Review

Bifunctional ligands of the bradykinin B_2 and B_1 receptors: an exercise in peptide hormone plasticity

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Abstract

Kinins are the small and fragile hydrophilic peptides related to bradykinin (BK) and derived from circulating kininogens via the action of kallikreins. Kinins bind to the preformed and widely distributed B_2 receptor (B_2R) and to the inducible B_1 receptor (B_1R). B_2Rs and B_1Rs are related G protein coupled receptors that possess natural agonist ligands of nanomolar affinity (BK and Lys BK for B_2Rs, Lys-des-Arg^9-BK for B_1R). Decades of structure-activity exploration have resulted in the production of peptide analogs that are antagonists, one of which is clinically used (the B_2R antagonist icatibant), and also non-peptide ligands for both receptor subtypes. The modification of kinin receptor ligands has made them resistant to extracellular or endosomal peptidases and/or produced bifunctional ligands, defined as agonist or antagonist peptide ligands conjugated with a chemical fluorophore (emitting in the whole spectrum, from the infrared to the ultraviolet), a drug-like moiety, an epitope, an isotope chelator/carrier, a cleavable sequence (thus forming a pro-drug) and even a fused protein. Dual molecular targets for specific modified peptides may be a source of side effects or of medically exploitable benefits. Biotechnological protein ligands for either receptor subtype have been produced: they are enhanced green fluorescent protein or the engineered peroxidase APEX2 fused to an agonist kinin sequence at their C-terminal terminus. Antibodies endowed with pharmacological actions (agonist, antagonist) at B_2R have been reported, though not monoclonal antibodies. These findings define classes of alternative ligands of the kinin receptor of potential therapeutic and diagnostic value.

Keywords: bradykinin, B_2 receptors, B_1 receptors, synthetic peptides, fluorescent ligands, biotechnological agents

List of major abbreviations

ACE, angiotensin converting enzyme

ε-ACA, ε-aminocaprolyl

APEX2, engineered soya bean peroxidase

Arg-CP, arginine carboxypeptidase

B_1R, B_1 receptor

B_2R, B_2 receptor

B_2R-GFP, rabbit B_2 receptor fused to green fluorescent protein

BK, bradykinin

CF, 5(6)-carboxyfluorescein

Cy7,cyanine dye 7

EGFP, enhanced green fluorescent protein

FTC, fluorescein-5-thiocarbamoyl

GPCR, G protein coupled receptor

GRK, G protein coupled receptor kinase

HSA, human serum albumin

KLK-1, tissue kallikrein

MK, maximakinin

NG, asparaginyl-glycyl

PTH, parathyroid hormone

TM, transmembrane (domain)

Other abbreviations are defined in the figure legends

1. Formation, signaling, metabolism and medical importance of kinins

The bradykinin (BK) sequence, H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH, is imbedded in domain 4 of 2 circulating proteins, the low and high molecular weight kininogens, produced by the liver from a single gene subjected to mRNA alternative splicing [1-3]. Kinins, the BK-related peptides, are generated from these kininogens, mainly by two major types of serine proteases, plasma and tissue kallikreins, that generate the native kinins BK and Lys-BK (also known as kallidin). Several constituents of the kallikrein-kinin system are found in the blood plasma, but also at the surface of cells or in their secretions (e.g., tissue kallikrein = KLK-1) [1, 3].

Two related receptors belong to the kallikrein-kinin system, the B_1 and B_2 receptors (B_1R, B_2R). Those are "type A" G protein coupled receptors (GPCRs). While the B_2R is expressed at a rather constant level in many tissues and cell types and submitted to a classical endocytosis/reexpression cycle following stimulation, the B_1R is highly regulated by tissue injury, notably via cytokines or the innate immune system [1, 2]. Both receptor types are mainly coupled to the G_q protein (also G_i), with ensuing stimulation of a phospholipase C and generation of second messengers such as calcium and diacylglycerol [2] (schematic representation, Fig. 1B). The B_2R is widely distributed, the vascular endothelial cells being a privileged physiological site of BK action, but the receptor is also expressed in sensory neurons, smooth muscle cells, epithelial (intestinal, respiratory) and some types of leukocytes. B_1R tend to be present in the same cell types when expressed.

Kinins are often considered inflammatory mediators, with such effects as edema, pain, diarrheic states [4] and flu-like airway irritation [5], but are as well compensatory vasodilator autacoids

that release nitric oxide (NO) and prostanoids from vascular endothelial cells, with salutary effects in the circulation of the heart, kidney, brain and the promotion of angiogenesis [1, 6]. Cultured human umbilical vein endothelial cells (HUVECs) are a conventional model where both NO production and actin reorganization, predictive of microvascular leakage, can be recorded in response to BK (Supplementary Figures 1 and 2).

Kinins are fragile peptides, being degraded by several metallopeptidases present both in blood plasma and at the cell surface. Angiotensin converting enzyme (ACE), present at the surface of endothelial cells and also circulating under a cleaved form, is by far the major kinin-destroying enzyme in the extracellular compartment both in human blood plasma and *in vivo* in rats [7, 8]. Second in importance for BK inactivation in both milieus is aminopeptidase P that cleaves Arg^1 and inactivates the peptides. Arginine carboxypeptidases (Arg-CPs), such as plasma carboxypeptidase N and membrane carboxypeptidase M, are minor pathways, but ones that have a particular importance: they generate des-Arg^9-BK and Lys-des-Arg^9-BK which have high affinity for the B_1R, the latter peptide being optimal at the human form of this receptor subtype. Aminopeptidase N is an ectopeptidase that removes the N-terminal Lys residue in Lys-BK and Lys-des-Arg^9-BK; the effect is pharmacologically neutral for the former B_2R agonist, but the latter peptide is partly inactivated by this reaction *vs.* the B_1R [9].

While a large body of literature suggests that interventions on the kallikrein-kinin systems are therapeutic in animals subjected to various experimental pathologies [2], clinical applications are presently minimal [10, 11]. The B_2R antagonist icatibant is approved to abort attacks of hereditary angioedema, an autosomal dominant condition where the serpin G1 is deficient in abundance or function, leading to explosive activation of the contact system and plasma kallikein

[12]. For the same rare indication, biotechnological inhibitors of plasma kallikreins are either approved (ecallantide) or under final clinical development (the neutralizing monoclonal antibody lanadelumab) [13]. Orally bioavailable, small molecule inhibitors of this serine protease are also considered for hereditary angioedema and various edematous retinopathies (e.g., KV123833 from Kalvista). However, these are not kinin receptor ligands. Clinical trials concerning B_2R and B_1R antagonists have been so far largely negative, despite positive effects in animal models. An early B_2R peptide antagonist, deltibant, failed to improve the condition of patients with sepsis [14]. Anatibant, a non-peptide B_2R antagonist, had no clear effect on post-traumatic brain edema [15]. At least 2 sophisticated non-peptide B_1R antagonists have failed to reduce inflammatory pain in humans [14].

ACE inhibitors, such as enalapril, lisinopril and many others, are used in cardiovascular conditions where the synthesis of the vasopressor hormone angiotensin II needs to be repressed; however this metallopeptidase is also the main BK-inactivating enzyme. A fraction of the antihypertensive effect of ACE inhibitors is attributed to the potentiation of the vasodilator action of endogenous kinins in humans (evidence based on blunting of their effect by icatibant co-administration) [16-18]. Recombinant active KLK-1 (DM-199) is being clinically developed as a parenterally administered drug to take advantage of the salutary effect of kinins on the circulation and metabolism [19]. The BK analog labradimil was designed to deliberately exploit the B_2R-mediated extravasation and open the blood-brain barrier, thus possibly facilitating the delivery of chemotherapeutic agents. However, a formal clinical trial failed to show a therapeutically favorable interaction between labradimil and carboplatin in patients with primary brain tumors [20].

The development of kinin receptor ligands mentioned in this section will be further reviewed below. The reader is referred to previous reviews for a full discussion of these issues and historical background [1, 2, 21]. We rather propose an excursion into the medicinal chemistry of kinins with emphasis on the plasticity of these small peptide hormones (overview, Fig. 1A). Many synthetic peptides and even biotechnological ligands of the B_2R and B_1R have now been produced and are case studies for the concept of bifunctional ligands for receptors of small peptide hormones. The first author has been an actor in the field for the past 40 years and there will be a certain autobiographical bias in the selection of the reviewed material.

2. Overview of the molecular pharmacology of kinins receptor ligands

Fig. 1A introduces the plasticity of the ligands by summarizing the critical regions of BK and Lys-des-Arg^9-BK and the steps to produce synthetic analogs. The current docking model of the agonist ligands to either receptor type involve the interaction of the N-terminal positive charges (NH_2^+-Arg- for BK, NH_2^+-Lys-Arg- for Lys-des-Arg^9-BK) with negatively charged residues in the extracellular loop 3 of the receptors [2]. The C-terminal region plunges into the rosette formed by the transmembrane (TM) domains and it is its interaction with TM6 and TM7 in particular that is believed to activate receptors. This is supported by the fact that specific residue substitutions in this region cause the transition from the agonist to the antagonist behavior (see below). Residues in the internal sequence (Pro^2 to Pro^7) have generally little tolerance for substitution.

Dynorphin A and specific fragments are cationic peptides unrelated to the BK sequence; however, they can bind both types of kinin receptors, based on radioligand competition assays applied to recombinant receptors [22]. This interaction of micromolar affinity is nevertheless proposed to be a basis on neuropathic pain via the stimulation of spinal B_2R by dynorphin. GPCR heterodimerization has also the potential to generate alternative pharmacology, as well as alterations of receptor stability, amplification or extension of signaling and alteration of cycling [23]. However, these issues are technically difficult to address, often involving heterologous systems involving highly expressed recombinant GPCRs and inconsistent application of approaches that prove close molecular interaction between partners, such as energy transfer techniques. The BK B_2R has been proposed to form heteromers with the B_1R, the dopamine D_2, κ-opioid, the angiotensin AT_1 and the catecholamine β_2 receptors [24-28]. In turn, the B_1R-apelin receptor heterodimer has been proposed [29]. Further, both B_1R and B_2R may associate with the non-receptor peptidase ACE in the presence of an ACE inhibitor to promote increased signaling [30], although this is controversial because protection from kinin inactivation by ACE is a major confounding factor. The B_2R has been also proposed to be one of the components of a multi-molecular complex that transactivates insulin receptors [31]. These reports have generally not documented changes in the structure-activity relationship of the kinin receptor ligands, which is the focus of the present review: this front of research remains open for future investigation.

3. B_2R agonists, antagonists and stabilization *vs.* peptidases

BK is the smallest sequence endowed with a nanomolar affinity for the B_2R and this nonapeptide is a convenient basic structure to discuss the development of peptide/protein ligands for this kinin

receptor subtype (Fig. 2). N-terminally extended peptides derived from the human kininogen sequence, Lys-BK and Met-Lys-BK, are approximately equipotent [2]. One of the well investigated kinins derived from animal toxins is the 19-mer peptide maximakinin (MK), also called bombinakinin M, (H-Asp-Leu-Pro-Lys-Ile-Asn-Arg-Lys-Gly-Pro-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH). MK possesses the C-terminal sequence of BK, is an agonist of the rabbit and rat B_2Rs (though not of the human form) and elicits prolonged signaling in cultured HEK 293 cells [32, 33]. MK has a lower affinity for ACE than BK and is also less susceptible than the latter nonapeptide to the endosomal inactivation that follows the endocytosis of the agonist-receptor complex [32], suggesting that this little studied inactivation pathway is mediated by cathepsin(s) that operate as aminopeptidase(s).

Nonetheless, the main kininase is ACE and efforts to protect BK for this and other carboxypeptidases included a modified peptide bond between residues 8 and 9 as in [Phe$^8\Psi$(CH_2-NH)Arg^9]BK and its derivative labradimil (Fig. 2, marker *1*). Exploiting the vascular leakage that is mediated by the B_2R at the level of endothelial cells, labradimil infusion has been developed as an adjuvant to open the blood-brain barrier, thus facilitating the delivery of chemotherapeutic agents to patients with brain tumors. However, as mentioned above, a clinical trial has disappointed [20].

The first generation of BK B_2R antagonists was based on the introduction of a peptide backbone structural constraint as in [D-Phe^7]-BK and further prototypes (Fig. 2, marker *2*) [34]. This structure-activity exploration has been subsequently intensely pursued and one of the very successful peptide B_2R antagonist is icatibant (Hoe 140) (Fig. 2), currently referenced in more than 1,200 articles in PubMed. Icatibant, possessing the backbone-orienting D-Tic^7 substitution,

is the first antagonist with a steric limitation of rotation conferred by the neighbouring bulky Oic^8 residue; this produced a remarkable gain of affinity, presumably by freezing an optimal docking conformation to the B_2R [35]. The N-terminal of the peptide is protected by its extention with D-Arg^0 from aminopeptidase P cleavage. Icatibant is the only kinin receptor ligand in clinical use, injected subcutaneously to abort attacks of hereditary angioedema [12]. Icatibant at micromolar concentration levels has some documented off-target actions: it is an inhibitor of the ubiquitous ectoenzyme aminopeptidase N [36], an effect of speculative significance, and an agonist of a peculiar GPCR found in connective tissue mast cells termed MRGPRX2. The latter mediates degranulation induced by various highly cationic peptides and the universal redness reaction at the site of subcutaneous icatibant injection was attributed to this [37].

B-9430 is an alternate B_2R antagonist of high potency that applies all the innovations found in icatibant (D-Arg^0, the conformationally locked combination D-Igl^7-Oic^8, Fig. 1). An exact isomer of this peptide, B-9972, lacks the D-conformation residue (Oic^7-Igl^8; Fig. 2, marker *3*) and is a highly selective B_2R full agonist with remarkable resistance to inactivation, including endosomal inactivation [38, 39]. Despite a certain loss of potency *vs.* BK at the B_2R level, B-9972 is as apt as BK to reorganize actin in cultured HUVECs and more potent to release NO from these cells (Supplementary Figs. 2 and 3). Both the antagonist version B-9430 and its agonist isomer B-9972 were the basis for developing several bifunctional ligands (Fig. 2, markers *4*, *5*). B-9870 (CU201) is a dimer of B-9430 held together by a linker and has been developed as an anti-cancer agent (Fig. 2, marker *6*). It has activity against various lung cancer lines, both small cells and non-small cells, head and neck squamous cell carcinoma and MDA-MB-231 breast cancer cells, *in vitro* or in xenotransplantation [41-43]. Although it has measurable nanomolar affinity for and antagonist action at the B_2R, its anti-mitotic effect is not likely to be related to B_2R; its polycationic

structure may make it a cell-penetrating peptide with undefined intracellular targets at micromolar concentrations [43].

Efforts to develop non-peptide ligands of the BK B_2R are exemplified in the Supplementary Fig. 3, its legend and supplementary references. The antagonists did not reach clinical use. A series of peculiar compounds derived from these antagonists consists of non-peptide partial B_2R agonists with a large intrinsic activity; compound 47a is one of them and parallels the pharmacological profile of the protected peptide agonist B-9972 [39].

Biased agonism, or functional selectivity, refers to compounds that preferably recruit one of the receptor signaling pathways over the others; this is currently a topic of high interest in the development of GPCR ligands [44]. For the kinin receptor ligands, there are yet no compelling examples of this, especially with proper quantification of selectivity [44]. In a sense, the inactivation-resistant agonists B-9972 and 47a induce biased signaling as a function of time in HEK 293 cells, because their calcium mobilization is as short as that induced by BK, but the recruitment of arrestins, ERK and c-Fos signaling induced by the former agents is much prolonged *vs.* the effects of BK [16]. Activated arrestins in persistent endosomal structures have been proposed as the nucleus of a signaling scaffold for the angiotensin AT_1 receptor [55], a possible (but undocumented) mechanism for biased signaling of peptidase-resistant agonists of the related B_2R.

4. B_1R agonists, antagonists and stabilization *vs.* peptidases

Starting from the optimal agonist of the human B_1R, Lys-des-Arg^9-BK, an agonist protected from both aminopeptidases and carboxypeptidases, Sar-[D-Phe^8]des-Arg^9-BK (Fig. 3, marker *1*), has been exploited in various experimental settings where inactivation is a concern, such as *in vivo* in LPS-treated rabbits (prolonged hypotensive effects *vs.* Lys-des-Arg^9-BK) [46, 47] and in long term cultures of human vascular smooth muscle cells (inhibition of cell migration [48]; "autoregulatory" slow induction of B_1R expression [49]). Obtaining an antagonist from a B_1R agonist was relatively easy: substituting Phe^8 by a residue possessing an aliphatic side chain, such as Leu, was successful [50] (Fig. 3, marker *2*). However, the removal of the Arg^9 residue in modern constrained B_2R antagonists, such as icatibant, B-9430, etc., also provided B_1R antagonists of high selectivity with better metabolic stability (some examples are shown in Fig. 3, marker *3*). The agonist Lys-des-Arg^9-BK and the antagonist B-9958 have both been prolonged at their N-terminus to confer resistance to aminopeptidase N or to produce bifunctional ligands (Fig. 3, markers *4* and *5*). B-10356 is B-9958 N-terminally prolonged with D-Arg: it antagonizes the B_1R at nanomolar levels but inhibits aminopeptidase N as well at micromolar concentrations [51]. Although not tested *in vivo*, B-10356 is a potential anti-angiogenic drug with a dual mode of action, targeting two relevant molecules [51]. Many non-peptide antagonists of the B_1R have been developed since the 1990's; some have reached clinical trials, but not clinical use [52, 53].

5. Conjugation of peptide ligands with chemical fluorophores

Bifunctional ligands of the kinin receptors include agonist or antagonist peptides conjugated with a chemical fluorophore. A whole range of chemical fluorophores, emitting from the infrared to the ultraviolet, have been included in sets of B_2R ligands that were pharmacologically

characterized (Fig. 2, markers *4*, *5* and *7*) [54, 55]. All such peptides were prolonged at their N-terminus, believed to be close to the receptor/extracellular fluid interface in the current docking model of ligands to B_2Rs [2]. Ligands that included 5(6)-carboxyfluorescein (CF) or AlexaFluor-350 were used with an ε-aminocaproyl (εACA) spacer condensed at the N-terminus of peptides, which was positively shown to increase the affinity in CF-εACA-BK, *vs.* CF-BK [56]. Other peptides protected from peptidases, such as the agonist B-9972 or the antagonist B-9430, were both directly conjugated with the infrared fluorophore Cy7 that possesses a "built-in" spacer [55]. All constructions involving a chemical fluorophore suffered from important losses of affinity *vs.* the parent peptide, but less so for antagonists. All were tested using the displacement of [^{3}H]BK binding from recombinant B_2Rs and for their agonist/antagonist behavior in the contractility assay based on the human isolated umbilical vein [54, 55]. These ligands were essentially used as visualization aids in epifluorescence or confocal microscopy, with some applications to cytofluorometry. Colocalization experiments were extensively conducted with fluorophore-labeled receptors, arrestins, Rab proteins and organelle markers by exploiting different combinations of emissions. The findings illustrated that the activated B_2R transports its agonists into endosomes in parallel to its well-known internalization behavior [54-56]. The antagonists label only the resting plasma membrane receptors in intact cells. Further, comparing the ligand structures opens an interesting window on the endosomal breakdown of the B_2R agonists which is required for receptor recycling at the cell surface: the endosomal fluorescence associated with CF-εACA-BK is progressively transferred to the cytosol of cells incubated at 37°C, but not in the presence of bafilomycin A1, an inhibitor of organelle acidification [56], suggesting release of fluorescein after the peptide breakdown. Further, the fluorescence of an alternate agonist of similar affinity, FTC-B-9972, remained in endosomes in incubated cells that express B_2Rs [54], suggesting that the intrinsic resistance to peptidases of B-9972 applied also to the endosomal

inactivation pathway. A possible drawback of fluorescent peptides based on the BK sequence is that they have an affinity for ACE, visualized as a plasma membrane protein, at concentrations that label B_2Rs [54, 56]. However, this should not apply to B-9972 derivatives, with their highly modified C-terminus.

Carboxyfluorescein-conjugated B_1R ligands have been developed based on the extension of the agonist Lys-des-Arg^9-BK or the antagonist B-9958 (Fig. 3, markers *5* and *6*) [54, 57]. In cells that express recombinant B_1Rs, the antagonist version retains a good affinity and labels the plasma membrane. The agonist version, B-10378, does not label endosomes in living cells, consistent with the lack of agonist-induced phosphorylation and internalization of the B_1R, but the labeling is subtly heterogenous at the cell surface, possibly consistent with some redistribution of activated B_1Rs to caveolae.

6. Conjugation of peptide ligands with other small N-terminal extensions (drugs, epitopes, isotope carriers)

N-terminal extension of the B_2R agonists BK or B-9972 with drug-like molecules to confer an additional pharmacological effect following release from endosomes of the regenerated drug has been attempted (Fig. 2, markers *5* and *7*) and has disappointed. Former realizations have been described by Gera et al. [54, 58] and literature cited herein. An unpublished attempt was the fusing of either the agonist B-9972, or the antagonist B-9430 for comparison, with emtansine, which consists of the highly cytotoxic drug maytansine with a labile coupler that releases the drug in endosomes (Suppl. Fig. 4; conjugates termed B-10648 and B-10647, respectively). This

scheme is clinically applied with transtuzumab emtansine, a drug-conjugated anti-HER2 monoclonal antibody [59]. HER2 is a tyrosine kinase receptor overexpressed at the surface of cancer cells in a subset of breast cancer cases and endocytosis is believed to follow antibody engagement. However, several emtansine substitutions are likely to be present in the drug-conjugated antibody, increasing the toxicity. This is not the case with the emtansine conjugates of B_2R ligands (a 1:1 molar ratio). Very typical of the N-terminally extended kinins, the agonist version emtansine-B-9972 exhibits an affinity ~100-fold lower than that of the parent peptide for B_2Rs, whereas the loss of affinity is modest in the antagonist version emtansine-B-9430 (Suppl. Fig. 5). At suitable concentrations, emtansine-B-9972 induced the endocytosis of the stably expressed B_2R-GFP construction in HEK 293 cells, and emtansine-B-9430 rather prevented the effect of BK (microscopy, Suppl. Fig. 6), confirming their pharmacological identities. However, in these cells that express a high density of functional B_2R-GFP or in other cells, such as the non-transfected HEK 293 or MBA-MD-231 breast cancer cells, the emtansine-conjugated peptides were not cytotoxic over 24 hrs (assayed using the cell impermeant DNA stain DRAQ7) and did not modify the cell cycle in a receptor-dependent manner (data not shown). The general problem with drug conjugates of the B_2R ligands may be the low efficiency of the transport mediated by agonist-induced receptor endocytosis, a few tens of femtomols per flask of cells densely expressing recombinant B_2Rs.

Other ligands were generated by the extension of the N-terminus of B_2R ligands with a moiety recognized by an antibody, as in myc-tagged B-9972 [60] and digoxigenin-εACA-BK [61], or by streptavidin, in biotinyl-B-9430 [62]. The digoxigenin conjugate was developed as a tracer to support an enzyme immunoassay of BK based on anti-BK antibodies, thus not as a receptor ligand. However, when assembled with a large macromolecule, such peptides do not label B_2Rs

at the surface of cells [62], or very barely so [60], certainly indicating steric hindrance due to the small distance between this GPCR and the protein. On the other hand, the receptor binding and cycling is easily visualized for larger myc-tagged peptides bound to fluorescent anti-myc antibodies, for instance parathyroid hormone-myc and the chemokine CCL19-myc [63, 64]; these peptide hormones protrude more than BK in the extracellular fluid when bound to their cognate receptors. The steric hindrance limitation will also be a concern with proteins fused with kinin receptor ligands (see below).

The B_1R is selectively expressed in inflamed tissues but, in addition, all tumors have a more or less inflammatory stroma and many transformed cell lines also express B_1Rs. The B_1R antagonist B-10324 includes an N-terminal pentafluorocinnamyl extension that logically makes it resistant to aminopeptidase N, *vs.* the parent peptide B-9958 that possesses a free Lys-Arg N-terminal sequence (Fig. 3) [54, 65]. B-10324 has anti-proliferative effects on some human tumor cell lines, but the mechanism is not clear at this time: the drug-like extension of the B_1R antagonist may or not participate to the cellular effect. An ambitious project is to map B_1R expression in mice that bear a transplanted tumor by virtue of an isotope-labelled B_1R ligand (examples of such peptides are cited in Fig. 3). DOTA and NODA are chelators of positron-emitting metal ions or metal-F complexes that are condensed with a spacer and with the antagonist B-9958 in the work of Zhang et al. [66]. Following injection into tumor-bearing animals, positron emission tomography neatly identified the tumoral mass in a specific manner (i.e., labeling prevented by the co-treatment with an unlabeled ligand). The antagonist versions were estimated superior to the agonist ones. However, the urinary tract (kidneys and urinary bladder) is heavily labeled in a non-specific manner, due to the effective glomerular clearance of the low molecular weight peptides. The

project elegantly shows the potential diagnostic value of detecting B_1R expression *in vivo*, but also the potential therapeutic perspective if a receptor ligand is a vector for a cytotoxic conjugate.

7. Conjugation of peptide ligands with cleavable sequences

A vast body of literature shows the protective effects of kinins, more often mediated by B_2Rs, in ischemic and renal disease [1]. KLK-1 administration, notably under the form of an expression vector, attenuates hypertension and ischemic renal injury [39]. Prolonged infusion of peptidase-resistant B_2R agonists have been attempted in animals submitted to experimental pathologies with beneficial results, but side effects were not reported, if any [68-70]. We explored in recent years the idea that prodrug B_2R agonist sequences may be developed by C-terminally extending BK with a cleavable sequence that regenerates BK following a definite cleavage rule by a specific peptidase (Fig. 2, marker *8*; Fig. 4, top). The concept also included the considerations that C-terminal extension of the BK sequence is not well tolerated for B_2R binding, that the chosen activating peptidases must be associated with the circulation to limit extravascular side effects (such as the stimulation of afferent nerve terminals, epithelia, etc.) and that the regenerated peptide, BK or an homolog, is itself fragile (a soft regenerated drug) and not likely to escape the circulation. The project was inspired by the characterization of a natural sequence derived from high molecular weight kininogen following its hydrolysis by purified neutrophil proteinase 3, Met-Lys-BK-Ser-Ser [71]. Indeed, this peptide was found to possess little affinity for recombinant B_2Rs, but an affinity for ACE comparable to that of BK [72]. The isolated human umbilical vein is a contractile bioassay for the ligands of the B_2R and, as a freshly isolated vascular system, possesses peptidases activities. In this system, Met-Lys-BK-Ser-Ser is a

contractile agonist, but it potency is reduced ~30-fold in the presence of an ACE inhibitor, supporting that this carboxydipeptidase removes the Ser-Ser extension at once, thus regenerating the known high-affinity B_2R agonist Met-Lys-BK [72].

Extending the idea to synthetic peptides, we developed BK-Arg as a potential kinin activated by Arg-CPs and other prodrugs prolonged with a dipeptides as potential ACE substrates, e.g., BK-His-Leu (Fig. 4) [73]. Plummer's inhibitor, a blocker of Arg-CPs, decreased 15-fold the apparent potency in the umbilical vein contractility assay, consistent with *in situ* regeneration of BK, and also nearly abolished the hypotensive effect of BK-Arg in rats, while leaving that of BK unaffected (Fig. 4) [73, 74]. While the ACE inhibitor enalaprilat neatly decreased the potency of BK-His-Leu in the vein [73], the cleavage rule involving ACE in anesthetised rats was much less clear, with possible alternate competing regenerating pathways [74]. A more recent realization is D-Arg^0-BK-Arg-Arg, a peptide protected from aminopeptidase P that is essentially regenerated *in vivo* by 2 cycles of Arg-CP catalysis, although ACE has an affinity for it *in vitro* [75]. Such agents might be intravenously infused to patients in intensive care settings.

8. Biotechnological ligands

The definite test of the ligand plasticity for small peptides such as kinins is the development of fusion proteins that include a kinin sequence at its C-terminus, with a functional protein protruding in the extracellular fluid (Fig. 5). A spacer sequence has been systematically used in the reported designs. For B_2Rs, an amphibian peptide possessing the intact BK sequence at its C-terminus and a 10-residue hydrophilic extension, MK, was part of the combined "spacer-agonist

ligand" module [76, 77] (see section 2). The compact enhanced green fluorescent protein (EGFP) was successfully fused to MK to produce a nanomolar potency fluorescent B_2R probe [76, 77]. A repeat of the relatively hydrophilic and flexible sequence Asn-Gly (NG) sequence was used as a spacer fused to the B_1R agonist Lys-des-Arg^9-BK in the construction enhanced green fluorescent protein (EGFP)-$(NG)_{15}$-Lys-des-Arg^9-BK [78]. It is not possible to encode a B_2R antagonist in an expression vector because all peptide antagonists include non-natural amino acid residues (see section 2). However, it is feasible in the B_1R antagonist ligand EGFP-$(NG)_5$-Lys-[Leu^8]des-Arg^9-BK, but it showed little receptor affinity. Consistent with microscopy results obtained with small agonist peptides conjugated with chemical fluorophores, EGFP-MK is internalized along with the B_2Rs in endosomes in live cells whereas EGFP-$(NG)_{15}$-Lys-des-Arg^9-BK stays mostly at the level of the plasma membrane in cells that express B_1Rs [76-78]. Fig. 5 and its legend show the exploitation of EGFP-MK to study the cycling of the agonist-stimulated B_2R with microscopy results that are in part original. Advantages of the EGFP fusion proteins as kinin receptor probes include a brighter, more stable fluorescence than that of many chemical fluorophores and the exclusion of the active sites of peptidases such as ACE, leading to a surprising stability of the agonist sequence both in the cytosol of producer cells and in the extracellular milieu. A disadvantage of EGFP-MK is its very low affinity for the human form of the B_2R, whereas rabbit and rat B_2Rs are readily labeled by this fusion protein. EGFP-$(NG)_{15}$-Lys-des-Arg^9-BK binds well to human recombinant B_1Rs with an estimated Ki of 7 nM [78].

Another construction that includes MK at its C-terminus involves the peroxidase APEX2 (36.7 kDa) with an extended spacer (Fig. 5): APEX2-$(NG)_{15}$-MK is a bona fide agonist of the rat B_2R and is compatible with various substrate/co-substrate commercial detection systems that allow receptor visualization (histochemistry) and luminescent or colorimetric quantification under a cell

well plate format [77]. As such, competition of APEX2-$(NG)_{15}$-MK binding to recombinant B_2Rs can be used for drug discovery in a non-isotopic ligand scheme. This is the perfect illustration of a bifunctional ligand. However, attempts to fuse conformationally correct human serum albumin (HSA, 66.5 kDa) to various C-terminally positioned spacer-MK or spacer-BK modules uniformly failed to produce high affinity ligands of the rat B_2R [77]. On the other hand, the myc-HSA-MK construction, synthesised in a denatured form in the cytosol of producer cells, was a B_2R agonist, indicating possible steric hindrance between the correctly folded and secreted protein HSA and the B_2R [77].

9. Antibodies

Anti-GPCR antibodies are perhaps counter-intuitive drug candidates since this class of receptor is amenable to the development of small ligands, including antagonists. However, there is a recent interest in this field: therapeutic human or humanized monoclonal antibodies are being actively developed as long-lived GPCR ligands that can be pharmacological antagonists, according to various types of molecular interactions with the receptor, or agonists by stabilizing the active receptor conformation; in addition some antibodies may promote GPCR internalization without signaling or promote dimerization [79]. As for the kinin receptors, the Müller-Esterl laboratory has experimented with polyclonal antibodies that exhibited affinity to the native BK B_2R, though not with monoclonal antibody ligands. Firstly, polyclonal antibodies raised against an anti-BK monoclonal behaved as an agonist anti-idiotype antibody, suggesting that a successive molding process may have retained some conformational features of BK in the final antibodies [80]. An ambitious project consisted of raising polyclonal antibodies to each extracellular domain of the rat B_2R, and to some intracellular domains as well [53]. Antibodies to extracellular loops 2 and 3

(or extracellular domains 3 and 4, respectively) behaved as pharmacologically active ligands, the former being agonists and the latter antagonists of the B_2R. Interestingly, both antisera displaced [^{3}H]BK binding from receptors, but only the anti-extracellular domain 4 antibodies displaced the radiolabeled form of the antagonist icatibant, suggesting differential orientation of the 2 types of ligands close to the extracellular fluid interface [81]. These experiments support the feasibility of developing therapeutic monoclonal antibodies directed to kinin receptor. Further, as high molecular weight proteins, those could theoretically be restricted to the circulation, thus preventing side effects that could originate from extravascular sites.

Fig. 7 illustrates the sequence of the rabbit B_2R with several structural features of functional significance identified; those were generally discovered in the human B_2Rs but are very well conserved in the rabbit sequence. A more prosaic application of antibodies that bind the kinin receptor rests on the introduction of an epitope in a recombinant construction, such as the myc tag at the N-terminus (Fig. 7, marker *3*). Excellent commercial anti-myc tag monoclonals, such as the clones 4A6 or 9E10, bind to rabbit myc-B_2Rs but exert no agonist or antagonist activities [33, 82], like the pharmacologically inert anti-N-terminal domain serum in another study [81]. However, agonist-stimulated myc-B_2R transports the anti-myc tag antibodies into endosomal structures in intact cells, as well as very large cargoes constructed around the 4A6 antibody, such Qdot nanomaterials coated with secondary antibodies [33, 82].

10. Is KLK-1 a B_2R ligand?

The extracellular domains of any GPCR can certainly include residue sequences susceptible to concentrated proteases; for instance, there is evidence that a micromolar concentration of sequencing grade trypsin or endoproteinase Lys-C applied to intact cells can partly degrade the construction B_2R-GFP, itself based on the rabbit receptor sequence (Fig. 7, marker *2*) [83, 84]. There are a few lysyl or arginyl residues in the extracellular loops of the rabbit receptor that may be the basis of these actions (Fig. 7). However, the specificity of KLK-1 is now better known, and it has a dual tryptic and chymotryptic action with sensitivity to the sequences flanking the cleavage site [85]. There is an acceptable chymotryptic site of cleavage for KLK-1 in the extracellular loop 2 of the rabbit B_2R (Y↓RD; Fig. 7, marker *5*); this site is not conserved in the human sequence [86]. Thus, KLK-1 can degrade in part the rabbit B_2R-GFP construction expressed at the cell surface, based on the generation of stable GFP-sized fragments recovered in the cell extract [84, 86].

While the serine protease KLK-1 indirectly stimulates kinin receptors by generating Lys-BK, that can be metabolized into alternate active ligands such as Lys-des-Arg^9-BK and BK, it has been proposed that the enzyme can bind directly to and activate B_2Rs with a nanomolar affinity and in in a kininogen-independent manner [87-89]. Basically, we did not obseve that a recombinant form of KLK-1 of high specific activity and purity could displace [^{3}H]BK binding to the human and the rat recombinant B_2Rs, but some minor competition was seen at the rabbit B_2R [86], possibly in line with its unique docking Y↓RD sequence. However, various pharmacologic activities of nanomolar and subnanomolar levels of KLK-1 are essentially dependent on the local generation of kinins from kininogen(s) often hidden in various experimental systems, including isolated organs and cultured cells [84, 86]. Other findings supporting indirect effects are that the catalytic activity of KLK-1 is always needed for a pharmacologic activity, that KLK-1 effects are

highly tachyphylactic but can be restored after kininogen replenishment and that the protease does not desensitize the B_2R population to the subsequent effects of the *bona fide* agonist BK.

11. Perspectives

Kinin receptor ligands presently have a very limited impact in medicine, with the B_2R antagonist icatibant used on a small scale. However, the reviewed material and much preclinical data suggest that antagonist may have a great potential as anti-inflammatory drugs [2, 21]. The detection of B_1R expression has a diagnostic interest. The cardiovascular and renal benefits of B_2R stimulation are already obtained in patients *via* ACE inhibition, but we have argued that a more direct approach based on biotechnological or prodrug agonists could be tried [73-75, 77]. Novel approaches promise to facilitate the design of original kinin receptor ligands in the future, such as the *in silico* definition of pharmacophores from the analysis of the docking of several non-peptide antagonists; this has been done for 3-dimensional models of both the B_1R and B_2R [90, 91]. The pro-drug agonist peptides may be further optimized for additional peptidases that will provide targeted actions on a specific tissue of a selective physiological function. As for the development of biotechnological ligands, let's note several considerations:

(1) Kinins are very small peptides without a stable conformation in aqueous solutions and that they are essentially engaged below the interface of the plasma membrane and extracellular fluid when bound to receptors (Figs. 1, 7) [2]. This situation is more challenging than that of large peptide hormones that dock to their cognate GPCRs with a sizeable fraction of the ligand protruding in the extracellular fluid. For instance, parathyroid hormone (PTH) binds to its

receptor$_1$ subtype with its C-terminus free in the extracellular space. Thus, the PTH sequence can be readily fused without a spacer to that of partner proteins to produce bifunctional ligands such as PTH$_{1\text{-}34}$-EGFP and PTH-peroxidases [76, 92].

(2) There is a need to produce high-molecular weight kinin receptor ligands that, such as HSA, resist to both glomerular and endothelial filtrations and remain for a long time in the circulation. These ligands could activate (as in cardio- and nephro-protection) or block (as in angioedema states) the vascular kinin receptors with selectivity, or support mapping of B_1Rs in tumors or inflammatory sites without a heavy labeling of the urinary tract. Such high molecular ligands could be monoclonal antibodies, but also the HSA or Fc immunoglobulin domain sequences fused to a suitable spacer and a kinin sequence.

(3) A "spacer-BK" module applicable to the human B_2R has yet to be found [33, 37]; a combinatorial approach may be needed to achieve this.

(4) Bifunctional ligands endowed with enzymatic activity, as in the prototype APEX2-(NG)$_{15}$-MK [77], may be improved in various ways. Peroxidases are "suicide" enzymes inactivated after a certain number of catalytic cycles; hydrolases such as β-glucuronidase, also supported by a range of commercial substrates, offer perspectives for increased sensitivity in histochemistry and non-isotopic receptor binding assays.

These approaches will define classes of alternative ligands of the kinin receptor of potential therapeutic and diagnostic value.

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Figure legends

Figure 1. A. Overview of the molecular pharmacology of natural and synthetic ligands of both B_2R and B_1R, related GPCRs. B. Overview of the signaling and cycling of those receptors and of selected ensuing physiological responses (*italics*). See main text for details. Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; DAG: diacylglycerol; eNOS: endothelial nitric oxide synthase; ERK: one of the mitogen-activated protein kinases; G_q: protein G_q; GRKs: G protein coupled receptor kinases; IP_3: inositol triphosphate; MEK: one of the mitogen-activated protein kinases; MLCK, myosin light chain kinase; PGI_2, prostacyclin; PKC, protein kinase C; PLC-β, phospholipase C-β; Ppases, phosphatases.

Figure 2. Some of the key modifications to BK structure to produce novel peptide ligands of the B_2R. Markers: ***1***, Protection from carboxypeptidases, including ACE, by an isosteric, non-hydrolysable bond between Phe^8 and Arg^9 [93, 94]. ***2***, Production of competitive antagonists by structural constraint of the peptide backbone in the C-terminal region and secondary gain of resistance *vs.* peptidases [35, 35, 95]. ***3***, Isomerization of the antagonist B-9430 that produced B-9972, a specific and full agonist highly resistant to inactivation [38, 96]. ***4***, N-terminal prolongation of B-9430 [55, 62]. ***5***, N-terminal extension of B-9972 by a fluorophore [54, 55, 97], emtansine (unpublished) or the myc tag-KPG sequence [60]. ***6***, Antagonist dimers developed as anti-neoplasic agents, the prototype, B-9870, being made from two B-9430 sequences joined at their N-terminus by a chemical linker [40, 43]. ***7***, N-terminal prolongation of the BK sequence as produced by alternate cleavage of kininogens [2], kinin homologues found in animal venoms (such as maximakinin) [32], selective protection against aminopeptidases ($D\text{-}Arg^0$-BK) [75], extension with fluorophore or drug-like moieties [54, 56] (emtansine unpublished) or fused

proteins [77] (see also Fig. 5). ***8***, Prodrug agonists that are activated by vascular peptidases such as ACE or arginine-carboxypeptidases [72-75]. * Peptide pro-drugs that releases BK-related agonist upon cleavage; † peptide modified for resistance or inherently resistant to peptidases; ‡ bifunctional ligand. Numbers that follow the peptide names are nanomolar IC_{50} values for displacing 3 nM [^{3}H]BK from human (hu), rabbit (rb) or rat (rt) B_2Rs in our previous studies when the experimental conditions were comparable. Abbreviations for non-conventional residues and peptides: AF350: AlexaFluor-350; CF, 5(6)-carboxyfluorescein; CpG, α-cyclopentylglycine; Cy7, cyanine dye 7; ε-ACA, ε-aminocaproic acid; EGFP, enhanced green fluorescent protein; FTC, fluorescein-5-thiocarbamoyl; HSA, human serum albumin; Hyp, trans-4-hydroxyproline; Igl, α-(2-indanyl)glycine; KPG, Lys-Pro-Gly spacer tripeptide; MK, maximakinin; Oic: (3as,7as)-octahydroindole-2-carboxylic acid; Sar, sarcosine; Thi, β-2-thienylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

Figure 3. Some of the key modifications to Lys-des-Arg^9-BK structure to produce novel peptide ligands of the B_1R. Markers: ***1***, Agonist protected from amino- and carboxypeptidases [46]. ***2***, First generation antagonists: replacement of Phe^8 by a residue possessing an aliphatic side chain [50]. ***3***, Some of the second generation peptide antagonists possessing a constrained structure [9, 98]. ***4***, N-terminal protection of Lys-[Leu^8]des-Arg^9-BK conferring resistance to aminopeptidase N [9]. ***5***, Further modification the antagonist B-9958 with N-terminal extensions to confer aminopeptidase resistance [51, 65], conjugation with a fluorophore of with an isotope-labeled moiety (the latter from Zhang et al., [66]). ***6***, N-terminal prolongation of Lys-des-Arg^9-BK to obtain bifunctional agonist ligands [57, 78]. Abbreviations for non-conventional residues and moieties: APEX2: soybean-derived peroxidase; CF, 5(6)-carboxyfluorescein; CpG, α-cyclopentylglycine; DOTA: chelator tetraazacyclododecane-1,4,7,10-tetraacetic acid; ε-ACA, ε-

aminocaproic acid; EGFP, enhanced green fluorescent protein; Hyp, trans-4-hydroxyproline; Igl, α-(2-indanyl)glycine; Mpaa: 4-methylphenylacetic acid; NODA: 1,4,7-triazacyclononane-1,4-diacetic acid; Oic: (3as,7as)-octahydroindole-2-carboxylic acid; Pip: 4-amino-(1-carboxymethyl)piperidine; Sar, sarcosine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid. † peptide modified for resistance or inherently resistant to peptidases; ‡ bifunctional ligand. Numbers that follow the peptide names are nanomolar IC_{50} values for displacing 1 nM [^{3}H]Lys-des-Arg^9-BK from human (hu) or rabbit (rb) B_1Rs in our previous studies when the experimental conditions were comparable.

Figure 4. The extended BK sequence BK-Arg as a potential "prodrug" agonist (*) of the B_2R activated by Arg-carboxypeptidases. A. Schematic representation of the prodrug concept. B. The C-terminal sequence extension determines a profound loss of potency at the B_2R, determined by a radioligand competition assay conducted at 0°C [73]. C. The human umbilical vein contractility assay shows that Arg carboxypeptidase activity (blocked by Plummer's inhibitor) confers a gain of function to BK-Arg, but not to BK. Reproduced in a modified form from Charest-Morin et al. [73]. D. The hypotensive effect of BK is not modified by Plummer's inhibitor, whereas this drug inhibits that of BK-Arg (* $P<0.05$, ** $P<0.01$). Reproduced in a modified form from Jean et al. [74].

Figure 5. Some biotechnological ligands of the BK B_2R and B_1R. Left: the docking of BK to the B_2R is schematically represented [2]; the N-terminus interacts with 2 residues of extracellular loop 3 (EL3). Maximakinin (MK), an amphibian sequence that contains BK at its C-terminus and a 10-residue N-terminal extension, is an agonist with a fair potency at the rabbit and rat B_2Rs [32] and the extension should protrude out in the extracellular fluid. EGFP-MK is a fluorescent fusion

protein based on MK that is a nanomolar agonist of the rabbit and rat B_2R and is translocated to endosomes with it [33, 76, 77]. APEX2-(Asn- Gly)$_{15}$-MK is an enzymatic ligand, endowed with peroxidase activity [77]. Right: the optimal agonist of the human B_1R is Lys-des-Arg^9-BK and its N-terminus is also closed to the receptor-extracellular fluid interface [2]. EGFP-(Asn-Gly)$_{15}$-Lys-des-Arg^9-BK is a nanomolar potency fluorescent ligand of the human B_1R that is not subjected to receptor-mediated endocytosis [78]. * construction inherently resistant to extracellular and cytosolic peptidases/proteases; ‡ bifunctional construction. Reproduced in part, in a modified manner, from Charest-Morin et al. [77] with the permission of the Editor.

Figure 6. BK B_2R cycling illustrated with the fluorescent agonist ligand EGFP-MK, a cytosolic fusion protein obtained from the lysate of producer cells. To support microphotography, the non-fluorescent rabbit myc-B_2R was transiently expressed in HEK 293a cells, in some cases with a red fluorescence emitting partner protein (indicated in red). The microphotographs (original magnification 1000 ×) are rendered with variable enlargement, the white bar being 10 μm long. The duration of incubation period of EGFP-MK with the cells at 37°C are indicated close to each microphotograph. Markers: ***1***, G protein-mediated signaling from the cell surface upon agonist binding. ***2***, B_2R phosphorylation by G protein coupled receptor kinases (GRKs) [99-101], a first step toward desensitization and internalization. AP2/clathrin is involved, at least in part, in B_2R endocytosis [102], that is largely inhibited by receptor truncation [56, 82, 103] or dominant negative dynamin (inset *a*, original microscopy of EGFP-MK; the dynK44n vector given by Dr. J. Benovic, Thomas Jefferson University, Philadelphia, PA). Dynamin is a GTPase that assists the "pinching off" of coated pits to form endocytic vesicles, a mechanism widely applicable to agonist-stimulated GPCRs [104, 105]. Co-expression of dominant negative dynamin significantly reduces the endocytotis of [^{3}H]BK mediated by myc-B_2R, but does not abate BK-induced

signaling in cells (data not shown). ***3***, Rab5-mediated formation of early ensodomes that travel toward the (-) end of microtubules and that contain trimolecular complexes agonist-receptor-β-arrestin [97] (inset *b*, original microscopy). The tubulin turnover inhibitor paclitaxel prevents the progression of EGFP-MK-containing endosomes but not endocytosis *per se* (inset *c*, original microscopy). At the endosomal stage, the B_2R is still bound to both the β-arrestins and ligand, as shown by the colocalization of EGFP-MK and mCherry-β-arrestin$_1$ (inset *d*, from [76]). The cellular expression of a mutated GTP-locked Rab5 protein causes the formation of giant endosomes in which the EGFP-MK cargo is visualized (inset *e*, from [76]). Agonist destruction and the action of phosphatases must precede the extensive receptor recycling [32, 101, 106]. In the case of EGFP-MK, a loss of BK immunoreactivity and of molecular mass was observed when this protein ligand progressed into the endosomal tract (immunoblots) [77]. ***4***, Pathways for the extensive B_2R recycling. While a Rab11-mediated pathway travels along the actin cytoskeleton, another less defined Rab4-facilitated mechanism has also been identified [97]. It is uncertain whether internalized B_2Rs are the source of signaling, e.g., via scaffolding of kinases on arrestin as in the related angiotensin AT_1 receptor [107], notably because chemical inhibitors of protein kinase C suppress the downstream ERK signaling [39, 108]. ***5***, The degraded EGFP-MK, now reduced to a GFP-sized protein, is sorted to LAMP1-positive lysosomes (inset *f*, from [77], with permission of the editor). General outlook reproduced from [97] with the permission of the editor.

Figure 7. Structure of the rabbit BK B_2R [109] and derived constructions and docking of BK. Transmembrane domains, the 2 sites of cysteinyl palmytoylation (from the conserved motif of the human B_2R) [110] and the 3 putative glycosylation sites are indicated, as well as the position of a conserved disulfide bond. Aspartic residues interacting with 2 positive charges at the N-terminus

of BK (H_3N^+-Arg^1) are indicated in red; those involved in a stabilizing bridge between TM3 and TM6 in green [111]. Markers: ***1***, GRK substrate region (boxed; sequence identical in the human B_2R) [103]. ***2***, sequence prolonged with GFP in B_2R-GFP [83]. ***3***, inserting 9 residues after the initial M residue in myc-B_2R constitutes the 10-residue *myc-tag* epitope (M*EQKLISEEDL*N) supporting the binding and internalization of anti-myc monoclonal antibodies [38, 82, 97]. ***4***, C-terminal 28-mer deleted in the endocytosis-resistant myc-B_2R_{trunc} construction [56]. EL2, EL3: extracellular loops 2 and 3. ***5***, Postulated chymotryptic site of hydrolysis of B_2R-GFP by tissue kallikrein (KLK-1) [86]; this site is not well conserved in other mammalian species. ***6***, Docking of BK to the B_2R with particular reference to the position of the peptide N-terminal sequence [2].

Figure 1
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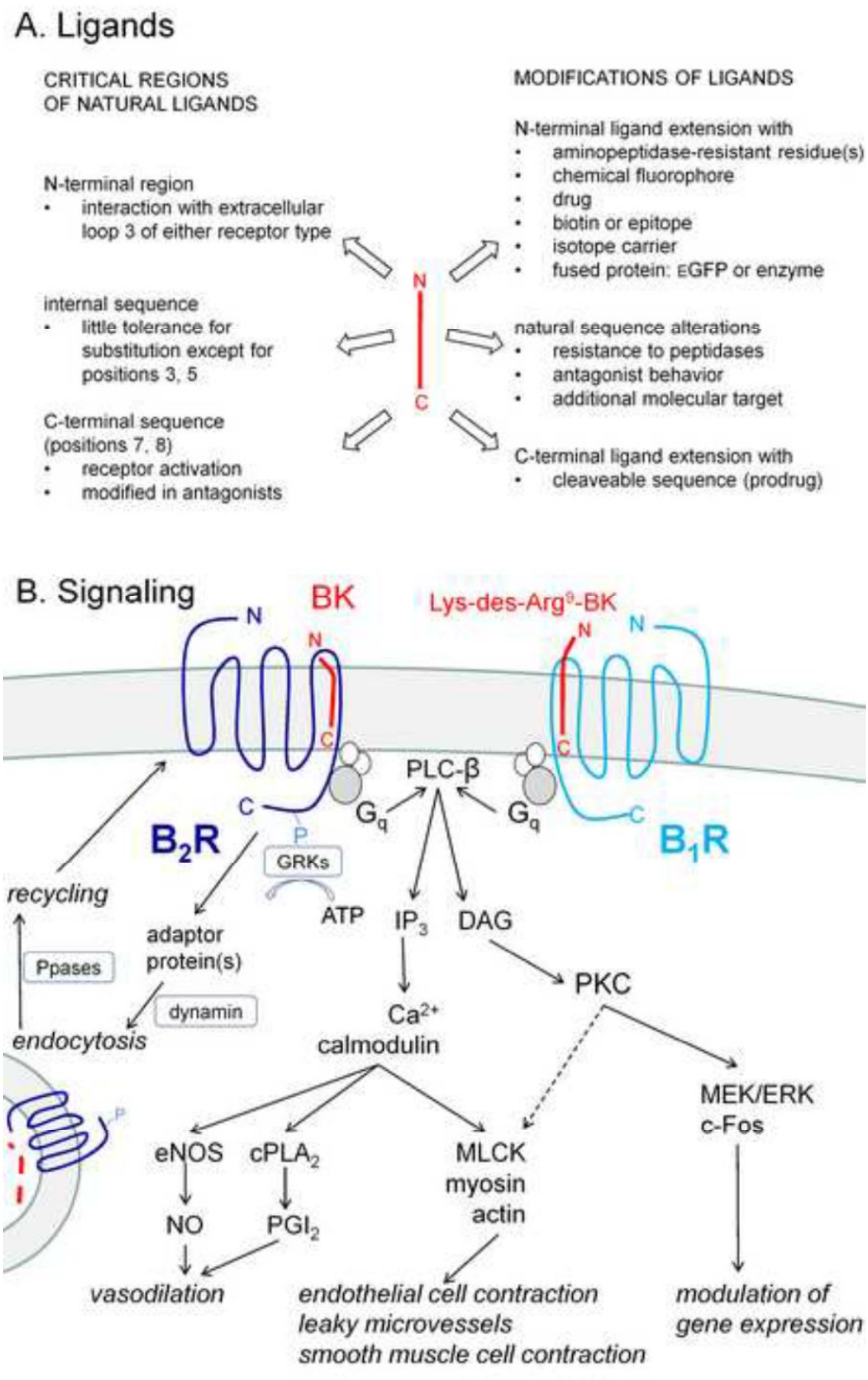

Figure 2
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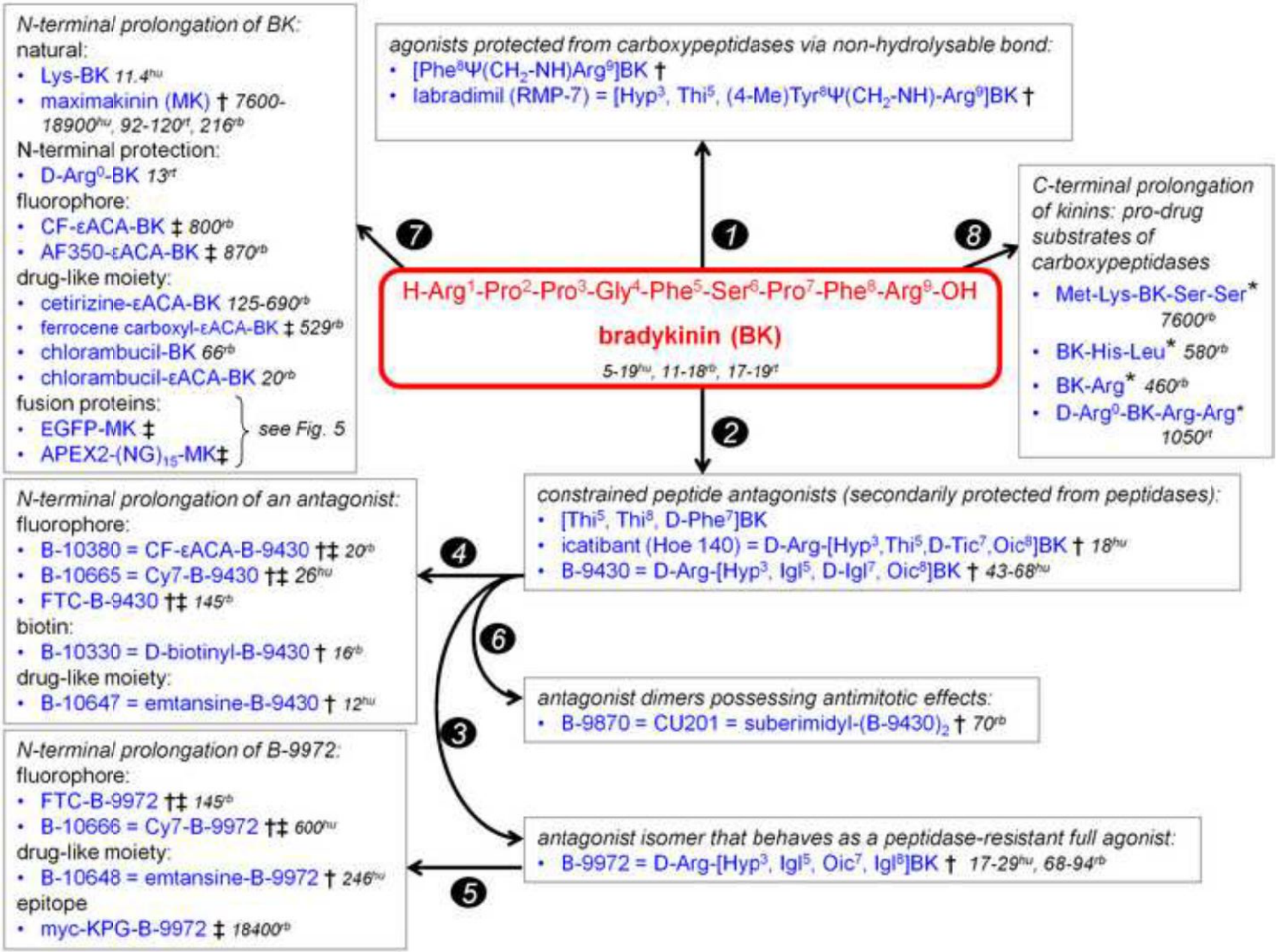

Figure 3
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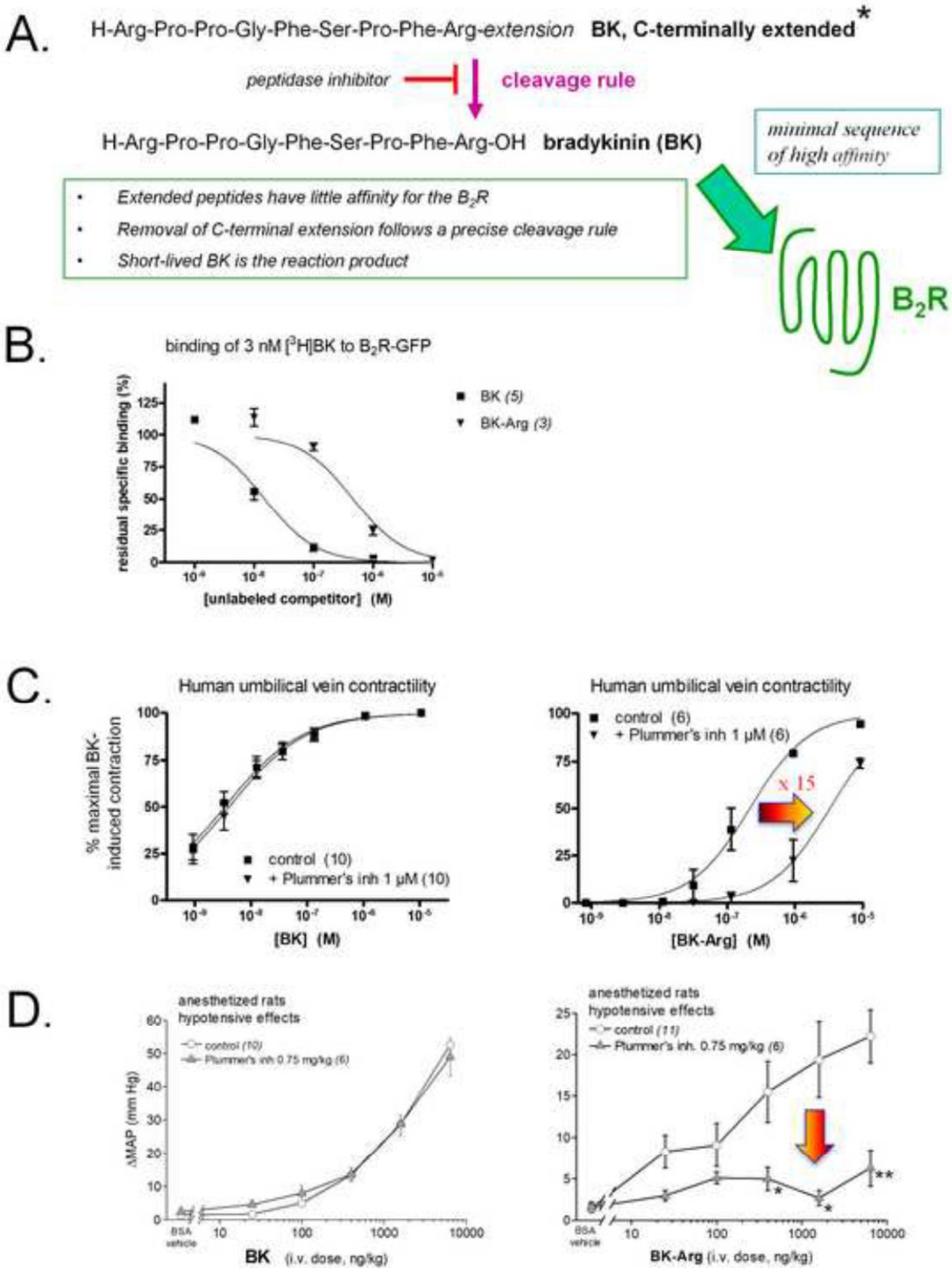

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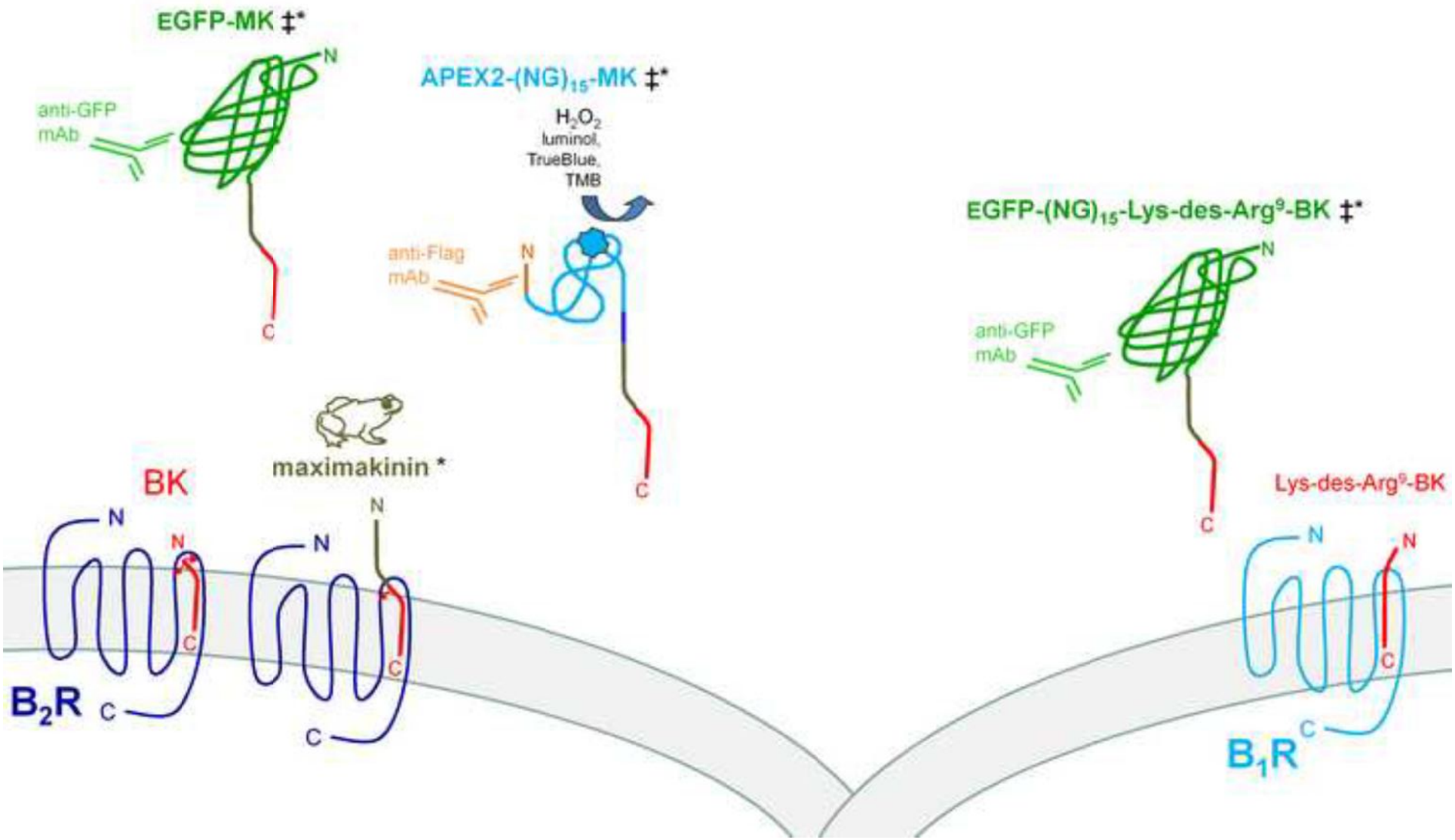

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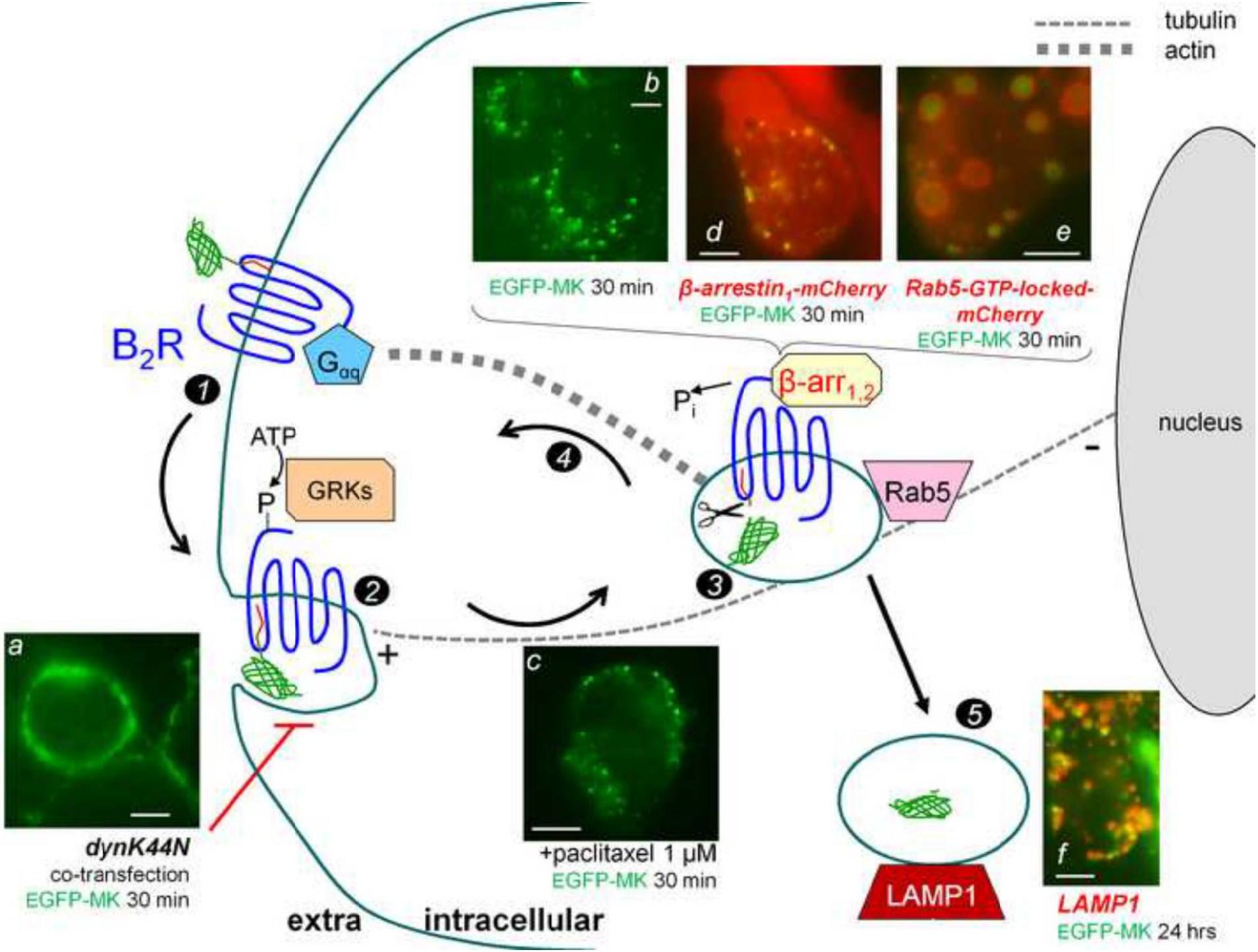

Figure 7
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