored [1]. In the last decade, studies concerning the interactions between tumor cells and the extracellular environment highlighted the possibility of identifying molecules that may represent interesting therapeutic targets for tumor cell proliferation and motility control [2]. Neoplastic growth is a complex process resulting from interactions among different cell types present in the tumor mass such as tumor cells, cells present in the tumoral niche, fibroblast and endothelial cells, that contribute to the composition of the extracellular matrix (ECM). ECM is a complex and dynamic structure that differs in protein composition and regulates cellular processes like cell migration, proliferation and differentiation [2].

ECM components can be broadly classified in glycosylated proteins and proteoglycans. The ECM proteins are represented by the fibronectin molecule and by two families, laminins and collagens, that include several distinct structural and functional members [3]. The ECM proteins exert their effect by binding cell surface receptors, ubiquitously expressed in almost any mammalian cell, denominated integrins.

The integrin receptor family have been identified as key regulators of different cellular processes related to tumoral growth like cell adhesion and migration, cell proliferation and neo-angiogenesis. Several integrin antagonists are in clinical trial for cancer therapy and in this review we will examine some features of the most promising small molecule integrin antagonists together with their potential therapeutic use.

THE INTEGRIN RECEPTOR FAMILY

Integrins are heterodimeric glycoprotein receptors formed by the combination of different 18 α–subunits and 8 β-subunits that give rise to 24 distinct receptors [4]. Integrin expression, though ubiquitous in human tissues, varies with cell types; α and β subunits are expressed in different combinations and exhibit ligand specificities [4]. The integrins expressed by cells, therefore, control the ECM components with which the cell can interact. Several types of integrin dimers have been identified in cancer cells [5] where they appear to play key roles mainly in proliferative and metastatic processes. The α5β1, αvβ3 and αvβ5 are widely expressed in several cancer types and recognize the tripeptide sequence Arg-Gly-Asp (RGD), found in many ECM proteins [6]. The structure of αvβ3, determined in unliganded [7] and ligand-bound states [8], showed a crevice formed by the ectodomain of the β chain [9], that is the RGD-like peptide binding site. Interestingly, integrin expression changes during the transition from a non-neoplastic to a neoplastic state suggesting that alterations of the adhesion properties in cancer cells may be involved in the early steps of metastasis formation [10,11].

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INTEGRIN MEDIATED SIGNALLING

Through the extracellular domain, integrins bind ECM proteins while inside the cell the short cytoplasmic tail binds to the cytoscheleton [9,12]. This is a bi-directional interaction because the cytoskeleton proteins modulate the affinity of the integrin-ECM binding and, conversely, perturbations of integrin binding to ECM change the shape and protein assembly of the cytoscheleton. This tight linkage between cells and ECM is achieved through the interaction of the short cytoplasmic domain with proteins like talin1, tensin, vinculin, α-actinin and filamin [13]. The adapter protein paxillin may link talin to the integrin α-tails contributing to the formation of adhesion complexes and enhancing resistence of ECM-integrin-talin to mechanical stresses [13]. Focal adhesions are integrin-mediated adhesion complexes that may vary in composition according to integrins and matrix components. The mechanism underlying cell motility has been clarified in detail, although not completely on the molecular structure level: actin filaments apply force on the ECM–integrin complex leading to the transformation of focal complexes into focal adhesions indicating the direction of cell movement. Afterwords, the integrins located on the trailing edge of the cell are internalized to be recycled to the leading edge where new interactions with ECM are formed [13]. This mechanism is required to cross tissue layers while other mechanisms regulate cell migration in the interstitial space [14].

Integrin binding to ECM ligands transduce signals to the tyrosin kinase Src that is constitutively bound to the integrin β cytoplasmic tail. In clusterd integrins, Src transphosphorylation leads to recruitment of tyrosine phosphatases [15,16] and to activation of kinases like phosphatidylinositol-4-phosphate 5 kinase type γ (PIPKIγ) or FAK [17]. These signals contribute to the activation and recruitment of focal adhesion proteins like vinculin and talin and, through phosphorylation of intracellular trasducers like p130Cas or caveolin, lead to the activation of the classical ERK pathways implicated in survival and proliferation signalling [18]. It should be considered that growth factors receptors (GFRs) are influenced by the adhesion state of the cells and FAK–stimulated signalling cascade has been shown to modulate the ability of GFRs to stimulate ERK phoshorylation and cell cycle phases transition [19]. This combination of adhesion and survival signals triggered by integrin-ECM binding is considered to be the mechanism by which integrin-mediated adhesion promotes cell survival.

When wrong interactions between cells and ECM proteins cause the cell detachment from ECM, a kind of apoptosis called "anoikis" may be induced. Anoikis, homelesness in greek, indicates a condition in which proliferation and survival signals are suppressed and apoptotic caspase-dependent and independent processes start [20]. It was clearly demonstrated that integrins suppress anoikis by FAK activation in a variety of cell types and that integrin attachment to fibronectin counteracts the effect of mitocondrial pro-apoptotic proteins [21]; on the other hand, cilengitide and RGD peptides induce anoikis in human brain cancer and edothelial cells [22-24], suggesting that blocking of αvβ3 and αvβ5 receptors may represent a promising strategy to suppress apoptotic resistance in cancer cells.

INTEGRINS AND CANCER

Classical chemotherapeutic anticancer therapies try to hit the tumoral mass or to inhibit the growth of satellite foci but the main point is that 90% of death of cancers patients is caused by metastasis and not by the solid tumors [25]. While the tumoral growth has been widely studied and the steps that lead cells towards cancerogenic transformation process have been deeply investigated, the metastasis formation has received less attention and the molecular events regulating this complex process are only partially known. The metastatic process requires several complex steps that can be summarized in loss of cellular adhesion, increased cell motility and invasiveness, entry and survival in the circulation, crossing new tissue barriers, and colonization of distant sites [26,27]. αvβ3 integrins have been implicated in metastasis formation, specifically during adhesion of circulating tumor cells to the vascular endothelium and crossing the endothelial cell layer [28,29]. αvβ3 and αvβ5 appear to be directly involved in metastasis formation in different cancer types like colon cancer, metastatic melanoma and breast cancer [2]. In glioma samples, αvβ3 expression has been detected in glial tumor cells and correlates with tumor grade, suggesting that these receptors may be important players in the higly infiltrative behaviour of high grade gliomas [30].

INTEGRIN ANTAGONISTS

Because of their role in metastatic and infiltrative processes, αvβ3 and αvβ5 represent interesting targets for new anticancer strategies. In addition, integrins expressed by endothelial cells participate in angiogenesis. Indeed, integrin αvβ3 expression was found to be increased in proliferating vascular endothelial cells and for this reason it was thought to be involved in pathological angiogenesis [31]. These findings suggested the potential use of RGD-like αvβ3 inhibitors as antiangiogenic agents in cancer therapy. However, results from *in vivo* studies are controversial because while the antiangiogenic effect was beneath expectations, RGD-like inhibitors appear to affect glioma cell survival directly [23]. Therefore, the effect of integrin antagonists on tumor progression is still difficult to define and phase I and phase II studies often propose different mechanisms for the observed effects.

A different family of integrin inhibitors is represented by antibodies that bind to αvβ3. In particular, two antibodies have been successfully developed and are currently in clinical trial: Vitaxin (MEDI-523) is in phase I for the treatment of stage IV metastatic melanoma and androgen-independent prostate cancer and Abegrin (MEDI-522), a modification of MEDI-523, is in phase I for patients with solid tumors (abegrin, vitaxin). However, since the discussion of these inhibitors is beyond the scope of this review, for a more detailed discussion we suggest to see Tucker, 2006 [32].

PEPTIDES AND PEPTIDE-MIMETIC "SMALL MOLECULES" INTEGRIN INHIBITORS.

These compounds are peptide-mimetics carrying in their structure the RGD sequence [33,34] or nonpeptidic derivatives capable to interact with the integrin receptors through functional groups mimicking the guanidine and carboxylic moieties present in the side chain of arginine and aspartic acid, respectively.

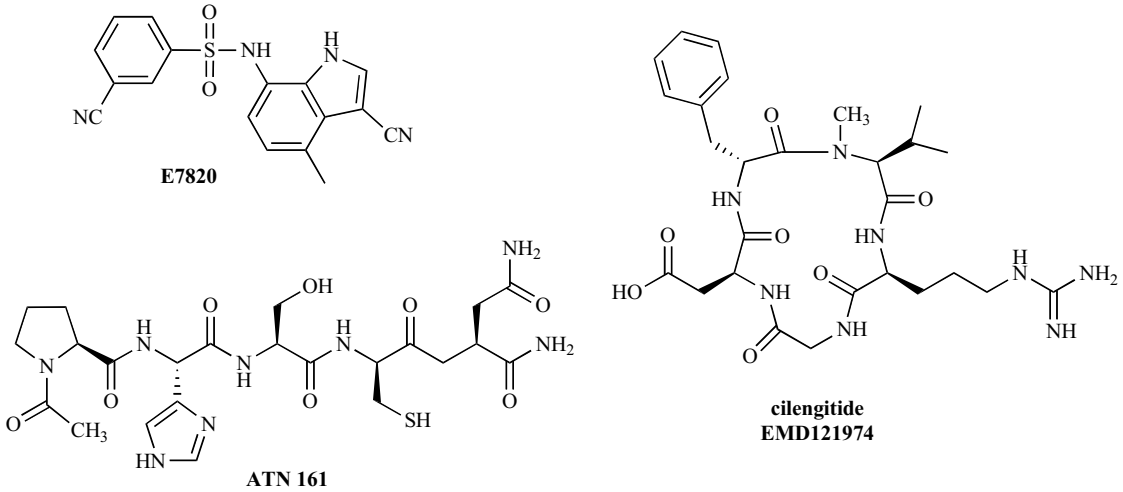

Fig. (1).

CILENGITIDE (EMD 121974)

The first integrin antagonist to be tested in humans has been Cilengitide (EMD 121974), a cyclic peptide belonging to the RGD-peptide family that binds to the described crevice of the β chain thus blocking the binding to ECM [35]. Cyclic RGD pentapeptides with constrained backbone conformations have first been developed as highly active and selective ligands for the αvβ3 integrin receptor [36-38]. Extensive ligand-based drug design studies led to the highly active αvβ3 selective first generation inhibitor *cyclo*(Arg-Gly-Asp-D-Phe-Val) [36,39-41]. The systematic derivatization of the lead peptide resulted in the *N*-methylated cyclopeptide EMD 121974, *cyclo*(Arg-Gly-Asp-D-Phe-NMeVal), as one of the most active compounds. The X-ray analysis of the complex αvβ3-EMD121974 shows that the ligand interacts mainly through electrostatic interactions [8,42]. Arg and Asp of the RGD form a "charged clamp" that binds regions with opposite charges in the protein: Asp interacts with a metal cation in the MIDAS region of the β subunit and Arg with two residues of aspartic acid (Asp218 and Asp150) located in the α subunit.

In preclinical studies, cilengitide has been shown to block glioblastoma (GBM) growth in nude mice [43] while other studies report that cilengitide in combination with radiotherapy decreased the tumor proliferation rate in ovarian cancer xenografts [44]. In this case, the decreased proliferation rate was not attributed to anti-angiogenic activity but to a pro-apoptotic effect. Cilengitide, in combination with temozolomide, was also reported to exert antiproliferative effects on melanoma cells *in vitro* and a reduction of melanoma growth, compared to the methylating agent alone, was also observed *in vivo* [45].

The efficacy of RGD peptides in clinical trials has been demonstrated (Table **1**), though in some cases results are contoversial. First data demonstrated good tolerability and low toxicity in patients, which prompted the initiation of further studies using a combination with chemo- or radiotherapy [45,46]. However, a recent study in patients with recurrent GBM has shown that cilengitide monotherapy, though well tolerated, has modest antitumor activity [47]. Another study reports that co-application of cilengitide and gemcitabina resulted in partial tumor remission in a patient with highly vascularized head and neck cancer and in this case the anticancer activity was attributed to an anti-angiogenic effect [48]. In patients with different solid tumors Cilengitide monotherapy gave no partial or complete responses [49], phase II studies have been designed to evaluate Cilengitide treatments in patients with non metastatic androgen-independent prostate cancer (NCC 6735) and patients with metastatic prostate cancer (NCC 6372). Recently, RGD cyclic peptides have been used as carriers for cytotoxic drugs

Table 1. Clinical Trials of Small Molecule avβ3 and αvβ5 Inhibitors

Name		Highest Phase Reached	Ref.
Cilengitide (EMD 121974)	glioblastoma	Phase III	[80]
ATN-161	malignant glioma	Phase I	[81]
	renal cell cancer	Phase II	[82]
E7820	solid tumor, lymphoma	Phase I	[83]
	colorectal cancer	Phase II	[84]

[50,51], to specifically target cancer cells and tumor blood vessels. Furthermore, suitably radiolabeled RGD peptides have been developed for non invasive imaging of integrin expression during tumor angiogenesis, growth, and spread in humans [52,53].

ATN-161

ATN-161 is an N-acetylated, amidated, non RGD-based, linear pentapeptide (Ac-PHSCN-NH2) in phase II clinical trial in patients with solid tumors (see Table **1**). It is a non-competitive inhibitor of the fibronectin "synergy region" PHSRN sequence. This fibronectin region increases the affinity and specificity of the RGD-mediated binding thus making integrin-fibronectin interaction stronger. In ATN-161 a cysteine residue has been introduced for arginine along with peptide acetylation and amidation, in order to yield a product with acceptable pharmaceutical properties.

An unregulated invasive response to the PHSRN sequence may contribute significantly to the growth, survival and metastasis of established tumors [54]. Recent studies show that ATN-161 binds to integrin beta subunits of $\alpha 5\beta 1$ and $\alpha v\beta 3$ subtypes and displays antitumorigenic and antimetastatic activities in different cancer types [55,56].

E7820

This molecule is an aromatic sulfonamide derivative (*N*-(3-Cyano-4-methyl-1H-indol-7-yl)-3-cyanobenzene-sulfonamide). E7820 cannot be considered a classical integrin antagonist since it does not bind to α or β integrin subunits, nevertheless an inhibitory effect on $\alpha 2$ integrin mRNA expression in endothelial cells was reported [57]. E7820 has demonstrated anti-angiogenic activity in both in-vitro and in-vivo angiogenesis models and this, in turn, leads to a decrease in cancer cell proliferation rate in different cancer models [57,58]. The antiangiogenic effects have been ascribed to inhibition of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) activity. Inhibition of $\alpha 2$ expression might be the mechanism underlying this effect. The drug is currently in phase II clinical development (see Table **1**).

NEW MOLECULES

Peptides

During the last decade other peptidomimetic compounds have been synthesized and tested for their ability in blocking RGD-mediated cell adhesion. Several turn mimetics [59,60] and sugar amino acids were incorporated into the lead structure cyclo(RGDf[NMe]V) by replacing the D-Phe-[NMe]Val dipeptide unit. A common feature of these derivatives was the presence of a 15 membered macrocycle, identical to that of EMD121974. Studies on peptide secondary structure lead to the synthesis of several 6,5- and 7,5-fused 2-oxo-1-azabicyclo[X.3.0]alkane amino acids possessing the general structure 1 (Fig. **2**) [61]. These scaffolds can be regarded as conformationally restricted substitutes for Ala-Pro and Phe-Pro dipeptide units [50,62]. All the bicyclic compounds include a natural L-Pro residue, but vary in the lactam ring size and in the configuration at the fusion carbon and at the NH2-bearing center. Computational and spectroscopic studies have revealed that these scaffolds can mimic reverse-turn motifs as those investigated by Haubner and coworkers [39], although there is a dependence of the turn-inducing ability on the lactam ring size and stereochemistry [63]. Replacement of the D-Phe-[NMe]Val dipeptide in the reference compound cyclo(RGDf[NMe]V) with such azabicycloalkane derivatives, showing different reverse-turn mimetic properties, constrained the RGD sequence into specific conformations and provided the required activity and selectivity for integrin antagonism. For a more complete figure of turn mimetics for the D-Phe-Val units see Weide *et al*. [33].

A library of RGD-containing cyclic pseudopentapeptides was then synthesized, allowing the achievement of some specific high-affinity $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin antagonists. Among the peptides tested, ST1646 and compound 2 showed the highest affinity towards $\alpha v\beta 3$ receptor, inhibiting [^{115}I]echistatin binding to $\alpha v\beta 3$ with an IC50 value of 3.7 ± 0.6 nM and of 6.4 ± 0.1 nM respectively, that are comparable to EMD121974 IC50 values (18.9 ± 3.1 nM) in the same assay.

In preliminary experiments, compound 2 has shown an inhibitory effect on cell attachment and cell migration assays, probably mediated by FAK inhibition, in human glioblastoma cells (Russo *et al*., manuscript in preparation). These data highlight the possibility of *in vivo* experiments to further clarify the anticancer activity of this compound.

Another series of peptide antagonists was recently patented [64]. These compounds are a hybrid cyclopeptide derivative embodying pyrrolidine or piperidine-based amino acid grafted onto a RGD tripeptide sequence and belongs to

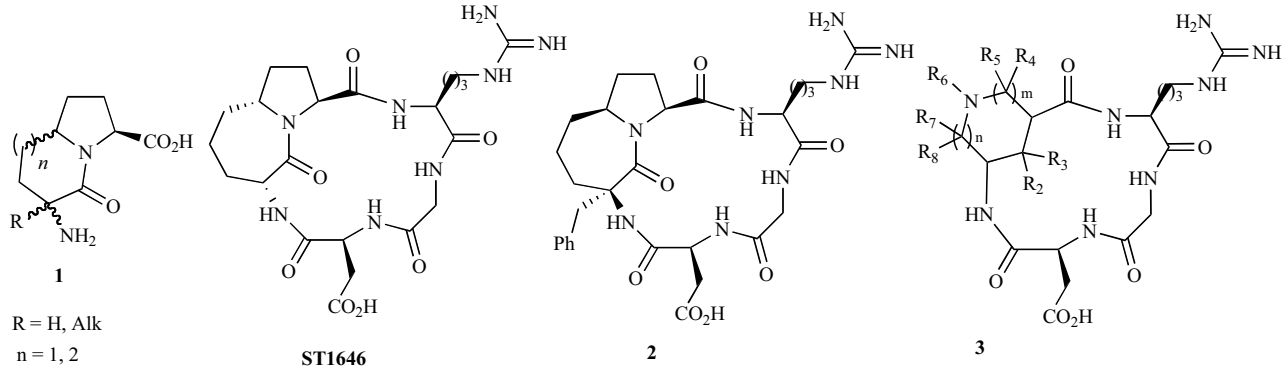

Fig. (2).

a family of compounds of general formula 3. The structural novelty is represented by pseudopetide 14-atom macrocycles more constrained than the aforementioned cyclopentapeptides and these compounds exhibited low-nanomolar and even picomolar antagonist activity towards the αvβ3 and αvβ5 integrins.

Non Peptidic

The need for antagonists with higher bioavailability and lower molecular weight has prompted several research groups to develop small constrained nonpeptidic molecules mimicking the RGD motif. Many of the structures proposed consist of a polyfunctionalized rigid core, linked to appendages corresponding to arginine and aspartic acid side chains. Both the basicity and the length of the arginine-mimic group showed to be essential for binding interaction with the integrin receptor. Moreover, the presence of a carboxylic function mimicking the aspartic acid of RGD is an essential feature to create anionic interaction with the MIDAS cationic region.

Many heterocyclic scaffolds have been employed to hold the acidic and the basic ends of the molecule at the appropriate distance and with the suitable conformation for binding interaction [34]. Here we only report compounds which strongly bind to integrins involved in the tumoral process and which therefore are promising candidates for future applications in cancer therapy.

Recently, the aforementioned azabicyclo[4.3.0]alkanes were functionalized with guanidinium or carboxylate groups on both cyclic components [65]. The most active lactam of the series was **4**, bearing a $-CH_2CO_2H$ group on the proline ring and an arginine residue linked to the amino group at the 3-position.

In their continuous efforts focused at finding highly active and selective αvβ3 integrin antagonists, Kessler and coworkers designed new RGD mimetics in which the glycine was replaced either by an aza-glycine residue (see **5a**, Fig. **3**) [66] or an azacarba analogue (compound **5b**) [67]. The aspartic acid was mimicked by various β-amino acids or glutaric acids carrying different substituents at the β-position. A guanidinic function linked to the GD-mimic by either aromatic or aliphatic spacers completed the structure. The most active compound of the series was compound **6**, that showed an excellent IC50 value in the subnanomolar range (0.1nM). By carrying out some structural modifications on compounds of general formula 5, the same research group showed that a set of integrin specific inhibitors can be designed around a conserved aza-glycine backbone [68]. Compound **7** was the first specific, low molecular weight αvβ6 peptidomimetic inhibitor described in the literature.

The αvβ6 integrin receptor is up-regulated in tumor proliferation [69,70] and in a number of pathological processes such as inflammatory events, wound healing, cell migration and proliferation. This class of inhibitors is therefore of great interest for future applications in human pathologies such as cancer and osteoporosis.

Another integrin which has recently been drawn into the focus of research is the α5β1 subtype, whose pro-angiogenic function has been clearly demonstrated [71]. Recently, the synthesis of new nonpeptide αvβ3/α5β1 integrin dual antagonists containing 5,6-dihydropyridin-2-one scaffolds was reported. The dihydropyridinone heterocycle was converted into a series of potential integrin antagonists changing the length, the structure and the position of the side chains bearing the acidic and the basic appendages. The biological activity of the various derivatives has been evaluated by testing their ability to inhibit cell attachment mediated by αvβ3 and α5β1 integrins in cell adhesion assays. The enantiomerically pure derivative **8** had excellent affinity towards both receptors [72].

The hint for the design of new α5β1 ligands came from the homology model of the α5β1 receptor [73]. On the basis of these studies Heckmann *et al*. [74] synthesized a series of compounds based on a tyrosine scaffold that has already been successfully employed in the integrin field [75]. Compound **9** showed the best α5β1 affinity (0.7 nM) and good selectivity against αvβ3 (300-fold). In another paper [76], the same authors used the structure-activity informations provided by a tyrosine-based compounds library to design new α5β1 aza-glycine-based ligands with affinities of ~1 nM and selectivities that exceed 10^4-fold. The activity on α5β1 and selectivity against αvβ3 seems to be strongly connected to the presence of a *S* configured, alpha-amino acid as carrier of the carboxyl moiety and an aromatic amide with an ortho-dimethyl-substitution pattern (compound **10**).

Stragies *et al*. synthesized a new class of α5β1 pyrrolidine-based antagonists of general formula **11** [77]. Integrin selectivity was tuned, in agreement with the observations reported by Hackmann *et al*., by switching from a sulphonamide moiety to a mesitylene amide moiety for R3. Moreover, an increase of activity by a factor of 4-6 was achieved by introducing 4-methoxy-2-aminopyridine as a basic group. Compounds **11a** and **11b** showed subnanomolar α5β1 activity and very high selectivity toward other related integrin receptors.

Different groups investigated the use of a central heterocycle core in order to maintain the aspartate and the guanidine mimetics in the appropriate orientation to modulate the pharmacokinetic profile of the αvβ3 antagonists.

Several indol-1-yl propionic acids, containing a variety of basic moieties at the 5-position as well as substitutions alpha and beta to the carboxy terminus, were reported [78]. The most active compound **12** carries a 3-pyridyl substituent on the carboxylic chain and a tetrahydronaphthyridine moiety at the 5-position of the indole ring. This derivative possesses a number of favorable properties including good oral bioavailability and a subnanomolar activity towards αvβ3 receptor.

In other studies the synthesis of benzazepine derivatives is reported (compound **13**). These molecules are highly potent, orally active (in rats) αvβ3 antagonists. An extensive study aimed at increasing the lipophilicity of these derivatives revealed that a trifluoro-ethyl-substituent placed on the 2-position considerable improved oral bioavailability in rats. Moreover, incorporation of the 6-(N-methylamino)pyridine Arg mimetic had a significant impact on biological activity [79].

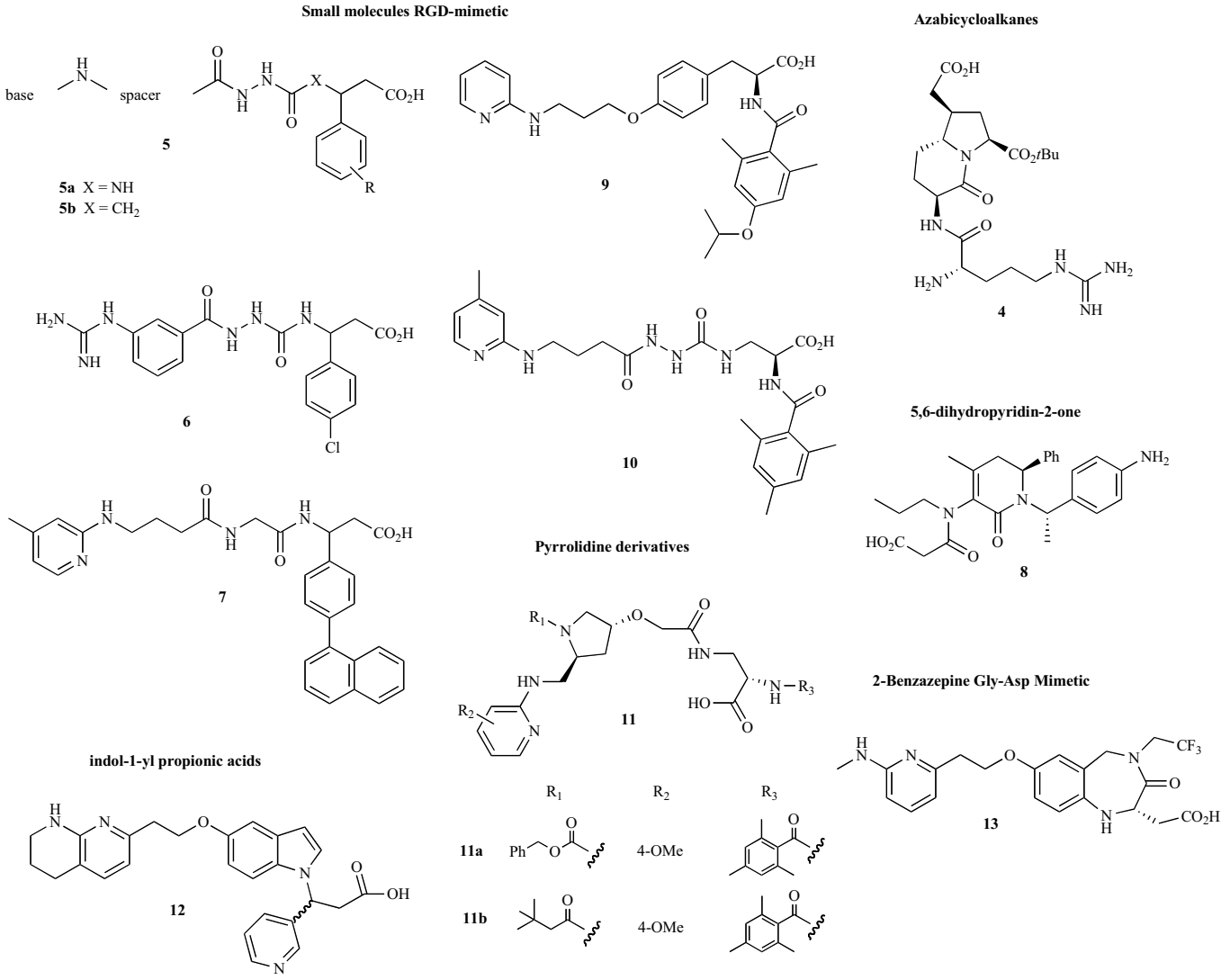

Fig. (3).

CONCLUSION

The results so far obtained from clinical trials with αvβ3 and αvβ5 antagonists suggest that these molecules, though exerting both anti-angiogenic and direct anticancer activity, when administered alone, achieve too week results as anticancer agents. On the contrary, very promising results come from trials in which the compounds are associated to chemo- or radio-therapy.

Ongoing studies highlight other interesting applications for αvβ3 and αvβ5 antagonist, like selective delivery of cytostatic drugs or diagnostic uses in combination with imaging techniques. The development of new small molecule integrin antagonists with good bioavaillability could give an important contribution to the most important target of anticancer therapy: the arrest of the metastasis spread.

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