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REVIEW

Targeting the adrenomedullin-2 receptor for the discovery and development of novel anti-cancer agents

Ameera B. A. Jailani [a,#], Kamilla J. A. Bigos [a,#], Paris Avgoustou [a,#], Joseph L. Egan [b], Robert A. Hathway [b], Timothy M. Skerry [a,#] and Gareth O. Richards [a,#]

[a]Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK; [b]Department of Chemistry, University of Sheffield, Sheffield, UK

ABSTRACT

Introduction: Adrenomedullin (AM) is a peptide responsible for many physiological processes including vascular health and hormone regulation. Dysregulation of AM signaling can stimulate cancers by promoting proliferation, angiogenesis and metastasis. Two AM receptors contribute to tumor progression in different ways. Adrenomedullin-1 receptor (AM_1R) regulates blood pressure and blocking AM signaling via AM_1R would be clinically unacceptable. Therefore, antagonizing adrenomedullin-2 receptor (AM_2R) presents as an avenue for anti-cancer drug development.

Areas covered: We review the literature to highlight AM's role in cancer as well as delineating the specific roles AM_1R and AM_2R mediate in the development of a pro-tumoral microenvironment. We highlight the importance of exploring the residue differences between the receptors that led to the development of first-in-class selective AM_2R small molecule antagonists. We also summarize the current approaches targeting AM and its receptors, their anti-tumor effects and their limitations.

Expert opinion: As tool compounds, AM_2R antagonists will allow the dissection of the functions of CGRPR (calcitonin gene-related peptide receptor), AM_1R and AM_2R, and has considerable potential as a first-in-class oncology therapy. Furthermore, the lack of detectable side effects and good drug-like pharmacokinetic properties of these AM_2R antagonists support the promise of this class of compounds as potential anti-cancer therapeutics.

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Adrenomedullin; angiogenesis; antagonists; cancer; calcitonin receptor-like receptor; receptor activity-modifying protein; therapeutic target; G protein-coupled receptor; hypoxia; metastasis

1. Introduction

Adrenomedullin (AM) is a multifunctional peptide belonging to the calcitonin gene-related peptide (CGRP) superfamily of peptide hormones. Originally identified as a hypotensive agent, AM is also a bronchodilator, hormone regulator and neurotransmitter [1]. AM has a closely related peptide called intermedin (IMD or adrenomedullin-2) with similar physiological effects and tissue expression [2].

AM is mainly secreted by endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) as important sources of circulating AM [3]. Serum levels of AM in humans normally range between 10 and 30 pg/mL [4–6]. An elevated serum AM level is associated with many diseases including heart failure (~50 pg/mL) [5] and sepsis (~33–500 pg/mL) [6,7], warranting investigations into AM being a potential biomarker [8,9].

AM mediates its activity through receptor complexes comprising a G protein-coupled receptor (GPCR) – calcitonin receptor-like receptor (CLR) – together with one of three accessory proteins known as receptor activity-modifying protein (RAMPs; Figure 1). RAMPs aid in GPCR trafficking, ligand selectivity and downstream signaling [10]. There are two AM receptors – adrenomedullin-1 receptor (AM_1R) and adrenomedullin-2 receptor (AM_2R) – which are formed by CLR/RAMP2 and CLR/RAMP3 heteromers respectively. CLR/RAMP1 forms the CGRP receptor (CGRPR) which AM binds to less potently compared to AM_1R and AM_2R. AM_1R regulates physiological roles including fetal development and cardiovascular health [11,12]. However, dysregulation of AM signaling via both AM_1R and AM_2R can mediate different types of pro-tumoral signaling within the tumor microenvironment.

2. AM in cancer

AM and its receptor components are expressed in many cancers and is usually associated with poorer prognosis (Table 1). AM and its receptors are secreted and expressed not only by cancer cells but also stromal cells including cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and mast cells [13–17]. Bidirectional AM signaling between cancer and stromal cells can result in various pro-tumoral effects within the microenvironment (Figure 2).

Overexpression and exogenous treatment of AM has been shown to increase proliferation of some cancer cells *in vitro* and *in vivo* [33–35] by promoting signaling pathways including ERK/MAPK and JNK/AP-1 [33,36]. Furthermore, AM also enables cancer cells to evade apoptosis by up-regulating NF-

CONTACT Gareth O. Richards g.richards@sheffield.ac.uk Department of Oncology and Metabolism, University of Sheffield, Sheffield, UK

[#]These authors contributed equally to the work

Article highlights

- Tumor hypoxia up-regulates many proangiogenic targets, one of which is adrenomedullin (AM).
- AM is secreted by cancer and stromal cells to mediate and sustain pro-tumoral processes including angiogenesis, metastasis and immune evasion.
- Overexpression of AM and its receptors is correlated with poorer prognosis in a variety of cancers.
- AM signals through two receptors which contribute to tumor progression and development in distinct ways.
- AM receptors are each comprised of the same G protein-coupled receptor coupled with a different accessory protein - receptor activity-modifying proteins (RAMPs).
- Current methods targeting AM and its receptors (CLR or RAMPs individually) show promising anti-cancer activity, but do not target the receptor complexes as a whole.
- To reduce off-target effects, new therapies should target the adrenomedullin-2 receptor complex, not the individual components.
- Key residue differences between AM receptors allow selective small molecule antagonists against adrenomedullin-2 receptor complex to be developed as potential anti-cancer agents.

κB and Bcl-2 and downregulating caspases-3 and −8 [35,37–39]. AM has also been shown to activate molecules associated with invasion and migration in several cancers, including up-regulating integrin α5β1 and phosphorylation of FAK and paxillin [37,40,41]. These signals can modify various functions of cancer cells including their shape and motility, encouraging them to migrate and invade neighboring tissues to eventually metastasize to distant sites.

The role of AM in angiogenesis has been extensively researched due to its expression in ECs and VSMCs. AM is one of several proangiogenic targets up-regulated in hypoxia to maintain tumor growth and progression [42]. Transcription factor HIF-1α synthesizes AM through hypoxic response elements [43,44]. Multiple studies have shown that AM treatment or overexpression in cancer cells resulted in increased capillary density and tumor weight *in vivo* [33,36,45–49]. AM is not only secreted by cancer cells, but also CAFs, TAMs and mast cells to induce and maintain angiogenesis within the tumor microenvironment [13–17].

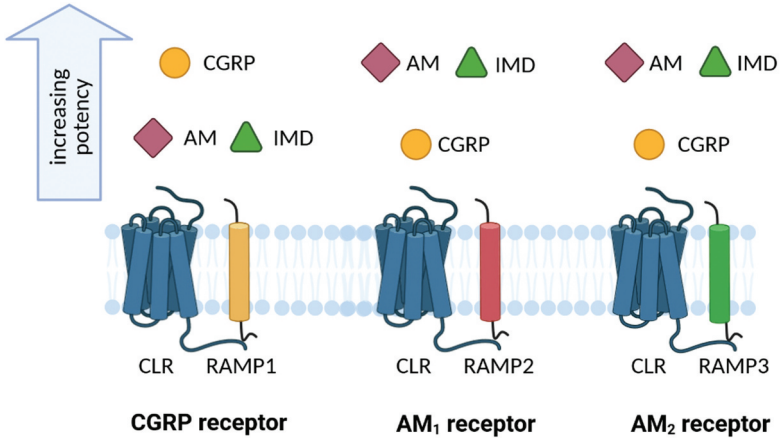
AM can protect tumors from immune surveillance by dampening pro-inflammatory cytokine signals [50]. Tumor-derived AM increases infiltration of pro-tumoral immune cells (myeloid-derived suppressor cells, mast cells, and M2 TAMs) [51]. AM also mediates macrophage polarization into M2 TAMs [52] which are not just immunosuppressive but also promote tumor growth and metastasis. Plasma and tissue AM expression are also correlated with neuroendocrine differentiation in tumors, which is associated with poorer prognosis [53].

Although there is a body of evidence for the role of AM in mediating pro-tumoral processes, it is less clear which AM receptors mediate each function.

3. AM receptors in cancer

Receptor components for both AM receptors are expressed in many cancers (Table 1), making it difficult to distinguish which AM receptor is responsible for pro-tumorigenic effects. Whilst CLR, RAMP2 and RAMP3 are all expressed in tumors, RAMP3 expression is particularly associated with stromal cells such as infiltrating endothelial and immune cells [37,51,54] as well as CAFs [14].

Knockout mouse studies have provided an insight into the specific roles of AM_1R and AM_2R in cancer. As complete RAMP2 knockout mice result in embryo lethality due to defects including lymphatic vascular development [55], tissue-specific RAMP2 knockout [56] or heterozygous mouse models [57] have been used to interrogate the role of RAMP2 in cancer. Tanaka *et al.* have shown that in mice with tamoxifen-inducible endothelial-specific RAMP2 knockout (DI-E-RAMP2-/-), sarcoma and melanoma *in vivo* tumor growth and angiogenesis were decreased compared with wild-type mice [56]. However, lung metastasis was increased in DI-E-RAMP2-/- mice because pulmonary ECs allowed infiltrating immune cells to express chemotactic factors,



AM: adrenomedullin; CGRP: calcitonin gene-related peptide; CLR: calcitonin receptor-like receptor; IMD: intermedin; RAMP: receptor activity-modifying protein

Figure 1. Calcitonin receptor-like receptor (CLR) and receptor activity-modifying protein (RAMP) receptor complexes. Interaction of CLR with one of three RAMPs leads to the formation of three CLR/RAMP receptor complexes with distinct pharmacology, trafficking and signaling consequences. Each CLR/RAMP receptor favors the calcitonin family of peptides at different potencies. Image created in Biorender.com.

Table 1. Expression of AM and its receptor components (CLR, RAMP2 and RAMP3) in different cancers.

Cancer type	AM	CLR	RAMP2	RAMP3	Clinical implication	References
Acute myeloid leukemia	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	↑ AM ↑ CLR → ↓ OS and DFS	[18,60]
Breast	$+^{(m)(p)}$	-	-	$+^{(m)}$	↑ AM → ↑ lymph node mets	[19,20,101]
Colorectal	$+^{(m)(p)}$	$+^{(p)}$	$+^{(p)}$	$+^{(p)}$	↑ AM, CLR, RAMP2, RAMP3 → ↑ lymph node and distant mets ↑ AM → ↓ DFS	[21,34]
Glioblastoma	$+^{(m)(p)}$	$+^{(m)}$	$+^{(m)}$	$+^{(m)}$	No prognostic data	[98]
Renal	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	↑ AM ↑ CLR → tumor vs. healthy tissues ↑ AM → ↑ risk of relapse following nephrectomy ↑ CLR → ↑ tumor grade	[22,23,37]
Liver	$+^{(m)(p)}$	$+^{(m)}$	$+^{(m)}$	$+^{(m)}$	↑ AM → ↑ intrahepatic mets	[24,25]
Lung	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	No correlation between AM expression, tumor stage and OS	[26,92]
Melanoma	$+^{(p)}$	$+^{(p)}$	$+^{(p)}$	$+^{(p)}$	No prognostic data	[13,27]
Osteosarcoma	$+^{(m)(p)}$	$+^{(m)}$	$+^{(m)}$	$+^{(m)}$	↑ AM → ↑ mets	[28,29]
Ovarian	$+^{(m)(p)}$	$+^{(m)}$	$+^{(m)}$	$+^{(m)}$	↑ AM → ↑ tumor stage	[30,40]
Pancreas	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)}$	↑ AM → ↓ DFS	[31,90]
Prostate	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	$+^{(m)(p)}$	↑ AM → ↑ Gleason score	[32,33]

All results presented were obtained from human cancer cell lines or tissues. +: positive expression; -: undetected; m: mRNA expression determined by endpoint or real-time PCR; p: protein expression determined by western blotting or immunohistochemistry; mets: metastasis; DFS: disease-free survival; OS: overall survival

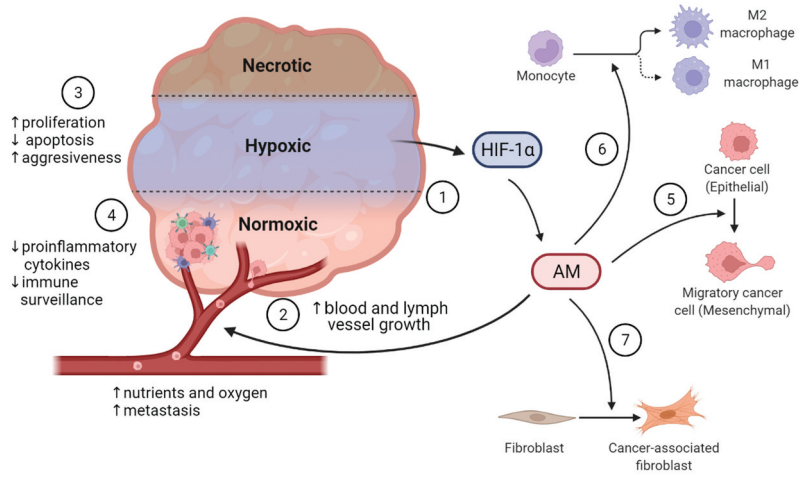


Figure 2. Bidirectional AM signaling between cancer and stromal cells can result in various pro-tumoral effects within the microenvironment. (1) Hypoxic tumors up-regulate transcription factor HIF-1α to increase angiogenesis for more oxygen supply to the area. Aside from VEGF, another of HIF-1α's targets is AM, which not only regulates blood vessel growth into the tumor, but also lymph vasculature (2). (3) By increasing nutrient and oxygen availability to the once hypoxic tumor, AM mediates cancer cells within the microenvironment to increase proliferation, evade apoptosis, become more aggressive (neuroendocrine phenotype). (4) AM also enables pro-tumoral immune cells to infiltrate the tumor and dampen the pro-inflammatory signals, protecting tumors from immune surveillance. (5) AM can induce cancer cells to undergo epithelial-to-mesenchymal transformation, becoming more migratory and metastatic. Aside from affecting cancer cells themselves, AM also promotes pro-tumoral phenotype in other stromal cells. AM promotes infiltrating monocytes to polarize into M2 tumor-associated macrophages (TAMs, 6) as well as activate normal fibroblasts into cancer-associated fibroblasts (CAFs, 7). TAMs and CAFs themselves secrete AM and other factors into the microenvironment to sustain cancer growth and development. Image created in Biorender.com.

forming a pre-metastatic niche. This shows how important the AM/RAMP2 system is for vascular integrity and to inhibit tumor metastasis.

The group also investigated the roles of both RAMP2 and RAMP3 in pancreatic tumors, using DI-E-RAMP2-/- mice and RAMP3-/- mice [58]. Overall, the study found that selective activation of AM/RAMP2 and inhibition of AM/RAMP3 suppresses both tumor growth and metastasis. As in the previous study, metastasis was increased in DI-E-RAMP2-/- mice. However, liver metastasis was decreased in RAMP3-/- mice, correlating with reduced infiltrating podoplanin-expressing CAFs. This subtype of CAFs has been demonstrated to be associated with poorer prognosis in cancer patients [59]. This is one of the first studies to show the significant role of AM_2 R in cancer.

Recently, elevated expression of AM and CLR was shown to correlate with adverse outcomes in acute myeloid leukemia (AML) [60]. AM/CLR axis is required for cell growth and survival of AML blasts. Most interestingly, the study revealed a critical role of AM/CLR in maintaining resistant stem cell populations that persist after chemotherapy. As a whole, these studies suggest that AM and its receptors not only mediate tumor growth and metastasis, but also drug resistance (Figure 3).

The field is still limited in available tools to clearly delineate the roles of each AM receptor. Many of these studies target individual receptor components (CLR, RAMP2 or RAMP3) by using peptide antagonists, small molecules, antibodies or genetic manipulation. However, those methods do not allow for targeting of CLR/RAMP receptors as heteromeric complexes and it is imperative to develop compounds that do

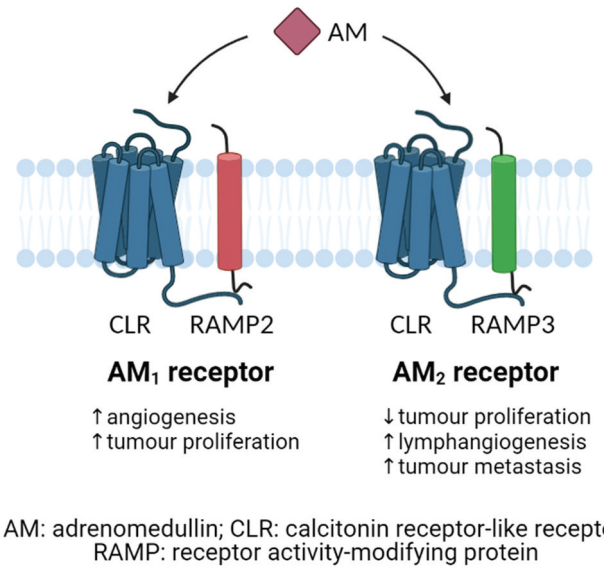


Figure 3. AM and its receptors contribute to tumor growth and development by different functions. AM_1 receptor is associated with increased tumor proliferation and regulation of endothelial cells for angiogenesis. AM_2 receptor, whilst it is linked with inhibition of tumor proliferation, regulates cancer-associated fibroblasts to promote lymphangiogenesis and induce metastasis. Image created in Biorender.com.

because heteromeric receptors comprising either the CLR or the RAMPs associated with other partners make interpretation of such studies complex. Selective antagonists of specific receptor/RAMP complexes provide more specific ability to explore such questions.

4. Differences between CLR/RAMP receptor complexes

Published crystal structures of CGRPR and AM_1R bound to truncated peptide antagonists $CGRP_{27–37}$ and $AM_{35–52}$ respectively, give insights into the association of ligands with CLR/RAMP receptors [61,62]. Different research groups have shown independently the presence of a hydrophobic patch and pocket separated by the Try72 shelf (W72 bulge), which largely form the binding pocket of the heteromers [61,62]. Furthermore, it was demonstrated that a β-turn on both CGRP and AM peptides enables them to occupy their binding pockets and modulate their interactions with CLR and RAMP residues [61].

CGRP interacts with CLR residues G71 and W72 as well as RAMP1 residue W84 via its F37 phenyl ring [62]. As described by ter Haar and coworkers, CGRP binds almost entirely to the CLR domain and makes only one critical contact with RAMP1 (residue W84) [62]. Also, hydrogen bonds are formed between CGRP residue V32 and CLR's W72 bulge and a main-chain to side-chain connection is made between CGRP T30 and CLR loop 3 D94 residues [61].

Similarly, AM residues Y52 and K46 interact with residues R97, E101 and E105 on RAMP2. An extension of a single helical turn allows AM K64 to contact the W72 bulge. It is worth mentioning that the equivalent residue on RAMP1 (W74) was unable to interact with AM. This was explained by the lack of a glutamine residue at position 74 of RAMP1 that discourages AM interaction [63].

Recent studies, including the published cryo-EM structures of CGRPR, AM_1R, and AM_2R, have given a deeper understanding to these receptor complexes and their mechanisms of activation [64–66]. An alanine substitution study on the AM_{15-52} identified several residues important for receptor signaling. More specifically, substitution of AM residues F18, T20, L26, and I30 showed a significant decrease in activity in all the three CLR/RAMP receptors [66]. Interestingly, alanine substitution on AM_{15-52} residue G19 showed an increase in activity in all pathways tested and in all receptors. More importantly, this substitution revealed a more CGRP-like profile to AM response whereby AM could activate IP1 production which was otherwise only restricted to CGRP [66].

5. Development of molecules targeting CLR/RAMP receptors

A notable example of pharmacological targeting of GPCR complexes and more importantly CLR/RAMP complexes (Table 2) is the modulation of CGRPR for migraine treatment [67]. Evidence of CGRP's key role in migraine [68,69] have been accumulating since its discovery [70], leading to the initiation of many drug development programs against the receptor complex. This led to the development of several small-molecule antagonists against CGRPR, collectively known as 'gepants' with promising preclinical and clinical indications [67].

Although many members of the family, including telcagepant [71] and olcegepant [72], have been shown to reduce migraine symptoms without the cardiovascular side-effects (such as myocardial infarction, cardiovascular death and ischemic heart disease) of existing treatments (i.e. triptans and DHE), they were later discontinued due to modest elevations of liver enzymes indicating potential liver toxicity [73]. Other gepants such as ubrogepant [74] and rimegepant [75] have not been associated with liver toxicity, leading to their FDA approval for acute migraine treatment in 2019 [76] and 2020 [77], respectively. Even though gepants have significant differences in their chemical structures, they act in a similar manner by blocking the CLR/RAMP1 interface preventing CGRP binding [62], indicating a distinct chemophore that can be utilized for targeting this receptor complex. This was further supported by structure–activity relationship studies that showed the presence of three important interactive regions on CGRPR antagonists that bind to CLR/RAMP heteromers that facilitate their binding and selectivity: 1) CLR-binding region; 2) interface region that binds close to the Trp72 bulge; 3) CLR/RAMP1 binding region that interacts with the CLR/RAMP1 hydrophobic patch [78].

Similar mode of action was shown by erenumab [79], the first FDA-approved monoclonal antibody which targets the ligand binding site of the CGRPR [80]. Three other recently approved human CGRP antibodies (fremanezumab [81], galcanezumab [82] and eptinezumab [83]), target the CGRP ligand itself, preventing it from accessing the CGRPR binding pocket.

The importance of the distinct chemophore described earlier, was further supported by the recent development of the first-in-class small molecule antagonist against AM_2R [84]. As the three receptors (CGRPR, AM_1R and AM_2R) comprise the

same receptor but a different RAMP, it is important to investigate the differences between RAMPs when developing potent and selective antagonists. Avgoustou *et al*. highlighted four key residues in the vicinity of the small molecule ligand-binding pocket that are different between each RAMP (Figure 4) [84]. By inspecting the different RAMP sequences and how these interact with known small molecule CGRPR antagonists, the authors were able to utilize specific interactions with RAMP3 that provided a drastic increase in potency for AM_2R (nanomolar range) with significant selectivity (1000-fold) over the AM_1R. These molecules, although still in the preclinical stage, have shown very promising anti-tumor effects against both pancreatic [84] and breast [85] cancers. These small molecules are the first selective AM_2R antagonists.

6. Targeting AM and its receptors in cancer

Multiple approaches have been used to target the AM system in cancer: 1) neutralizing the AM ligand itself; 2) antagonizing AM receptor complexes (i.e. CLR/RAMP2 or CLR/RAMP3); 3) antagonizing individual components of the AM receptors (i.e. CLR, RAMP2 or RAMP3). To date, several peptides, antibodies and small molecules have been developed to antagonize either AM or its receptors. Their therapeutic effects have not only been shown against various cancers but also in other disease models including sepsis and heart failure [86,87]. The potential of targeting AM in oncology has been reviewed elsewhere [88]. This review will focus on how these molecules and antibodies (Table 2) may specifically target the human AM_2R in cancer.

Table 2. Drugs targeting CLR family of receptors.

Name	Target	Discovered by	Developed by	Developmental status	Disease indication	Ref
Small molecule antagonists						
Telcagepant	CGRPR	Merck & Co	N/A	Failed due to hepatic toxicity [73]	Prophylaxis of episodic migraine	[71]
Olcegepant	CGRPR	Boehringer Ingelheim	N/A	Failed due to hepatic toxicity [73]	Migraine treatment	[72]
Ubrogepant	CGRPR	Merck & Co	Allergan USA, Inc.	FDA approved, Ubrelvy [76]	Acute and preventative treatment of migraine	[74]
Rimegepant	CGRPR	Bristol-Myers Squibb	Biohaven Pharmaceuticals	FDA approved, NURTEC [77]	Acute and preventative treatment of migraine	[75]
NSC 16311	AM ligand	Siclari *et al.*	N/A	N/A	Anti-tumor and osteolytic effects in *in vitro* breast cancer model	[101]
NSC 37133	AM ligand	Fang *et al.*	N/A	N/A	Neutralizes lymphatic endothelial cell tube formation *in vitro*	[102]
2,2-dimethyl-N-[[2-(methylaminomethyl)phenyl] methyl]-N-[2-oxo-2-[[(2 R)-2′-oxospiro[1,3-dihydroindene-2,3′-1 H-pyrrolo[2,3-b]pyridine]-5-yl] amino]ethyl]propanamide	AM_2R	University of Sheffield	N/A	Preclinical	Anti-tumor effects in breast and pancreatic cancer preclinical models	[84,85]
Antibodies						
Erenumab	CGRPR	Amgen Inc.	Amgen Inc. & Novartis	FDA approved, Aimovig [80]	Preventative treatment of migraine	[79]
Fremanezumab	CGRP ligand	Rinat Neuroscience/ Pfizer	Rinat Neuroscience/ Pfizer & Teva	FDA approved, Ajovy [81]	Preventative treatment of migraine	[81]
Galcanezumab	CGRP ligand	Eli Lilly and Company	Eli Lilly and Company	FDA approved, Emgality [82]	Preventative treatment of migraine and cluster headaches treatment	[82]
Eptinezumab	CGRP ligand	Lundbeck Seattle BioPharmaceuticals	Lundbeck Seattle BioPharmaceuticals	FDA approved, Vyepti [83]	Preventative treatment of migraine	[83]
Cocktail of antibodies against CLR, RAMP2 and RAMP3	Individual AM receptor components	Kaafarani *et al.* Ouafik *et al.*	N/A	N/A	Anti-tumor effects in preclinical cancer models of glioblastoma, lung, colon and mesothelioma	[54,98]
Peptide antagonists						
AM_{22-52}	AM_1R	Eguchi *et al.*	N/A	N/A	Anti-tumor effects in preclinical cancer models including melanoma, pancreatic, breast, ovarian, renal and mesothelioma	[89]
$CGRP_{8-37}$	CGRPR	Chiba *et al.*	N/A	N/A	Anti-cancer effect in *in vitro* prostate cancer model	[95]
C7	AM_1R & AM_2R	Robinson *et al.*	N/A	N/A	N/A	[93]
Acylated truncated AM/intermedin analogs	CGRPR & AM_1R	Chang *et al.*	N/A	N/A	N/A	[97]

AM: adrenomedullin; AM_1R: adrenomedullin-1 receptor (CLR/RAMP2); AM_2R: adrenomedullin-2 receptor (CLR/RAMP3); CGRP: calcitonin gene-related peptide; CGRPR: calcitonin gene-related peptide receptor (CLR/RAMP1); N/A not applicableLegends:

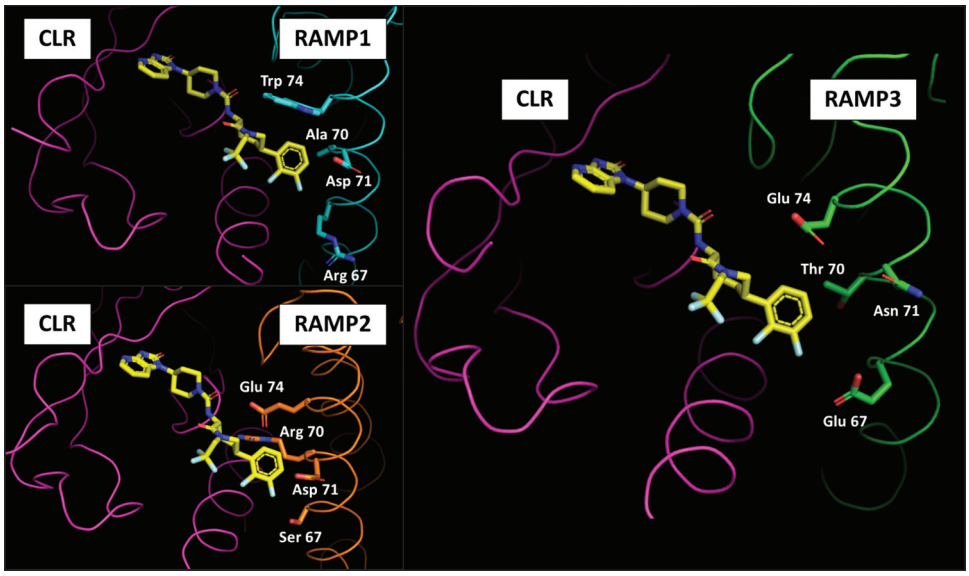


Figure 4. Models of CLR/RAMP complexes with telcagepant, based on crystal structures of CGRP and AM_1 receptors (PDB codes: 3N7R and 3AQF, respectively). For the AM_2 receptor, a hybrid model combining information from the published crystal structures of the CLR domains of the CGRP and AM_1 receptors with a predicted structure for the RAMP3 domain was used. CLR is rendered in magenta, RAMP1 in cyan, RAMP2 in orange and RAMP3 in green. RAMP residues (at telcagepant binding site) that differ across three RAMPs are labeled.

6.1. Peptide antagonists

AM_{22-52}, also known as AM antagonist (AMA), is a truncated version of AM developed to compete with AM for its receptors [89]. AM_{22-52} has been used to antagonize AM in many cancer models including melanoma, pancreatic, breast, ovarian, renal and mesothelioma [13,47,51,52,90–92]. AM_{22-52} has been shown to exert its anti-tumor effects by decreasing tumor cell proliferation, angiogenesis [47,51,91,92], lymphangiogenesis [92], pro-tumoral macrophage differentiation [13,52] and recruitment of myelomonocytic cells [51]. AM_{22-52} is slightly selective for AM_1R (pA_2 7.34 ± 0.14) over AM_2R (pA_2 6.73 ± 0.14) [93,94] making it hard to distinguish its effect in antagonizing either of these AM receptors in disease models.

$CGRP_{8-37}$ is a truncated version of CGRP, and was initially developed as a selective antagonist for CGRPR [95]. Interestingly, $CGRP_{8-37}$ is slightly more selective for AM_2R than AM_{22-52}, although it appears to antagonize both AM receptors equally [94]. While $CGRP_{8-37}$ has not been as extensively used as an AM antagonist compared to AM_{22-52}, $CGRP_{8-37}$ has been shown to exert anti-tumor effects in prostate cancer *in vitro* models [96]. $CGRP_{8-37}$ was slightly more effective in inhibiting proliferation and enhancing apoptosis in DU-145 prostate cancer cell line *in vitro*, as compared to AM_{22-52}, leading the authors to conclude that AM's tumor-promoting effect in prostate cancer is primarily mediated by AM_2R. However, due to $CGRP_{8-37}$'s limited selectivity between the AM receptors, it is again difficult to discriminate between its effects on either receptor. $CGRP_{8-37}$'s anti-tumor effect has also not been reported in more complex cancer models.

Peptide chimeras were also developed using fragments of AM and its related ligands (intermedin and CGRP) to identify molecules with a higher affinity for AM receptors compared to the aforementioned truncated peptides [93]. While the peptide 'C7' had a similar affinity to AM_{22-52} in inhibiting both AM_1R and AM_2R (~100 nM), 'C7' showed a higher selectivity for AM_2R (pA_2 7.81 ± 0.20) compared to AM_1R (pA_2 7.25 ± 0.13), albeit modest. Unfortunately, the therapeutic potential of these peptide chimeras in cancer or other applications has not been reported.

Most recently, chimeric and bifunctional antagonists have also been developed against CLR/RAMP receptors [97]. These include acylated truncated AM/intermedin analogs with potent antagonistic activity against CGRPR and AM_1R and chimeric analogs (comprising a somatostatin analog and AM antagonist) which exhibit dual antagonistic activities on both somatostatin and CLR/RAMP receptors (specifically CGRPR and AM_1R). However, the potency and affinity of these compounds against AM_2R, as well as their therapeutic potential, were not reported and are therefore currently unknown.

6.2. Antibodies

Ouafik's group developed the first antibody-based method to target AM receptors involving a combination of antibodies (αAMR) against individual AM receptor components (i.e. CLR, RAMP2 and RAMP3) [54,98]. This method is limited in its selectivity in targeting specific AM receptors as it may also antagonize other receptors that bind to these individual components including CGRPR, amylin receptors and other RAMP-interacting receptors. However, despite there being several FDA-approved CGRPR antibodies for migraine treatment, there are currently no antibodies developed to bind to AM receptor complexes. Regardless, αAMR treatment has been used to demonstrate anti-tumor activity in preclinical cancer models of glioblastoma, lung, colon and mesothelioma by suppressing not only tumor burden but also disrupting tumor vasculature [34,54,92,98]. However, due to the aforementioned limitation of αAMR possibly antagonizing other related receptors and lack of pharmacology characterization

data, it is unclear whether the observed anti-tumor effects can be attributed directly to antagonism of only the AM receptors.

6.3. Small molecules

Chemical library screening identified some small molecule AM antagonists including NSC 16311 and NSC 37133 [99]. NSC 16311 has been shown *in vivo* to efficiently inhibit tobacco-induced lung cancer growth [100] and may also be effective against breast cancer metastasis by blocking AM's pro-tumoral and osteolytic effects [101]. NSC 16311 is also able to neutralize AM-induced tube formation of lymphatic ECs *in vitro*, suggesting it may have possible therapeutic applications in edema and metastatic diseases [102]. It is worth noting that these compounds bind to AM and not the receptors. To date, there are no clinical trials using small molecule AM antagonists.

In 2020, the development of first-in-class potent and selective small molecule antagonists of AM_2Rs was reported [84]. Interestingly, these molecules have anti-tumor effects *in vitro* and *in vivo* in both breast and pancreatic cancer models [84,85]. Immunohistochemical analysis of pancreatic cancer xenografts from AM_2R antagonist-treated animals revealed significant decreases in markers of proliferation, blood vasculature and CAFs [84], suggesting that AM_2R antagonists exert anti-tumor effects on both cancer and stromal cells within the tumor microenvironment. The good selectivity of these antagonists over the AM_1R (1000-fold) is also of particular importance as AM_1R is essential for physiological processes such as cardiovascular health [57]. It is worth noting that the current class of AM_2R antagonists are equipotent in inhibiting CGRPR. However, the anti-proliferative actions were shown to be mediated through the inhibition of the AM_2R, by use of selective CGRPR antagonist control which showed no anti-proliferative effect. The lack of detectable side effects and good drug-like pharmacokinetic properties of these AM_2R antagonists support the promise of this class of compounds as potential anti-cancer therapeutics.

7. Expert opinion

This review has addressed specifically the developments and strength of evidence for AM_2R's roles in physiology and as potential therapeutic targets. The article has two broad implications. First, it is now recognized that AM_2R has important roles in both physiology and pathology that can be distinguished from that of AM_1R. This comes from studies across a range of disciplines, so that structural information combined with pharmacological studies [65,66], knockout mouse research [55–57] and the development of selective antagonists for CLR/RAMP receptors [84,85] provide a compelling (but as yet incomplete) explanation of the way AM mediates its functions.

The second implication is broader. The development of selective antagonists for GPCR/RAMP receptor complexes began with the CGRPR antagonists, as a search for agents to prevent or treat migraines. However, since those programmes which started over 20 years ago, progress had slowed until we demonstrated the ability to design selective AM_2R antagonists capable of discriminating between AM receptors (albeit with small discrimination over CGRPR) [84,85]. Development of small molecule AM_1R antagonists appears to be more challenging, although progress is being made [103]. AM_2R antagonists are likely to provide the tools to aid in the understanding of AM biology in both physiology and disease, as well as a potential therapeutic intervention in diseases involving AM_2R, including cancer.

There is growing knowledge of other GPCRs that associate with RAMPs and other accessory proteins. RAMP interactions have historically been associated Class B GPCRs, but emerging data suggest that GPCR/RAMP interactions are far more common, with new partner GPCRs being discovered [104]. A greater understanding of the consequences of RAMP-interactions on signaling and cellular events [105] appears certain to increase importance of GPCR/RAMP interactions as potential drug targets.

Development of highly selective compounds against specific CLR/RAMP heteromers is tractable and may spark interest in development of compounds against the wider GPCR/RAMP interactions previously considered either undruggable or too challenging to be considered worthwhile targets. Like most science, each step forward that we hope will answer questions often raises more uncertainties. However, we believe it is likely that RAMP-interacting GPCR biology will see an upturn in interest and activity. Exciting times are ahead.

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Declaration of Interest

A Jailani, P Avgoustou, T Skerry (Founder director) and G Richards (Founder director) have financial interests (shareholdings) in Modulus Oncology, a University of Sheffield spinout company involved in the development of AM_2R antagonists for the treatment of cancer. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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ORCID

Ameera B. A. Jailani http://orcid.org/0000-0001-9837-6062
Kamilla J. A. Bigos http://orcid.org/0000-0002-7824-6479
Paris Avgoustou http://orcid.org/0000-0002-1642-1083
Joseph L. Egan http://orcid.org/0000-0003-2812-0074
Robert A. Hathway http://orcid.org/0000-0002-1948-6472
Timothy M. Skerry http://orcid.org/0000-0003-1319-5575
Gareth O. Richards http://orcid.org/0000-0001-7984-6882

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