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Allostery: The Good, the Bad and the Ugly

Terry Kenakin Ph.D.

Department of Pharmacology

University of North Carolina School of Medicine

Chapel Hill, N.C.

'Whatever affects one directly, affects all indirectly. This is the interrelated structure of reality'.

Martin Luther King Jr.

Corresponding author: **Terry Kenakin (kenakin@email.unc.edu)**

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Running Title: The Good and Bad of Allosteric Modulation

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Abbreviations: AIDS: acquired immunodeficiency syndrome / **Alvameline:** 3-(2-ethyltetrazol-5-yl)-1-methyl-5,6-dihydro-2H-pyridine / **Aplaviroc:** 4-[4-({(3R)-1-Butyl-3-[(R)-cyclohexyl(hydroxy)methyl]-2,5-dioxo-1,4,9-triazaspiro[5.5]undec-9-yl}methyl)phenoxy]benzoic acid / **BQCA,** benzyl quinolone carboxylic acid [1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid] / **CCL3L1**: Chemokine (C-C motif) ligand 3-like 1/ **CCR5**: Cysteine-Cysteine Chemokine Receptor 5 / **CRTH2** receptor: Chemoattractant receptor-homologous receptor / **Dynorphin 1-11**: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys / **ERK1/2:** extracellular signal-regulated kinase ½ / **GLP-1:** Glucagon-like Peptide 1 / **gp-120:** a glycoprotein on the HIV envelope / **GTPγS**: (guanosine 5'-O-[γ-thio]triphosphate) / **HIV-1**: Human Immunodeficiency Virus / **Ifenprodil:** 4-[1-hydroxy-2-[4-(phenylmethyl)-1-piperidinyl]propyl]phenol / **LP1805:** N,N-(2-methylnaphthyl-benzyl)-2-aminoacetonitrile / **LY2033298**: 3-Amino-5-chloro-N-cyclopropyl-6-methoxy-4-methylthieno[2,3-b]pyridine-2-carboxamide / **MC4R:** melanocortin receptor 4 / **NOVO2:** (6,7-dichloro2-methylsulfonyl-3-tert-butylaminoquinoxaline; / **MK-866**: 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]-α,α-dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid, / **NMDA receptor**: N-methyl-D-aspartate receptor / **Oxyntomodulin:** L-histidyl-L-seryl-L-glutaminylglycyl-L-threonyl-L-phenylalanyl-L-threonyl-L-seryl-L-α-aspartyl-L-tyrosyl-L-seryl-L-lysyl-L-tyrosyl-L-leucyl-L-α-aspartyl-L-seryl-L-arginyl-L-arginyl-L-alanyl-L-glutaminyl-L-α-aspartyl-L-phenylalanyl-L-valyl-L-glutaminyl-L-tryptophyl-L-leucyl-L-methionyl-L-α-aspartyl-L-threonyl-L-lysyl-L-arginyl-L-asparaginyl-L-lysyl-L-asparaginyl-L-asparaginyl-L-isoleucyl-L-alanine / / **Palonosetron** : (3aS)-2-[(3S)-1-azabicyclo[2.2. 2]octan-3-yl]-3a,4,5,6-tetrahydro-3H-benzo[de]isoquinolin-1-one;hydrochloride / **PAM**: Positive Allosteric Modulator / **PDG2:** Prostaglandin D2 receptor / **Pentylthio-TZTP**: 3-(1-methyl-3,6-dihydro-2H-pyridin-5-yl)-4-

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pentylsulfanyl-1,2,5-thiadiazole / **Quercetin** : 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one / **Salvinorin**: Methyl (2S,4aR,6aR,7R,9S,10aS,10bR)-9-acetoxy-2-(3-furyl)-6a,10b-dimethyl-4,10-dioxododecahydro-2H-benzo[f]isochromene-7-carboxylate / **SCH-3511254** [(Z)-(4-bromophenyl)- (ethoxyimino)methyl]-1'-[(2,4-dimethyl-3- pyridinyl)carbonyl]-4'-methyl-1,4'- bipiperidine N-oxide / **TAK652:** (S,E)-8-(4-(2-Butoxyethoxy)phenyl)-1-isobutyl-N-(4-(((1-propyl-1H-imidazol-5-yl)methyl)sulfinyl)phenyl)-1,2,3,4-tetrahydrobenzo[b]azocine-5-carboxamide / **TAK779**: N-[[4-[[[6,7-Dihydro-2-(4-methylphenyl)-5H-benzocyclohepten-8-yl]carbonyl]amino]phenyl]methyl]tetrahydro-N,N-dimethyl-2H-pyran-4-aminium chloride / **TAK-875**: (3S)-6-[[2',6'-dimethyl-4'-[3-(methylsulfonyl)propoxy][1,1'-biphenyl]-3-yl]methoxy]-2,3-dihydro-3-benzofuranacetic acid / **(+)U50,488**: 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide

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Allostery: The Good, the Bad and the Ugly

Terry Kenakin Ph.D., Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, N.C.

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Abstract: *With the advent of functional screening, more allosteric molecules are being discovered and developed as possible therapeutic entities. Allosteric proteins are unique because of two specific properties: (1) separate binding sites for allosteric modulators and guests, and (2) mandatory alteration of receptor conformation upon binding of allosteric modulators. For GPCRs, these properties produce many beneficial effects on pharmacologic systems that are described here. Allosteric discovery campaigns also bring with them added considerations that must be addressed for the endeavor to be successful and these are described herein as well.*

Significance Statement: Recent years have seen the increasing presence of allosteric molecules as possible therapeutic drug candidates. The scientific procedures to characterize these are unique and require special techniques so it is imperative that scientists understand the new concepts involved in allosteric function. This review reviews the reasons why allosteric molecules should be considered as new drug entities and the techniques required to optimize the discovery process for allosteric molecules.

I. Introduction

G Protein Coupled Receptors (GPCRs) are Nature's prototype allosteric protein; their one job in natural physiology is to transmit information through changes in protein conformation.

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Their allosteric nature is made manifest in the transmission of allosteric energy from a bound ligand (modulator) through the conduit of the protein (receptor) to a guest (which could be another protein or a binding locus for another ligand). In this sense, ***every*** ligand binding to a GPCR is an allosteric ligand. Natural hormone and neurotransmitter agonists are allosteric modulators of GPCRs by virtue of the fact that they bind to a natural binding site on the receptor to alter receptor conformation to one that subsequently changes the interaction of the receptor with cellular signaling proteins. For the purpose of this review, these natural interactions at the endogenous agonist binding site will be defined as being orthosteric and competition of ligands at these natural binding sites will be defined as orthosteric interactions (i.e. steric hindrance). These effects will be differentiated from interactions of molecules at other binding sites on the receptor that modify receptor behavior toward all other ligands and signaling proteins through alteration of protein conformation; these will be designated as ***allosteric*** modulator effects. This paper will discuss the unique ways in which allosteric modulators can modify receptor pharmacology for therapeutic benefit.

II. Beneficial Properties of Allosteric Proteins (The Good)

There are two basic properties of GPCRs as allosteric proteins that lead to uniquely beneficial effects of drugs exploitable for therapy; these are (1) geographical separation of binding sites (orthosteric vs allosteric) on the protein and (2) mandatory alteration of tertiary protein conformation upon binding of the allosteric ligand. These two general behaviors form the basis of all pharmacologic properties of allosteric modulators- see Fig 1. It should be noted that many of these effects of modulators **cannot** be duplicated by standard conventional orthosteric agonists and antagonists and thus they constitute a unique battery of new exploitable pharmacologic effects. Accepting the premise that pharmacology is the

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chemical control of physiology, allosteric modulators have taken this chemical control to a new level of novelty and value. It is worth considering the unique properties of allosteric modulators within the context of these two main protein behaviors. As a preface, the different types of allosteric modulators are listed and defined in Table I for reference.

A. Orthosteric vs Allosteric Binding Sites

The first major property of allosteric proteins is that they bind ligands (or other bodies such as protein) to two separate sites. Thus, there is no physical encumbrance to the binding of each by the other although the relative affinities of the two sites for their binding partners can be affected by allosteric energy through the protein (*vide infra*).

1. **Saturation of Effect:** In a competitive orthosteric situation where two ligands compete for the same binding site, the relative dominance will be attained by the ligand with the higher concentration relative to its own affinity. Under these circumstances, there is no limit to the extent of relative activity, i.e. as long as the concentrations are varied, the effect will not come to saturation. However, with separate binding sites, this is not the case as further allosteric effect (change in agonist affinity and/or efficacy) will cease once the allosteric site is fully occupied (see Fig 2A). Thus, whatever the allosteric effect, there will be a maximum reached and the receptor will in essence be re-set to a different level of activity. As in the example shown in Fig 2B, an allosteric modulator produces a maximal dextral displacement of the acetylcholine curve of 10-fold. This can be extremely useful therapeutically as a given target may just need modulation (a reduction or augmentation) of normal activity but otherwise remain operative. Limiting the maximal effects of receptors with different positive allosteric modulators (PAMs) also can be a useful strategy to prevent overdose. If the increases are limited to levels that

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never elevate signals into the toxic range, then essentially such a PAM would be an extremely safe way to augment response without threat of over-stimulation; an example is shown in Fig 3.

2. **Co-binding with natural agonists:** The separation of binding sites allows modulators to influence the natural signaling of the system. Specifically, an orthosteric partial agonist will 'hijack' the receptor, precluding natural agonist binding and impart the efficacy of the partial agonist onto the system. In contrast, an allosteric partial agonist will co-bind with the natural agonist (essentially become a 'hitchhiker') but still may allow natural signaling (albeit altered, either reduced or augmented) to occur thereby giving an amalgam of signals *in vivo*; these differing effects are illustrated by the orthosteric muscarinic agonist alvameline and allosteric modulator BQCA (Bdioui et al, 2018)- see Fig 4. This also makes the effects of allosteric agonists more complex in vivo in that overall response may then be an amalgam of the modulator and natural agonist (and thus depend on the level of agonism in the system).
3. **Multiplicity of binding sites to GPCRs:** A screening campaign restricted to interactions at the orthosteric binding site will detect ligands that interfere with probe binding at this site and no other. In contrast, the entire surface of the GPCR may be considered to contain potential binding sites for allosteric ligands and this may increase the hit rates in allosteric, over orthosteric, screens. There are reports of 'pocketomes' of various allosteric sites on a number GPCRs (Hedderich et al, 2022; Wakefield et al, 2019) For example, Fig 5 shows three separate binding sites on GPR40 for allosteric modulators (Wang et al, 2021).

4. **Druglike Molecules for Peptide Receptors:** Historically it has been difficult to find small orally bioavailable druglike molecules for peptide receptors. It is not clear whether peptide receptor binding pockets require a greater number of interactions than a small molecule is capable of or if multiple binding loci are required to stabilize a peptide receptor into an active state. However, a viable strategy around this limitation is to find druglike allosteric modulators that may bind at sites that do not have the constraint of the natural peptide binding site (Bartfai and Wang, 2013). Thus therapeutic molecules for peptide receptors such as GLP-1 (Koole et al, 2010) and CCR5 (Muniz-Medina et al, 2009) have been discovered which modify peptide behavior with favorable pharmacokinetic profiles.
5. **Overcome Mutation of Natural Binding Site Recognition:** Allosteric agonism is mediated through the binding of the allosteric agonist to a site separate from that utilized by the natural agonist for the receptor. Therefore, if a disease mutation for a receptor renders it inoperable to natural agonism through aberration of the natural agonist binding site, an allosteric agonist may salvage the response. For example, the allosteric agonist alcuronium produces potent activation of muscarinic m2 receptors under conditions where the natural binding site is made inoperable by high concentrations of the orthosteric antagonist QNB (Jakubic et al, 1996).

B. **Conformational Changes with Ligand Binding:** The other major allosteric property of GPCRs is the change(s) in protein conformation produced by ligand binding. Seen within the context of molecular dynamics, GPCRs exist in ensembles of slightly different tertiary conformations of similar free energy. Ligands preferentially bind to those conformations for which they have the highest affinity and drive the ensemble toward those preferred

conformations (Le Chatelier's Principle). Thus, cells deal with an intrinsic ensemble in the absence of a ligand and a different ligand-bound ensemble after ligand binding (Amadei et a, 1993; Hilser and Freire ,1997a,b; Hilser et al, 1997; 1998). These ligand bound ensembles then deal with pleiotropic signaling pathways in the cell to bias stimulus to various pathways. Molecular dynamics also dictates that the same molecular forces that govern ligand affinity also control changes in the protein conformation upon ligand binding. This mandates that binding is not a passive process (Kenakin and Onaran, 2002) and no matter how physically small a ligand may be in relation to the protein, the protein will discern it's presence and change it's energy; as stated by Sir Francis Bacon in the year 1620: *'It is certain that all bodies whatsoever….have perception; for when one body is applied to another…. Evermore a perception precedeth operation…'.* Therefore, allosteric ligands will necessarily produce changes in the tertiary conformation of GPCRs although the practical outcome of this behavior may vary in different systems. These changes in conformation augur distinct natural ligand-dependent behaviors for some allosteric modulators and these can be beneficial in drug therapy.

1. Alteration of Protein-protein Interactions for Huge Proteins: Linking ligand affinity with global protein conformation predicts that the binding of allosteric ligands will modify a range of regions in the receptor. This, in turn, may alter the receptor's interaction with proteins that interact at a number of loci. An example of where this is therapeutically relevant is the binding of the HIV-1 viral coat protein gp120 to the CCR5 chemokine receptor. Once it was discovered that the mediator of HIV-1 infection in AIDS is the CCR5 receptor / gp120 binding interaction, a great deal of mutation work was done in an attempt to identify a 'hotspot' where a small molecule could orthosterically interfere with CCR5 / gp120 binding. However, blockade of HIV infection was shown not to be

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amenable to single point mutations (Doranz et al, 1997) and in fact, all four extracellular domains of CCR5 appear to be involved in the fusion process (Bieniassz et al, 1997; Kwong et al, 1998; Tong et al, 2002). Yet several small molecules subsequently were found that are extremely potent (nanomolar potency) inhibitors of HIV-1 infection. These are all allosteric modulators that appear to globally alter CCR5 conformation with the result that numerous interacting regions with gp120 are disrupted- see Fig 6.

2. Allosteric Probe Dependence: Conformational changes produced by allosteric molecules cause regionally separate alterations in binding loci throughout the receptor. Therefore, if two probes (anything that senses receptor conformation such as a ligand or a signaling protein) interact with the receptor at two different geographical binding sites, there is no rule that dictates the effects of the allosteric change will be uniform for the two probes (and indeed, prevailing evidence shows it rarely if ever is). This is true for all allosteric effects including orthosteric agonism. Thus, when an orthosteric agonist stabilizes a new active conformation of the receptor, changes at separate binding loci such as those mediating G protein and β-arrestin interaction, will be affected; probe dependence dictates that these effects will not be uniform and this leads to the now well known phenomenon of biased signaling (Kenakin, 2019). In general, biased signaling should be *expected* as it is the consequence of natural receptor allosteric probe dependence. A general outcome of this is that the signaling pattern for synthetic agonists should not be expected to be the same as that for the natural endogenous agonist(s). In fact, Nature uses this mechanism to fine tune GPCR signaling for receptors with multiple natural agonists (Kohout et al, 2004)

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These same principles hold for guest allostery mediating the interaction of any two bodies interacting with separate sites on the receptor and this effect can yield therapeutic advantages. For instance, as stated earlier, HIV-1 infection leading to AIDS is mediated through the interaction of the chemokine receptor CCR5 and gp120 on the HIV viral coat. Minimization of HIV-1 binding is a viable therapy against progression of HIV-1 infection to AIDS and there are two mechanisms to eliminate CCR5 as a viable binding entity for HIV-1: (1) allosteric blockade of HIV-1 binding and (2) internalization of CCR5 into the cell cytosol. This latter mechanism has therapeutic relevance with the finding that in an extensive study of 1064 patients with HIV-1 infection, survival was linked to the gene copy number for the natural ligand for CCR5 CCL3L1, the implication being that a high gene copy number is linked to high levels of CCL3L1 and a greater ambient level of internalization of CCR5 (Gonzalez et al, 2005). The regions of CCR5 that mediate chemokine binding and binding of HIV-1 are physically separate therefore probe dependence offers an opportunity to improve anti HIV-1 therapy by making allosteric modulators that perturb HIV binding (to block infection) but otherwise do not preclude CCL3L1 mediated internalization. Fig 7 shows such a molecule (TAK 652) which has an 8-fold higher potency for blocking HIV than CCL3L1 internalization (Muniz-Medina et al, 2009).

3. **Allosteric Modulators can Overcome Mutation:** In keeping with the dependence of drug effect on GPCR conformation, mutations in the receptor can lead to debilitating inhibition of drug response through inhibition of receptor stimulus-response transduction. For example, a number of mutations in MC4R, a receptor intimately involved in energy homeostasis, lead to early onset morbid obesity. The primary signaling pathway for

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MC4R is Gαs-mediated increases in cyclic AMP and aberration of this signaling leads to high BMI values for patients. For example, the D90N mutation of MC4R completely eliminates cAMP signaling to MC4R agonists and results in an early onset obese phenotype (Buch et al, 2009; Xiang et al (2010). Allosteric modulators have the ability to modify receptor efficacy and revive function. For example, it has been shown that the positive muscarinic ~~positive~~ allosteric modulator LY2033298 produces a complete reversal of the loss of function mutation $D112^{3.32}E$ of the muscarinic m4 receptor (Leach et al, 2011).

Allostery also can overcome drug resistance due to mutation as in the case of HIV-1 infection leading to AIDS. The prevailing evidence shows that different allosteric modulators such as aplaviroc, SCH-3511254 and TAK779 stabilize different tertiary conformations of receptors. For example it has been shown that the antibody binding profiles of Ab45531 and Ab45523 differ for CCR5 in the presence of the allosteric HIV-1 entry inhibitors TAK779, SCH-3511254, and aplaviroc (Watson et al, 2006; Kenakin 2007). This may provide a unique way to avoid the viral resistance expected from the constant mutation of the HIV-1 envelope. ~~the modulator.~~ Specifically, the HIV-1 virus is known to continually mutate and alter the composition and conformation of gp120 in its routine realm of existence (Wyatt and Sodroski, 1998; Poignard et al., 2001). Given this, a mutated virus conceivably could learn to use whatever form various HIV-1 entry inhibitors impose on the CCR5 receptor leading to viral resistance (Trkola et al, 2002; Kuhmann et al., 2004). Thus stabilization of different tertiary conformations of CCR5 could overcome such resistance.

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4. **Increased Receptor Subtype Selectivity:** Selectivity of effect through binding at the orthosteric natural agonist recognition site for families of receptors with a common agonist (i.e. five subtypes of muscarinic acetylcholine receptor all bind acetylcholine) is difficult due to the similarity of the conserved acetylcholine binding sites (Gentry et al, 2013; Myslivecek, 2022). It has been reported that while residues required for structural integrity of receptors are highly conserved, residues critical for allosteric signaling are poorly conserved (Leandera et al, 2020). The fact that allosteric sites are much less evolutionarily conserved allows strategies to find subtype selectivity due to allosteric binding sites. For example, selective antagonism of the elusive selective muscarinic m5 receptor employs a negative allosteric modulator (Bender et al, 2019).
5. **Preservation of Complex Signaling Patterns:** In systems where stimulation is a therapeutic requirement, global activation of the entire system without consideration of geographical receptor distribution and regional sensitivity of cellular transduction systems is untenable and, in fact, could be disastrous. Allosteric modulation, however, allows the system to dictate where and how intense the signals occur and modifies them in relation to this complex wiring accordingly. In general, many allosteric modulators have been postulated to be of value in especially, CNS disorders such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and Huntington's disease) and psychiatric or neurobehavioral diseases (anxiety, schizophrenia, and addiction) (Engers and Lindsley, 2013; Nickols and Conn, 2014). The added selectivity seen with allosteric modulators plays an integral role in these therapeutic applications. For example, acetylcholine receptors have been implicated in a vast array of behavioral disorders (Bender et al, 2019; Teal et al, 2023) but selectivity of effect is essential in

approaches to modify these ubiquitous receptors. An approach to achieve receptor subtype selectivity and to preserve complex receptor brain distribution patterns has been to link therapeutic effect of synthetic ligands to the natural ongoing physiological signal present in the system, i.e. allosteric modification of an ongoing signal. This has been utilized for nicotinic receptors in Alzheimer's disease (Krause et al, 1998; Wang et al, 2020) and muscarinic acetylcholine receptors in schizophrenia, cognition, and addiction (Moran et al, 2019).

6. **Separate Modulation of Affinity and Efficacy**: The functional allosteric model describes modulator-specific effects on receptors in terms of two parameters; α which is the change in agonist affinity produced by the modulator and β, the change in the efficacy of the natural agonist produced by the modulator (Kenakin, 2005; Ehlert, 2005; Price et al, 2005). As with the heterogeneous nature of protein conformational changes, there is no ordained order of which of these effects (α or β) will be operative for any modulator-agonist pair. In general, values of α and/or $\beta > 1$ will denote respective increased affinity and efficacy and values< 1 denote inhibition. Particularly interesting effects can be seen with modulators that have diverse effects on affinity and efficacy. Specifically, PAM-Antagonists increase agonist affinity ($\alpha>1$) but decrease efficacy ($\beta<1$) to produce a unique functional negative allosteric modulation (NAM effect) (Kenakin and Strachan, 2018)-see Table I. The discerning property of these modulators is that they increase their antagonist potency with greater agonism, i.e. the harder the functional system is driven by agonist, the more antagonism is produced. This is because of the reciprocity of allosteric energy. Specifically, just as the modulator increases the affinity of the agonist, so too does the agonist increase the affinity of the modulator. With increasing affinity of the

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modulator comes greater receptor occupancy and since the presence of the modulator decreases the signaling efficacy of the agonist, the greater the blockade of agonism. The first PAM Antagonist described is ifenprodil, an allosteric antagonist of NMDA receptors. The reverse potency on blockade with agonism is made manifest by the fact that the IC_{50} for inhibition of 10 μM NMDA is 2.5μM whereas the IC_{50} for inhibition of a 10-fold higher concentration of NMDA (100 μM) is 0.3 μM; thus, ifenprodil becomes 8.3-fold more potent as an NMDA antagonist with the increased concentration of agonist (Kew et al, 1996). PAM Antagonists represent a new class of drug that can be used to preferentially target persistently activated receptors. For instance, vascular constriction by endothelin is persistent ($t_{1/2}$ =7 to 77 hr) and resistant to standard antagonists (De Mey et al, 2011). The difficulty in reversing endothelin-induced vasoconstriction is shown by difference in the ability of endothelin antagonists to reverse endothelin vascular effects after contraction have been induced compared to the potency needed to prevent vascular endothelin-mediated contraction when pre-equilibrated in the absence of endothelin (Compeer et al, 2012). The preferential antagonism of agonist-activated receptors by PAM Antagonists would predict selective blockade of agonist-bound receptors, i.e. PAM Antagonists seek and destroy activated receptors. These effects could be useful in conditions such as preeclampsia (George and Granger 2011; Jain, 2012; George et al,2012), pulmonary arterial hypertension (Clozel, 2016; Kuntz et al, 2016; Rivera-Lebron and Risbano, 2017), tumorigenesis and metastasis (Said and Teodorescu, 2012; Rosano et al, 2013), and diabetes (Andress et al, 2012; Pernow et al, 2012).

7. **Greater Target Residence Time In Vivo:** Another effect of PAM-Antagonism is the added persistence of binding of the modulator to the target receptor in the presence of the

endogenous agonist. As with the effects discussed for ifenprodil, the presence of an agonist in vivo elevates the affinity of the receptor for the antagonist with resulting prolonged target residence time for the antagonist (produced by the presence of endogenous agonist). This effect is seen with the 5-HT_3 antagonist for chemotherapy therapy-induced nausea palonosetron (Saito and Tsukuda, 2010). Although structural studies suggest palonosetron binds to or near the orthosteric site (Zarkadas et al, 2020) , functional kinetic studies indicate palonosetron is an allosteric modulator having co-operative effects with 5-HT (Rojas et al, 2008). The increased affinity of palonosetron in the presence of 5-HT increases the $t_{1/2}$ for dissociation from the receptor by a factor of nearly 10 ($t_{1/2}$ for no 5-HT present = 2.3 h: 5-HT present $t_{1/2}$= 21,9 h) (Lummis and Thompson, 2013). This produces a much longer therapeutic target coverage for treatment of nausea.

8. **Allosteric Salvage of Forbidden Drug Targets:** GPCRs are pleiotropic with respect to the signaling pathways they activate in cells. The combination of signals imparted by a given agonist thus presents a quality of efficacy that is unique to the receptor and the ligand. Within these mixtures of signals can be some that are detrimental to physiology or drug therapy and this fact may preclude the target from consideration as viable therapy. For example, *K*-opioid receptors activate G protein and β-arrestin signaling in cells and produce effects postulated to be involved in cognition, reward, mood and perception. Such a profile suggests K-opioid agonism of possible utility as antidepressants and anxiolytics in affective disorders, drug addiction, and psychotic disorders. However, an important effect of K-opioid agonists, such as salvinorin, is vivid and disturbing hallucinations, an effect that precludes conventional K-opioid agonism for therapeutic

effect. Studies suggest that the hallucinogenic activity is primarily dependent on β-arrestin (Che et al, 2021) therefore this condition lends itself to elimination of these signals through biased agonism. Specifically, a G protein biased ligand will not only not produce debillitating β-arrestin effects, but will be an antagonist of the natural agonist producing these same effects. Analyses of biased signaling for *K*-opioid receptors reveal agonists that are 8-fold biased toward β-arrestin ((+)U50,488) and notably 44-fold biased toward G-protein (dynorphin 1-11) (White et al, 2014). This suggests that the natural probe dependent allostery that mediates biased signaling can be employed to edit signaling signatures of previously forbidden receptors and convert them to be viable therapeutic targets.

9. **Reduced on Target Side Effects Through Temporal Selectivity:** Clearly selectivity contributes to a lack of side effects but also, the temporal pattern of drug treatment can contribute to selective drug effect as well. An important feature of positive allosteric modulators is their lack of effect when the system is not stimulated and this property can improve GLP-1 therapy for diabetes. Specifically, post-prandial GLP-1 (release of GLP-1 after a meal) is known to potentiate insulin secretion and the augmented insulin released assists in diabetes. However, in pre-prandial periods, no GLP-1 receptor stimulation is produced since no GLP-1 is released; the increased GLP-1 signal is elevated only periodically. This alleviates a known side effect of direct GLP-1 agonism, namely nausea which is seen in standard GLP-1 agonist treatment for diabetes where receptor stimulation is constant (Theodosios et al, 2014). In general, such periodic stimulation with PAMs may reduce such side effects and also reduce desensitization caused by prolonged stimulation of receptors.

III. Possible Deleterious Effects of Allostery (The Bad)

Probe dependence dictates which probes will and which will not be affected by an allosteric modulator and for receptors with multiple natural agonists, the important agonists need to be the ones affected. Fig 8 shows the effect of the PAM NOVO2 on GLP-1 agonism expressed as a fold-potentiation of the concentration response of the agonist (Koole et al, 2010). As can be seen, of the four natural GLP-1 agonists, only oxyntomodulin is significantly potentiated (25-fold); the dissimulating aspect of these data is that GLP-1(7-36)NH_2 is the most important natural agonist operative in this system whereas oxyntomodulin is a minor player. Such losses at 'agonist-roulette' can be costly in the clinic if not detected early *in vitro*.

IV. Project Derailment Allosteric Issues (The Ugly)

The fact that allosteric sites are less evolutionarily conserved leads to improved selectivity but when examined over different animal species, this can be deleterious specifically with animal orthologues of therapeutic target receptors. In general there is homogeneity between human and animal endogenous binding sites for receptors due to the fact that the same chemical hormones and transmitters are operative but this is not the case for allosteric sites and a great deal more heterogeneity can be seen between human and animal receptors. Fig 9 shows the potentiation of dopamine D1 receptor effects, thought to be beneficial for cognitive defects in schizophrenia, by the PAM [rel-(9R,10R,12S)-N-(2,6-dichloro-3 methylphenyl)-12-methyl-9,10-dihydro-9,10-ethanoanthracene-12-carboxamide] (Lewis et al, 2015). With the observance of such favorable target activity, the next step would be to validate the effect as a possible therapeutic response with an animal model, in this case, the rat novel object recognition assay (Mathiasen and DiCamillo, 2010). Unfortunately, lack of corresponding allosteric effects at the rat D1 receptor (Fig 9) precludes prediction of successful corroboration of cognitive effects in the rat model

(Lewis et al, 2015). In general, there are a number of studies showing how allosteric effects seen in human receptor systems do not correspond to animal receptors and vice versa (Cho et al, 2014; Wenthur et al, 2014).

V. An Allosteric Checklist (Additional Data for Characterizing Allosterism)

While the list of allosteric properties seems to lie far towards the positive, it should be noted that an allosteric drug campaign brings with it a shortlist of additional requirements to fully characterize a modulator (as opposed to conventional orthosteric ligands). Usually, the affinity and efficacy of new ligand needs to be measured and for orthosteric ligands, this is readily accomplished through binding and fitting functional concentration-response curves to the Black/Leff operational model (Black and Leff, 1983). While the latter model works well for characterizing the efficacy of allosteric agonism, affinity may be problematic, at least for PAMs where the effective affinity of the modulator is dependent upon the nature and concentration of the co-binding ligand (agonist). Considering binding, it should be recognized that allosteric models are fundamentally different from standard mass action orthosteric models which describe receptor occupancy in terms of stochastic presence and replacement (Hall, 2006). Allosteric models describe the conversion of protein species through ligand binding and allosteric effect. For instance, a conventional orthosteric 'displacement' curve describes a competitive situation whereby a non-radioactive ligand competes with a radioactive ligand for free receptors to diminish the signal. An allosteric counterpart 'displacement curve' does not characterize physical competition but rather describes a negative allosteric molecule binding to its allosteric site to lower the natural affinity of the receptor for the radioligand which then dissociates because of the lower affinity. The observed potency (C_{50}, concentration producing half maximal effect) of an allosteric ligand is given by:

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$$C_{50} = K_B \frac{(1 + [A]/K_A)}{(1 + \alpha[A]/K_A)} \quad \ldots[1]$$

Where [A] is the concentration of the co-binding ligand, K_A and K_B the equilibrium dissociation constants of the co-binding ligand and modulator-receptor complexes, and α the co-operativity constant imposed on binding by the modulator. For negative allosteric modulators (NAMs) α usually is small (i.e. $\alpha = 0.01$; this means the modulator imposes a 100-fold reduction in the affinity of the receptor for the co-binding ligand). It can be seen from eqn 1 that as $\alpha \rightarrow 0$, the C_{50} equation devolves to the Cheng/ Prusoff correction (Cheng and Prusoff, 1973) common to orthosteric ligands and no special provision need be made for such NAMs. This is not true for positive allosteric modulators (PAMs) as in these cases, $\alpha >> 0$ and the C_{50} can be considerably different from the affinity (K_B). Therefore, a practical issue for studies with PAMs is determining receptor occupancy since this will be affected by the concentration of the co-binding ligand. The problem is compounded for functional response as the effect of the modulator on agonist efficacy becomes involved (denoted as β) and also the efficacy of the agonist and sensitivity of the system plays a role (denoted by τ_A). Under these circumstances, the potency of the modulator is given by:

$$C_{50} = K_B \frac{([A]/K_A)\,(1+\tau_A) + 1}{(\alpha[A]/K_A)(1+\beta\tau_A)} \quad \ldots..[2]$$

This can make estimation of receptor occupancy and effective concentrations problematic *in vivo* (Gregory et al, 2019).

The other unique area of characterization for allosteric modulators is the assessment of their relative interaction with multiple component systems and also their impact on pleiotropic

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signaling. The former issue concerns probe dependence with multiple natural agonists as discussed with NOVO2 and the GLP-1 receptor; all possible interactants should be tested to assess the scope of activity of the modulator. Probe dependence also can play a role in screening for new ligands. Unlike orthosteric ligands, the probe dependence practiced by allosteric molecules can confound screening efforts to find useful therapeutic entities. For example, a theoretical approach to revitalizing failing cholinergic neurotransmission in Alzheimer's disease is allosteric augmentation of acetylcholine receptor response (Maelicke and Albuquerque, 1996; Krause et al,1998). Screening for acetylcholine receptor PAMs requires agonism but acetylcholine is an unstable and unwieldy agonist to use in a screening assay and stable surrogate agonists often are employed. However, allosteric probe dependence augurs risks with these stable muscarinic agonists as surrogates; Table II shows how allosteric modulators potentiate the surrogate agonists but actually produce antagonism of acetylcholine. In general, these data support the notion that the natural agonist should be used for screening campaigns whenever possible.

As with all agonists, biased signaling should be assessed as it would be an expected outcome for any synthetic agonist. In the case of allosteric agonism, however, it may be more relevant as there are data to show that allosteric agonists tend to have a different signaling bias from standard orthosteric agonists. In a study of muscarinic m4 receptors in CHO cells, bias plots comparing [^{35}S]GTPγS and ERK1/2 responses, a clear distinction has been observed between orthosteric and allosteric agonists. Specifically, conventional orthosteric agonists are biased toward GTPγS, the allosteric agonists conversely are biased toward ERK1/2 (Gregory et al, 2010).

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An added consideration with allosteric PAMs and NAMs is induced bias with natural signaling, i.e. an allosterically-induced change in the quality of natural efficacy. Thus, a PAM or a NAM may change the nature of the natural agonist signal and change the physiology accordingly. For example, neurokinin produces Gαq and Gαs protein activation through the NK2 receptor; the NAM LP1805 blocks only the Gαs response and leaves the Gαq response intact thereby changing the nature of neurokinin signals in vivo (Maillet et al, 2007). Similarly PDG2 activates CRTH2 receptors to activate Gαi protein and β-arrestin; the NAM Indole[1] blocks the β-arrestin response but leaves the Gαi signal unblocked thereby changing the nature of natural PDG2 signaling (Mathiesen et al, 2005). The same effect can be seen with PAMs; for example, the PAM Novo2 potentiates cyclic AMP signaling, but does not potentiate calcium signaling; in contrast, the PAM quercetin, reverses this effect and potentiates calcium but not cyclic AMP (Koole et al, 2010)

Once a molecule has been identified, it is important to characterize its pharmacologic properties; this is because drugs work in concert with physiology and give different profiles of activity depending on the physiological state of the organs with which they interact (i.e. receptor expression levels, relative stoichiometry of receptors to signaling proteins). Characterizing drug properties in terms of system independent parameters (i.e. affinity, efficacy) allows use of these numbers to predict drug response in a range of systems, not only the system where the initial measurements are made. While quantification of affinity and efficacy can be used to characterize orthosteric molecules, these parameters fall short for allosteric molecules. This is because of the interactive nature of allosteric systems since in these, the parameters that measure the relative effects of natural agonists in the absence and presence of allosteric modulators are relevant to in vivo response. These are α (changes in affinity) and β (changes in efficacy) alterations and once

these are measured, then the effects of a modulator, on a wide range of concentrations of natural agonist, can be predicted. Fitting experimental dose-response data with agonists and allosteric modulators to the functional allosteric model (Kenakin, 2005; Ehlert, 2005; Price et al, 2005; Gregory et al, 2012) enables this to be done. It can be shown that very different behaviors of PAMs seen in systems of extremely varying sensitivities can be uniformly quantified with a single set of allosteric parameters. Thus, in cells of high sensitivity, the PAM-Agonist BQCA produces direct agonism and potentiation of acetylcholine response whereas in tissues of lower sensitivity, BQCA produces only potentiation with no agonism; in a very low sensitivity systems, BQCA produces elevation of acetylcholine maximal response (Bdioui et al, 2018). These very different activities all can be modeled with the functional allosteric model with a single set of allosteric parameters (α=10 to 15, β=2, τ_B=1.2 to 1.4% τ_A, K_B-5μM) thereby giving medicinal chemists a stable set of parameters on which to base structure activity relationships (Bdioui et al, 2018).

VI. Conclusions

In general it can be seen that allosteric modulation is a flexible strategy for modifying physiology for therapeutic effect with a wide range of applications. However, additional experimentation, with attention to measuring quantitative parameters in the presence of co-binding ligands, probe dependence for natural multiple probes, signaling bias and induced signaling bias, are also important aspects of programs aimed at characterizing allosteric ligands.

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	Table I: Types of Allosteric Modulator	
Ligand	**Definition**	**Pharmacologic Effect**
Allosteric Agonist	Molecule that binds to a site on the receptor separate from the natural agonist (orthosteric) binding site that produces agonist response	Production of cellular response with possible modifications by natural agonism (either potentiation, additivity, or inhibition)
PAM (Positive Allosteric Modulator)	Molecule that binds to a site on the receptor separate from the natural agonist (orthosteric) binding site to potentiate the natural agonist response	Produces no direct effect until the natural agonist produces response; then the natural agonist response is increased.
PAM-Agonist	Positive Allosteric Modulator (as opposed to agonist) with added direct efficacy to produce response with no natural agonist present.	In sensitive tissues a Pam-Agonist will produce direct agonist response; when the natural agonist is present it will augment natural agonist response as well
NAM (Negative Allosteric Modulator)	Molecule that binds to a site on the receptor separate from the natural agonist (orthosteric) binding site to inhibit the natural agonist response	Produces no direct effect until the natural agonist produces response; then the natural agonist response is blocked.
NAM-Agonist	Negative Allosteric Modulator (as opposed to agonist) with added direct efficacy to produce response with no natural agonist present.	In sensitive tissues a Nam-Agonist will produce direct agonist response; when the natural agonist is present it will block natural agonist response as well
PAM-Antagonist	NAM with increasing affinity for the receptor n the presence of the agonist	PAM-Antagonists increase the affinity for the agonist but decrease the efficacy of the agonist

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Table II Effects of Allosteric Modulators on Responses to Acetylcholine and Stable Surrogate Acetylcholine Receptor Agonists

Target	Modulator	Surrogate Agonist	Effect on Surrogate Agonist	Effect on Natural Agonist (Acetylcholine)
Muscarnic m2	Alcuronium	Pilocarpine	2.7x potentiation	***10x Antagonism***
Muscarinic m4	Strychnine	Pentylthio-TZTP	3x potentiation	***4x Antagonism***
Muscarinic m2	Brucine	Pentylthio-TZTP	8x potentiation	***3.5x Antagonism***

Probe dependence at the screening stage for PAMs aimed at potentiating failing acetylcholine response in Alzheimer's disease. Stable muscarinic agonists used for screening indicate potentially useful potentiation but subsequent study with the natural agonist acetylcholine demonstrates antagonism. Data from Jakubic et al, 1997.

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Figure Legends:

Fig 1 Summary of the unique properties of allosteric modulators resulting from the two main properties of allosteric proteins. Letters and numbers refer to positions of discussion of the various properties in the text.

Fig 2 Saturation of allosteric effect due to full occupancy of the allosteric site. A. Schematic diagram of two binding sites on the receptor, one for the natural agonist (orthosteric site) and the other for the allosteric modulator. B. Limited antagonism of agonist responses by a NAM. The response in the presence of increasing concentrations of NAM B are shown in red moving to the right as the concentration of B increases ($[B]/K_B$ = 3, 10, 30, 100, 300). Black dotted lines represent analogous DR curves in the presence of an orthosteric competitive antagonist at the same concentrations. Arrows show the respective dose ratios for each concentration.

Fig 3. A. Dose response curve in vivo to a natural agonist in black; same curve in the presence of PAM1 (which produces a 50-fold sensitization to natural agonism) in red. Blue vertical arrow indicates the in vivo effect of PAM_1 (above toxicity level). B. Red curve is for a different PAM (PAM_2 which produces a 5-fold sensitization); blue arrows shows the maximal effect of PAM_2 never will attain toxicity. In this case it is assumed that the basal physiological response is produced by 1 μM natural agonist.

Fig 4. Effect of an orthosteric (alvameline) and allosteric (BQCA) partial agonist on acetylcholine responses. A. Schematic diagram of the relative binding geography of BQCA, acetylcholine (ACh) and alvameline. B. Concentration response curves for IP1 production mediated through muscarinic m1 receptors in CHO cells by ACh in the absence (filled circles)

and presence of alvameline (100 μM; open circles) and BQCA (10 μM, open triangles) dotted line curve. Assuming a basal muscarinic receptor tone in vivo of 46%, it can be seen that alvameline would produce an inhibition of basal tone and BQCA an augmentation of basal tone. Data for panel B simulation from Bdioui et al, 2018.

Fig 5. Schematic diagram demonstrating three separate functional allosteric binding sites on GPR40. Binding locations from Wang et al (2021).

Fig 6. A: Schematic diagram of two proteins binding at multiple loci; an orthosteric ligand can interfere with only one of those whereas an allosteric ligand producing a global change in conformation may interfere with more than one site.

Fig 7 Selective inhibition of HV-1 entry over CCL3L1-induced CCR5 internalization by the allosteric modulator TAK 652. Data from Muniz-Medina et al, 2009.

Fig 8 Bargraph shows potentiation of EC_{50} values of GLP-1 receptor agonists produced by NOVO2. With the exception of exendin-4, peptide fragments are all natural agonists of the receptor with GLP-1(7-36)NH_2 being the most important. It can be seen that whereas NOVO2 produces a powerful potentiation of the minor agonist oxyntomodulin, the effects on the most relevant agonist are mild. Data from Koole et al, 2010.

Fig 9 Preclusion of verification of human dopamine receptor PAM activity in a therapeutic animal model. The potentiation of human dopamine D1 receptor responses seen for human receptors (control = filled black circles; presence of 15 μM CMPD B open circles) indicates an 11-fold sensitization to dopamine. In contrast, the same concentration of CMPD B (15 μM) has no effect on the rat dopamine receptor. This makes verification of cognitive effects in a rat model futile. Data for human and rat DR simulated curves from Lewis et al, 2015.

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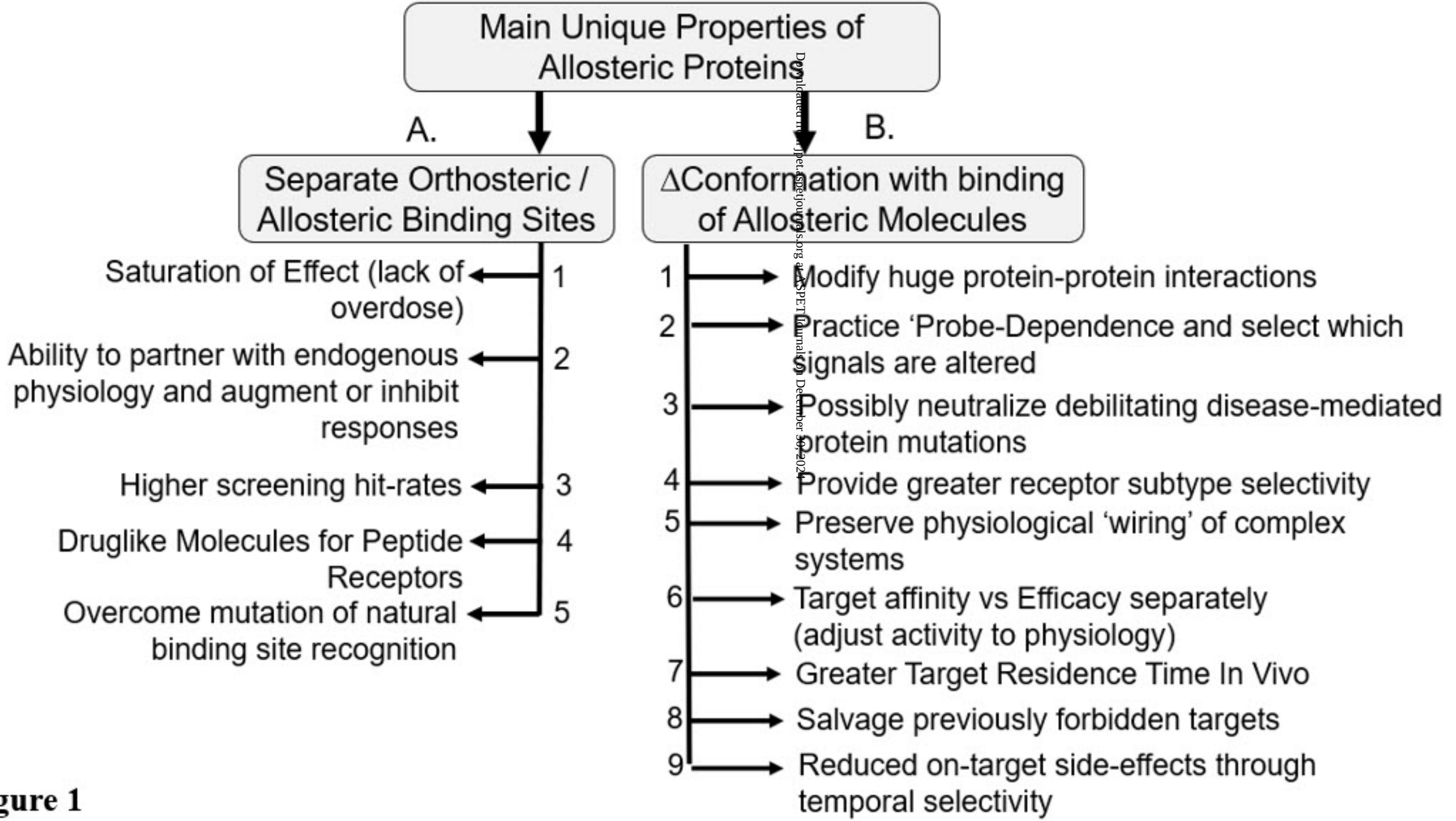

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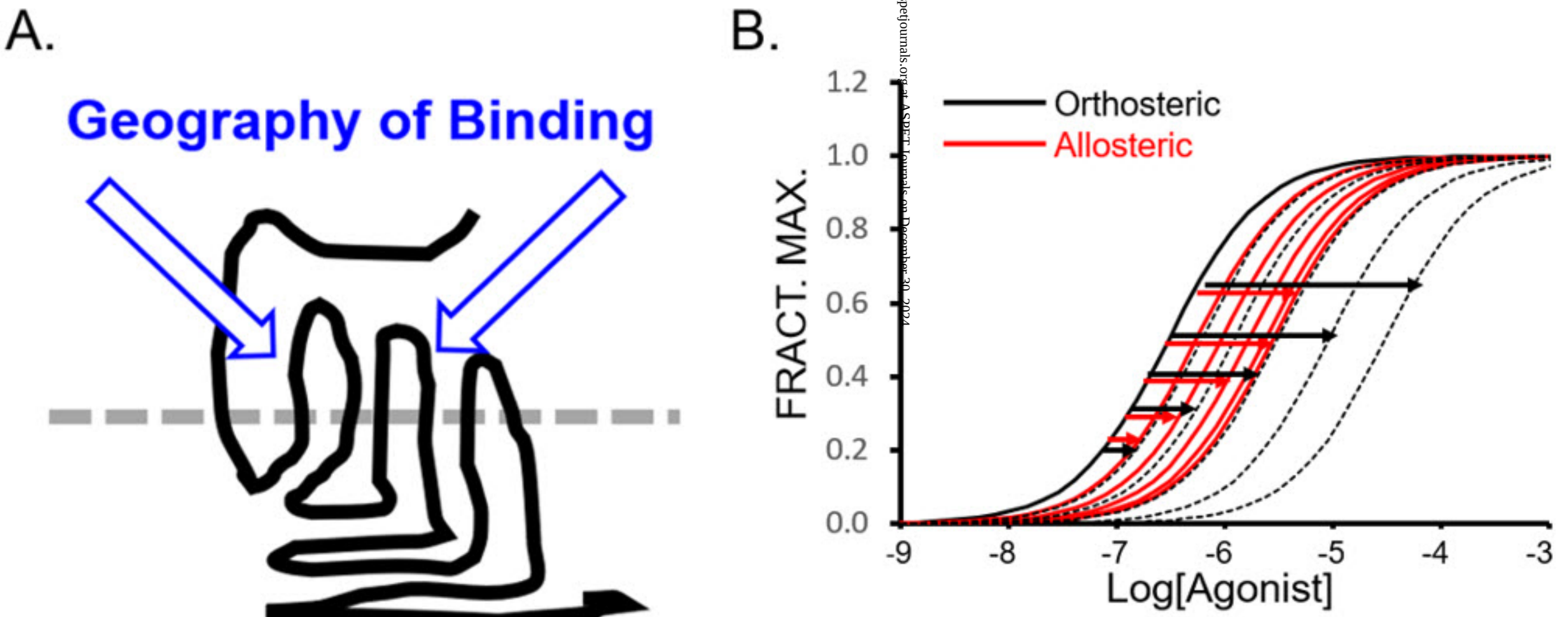

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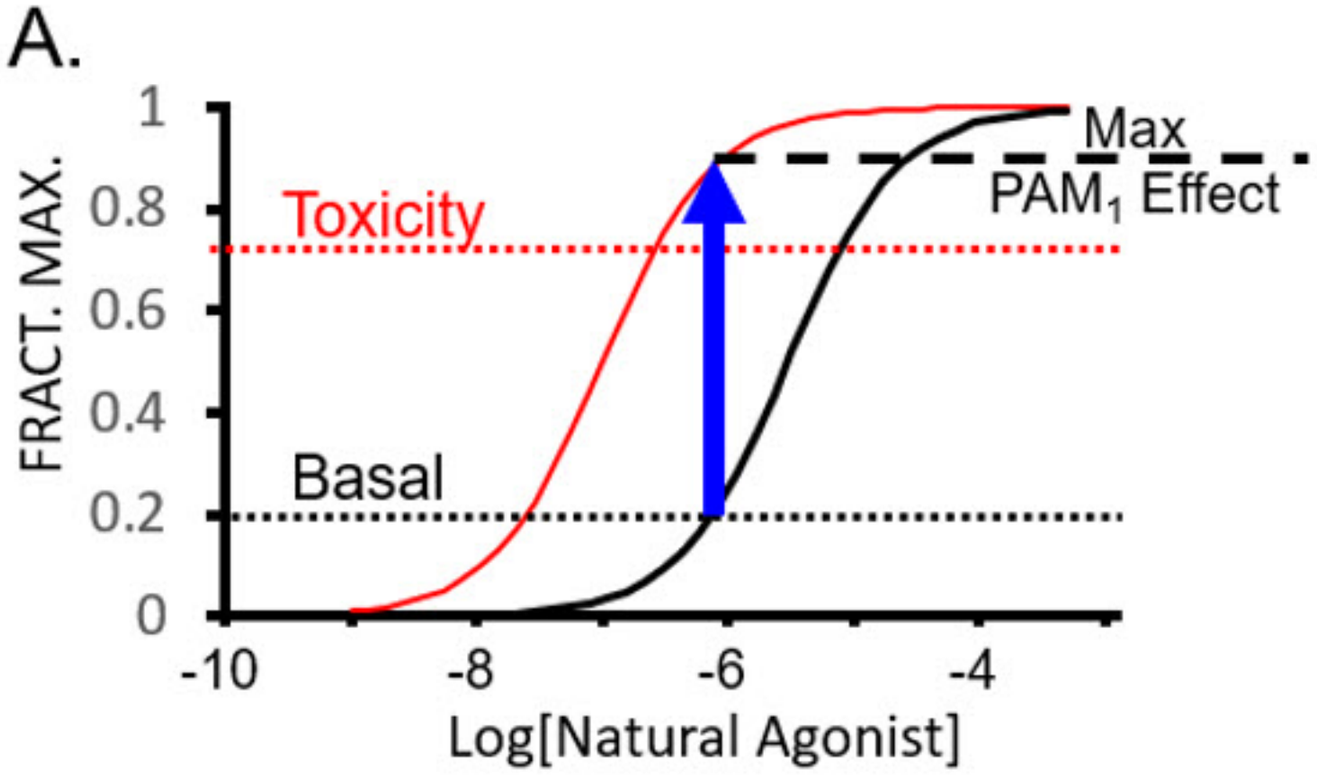

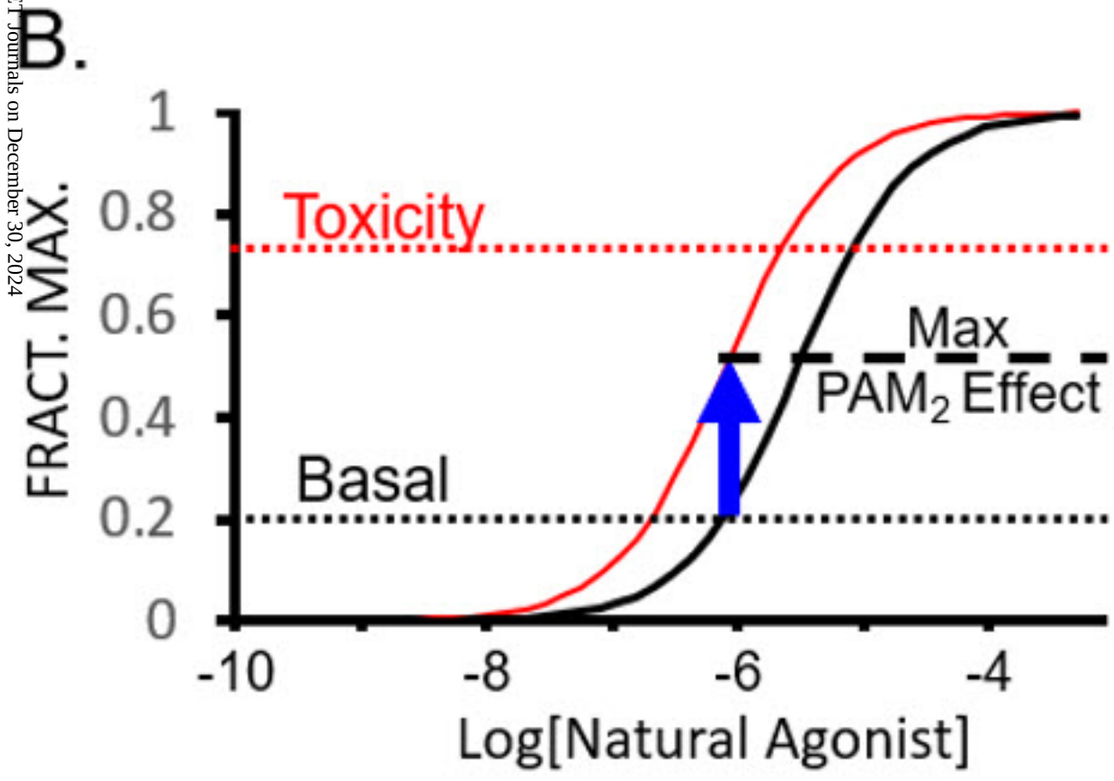

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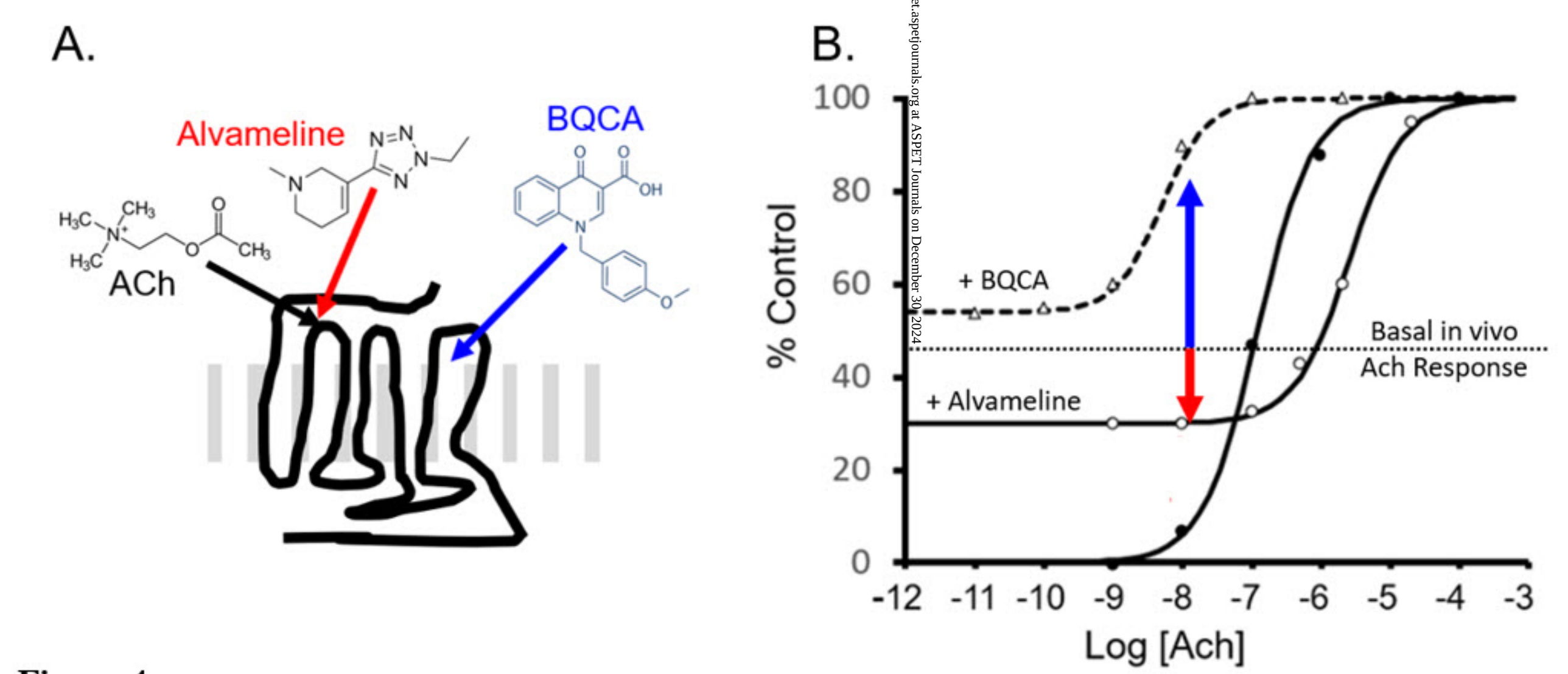

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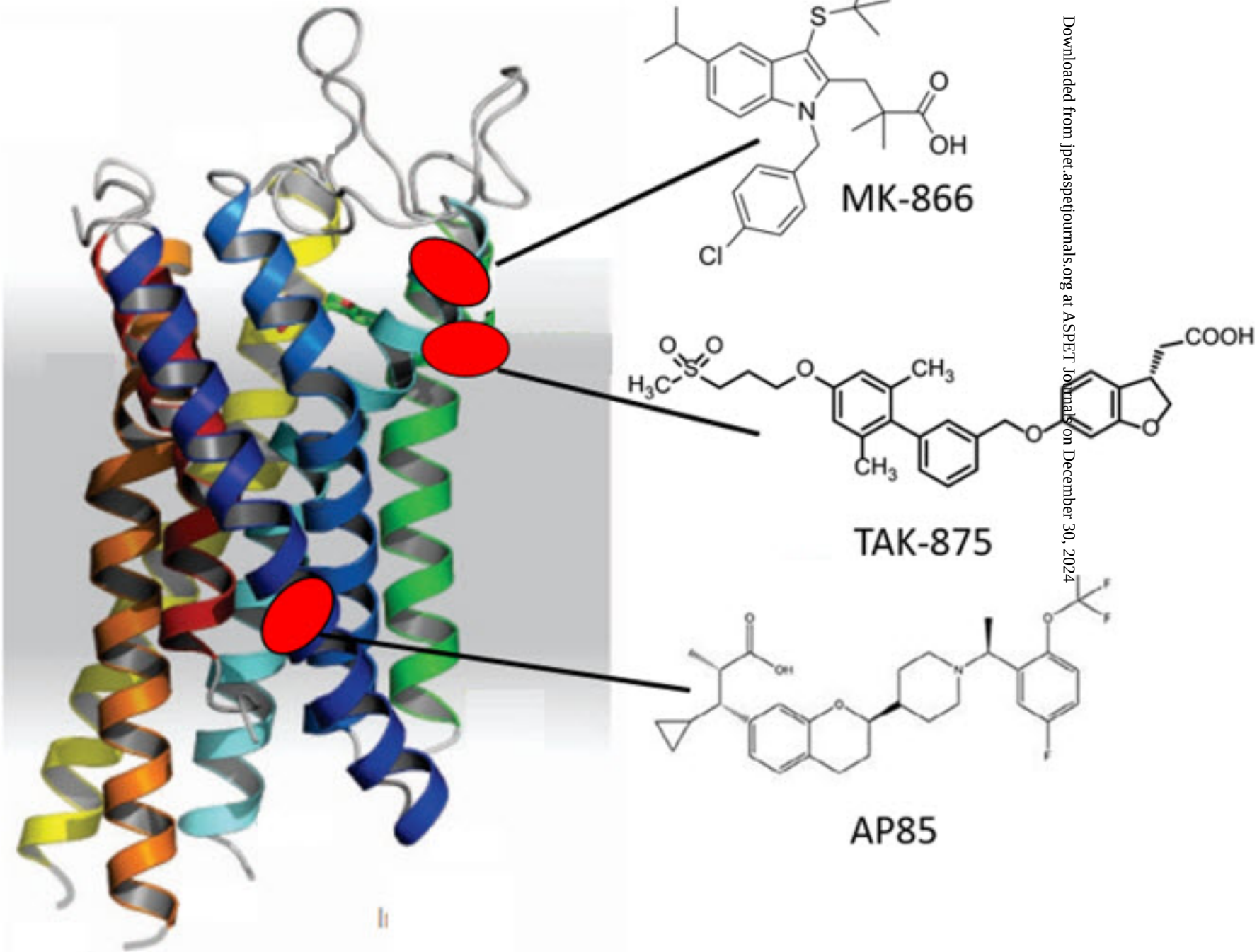

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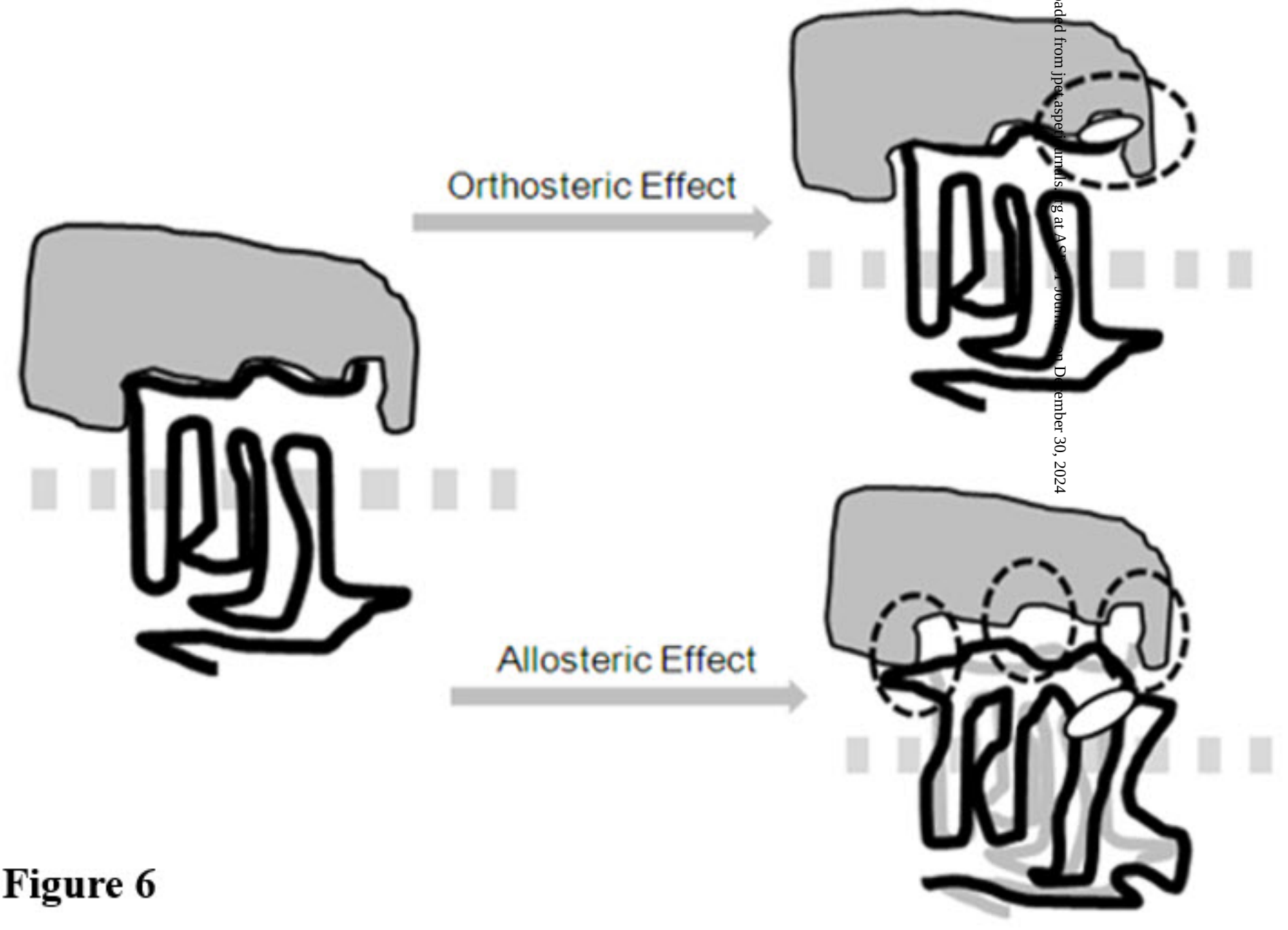

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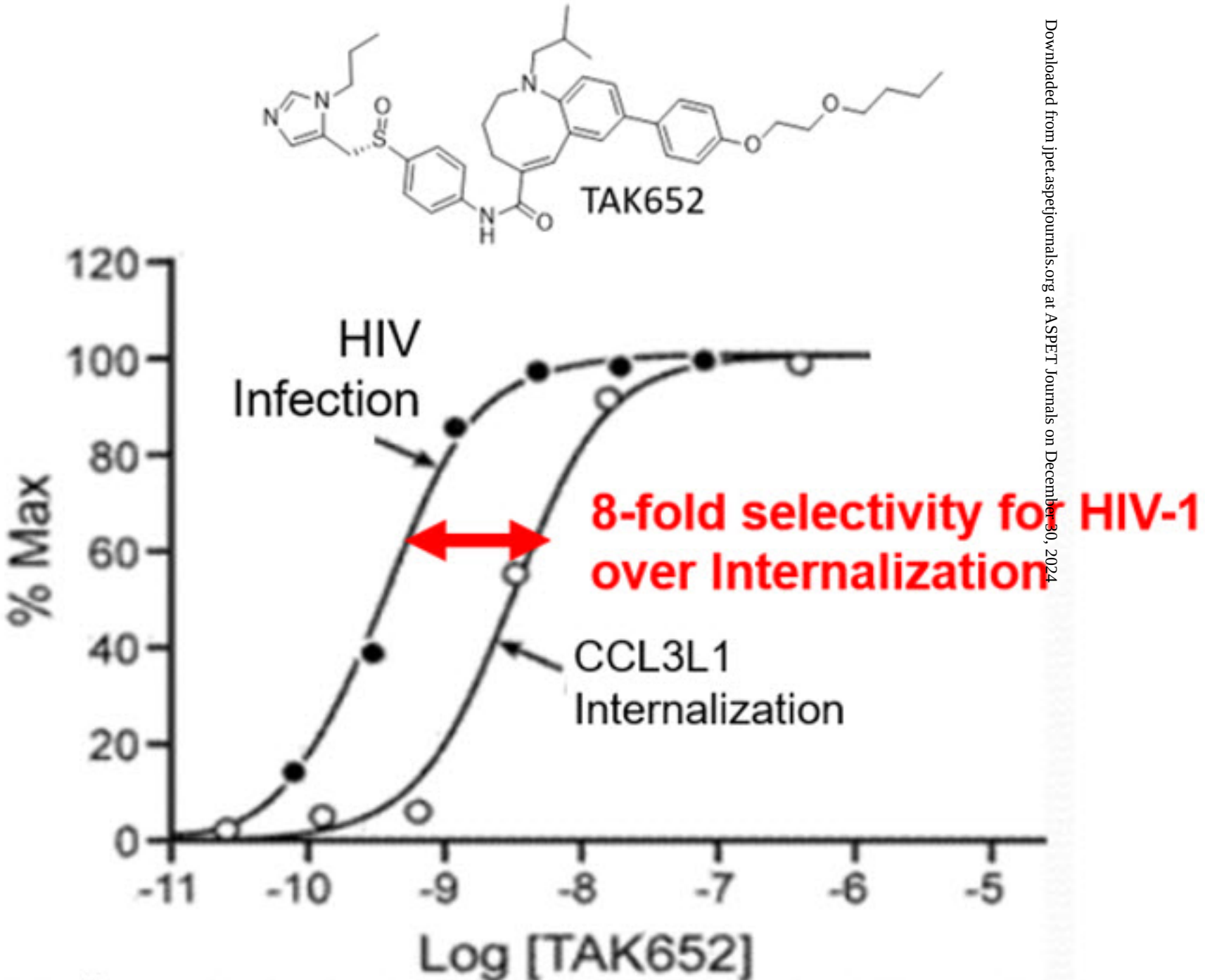

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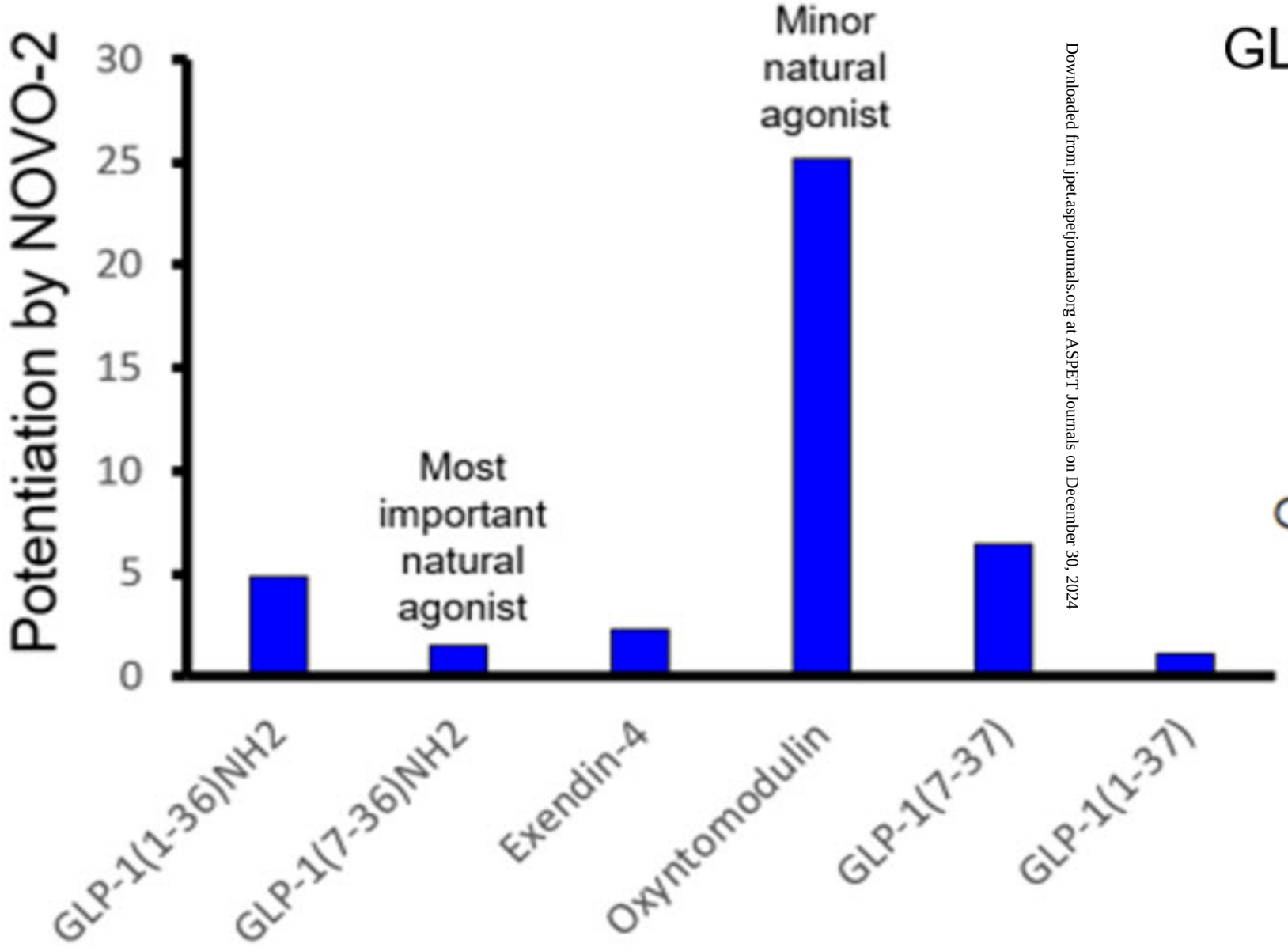

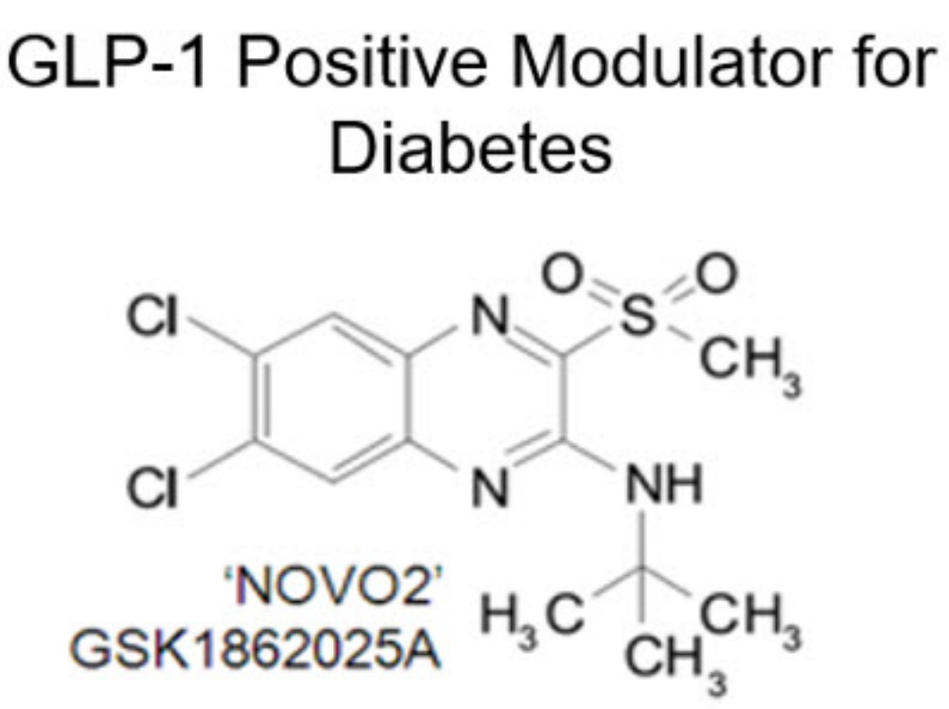

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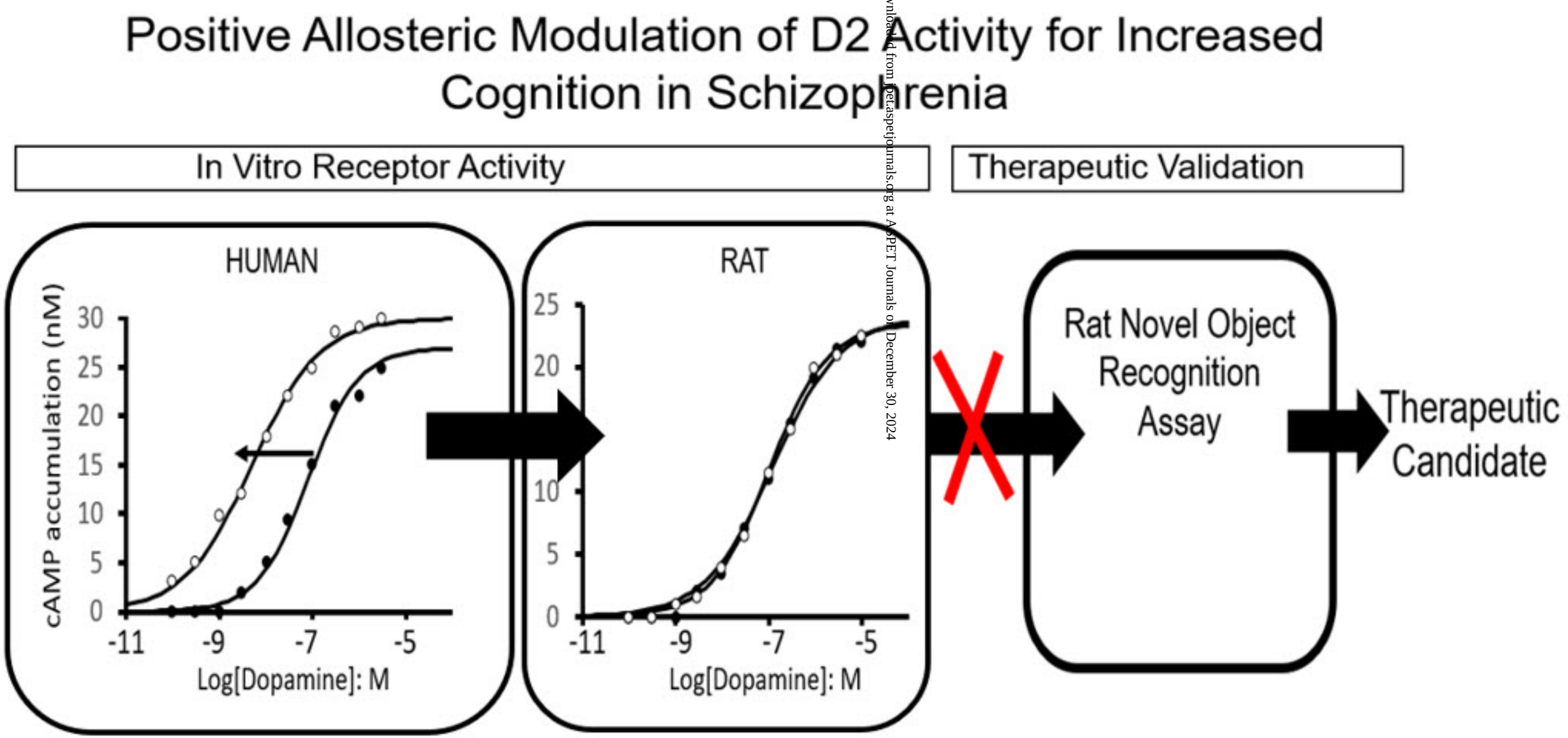

Figure 9