Therapeutic Targeting of G-Protein Coupled Receptor-Mediated Epidermal Growth Factor Receptor Transactivation in Human Glioma Brain Tumors

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Abstract: The epidermal growth factor receptor (EGFR) is the main tyrosine kinase receptor dysregulated or overexpressed in brain cancer types and its expression is directly correlated with tumor malignancy and unfavorable prognosis. Recently, the availability of endogenous EGFR ligands has been reported to be also regulated indirectly by the activation of several G-protein-coupled receptors (GPCRs) in many cancer cell types. This EGFR transactivation mechanism requires the initial activation of a GPCR that in turn induces the cleavage of membrane-bound EGFR ligands precursors *via* the involvement of the family of disintegrin and metalloproteases (ADAMs). The discovery of ADAMs in this transactivation mechanism led to the development of small molecule inhibitors. In this minireview we describe the expression of GPCR, ADAMs and EGFR ligands in human glioma brain tumors and the characteristics of small molecule ADAMs inhibitors. The addition of ADAM inhibitors to our pharmacological arsenal could enhance the outcome of combination therapies when using EGFR inhibitors against human brain tumors.

Key Words: Glioma, transactivation, EGFR, GPCR, sheddase, ADAM, brain tumor.

INTRODUCTION

One of the main challenges encountered in the development of therapeutic agents in cancer is the plethora of aberrant signaling tranduction pathways that should be simultaneously inhibited or downregulated to obtain an efficient therapeutic response. Several elegant studies have clearly identified a novel mechanism by which the activation of G-protein coupled receptors (GPCRs) might indirectly regulate functional effects such as cell proliferation and apoptosis either in physiology or disease, e.g. cancer. The stimulation of GPCRs induces the release of epidermal growth factor receptor (EGFR) agonists *via* the activation of specific disintegrin and metalloproteases (ADAMs). Interestingly, components of this mechanism have been shown to be expressed and even upregulated in several cancer type including human brain cancer cells, particularly malignant human gliomas [1]. Agents that inhibit the function of either membrane GPCR or EGFRs alone have shown modest therapeutic efficacy in patients with cancer. Recently, ADAMs metalloproteases have emerged as upstream activators of EGFR ligands and therefore ADAMs inhibitors that block the cleavage of multiple EGFR ligands would inhibit the activation of multiple EGFR-dependent pathways. Strategies targeting the ADAMs might complement existing pharmacological therapies and, when used in combination with various EGFR antagonists and other chemotherapeutic agents, could enhance the efficacy of these agents to reduce cancer cell invasion and malignancy. In this mini-review, the various components of the EGFR transactivation system in human glioma cells, together with compounds affecting ADAM functionality, will be described.

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THE GPCR-INDUCED EGFR TRANSACTIVATION MECHANISM

The GPCR-induced EGFR transactivation mechanism, sometimes called a "triple trans-membrane mechanism", includes several distinct steps:

1) activation of membrane GPCRs by endogenous agonists with second messenger formation, activation of protein kinases and cytosolic non-receptor tyrosine kinases (RTK). Distinct heterotrimeric G protein subunits participate in EGFR transactivation linking the GPCRs activation to ADAMs-dependent EGFR ligands shedding. In COS7 cells, EGFR-dependent activation of the ERKs cascade and the cytosolic tyrosine kinase c-Src after a_2-adrenergic receptor stimulation requires G_{bg}-subunits upstream of HB-EGF shedding [2]. The $G_{i/o}$ inhibitor pertussis toxin inhibits lysophosphatidic acid (LPA)-induced EGFR transactivation *via* ADAM-17-dependent generation of the EGFR ligand amphiregulin (AR) in squamous carcinoma cells, indicating a role of $G_{i/o}$ in this mechanism [3]. Another G protein, such as the G_q subunit that is directly linked to phospholipase C (PLC) activation, have been shown to modulate EGFR transactivation. In breast cancer cells, the G_q subunit participates in the EGFR transactivation mediated by HB-EGF shedding *via* ADAM17 upon stimulation of angiotensin(AT)1 receptors [4]. Different isoforms of the PKC family members have been implicated in EGFR transactivation. In ADAM17-/- and ADAM12-/- mouse embryonic fibroblasts the PKC activator phorbol-12-myristate acetate (PMA)-induced EGFR ligands shedding was greatly decreased [5] and in other cell lines the PKC-dependent metalloprotease activation *via* Src mediates HB-EGF shedding

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and subsequent EGFR transactivation following gonadotropin-releasing hormone and AT1 receptors activation [6]. In addition, Ca^{2+} and reactive oxygen species (ROS) may play a role in EGFR transactivation. In human glioma cell lines, CD44 cleavage by ADAM10 was stimulated *via* a Ca^{2+}-dependent mechanism but the same cleavage by ADAM17 was regulated *via* a Ras/PKC mechanism [7]. In COS7 cells, Ca^{2+} elevation and ROS generation mediates HB-EGF shedding *via* ADAM17 upon stimulation of AT1 receptors [4]. In addition, activation of GPCRs may also lead to the recruitment of scaffold proteins, such as Shc, Grb2, and Sos in addition to mitogen-activated protein kinase activation [8]. In other cases, RTKs use different components of GPCR-mediated signaling, such as β-arrestin, G protein-receptor kinases, and regulator of G protein signaling to integrate signaling pathways [9].

2) activation of membrane-bound matrix metalloproteases (MMP and ADAMs) and release of a membrane peptidergic bound-ligands into the extracellular space. The metalloproteases ADAMs belong to the metzincin superfamily and to date, about 40 ADAM family members have been identified that display either a broad or restricted tissue expression. In the brain ADAMs are involved in different role such as neural development, axon guidance and inflammatory responses [10]. Structurally the ADAMs are formed by a series of conserved protein domains: an NH2-terminal signal sequence, a pro-domain, a metalloprotease domain, a disintegrin domain, a cysteine rich region, an EGF-like domain, a trans-membrane domain and a cytoplasmic domain. A simplified model poses that inactive precursor of ADAM, upon stimulation of GPCR, undergoes activation by proteolytic cleavage to the catalytically active ADAM enzyme involved in EGFR ligands shedding [11]. In the following chapters we will discuss the features of catalitically active ADAMs subgroup expressed in human brain that includes ADAM 8,9,10,12,15,17 and 19. Other catalitically inactive ADAMs found in human brain , e.g. ADAM 11, 22 and 23, do not participate in EGFR ligand shedding because they play a major role in brain development and function [10]. The mechanism of ADAM activation is currently a field of active research, and although the molecular events are far from being fully elucidated, two proposed mechanisms have emerged from recent studies. According to the first mechanism, the phosphorylation of ADAM at certain residues by specific cytosolic protein kinases or tyrosine kinases leads to ADAM activation. In some cells the extracellular regulated kinases (ERKs)-dependent pathway [12,13] and the p38MAPK-dependent pathway [14] induce ADAM phosphorylation at residues Thr735 and Ser819 acting upstream of ADAM in HB-EGF shedding. In other cell model, the cytosolic tyrosine kinase c-Src has been implicated in metalloprotease-dependent EGFR transactivation induced by α_2A adrenergic receptors [2]. The second proposed mechanism involves a protein-protein interaction between the cytoplasmic domain of ADAMs and other interacting proteins such as kinases, adaptors proteins and substrates. For example the protein PACSIN3 associates with ADAM9, 10, 12, 15 and 19 *via* its SH3 domain and is required for HB-EGF shedding induced by PMA [15]. In this context, it is evident that the compartmentalization of different components of the EGFR transactivation machinery are crucial for a temporal and spatial control of ectodomain shedding. The new working hypotheis, supported by preliminary experimental data, states that all these proteins interact with each other at lipid rafts, which are functional microdomains of the local cholesterol-enriched plasma membranes.

3) stimulation of EGFR by their ligands, activation of downstream signaling pathways, induction of target genes and eventually modification of cellular functions. The EGFR itself and the related EGFR-dependent signaling transduction pathways play a pivotal role in the genesis and maintenance of glial tumorigenesis especially in human gliomas [16]. The EGFR downstream pathways include, but are not limited to, the Ras/Raf/MEK/ERK-, PI3K/Akt-, p38MAPK- and JNK-dependent pathways that in turn regulate first the expression of immediate early genes (IEG) such as *c-fos* and *c-jun* and then those of delayed response genes (DRG). These latter genes modulate cellular functions such as proliferation, cell growth, cell migration and invasion. ADAM inhibitors, blocking the formation of EGFR agonists and indirectly inhibiting these EGFR downstream pathways, are an instrumental tool to demonstrate ADAM-dependent EGFR transactivation. Indeed, pharmacological ADAMs inhibitors block EGFR transactivation and subsequent ERK activation induced by several GPCR agonists [17]. In squamous carcinoma (SCC-9) cells, the activation of the Akt/PKB pathway induced by LPA and carbachol was inhibited by the metalloprotease inhibitor BB94 or siRNA directed against ADAM17 [3] and in bladder cancer cells BB94 blocks the LPA-induced JNK and p38MAPK phosphorylation [18]. Finally, cell migration appears to be regulated by an ADAM17/ERK/PI3K-dependent mechanism because the MEK inhibitor PD98059 and the PI3K inhibitor LY294002 block cell migration induced by LPA in MDA-MB-231 cells [19].

GPCRs EXPRESSION IN HUMAN GLIOMA

Gliomas represent the most diffuse brain tumors and they are classified on the basis of their supposed tumorigenic differentiation from astrocytic, oligodendroglial or ependymal cells. Moreover, they are graded on a scale of I to IV according to their malignancy on the basis of histological, immunohistochemical and morphological features [20]. An aberrant expression of GPCRs or an exagerate activation of their downstream signaling pathways are key players in cancer cell migration, invasion and proliferation. Here we will describe the expression, in human glioma samples or glioma cell lines, of those GPCRs that have been shown to modulate EGFR transactivation in other cell types. However, it should be emphasized that only for few GPCRs a functional link between their activation and EGFR transactivation has been reported. This fact does not necessarily imply that GPCR-dependent EGFR transactivation does not occur in these cells but it might also mean that this mechanism has not been thoroughly investigated or, alternatively, that observation of this mechanism was hampered by intrinsic limitations of used or currently available methods. The cannabinoid system exerts its effects *via* the activation of two GPCRs (CB1 and CB2) linked to the activation of several signaling transduction pathways such as the ERK-, p38MAPK- and Akt-dependent pathways [21]. Glioblastoma cells may produce endogenous cannabinoids [22] that in turn stimulate the cannabinoid receptors expressed on the same cells [23]. Activa-

tion of CB receptors leads to a wide variety of cellular effects including inhibition of cell proliferation [24], inhibition of tumor growth and angiogenesis [25], induction of apoptosis [26], caspase activation and increase in oxidative stress [27]. The involvement of two EGFR ligands shed by one ADAM isoform has been documented in the cellular effetcs mediated by cannabinoids in glioma cell lines. In U373-MG glioblastoma and 1321N1 astrocytoma cells the cannabinoid agonists induce cell proliferation *via* the shedding of proamphiregulin and HB-EGF *via* the involvement of ADAM17 [18]. Several reports have described the expression of CXCR receptors and their ligands in glioma cell lines and human glioma specimen [28]. The activation of the CXCR4 receptors by its endogenous ligand SDF-1 increases cell proliferation and migration in glioblastoma cell lines *via* activation of ERKs- and AKT-dependent pathways [29]. In breast cancer cell lines SDF-1 induces cell proliferation *via* EGFR transactivation [30], but at the present time there is no published report of CXCR4-mediated EGFR transactivation in brain tumor cell lines.

The components of the endothelinergic system, such as the peptides endothelin (ET)-1, ET-2 and ET-3, are well characterized. ET-1 mRNA expression and ET-1 peptide have been found in T98G glioblastoma cells and in extracellular medium [31]. The expression of ET-1, endothelin converting enzyme (ECE)-1, ET-A and ET-B receptors has been detected in surgical samples of diffuse astrocytoma by immunocytochemistry [32], suggesting local ETs synthesis and processing. In addition, ET-A receptors are expressed by 1321N1 human glioma cell lines [33], and human oligodendrogliomas express ET-B receptors functionally coupled to intracellular signaling pathways involved in cell survival and/or proliferation [34]. Finally, ET-1, ET-B and ET-A receptor mRNA, together with IR-like ET-1, are expressed in three different cell lines [35] and the ET-B receptor is functionally coupled to ERK-dependent signaling pathway. The group I metabotropic glutamate receptors (mGluRs) represent another class of GPCR widely expressed in brain cells and potentially involved in the EGFR transactivation. The induction of cell proliferation through activated mGluRs in brain tumor cell lines has led some authors to propose mGluRs as a new promising target for the control of brain tumor growth [36]. The precise molecular mechanisms involved in cell proliferation are not completely understood, but it is noteworthy that the stimulation of mGluR5 *via* EGFR transactivation induces phoshorylation events previously associated with cell proliferation, i.e. ERKs phosphorylation in rat cultured astrocytes [37] and JNK phosphorylation and *c-jun* expression in cultured neurons [38]. LPA is a phospholipid with diverse biological functions that in several types of cancer cells has been shown to stimulate GPCR and EGFR transactivation [3,6]. Several reports have found that human glioma cell lines [39] or rat glioma C6 cell lines [40] synthesize LPA and might induce cell proliferation [41] or cytoskeletal rearrangement [42] *via* the activation of several signaling pathways. However, although the LPA system appears to be widely expressed in brain tumor cells, at the present time there is no report in the literature on a possible EGFR transactivation induced by LPA in brain tumor cell lines. Other GPCRs, that are expressed in human glioma cells and that have been shown to transactivate the EGFR in other cell types, include the formylpeptide receptor (FPR) [43], the angiotensin AT-1 and AT-2 receptors [44,45], the urokinase-type plasminogen activator receptor (uPAR) [46,47], the thrombin receptor PAR-1 [48,49], the bombesin/gastrin releasing peptide receptors [50,51], the braykinin receptors [52,53] and the prostaglandin receptors [54,55].

ADAMs EXPRESSION IN CANCER CELLS

The ADAM family members are widely expressed in various types of brain cancer cells (see Table **1**), including human glioma [56]. The capacity of ADAMs to affect EGFR ligand shedding have been studied in several experimental models using different approaches and techniques. These functional studies have clearly shown that only catalitically active ADAMs, e.g. ADAM8, 9, 10, 12, 15, 17 and 19, are directly involved in EGFR ligands shedding. The specificity of distinct ADAMs in their sheddase activity towards EGFR ligands has been tested in mouse embryonic cells lacking specific ADAM isoforms. The conclusion is that ADAM10 represents the main sheddase for EGF and BC whilst ADAM 17 mainly sheds ER, TGFα, AR and HB-EGF [57]. While ligand processing appears necessary for signaling *via* an autocrine or paracrine mechanism, membrane-bound EGFR ligands can also act *via* a juxtacrine mechanism in some model systems [58]. A recent report suggests that even juxtacrine activation of the EGFR by TGF-α requires ADAM activity, perhaps because ligands that remain tethered to the plasma membrane do not have the conformational freedom to assume a functional orientation [59]. ADAM8 has been found in lung carcinoma cells [60] but its substrate specificitiy is still not well characterized. In primary brain tumors and ADAM8 levels and activities are associated with brain tumor invasiveness [61]. ADAM9 is expressed in various cancer specimens and cell lines [57], where it appears to shed mainly HB-EGF. However, ADAM10 is potentially the main EGF and betacellulin (BTC) sheddase in mouse embryonic fibroblasts. ADAM11 has been found to be expressed in low- and high grade gliomas [62], but at the present time the functional effects of its activation on cell proliferation and its sheddase specificity are still unknown. ADAM12 is ubiquitously expressed in several cancer types where it appears to shed mainly HB-EGF [63]. One report found that ADAM12 is expressed at higher levels in glioblastoma compared to other brain tumors and that its mRNA levels are correlated to proliferactive activity [64]. ADAM17 is upregulated in U87 human glioma cells under hypoxia and its activity is associated with tumor invasion [65]. In the same study, ADAM17 activates several EGFR-dependent pathways, strongly suggesting that ADAM17 modulates glioma invasiveness *via* shedding of ligands acting on the EGFR under hypoxic conditions. Finally, ADAM19 is expressed in normal brain and in different gliomas [61] and although its precise functional role in brain cancer is poorly understood, its substrate specificity seems to be restricted to members of the NR family [61].

EGFR LIGANDS SHED BY ADAMs

As mentioned before, a dysregulation of the EGFR-dependent signaling transduction pathways is a common hallmark of almost every cancer type. The ErbB family of

Table 1. Features of Catalitically Active Adams Expressed in Human Brain Cancer

ADAM	MW (KDa)	Activating GPCR Agonist	EGFR Ligand Shed	Brain Cancer Types	Inhibitor
ADAM 8	88.7	-	-	GBM, AA, OA, OD, EM, PNT	batimastat
ADAM 9	72.3 (s) 90.5	-	HB-EGF,	-	batimastat, marimastat, CGS27023A
ADAM 10	84.1	Bombesin, LPA	HB-EGF, EGF, BTC,	-	GI254023X, INCB3619, compound (1)
ADAM 12	80.4 (s) 99.5(m)	PE, angiotensin II, ET-1	HB-EGF	GBM, AA, OA, OD, EM	KB-R7785, marimastat
ADAM 15	87.7	-	HB-EGF, AR, TGF-α	GBM, AA, OA, OD, EM	-
ADAM 17	93	LPA	HB-EGF, AR, EPR, NRG	GBM, AA, OA, OD, EM, U87 cell line	INCB3619, batimastat, marimastat, CGS27023, DPC333, compound (6)(13), GW280264X, GCS27023A PKF242-484, PKF241-466,
ADAM 19	105	-	NRG,	GBM, AA, OA, OD, EM	batimastat

ABBREVIATIONS: HB-EGF: heparin-binding EGF, EGF: epidermal growth factor, BTC: betacellulin, AR: amphiregulin, TGF-α: transforming growth factor α, EPR: epiregulin, NRG: neuregulin, LPA: lysophosphatidic acid, PE: phenylephrine, ET-1: endothelin-1, GBM: glioblastoma, AA: anaplastic astrocytoma, OA: oligoastrocytoma, OD: oligodendroglioma, EM: ependymoma.

receptors tyrosine kinase comprises four distinct receptors: EGFR (also termed Erb-1/HER1), Erb-2 (neu, HER2), Erb-3 (HER3) and Erb-4 (HER4). ErbB receptors are activated by binding to growth factors of the EGF-like family that include about 16 ligands that can be divided in three groups on the basis of their relative specificity toward the four different ErbB receptor subtypes. The first group includes epidermal growth factor (EGF), transforming growth factor α (TGFα) and amphiregulin (AR) that bind specifically to EGFR. The second group includes heparin-binding EGF-like ligand (HB-EGF), betacellulin (BTC) and epiregulin (EPR) that show dual specificity by binding both EGFR and ErbB-4. The third group includes members of the heregulin (HRG)/neuregulin (NRG) family that bind exclusively ErbB-3 and ErbB-4 [66]. The EGF-like ligands, synthesized as proprecursors and then delivered to the cell membranes, undergo a proteolytic cleavage in the extracellular domain by activated ADAMs. Studies in a wide variety of experimental models have demonstrated that the cleaved fragments interact with EGFRs *via* either autocrine and paracrine mechanisms. In the juxtacrine mechanism, the membrane-associated EGFR ligands act as bridging molecules between adiacent cells and therefore EGFR ligands shedding appears to be critical because the net balance between membrane-associated and soluble EGFR ligands leads to distinct cellular responses. The EGFR ligands bind directly to EGFR, Her-3 or Her-4, resulting in the formation of hetero- or homodimers, activation of the tyrosine kinase domain(s), and receptor autophosphorylation. After ligand binding, the activated ErbB receptors can trigger multiple downstream signaling cascades, including MEK-MAPK, PI3K-Akt, and STATs, which areoncogenic when dysregulated [8]. The ligand AR is expressed in some cancer cells [67] and its mRNA has been detected in one glioma cell line [68]. In adult mouse neural stem cells, AR is as potent as EGF in mitogenic activity, but few other effects of AR have been reported [69]. There is no report on the expression of betacellulin in brain cancer but interestingly in vascular smooth cells ET-1 induces the betacellulin ectodomain shedding [70] *via* ADAM10 and ADAM17 suggesting that this mechanism might also be present in brain cancer cells. The ligand epiregulin (ER) and neuroregulins (NRG) are expressed in several types of cancer cells [71] and at the present time only NRG1 and NRG2 are thought to promote schwannoma tumorigenesis *via* a combination of autocrine, paracrine and juxtacrine mechanisms [72]. The functional role of HB-EGF has been investigated in glioma specimen and in cell cultures. The membrane-associated proHB-EGF promotes cell-cell interactions and decreases migration, whilst the soluble HB-EGF enhances cell proliferation *via* activation of cyclin D1 and MMP induction [73]. The ADAM-dependent proteolytic cleavage of proHB-EGF yields not only the amino-terminal soluble HB-EGF but also the carboxyl-terminal cell-associated fragment HB-EGFc. This latter molecule translocates to the nucleus to interact with the transcriptional repressor promyelocytic leukemia zinc finger (PLZF) resulting in the CRM1-dependent nuclear export that eventually leads to increased cyclin A and Hox genes expression [73]. In glioma cell lines, exogenous HB-EGF increases cell proliferation indicating an autocrine loop or juxtacrine mechanism [68]. The same proliferative action of HB-EGF by an autocrine loop was confirmed in U251 glioma cell lines overepressing exogenous mutant EGFRvIII [74]. The key role of

HB-EGF in tumorigenicity, invasion, metastasis and resistance to chemotherapy in several cancer types makes it a promising target in cancer therapy as alternate or complementary strategy to block the EGFR-dependent malignant effects [75]. Because of its importance in tumorigenicity, invasion, metastasis and resistance to chemotherapy in different cancer types, HB-EGF has become a promising therapeutic target in strategies directed at inhibiting EGFR-dependent malignancies [75].

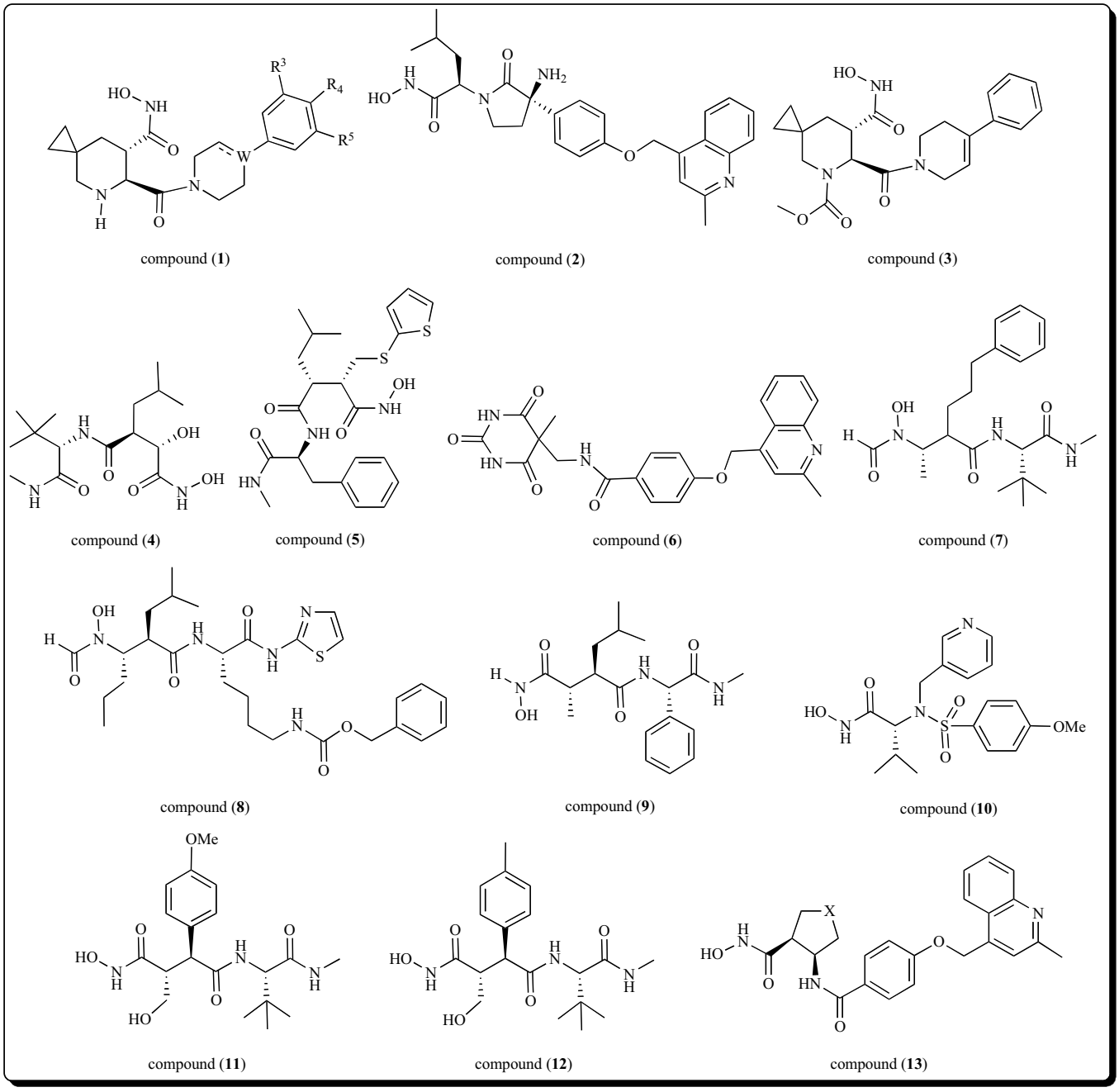
ADAMs INHIBITORS

Therapeutic agents that inhibit membrane GPCR or EGFR function alone have shown modest success in patients with cancer. Readers interested in EGFR inhibitors should refer to excellent published reviews discussing the EGFR inhibitors in oncology [76] and more specifically in brain cancer therapy [77]. Recently, ADAMs metalloproteases have emerged as upstream activators of EGFR ligands and therefore ADAMs inhibitors that block the cleavage of multiple EGFR ligands might inhibit the activation of multiple downstream EGFR-dependent pathways. The search for specific ADAMs inhibitors is still in its infancy and therefore studies aimed at blocking ADAMs activity have been carried out using broad spectrum compounds already known to block MMP (matrix metalloprotease) activity mainly in different cell types. It should be noted that at the present time there is only one report describing the effect of an ADAM inhibitor in brain cancer cells [61]. In the modern cancer drug development process, preclinical studies are focused on the identification of an accurate working models that mimics the disease to be targeted which will be used to screen new compounds in the preclinical trials. For brain tumors, a multidirectionl approach that includes a combination of *in vitro* techniques and *in vivo* use of animal models [78] has become an accepted strategy. The most commonly used *in vitro* models of brain tumors are cell-free systems and cell cultures. Cell-free systems are fundamental in the first steps to define the binding affinity or substrate specificity of new compounds toward purified or recombinant target cellular components such as enzymes and receptors. Cultured brain tumor cell lines mimic the basic behaviour of the original parental cells and therefore they are used to investigate the modification elicited by compounds in signaling transduction pathways and in cellular effects. Notably, these brain tumor cell lines can be stably or transiently modified by molecular biology techniques, such as eukaryotic expression vectors, retroviral vectors and small interfering (si)RNA gene silencing, to overexpress or downregulate a specific protein whose activity or functionality is supposed to be affected by screened compounds. However, this model fails to reproduce the complex and redundant nature of *in situ* human brain tumors and therefore suffers of two major drawbacks. Because the *in vitro* conditions do not reproduce the *in vivo* extracellular environment, artifacts such as altered gene expression, new mutations and differential selection resulting in clonal expansion have been reported in several *in vitro* experiments. More importantly, because tumor-stromal cell interactions cannot be replicated *in vitro*, many processes linked to tumor/niche crosstalk, such as angiogenesis, invasiveness and metastasis, cannot be studied. For this reason, *in vivo* animal models represent the gold standard to investigate tumor-host interactions, toxicity and antitumor effectiveness in brain cancer. These mouse animal models can be divided two broad categories: a) xenograft tumor model and b) genetically engineered mice with spontaneous tumor formation (GEM). The first model, obtained by injection or implantation of primary tumor cells or cell lines into immunodepleted mice, facilitates tumor visualization upon treatment but on the other hand lacks genetic stepwise changes occurring at the beginning or during tumor development. Transgenic or GEM animals provide useful model systems to examine the pathophysiology of brain tumors in immunocompetent models *in vivo*. The tumors are genetically and histologically similar to human gliomas; the causality and genetic or signalling pathway alterations are reminiscent of human gliomas and finally new therapies can be tested to evaluate their potential efficacy and safety. GEM models are obtained by germline modification strategies or somatic cell transfer and can give rise to a wide variety of animals displaying modified expression of a specific target such as gain of function/conditional gain of functions (knock in), loss of function (knock out), chromosome engineering and siRNA. Whilst knock out animals are excellent to delineate the role of a specific proteins in development, knock in animals are the ideal tool to investigate the functional effects of inhibitors on an overexpressed target in pathological conditions. The majority of studies on specificity and effects of various ADAMs inhibitors reported here, are largely based on *in vitro* cell-free or cellular models. The main reasons of the lack of additional *in vivo* studies on the efficacy of ADAMs inhibitors are the limited number of *in vitro* preliminary results and the low availability of valid GEM animal models to test ADAMs inhibitors. This consideration strongly suggests that suitable *in vivo* glioma animal models are absolutely mandatory for the development and screening of not only ADAMs inhibitors but also for a general pharmacological therapy directed against glioma. Finally, it should be kept in mind that the demonstration of efficacy of ADAM inhibitors in preclinical trial is only the first step in the their translation from bench to bedside. The most challenging aspects for the optimization of ADAM inhibitors lies in finding candidates with acceptable pharmacological, pharmacokinetical and selectivity profiles maximizing efficacy and bioavailability and minimizing heavy side effects. The majority of ADAMs inhibitors mentioned here and tested in animal models are small molecules with acceptable acqueous solubility for intravenous administration, but their bioavailability is probably the most challenging issue to be addressed. A possible clinical limitation of these compounds might be their poor oral bioavailability and short half-life that require frequent and continuous parenteral administration with costly treatments and great discomfort experienced by patients. Recent advances in pharmaceutical chemistry procedures aimed at modifying the biopharmacological properties of selected molecules, such as the conjugation with specific polymers and formation of micelles, may be used to enhance the oral disposition and stability of potential ADAMs inhibitors. However, it is evident that only bioavailability data obtained from clinical trial will lead to improvements in the pharmacokinetic and pharmacodynamic properties of lead compounds.

Efforts aimed at obtaining selective and potent ADAM-10 inhibitors led to the synthesis of a novel molecule belong-

ing to the class of (6S,7S)-7-[8hydroxyamino)carbonyl]-6-carboxyamide-5-azaspirol[2.5]octane-5-carboxylates, compound (1), that display different efficacy and binding affinity on the basis of substituents on P1' and P2' positions [79]. Another study found that the agent DPC333, also known as BMS-561392, ((2R)-2-((3R)-3-amino-3{4-[2-methyl-4-quinolinyl) methoxy] phenyl}-2-oxopyrrolidinyl)-N-hydroxy-4-methylpentan-amide)), compound (2), is a potent ADAM17 inhibitor in rodents, dogs, chimpanzees and human [80]. This inhibitor might provide the basis for a novel approach in the treatment of brain tumors with excessive TNF-α production. The selective, potent orally active small molecule ADAM inhibitor INCB3619 (methyl (6S,7S)-7-[(hydroxyamino) carbonyl]-6-[(4-phenyl-3,6-dihydropyridin-1(2H)-yl)carbonyl]-5-azaspiro[2.5]-octane-5-carboxylate), compound (3), prevents the processing and activation of multiple EGFR ligands and inhibits the activation of EGFR ligand-dependent signaling pathways in non-small cell lung cancer (NSCLC) cell lines [81].

The effect of INCB3619 was also investigated in a tumor-bearing mouse model induced by injecting subcutaneously human breast adenocarcinoma cell line (MCF-7) suspended in culture medium [82]. Administration of INCB3619 (120 mg/Kg) by implantation of subcutaneously Alzet pump reduced EGFR ligand shedding, inhibited the phosphorylation of Akt kinase, blocked tumor cell proliferation and survival. In addition, the antitumoral effect of INCB3619 synergized with the activity of the EGFR inhibitor gefitinib without causing toxic relevant side effects. Although the authors cannot directly demonstrate that inhibition of other ADAMin addition to ADAM10 and 17 can contribute to the INCB3619-dependent observed effect, this preclincial finding suggests that that INCB3619 its potential use in combination therapy with other anticancer agents. The broad-spectrum metalloproteinase inhibitor marimastat (N-[2,2-dimethyl-1-(methyl-carbamoyl)propyl]-2-[hydroxy-(hydroxycarbamoyl)methyl]-4-methyl-penta-namide), compound (4), partially inhibited the gelatinase activity of ADAM12, an enzyme involved in HB-EGF shedding [64], extracted and purified from urine of breast cancer patients [83]. Another broad-spectrum metalloproteinase inhibitor batimastat (2S,-3R)-5-methyl-3-[[(αS)-α-(methylcarbamoyl)phenethyl]-carbamoyl]-2-[(2-thienylthio)methyl]hexano-hydroxamic acid), compound (5), abolishes the IGF-1-induced ERK phosphorylation mediated by HB-EGF shedding in several cultured cell lines [84] and the ET-1-induced calcium response linked to proHB-EGF shedding in vascular smooth muscle cells [85]. The search for ADAM17 inhibitors by screening a library of pyrimidine-2,4,6-trione derivatives resulted in the synthesis of compound 51 (N-((5-(4-(methyl-ssulfonyl)piperazin-1-yl)-2,4,6-trioxohexahydropyrimidin-5yl)methyl)-4-(quinolin-4-yl-methoxy)benzamide), compound (6). This molecule displays an IC50 of 2nM in a porcine ADAM17 assay and therefore might represent the first examples of high affinity non-hydroxamate-based ADAM17 inhibitor [86]. Two other hydroxamate molecules, GI254023X ((2R,3S)-3-(Formyl-hydroxyamino)-2-(3-phenyl-1-propyl)butanoic acid [(1S)-2,2-dimethyl-1-methylcarba-moyl-1-propyl] amide), compound (7) and GW280264X ((2R,3S)-3-(Formyl-hydroxy-amino)-2-(2-methyl-1-propyl) hexanoic acid [(1S)-5-benzy-loxycarbamoylamino-1-(1,3-thiazol-2-ylcarbamoyl)-1-pentyl]-amide), compound (8), have shown a relative inhibiting activity toward different ADAM isoforms. The former agent blocked the constitutive shedding of IL6R, CX3CL1 and CXCL16 cytokines mediated by ADAM10 in cell-based cleavage experiments, whilst the latter agent blocked the PMA-induced shedding of cytokines mediated by ADAM17 [87]. The use of selective ADAMs inhibitors might be instrumental in deciphering the substrate specificity of different ADAM isoforms either *in vitro* and *in vivo* model systems. The molecule KB-R7785 ([4-(N-hydroxyamino)-2R-isobutyl-3S-methylsuccinyl]-L-phenylglycine-N-methylamide), compound (9), was first identified by screening experiments aimed at identifying new compounds with high affinity binding toward ADAM12. The effect of compound (9) (KB-R7785) was elegantly demonstrated in a study that employed *in vitro* and *in vivo* models [63]. In cultured rat neonatal cardiomyocytes, the PE-, AngII- and ET-1-induced EGFR transactivation mediated by HB-EGF shedding was blocked by a neutralizing specific for HB-EGF or by treatment with KB-R7785. In the *in vivo* C57BL/6J male mouse animal model, the pharmacological induction of cardiac hypertrophy was induced by treating mice with the GPCRs agonists PE and Ang II by osmotic pump and then the animals were administered daily intraperitoneally for 1 to 4 weeks with KB-R7785 (100 mg/kg). The finding that the dominant negative form of ADAM12 abolished this EGFR transactivation indicates that in the heart ADAM12 is mainly involved in this pathway and in addition identifies ADAM12 as potential target of KB-R7785. However, because in this study others ADAMs' activity was not downregulated, the authors suggest that ADAM12 is the main target of KB-R7785 without ruling the possibility that KB-R7785 may interfere with others ADAMs. The sulfonamide derivative GCS27023A (N-hydroxy-2(R)-[[(4-methoxy-phenyl)sulfonyl](3-picolyl)-amino]-3-methyl-butanamide), compound (10), displays a broad spectrum of inhibition toward different sheddases because it blocks ADAM17 in a cell-free assay, cellular TNF-α secretion and ADAM9 activity [88]. The effect of PKF242-484 ([(2S,3R)-N4-((S)-2,2-Dimethyl-1-methylcarbamoyl-propyl)-N1-hydroxy-2-hydroxy-methyl-3-(4-methoxyphenyl)-succinamide), compound (11), and PKF241-466 ([(2S,3R)-N4-((S)-2,2-Dimethyl- 1-methylcarbamoyl-propyl)-N1-hydroxy-2-hydroxy-methyl-3-phenyl-succin-amide]), compound (12), two dual inhibitors of ADAM17 and MMPs, was investigated in a mouse model of ischemia and reperfusion injury [89]. These two compounds elicited a dose-dependent (maximal dose 10 mg/Kg, administered intravenously) reduction of TNF-α concentrations in serum and a concomitant decrease of the chemokines CXCL1 (keranocyte-derived chemokine, KC) and CCL2 (monocyte chemoattrant protein-1, MCP-1) in intestine and lungs of reperfused mice. In addition, the two ADAM17 inhibitors reduced reperfusion-assciated local and remote tissue injury assessed by different paramaters such as changes in vascular permeability, neutrophil recruitment and hemorrahge. Given the wide substrate specificity of ADAM17 and its documented expression in several brain tumors, studies aimed at assessing the effects of these two compounds in animal model of brain tumor deserve further consideration. Finally, other researchers [90] have synthesized ADAM17 selective inhibitors based on a (1R,2S)-cyclopentyl, (3S,4S)-pyrrolidinyl, and (3R,4S)-tetrahydrofuranyl beta-benzamido



compound (**1**) compound (**2**) compound (**3**)

compound (**4**) compound (**5**) compound (**6**) compound (**7**)

compound (**8**) compound (**9**) compound (**10**)

compound (**11**) compound (**12**) compound (**13**)

hydroxamic acids structure backbone, compound (13). Some of these novel inhibitors have been found to be very active in an ADAM17 enzyme assay and very potent in the suppression of LPS-stimulated TNF-α release in human whole blood.

BRAIN CANCER STEM CELLS

One of the major challenges in cancer chemotherapy chemotherapy is the cellular heterogeneity of cancer cells. This problem could be potentially overcome by a new paradigm that has recently emerged in basic and clinical oncology. A small population of cells, termed cancer stem cells (CSC) has been identified and isolated from a variety of blood, breast, central nervous system, pancreas, skin, head, neck, colon, and prostate cancers. These CSC possess unique properties such as extensive self-renewal capability *in vitro* and *in vivo* and can recapitulate tumor pathophysiology in an immune-compromised animal model. Several research teams succeeded in characterizing CSC from various brain tumors, and these cells, also known as brain cancer stem cells (BCSC), are described in excellent reviews [91,92]. Although at the present time no report is available on the EGFR transactivation in BCSC, several studies suggest that this mechanism is likely to occur in these cells. When cultured and propagated *in vitro*, BCSC are usually grown in serum-free medium containing EGF and FGF to stimulate the EGFR and other TKR. In addition, some reports have demonstrated that BCSC express functional GPCRs such as glutamate metabotropic receptors [36], cannabinoid receptors [93] and specific chemokine receptors [94] whose activation

induces important cellular effects such as proliferation and ability to form neurospheres. Other authors have demonstrated that indeed a wide variety of additional GPCRs are expressed either on the non tumor neural stem cells (NSC) and BCSC membranes [95]. Therefore the *in vitro* BCSC model might represent a reliable drug screening assay in cancer chemotherapy [96]. Although it is probably too early to draw any firm conclusion about the reliability of this experimental model for new drug screening, it is evident that the possibility to purify and grow CSC offers great promise. The concept of CSC may have profound implications for our understanding of tumor biology and for the design of novel treatments targeted toward not only CSC but also toward cells in the tumor microenvironmental niche.

CONCLUSION

Despite the spectacular successes of several antibodies and small molecule kinase inhibitors targeting the EGFR receptor, the majority of patients in clinical trials treated with the new and most recent EGFR inhibitors do not display reliable long-tern benefits, with the best outcome being limited to an increase in progression-free survival. Two main factors likely account for this failure: drug resistance and more importantly heterogeneity and redundancy of aberrant signal transduction pathways in cancer cells. This latter problem could be overcome by alternative strategies including the combined administration of tyrosine kinases inhibitors or the use of multikinase inhibitors [97]. The screening of broad-spectrum or selective ADAMs inhibitors, with their potential to reduce the availability of EGFR and other tyrosine kinases receptor ligands, fits well with this option and might open new avenues in cancer drugs research. However, before taking this step, several issues need to be resolved. The precise functional role of different ADAM isoforms in brain cancers, using different *in vitro* and especially new animal *in vivo* models, should be further characterized in greater detail at the molecular level. Moreover, tumor samples should be analyzed by genome wide techniques such as microarray and proteomics in order to assess which ADAM isoform is specifically overexpressed in a specific subset of tumors. Unfortunately, the wide variety of substrates shed by ADAMs, their expression in tumor and non tumor cells and finally their multimodal mechanism of activation represent a double-edged sword. The lessons learned from the failure of MMP inhibitors [98], should warn against premature enthusiasm or confidence. On the basis of these premises, it is tempting to speculate that efforts aimed at developing ADAMs inhibitors have the potential to become an important complement to existing conventional anti-EGFR pharmacological therapies.

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