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REVIEW ARTICLE

Dopamine D_2 Receptors Dimers: How can we Pharmacologically Target Them?

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Abstract: ***Background*:** Dopamine D_2 and D_3 receptors can form homo- and heterodimers and are important targets in Schizophrenia and Parkinson's. Recently, many efforts have been made to pharmacologically target these receptor complexes. This review focuses on various strategies to act specifically on dopamine receptor dimers, that are transiently formed.

***Methods*:** Various binding and functional assays were reviewed to study the properties of bivalent ligands, particularly for the dualsteric compound SB269,652. The dimerization of D_2 and D_3 receptors were analyzed by using single particle tracking microscopy.

***Results*:** The specific targeting of dopamine D_2 and D_3 dimers can be achieved with bifunctional ligands, composed of two pharmacophores binding the two orthosteric sites of the dimeric complex. If the target is a homodimer, then the ligand is homobivalent. Instead, if the target is a heterodimer, then the ligand is heterobivalent. However, there is some concern regarding pharmacokinetics and binding properties of such drugs. Recently, a new generation of bitopic compounds with dualsteric properties have been discovered that bind to the orthosteric and the allosteric sites in one monomeric receptor. Regarding dopamine D_2 and D_3 receptors, a new dualsteric molecule SB269,652 was shown to have selective negative allosteric properties across D_2 and D_3 homodimers, but it behaves as an orthosteric antagonist on receptor monomer. Targeting dimers is also complicated as they are transiently formed with varying monomer/dimer ratio. Furthermore, this ratio can be altered by administering an agonist or a bifunctional antagonist.

***Conclusion*:** Last 15 years have witnessed an explosive amount of work aimed at generating bifunctional compounds as a novel strategy to target GPCR homo- and heterodimers, including dopamine receptors. Their clinical use is far from trivial, but, at least, they have been used to validate the existence of receptor dimers *in-vitro* and *in-vivo*. The dualsteric compound SB269, 652, with its peculiar pharmacological profile, may offer therapeutic advantages and a better tolerability in comparison with pure antagonists at D_2 and D_3 receptors and pave the way for a new generation of antipsychotic drugs.

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1. INTRODUCTION

Dopamine receptors belong to the monoaminergic G protein-coupled receptor (GPCR) family and are an important pharmacological target in Schizophrenia, Parkinson's disease, ADHD and drug abuse [1]. These receptors play a key role in the dopamine homeostasis that is relevant for motor activity, cognitive function, memory and reward [2]. Other neurotransmitters modulate dopamine release, *i.e.* in the reward system of the brain (brain reward cascade, BRC) [3]. The five dopamine receptors are divided into D1-like (D_1 and D_5) and D2-like (D_2, D_3 and D_4) receptors in relation to their functional and pharmacological properties, where the D_2 receptor generates, by alternative splicing, the long and the short isoforms (D2Long and D2Short).

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In addition, the GPCR family seems to diversify and amplify its repertoire of signaling by forming homo- and heterodimers, or even higher order oligomers.

In fact, the dopamine receptors follow molecular behavior of generating receptor complexes, and their homo- and heterodimers are endowed with new functional and pharmacological characteristics [4-6]. The existence of distinct receptor complexes in specific tissues makes them a pharmacological target for new compounds with improved efficacy and/or lesser side effects. Consistent with this concept, many efforts were made to develop "magic bullets" drugs targeting receptor dimers, and among these, the bifunctional compounds have gained a relevant consideration within the scientific community [7]. One such class of bifunctional compounds are the bivalent ligands that are composed of two pharmacophores, linked together by a spacer, which are able to simultaneously bind the two orthosteric sites of the dimeric complex [8]. These compounds are a powerful tool to identify the presence of dimers in different biological systems. Additionally, they may restrict their action in specific tissues expressing receptor homo- and/or heterodimers that may induce specific receptor signaling pathways [9]. Portoghese *et al*. initially introduced the concept of bivalent ligands for opioid receptors [10]. However, the dual targeting of the dimer requires important characteristics for the bivalent ligand and some studies have indeed questioned their actual capability of binding to both the receptors simultaneously [11]. In addition, these ligands, by having usually rather high molecular weights, show a poor oral absorption, the tendency to get rapidly degraded and a limited accessibility to the brain because of the blood-brain barrier [12]. Another elegant strategy to target dopamine receptors, including the dimeric complexes, is a bifunctional ligand that lays down extensively in a bitopic mode from the orthosteric site into a secondary allosteric pocket in the monomeric receptor. One of the most extensively studied bitopic ligand is the compound SB269,652 that has a very peculiar pharmacological behavior, such as though being an orthosteric antagonist for the monomer, it behaves as a negative allosteric across receptor dimers [13]. Hence, their therapeutic properties are versatile depending on the quaternary state of dopamine receptors. Considering the dynamics and reversible nature of dopamine receptor dimers, dualsteric ligands such as SB269,652 can have different pharmacological properties in different areas of the brain based on the percentage of dimers with respect to their monomeric fraction [14]. New scientific evidence based on single-molecule microscopy (SMM) has in fact revealed the transient dynamics of many GPCR dimers, including dopamine receptor family, where receptors display a monomer-dimer equilibrium characterized by rapid association and dissociation [15]. This complicates the picture about targeting receptor dimers, but it also adds an important variable that can be pharmacologically modulated. Indeed, at steady state, 30% of dopamine receptors are part of dimeric complexes and this percentage increases in the presence of some ligands, including bifunctional compounds [16]. These conclusions have been reached through a multidisciplinary approach based on different methodologies such as SMM, medicinal chemistry and molecular modeling. Today, this integrative strategy seems mandatory in order to understand the complexity of receptor dimerization and to find new drugs that are able to target receptor dimers.

2. THE STRATEGY OF BIVALENT LIGANDS TO TARGET DOPAMINE RECEPTOR DIMERS

Since the discovery of the existence of GPCR dimers, many efforts have been made to find new drugs that are able to target these receptor complexes. One particular strategy relies on bivalent ligands composed of two pharmacophores binding the two orthosteric sites of the dimeric complex. If the target is a homodimer (Fig. **1A**), then the two pharmacophores are the same, and the ligand is referred as homobivalent ligand. However, if the target is a heterodimer (Fig. **1B**), the two pharmacophores are different, and the ligand is called heterobivalent ligand [17]. An informative research work pioneered by Portoghese *et al*. resulted in successfully developing the first bivalent ligands for opioid receptor dimers [18]. Generally, bivalent ligands may restrict their action in specific tissues expressing receptor homo- or heterodimers, offering significant therapeutic advantages and they can be used as molecular tools to investigate GPCR dimerization [9]. In fact, in many cases, the quantity and type of these receptor complexes not only change among organs, but also within the same tissues. For instance, certain areas of the brain may express different amounts of dopamine receptor dimers. After the development of bivalent ligands for opioid receptors, a relevant number of bifunctional molecules have been synthesized to act on GPCR dimers, including dopamine receptors.

An early attempt to synthesize bivalent ligands for dopamine D_2 receptor was made by Huber *et al*. using ferrocene-derivatives formed by two pharmacophores, two linkers and a spacer [19]. The ferrocene part provides the required flexibility to the bivalent molecule that permits high affinity binding of the phenylpiperazine pharmacophores to the two orthosteric sites of D_2 dimer. After these early attempts, several bivalent ligands for D_2 dimers were designed using as a pharmacophore: 1,4-disubstituted aromatic piperazine (1,4-DAP) [20], propylaminoindane- and phenylpiperazine-derivative [21], amidoalkyl substituted phenylpiperazine [22], Clozapine [23], 5-hydroxy-2-(dipropylamino) tetralin (5-OH-DPAT) [24], Apomorphine [25], Haloperidol [26] and Ropirinole [27].

A gain in affinity or activity may be found for a bivalent ligand compared to its monovalent counterpart. The binding of the first pharmacophore increases the local concentration of the second tethered unit in proximity of the other orthosteric binding site and this significantly increases the second binding event. Moreover, the binding of a ligand to one monomer can influence the binding of the second tethered ligand to the second monomer in a positive fashion called positive allosterism. Positive allosterism was nicely shown for doubling the dopamine agonist 5-OH-DPAT moiety with a 95-fold increase in potency and a 30-fold increase of affinity compared to the parent compound [24]. Similar results were observed with bivalent ligands made of Clozapine with a relevant increase of affinity (80-fold) and a mild increase of potency (5-fold) [23]. Jorg *et al*. synthesized a series of homobivalent ligands of Ropirinole using spacers of different lengths [27]. The best compounds showed a 20 to 80-fold

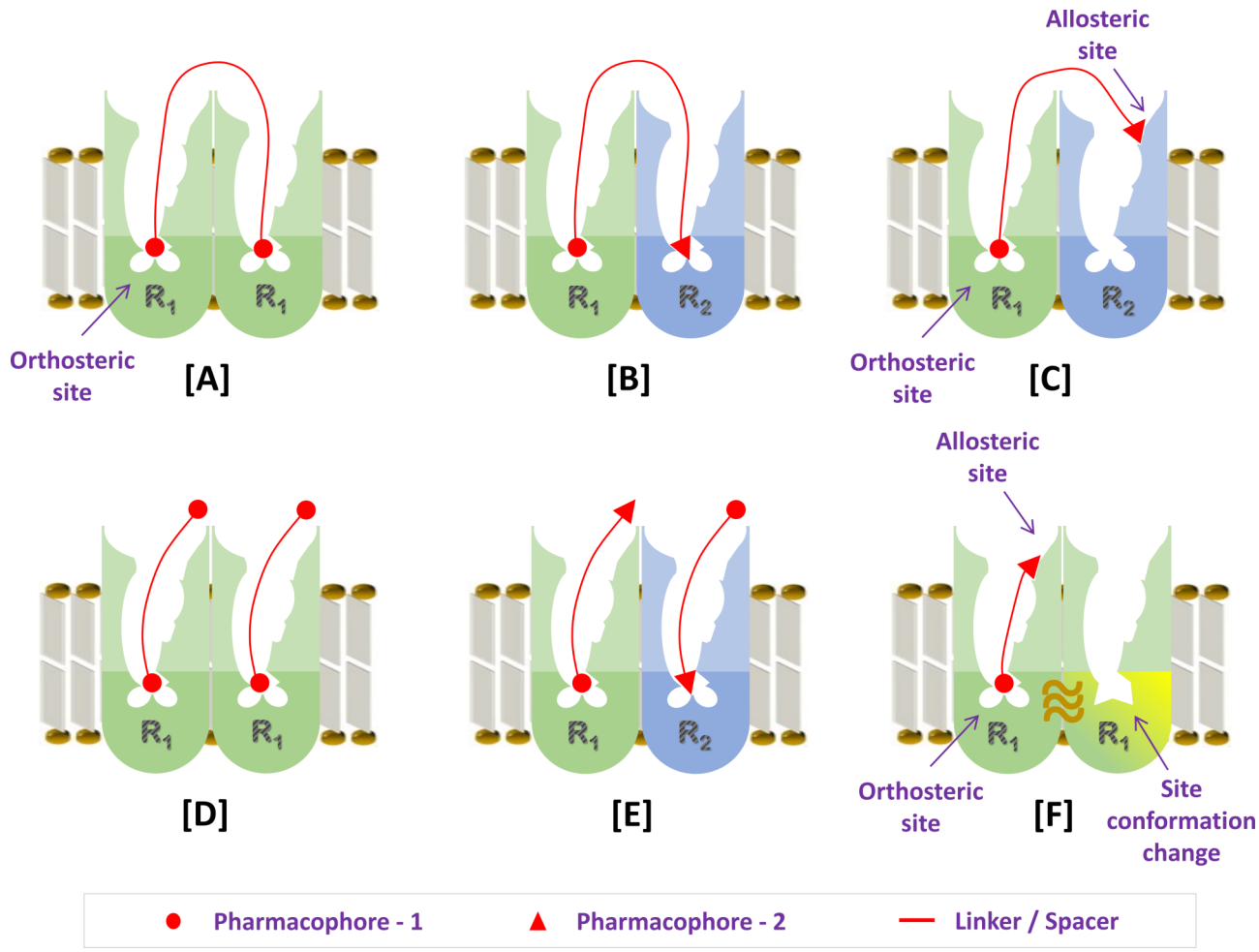

Fig. (1). A schematic view of bivalent-ligand binding modalities for GPCR dimers. (**A**) A *homobivalent ligand* consisting of two identical pharmacophores attached to respective linkers and connected by a spacer of variable length targeting simultaneously the orthosteric sites of a homodimer complex. (**B**) A *heterobivalent ligand* consisting of two different pharmacophores targeting simultaneously the orthosteric sites of a heterodimer complex. (**C**) A *heterobivalent ligand* consisting of two different pharmacophores targeting simultaneously an orthosteric site of one monomer and an allosteric site of other monomer of a heterodimer complex. (**D**) Dual acting *homobivalent ligands* consisting of two identical pharmacophores targeting the orthosteric sites of a homodimer complex with two different molecules and the simultaneous binding is not required. (**E**) Dual acting *heterobivalent ligands* consisting of two different pharmacophores targeting the orthosteric sites of a heterodimer complex with two different molecules and the simultaneous binding is not required. (**F**) A *bitopic compound* consisting of two different pharmacophores connected by a linker of variable length targeting both the orthosteric and the allosteric sites of the same monomer of a homodimeric complex. The binding of the dualsteric ligand induces a negative allosterism on the other monomer.

increase in potency and the efficacy was more or less same or slightly augmented. Finally, Huber *et al*. found few amidoalkylphenylpiperazine based bivalent ligands with increased affinity for D_2 receptor (7 to 15-fold respect to the monovalent counterpart), while the gain in affinity was greater for D_3 receptor [22].

Though the examples above have shown an increase in the affinity and potency for some bivalent ligands, this was not seen in other compounds [20, 21, 25, 26]. This could possibly be explained due to a reduction in the affinity of the original monovalent ligand owing to molecular changes made in the bifunctional compound, that somehow impairs its interaction with the orthosteric site and/or due to negative cooperation between the two monomers in that specific dimer complex [28]. For example, bivalent ligands based on aminoindane and phenylpiperazine derivatives did not show any gain in affinity for D_2 receptor [21]. In addition, compounds containing two Apomorphine pharmacophores linked by different spacers (12, 14 and 16 atoms) showed a reduction in affinity and efficacy with respect to Apomorphine. Interestingly, with a spacer length of 18 to 28 atoms, the bifunctional compounds regained affinity or efficacy similar to Apomorphine [25]. Bivalent compounds formed with two Haloperidol molecules designed by Salama *et al*. did not show any gain in affinity or potency compared to the parent compound [26].

Positive or negative types of cooperation among receptor monomers can be quantified by the Hill coefficient that is extrapolated by ligand binding curves. In experiments involving competitive radioligands, the use of a pure orthos-

teric antagonist binding to a single receptor should have a Hill coefficient around 1. Conversely, it has been shown that some bivalent ligands, such as compounds containing the two 1,4-DAP pharmacophores, have a Hill coefficient between 1.6 and 2.0. Salama *et al*. observed the Hill coefficient of 1.8 with Haloperidol bivalent compounds [26]. These indicate a positive cooperative binding within the dopamine dimer, where the vicinity of the second tethered pharmacophore facilitates its simultaneous binding to the second site, equated to liberation of two equivalents of radioligand. This brings to a steepening of the competition curve with a Hill coefficient higher than 1.

Strikingly, as already mentioned before, GPCRs are able to interact with other GPCR subtypes to form heterodimers. For targeting heterodimer complexes, Portoghese's work has inspired other groups for synthesizing many compounds such as ligand KDN-21, which belongs to a series of bivalent ligands containing δ-opioid and κ-opioid antagonist pharmacophores attached to variable-length spacers [29]. Regarding heterobivalent ligands targeting dopamine heterodimers, indeed their role in certain diseases seems promising [30]. Saveanu *et al*. were first to report on bivalent ligand where they synthesized a somatostatin-dopamine molecule (BIM-23A387) directed against the D_2/SST_2 receptor complexes [31]. Recently, a series of bivalent ligands for D_2-A_{2A} receptors were developed as a pharmacological strategy for Parkinson's disease. The dopamine-adenosine heterobivalent ligands have been useful in determining the presence of heteromers in striatal tissue, and they showed an increase in affinity in the presence of both receptors [32]. The quaternary structure of the D_2-A_{2A} receptor complex is believed to be a heterotetramer, *i.e.* dimers of the dimers [33]. This oligomeric complex can explain the modulatory effect reported for both A_{2A} agonists and antagonists on D_2 receptor. The binding of an agonist or an antagonist to the A_{2A} homodimer determines the same allosteric modulation to D_2 receptor activity. The existence of these complexes has been demonstrated by bioluminescence resonance energy transfer, bimolecular fluorescence and bioluminescence complementation [34]. It would be interesting to verify if other heterotetramers exist among dopamine receptor heteromers.

The simultaneous dual targeting of the homo- and heterodimers (Fig. **1D-E**) is however far from trivial, as some studies have suggested that different bivalent ligands might simply interact with their monomeric counterparts separately through a dual acting mechanism [11]. This is further complicated by the fact that affinities of bivalent ligands are often determined in comparison with the monovalent ligand parent, while the use of 'dummy' bivalent ligands (where one of the pharmacophores should be replaced with a non-binding moiety structurally related) would be a better control. Kühhorn *et al*. have shown this by using 'dummy' bivalent ligands as a negative control for homobivalent ligands binding D_2 receptor [20]. Recently while studying CB_1 receptor dimers, Glass *et al*. proposed that several bifunctional ligands, which were supposed to bind the dimer, might not be able to bind to CB_1 receptor complexes simultaneously [11]. Even if they do so, they might only be able to pass through the lipid bilayer and not through an external path. This is something that should be kept in mind when studying bifunctional ligands, especially for those ligands targeting lipid receptors.

Other important issues that must be taken into consideration in the synthesis of bivalent ligands are their large size and high molecular weight [12]. All these can severely reduce bioavailability and impair the ability to target the brain because of the blood-brain barrier, which therefore limits their usage in clinical and *in-vivo* studies. In fact, a bifunctional molecule is a complex structure made of two pharmacophores, two linkers and a spacer of variable length, built in such a way that it still keeps its dual binding properties with high affinity. Hence, the molecular weight of bivalent compounds is considerably higher than that of marketed oral drugs. Larger, more lipophilic and more flexible molecules are often associated with poorer oral absorption profiles. In fact, though many bivalent compounds have the desired *in-vitro* profile, they lack the *in-vivo* pharmacokinetic characteristics required for their further development as oral drugs [17]. Jorg *et al*. have also criticized the potential clinical use of such bivalent ligands [27]. However, these compounds may still represent a useful pharmacological tool to validate dimer existence *in-vivo*, and more importantly could set a path for the development of novel and more efficacious strategies for the treatment of complex diseases [8].

3. A NEW APPROACH TO TARGET DOPAMINE RECEPTOR DIMERS: BITOPIC LIGANDS WITH DUALSTERIC PROPERTIES

Another important avenue for drug discovery derives from the fact that some GPCRs contain a distinct allosteric binding site in the monomeric structure, and this allows the modulation of receptor function not only by orthosteric drugs, but also by specific allosteric compounds [35]. Allosteric sites are typically less conserved among receptor subtypes, and this may therefore offer the possibility of developing more selective ligands toward specific GPCRs [36]. Related to this, recently a new generation of bitopic compounds with dualsteric properties has been discovered that binds simultaneously to the orthosteric and the allosteric sites in one monomeric receptor [6, 13]. The concept of the bitopic compounds may be considered as a diversification or extension of the bivalent ligands strategy explained above. The bitopic compounds or the dualsteric ligands (Fig. **1F**) are a new class of drugs that are designed to achieve receptor subtype selectivity and higher affinity when compared to orthosteric drugs. Specifically, the allosteric part of the ligand should be able to recognize sites that are less conserved among receptor subtypes, thereby providing an increased specificity. Importantly, while generating a bitopic compound, two paramount steps need to be carefully evaluated: the size of the linker, which would determine the ability to simultaneously bind the orthosteric and the allosteric sites and, where and how the linker should be attached to the respective pharmacophores so as not to impair the pharmacological properties of each pharmacophore. In principle, dualsteric ligands can be either agonists or antagonists on the orthosteric site and have a positive or negative allosterism on the allosteric site. Hence, their pharmacodynamics depends on the orthosteric-allosteric combination of the two pharmacophores. By targeting allosteric receptor sites, these ligands

not only have higher specificity for GPCR family subtypes, but they might also stabilize specific receptor active conformations that are linked to biased agonism switching on specific routes of intracellular signaling. For instance, Grossmüller *et al*. demonstrate this switching properties using synthesized bitopic muscarinic M_2 acetylcholine receptor (M_2 mAChR) ligands [37]. As described previously for bivalent ligands, it is important the size of the bitopic molecule and how the three parts *i.e*. the two pharmacophores and the linker are assembled. Compounds that exceed 500 Dalton in molecular mass are usually poorly absorbed [38], and hence might perish in clinical studies. This means that the allosteric and orthosteric building blocks should be as small as possible.

Regarding dopamine D_2 and D_3 receptors, a new dualsteric molecule SB269,652 (Fig. **2**) has been discovered to have selective negative allosteric properties across D_2 and D_3 homodimers, but it behaves as an orthosteric antagonist on receptor monomer [39]. Originally, our group demonstrated for the first time that SB269,652 behaves as an allosteric compound [39], besides being an orthosteric antagonist as previously proposed by Taylor *et al*. [40]. In fact, SB269,652 only partially suppressed D_2 and D_3 receptor-mediated stimulation of $G_{\alpha i}$ or the phosphorylation of ERK 1/2. In addition, Schild analysis using $G_{\alpha i}$ assays and studies of radioligand dissociation kinetics supported allosteric actions of SB269,652 at D_3 and D_2 receptors [39]. Recently, Lane *et al*. identified the mechanism of action of SB269,652 characterized by a 'switch' in its pharmacological properties, from being an antagonist for the monomer to behave as a negative allosteric ligand within the D_2 dimeric complex [13]. Due to this pharmacological discrimination Wang *et al*. were able to reveal the existence of D_2 receptor homodimers *in-vivo* in tissues derived from rat striatum [41]. The allosteric characteristics of this compound were also demonstrated by using chimeric D_2/D_3 receptors with their second extracellular loops being switched between the two dopamine receptors [39]. This loop has a pivotal role for the binding of SB269,652 with high affinity, particularly for dopamine D_3 receptor, and crystallographic analysis has shown that together with the extracellular loop I and the junction of transmembrane helices I, II, and VII, loop II delimits the allosteric site [42]. The dualsteric nature of SB269,652 was also confirmed by analyzing its structure-activity properties. First, truncated fragments containing the 7-cyano-tetrahydroisoquinoline (7-CN-THIQ) moiety of SB269,652 that include the tertiary amine were progressively generated and, irrespective of their concentration, all these fragments behaved as orthosteric competitive antagonists on dopamine activity. On the contrary, the fragments containing the indole-2-carboxamide portion of SB269,652 inhibited dopamine action in a non-competitive manner. These properties were determined in functional and radioligand binding experiments [13]. Taken together, these results indicate that the 7-CN-THIQ moiety of SB269,652 binds directly to the orthosteric site, while the indole-2-carboxamide portion determines the binding to the second allosteric site. Interestingly, the indole-2-carboxamide part of SB269,652 has a structure similar to another recently discovered positive allosteric modulator for D_2 and D_3 receptors, suggesting the relevance

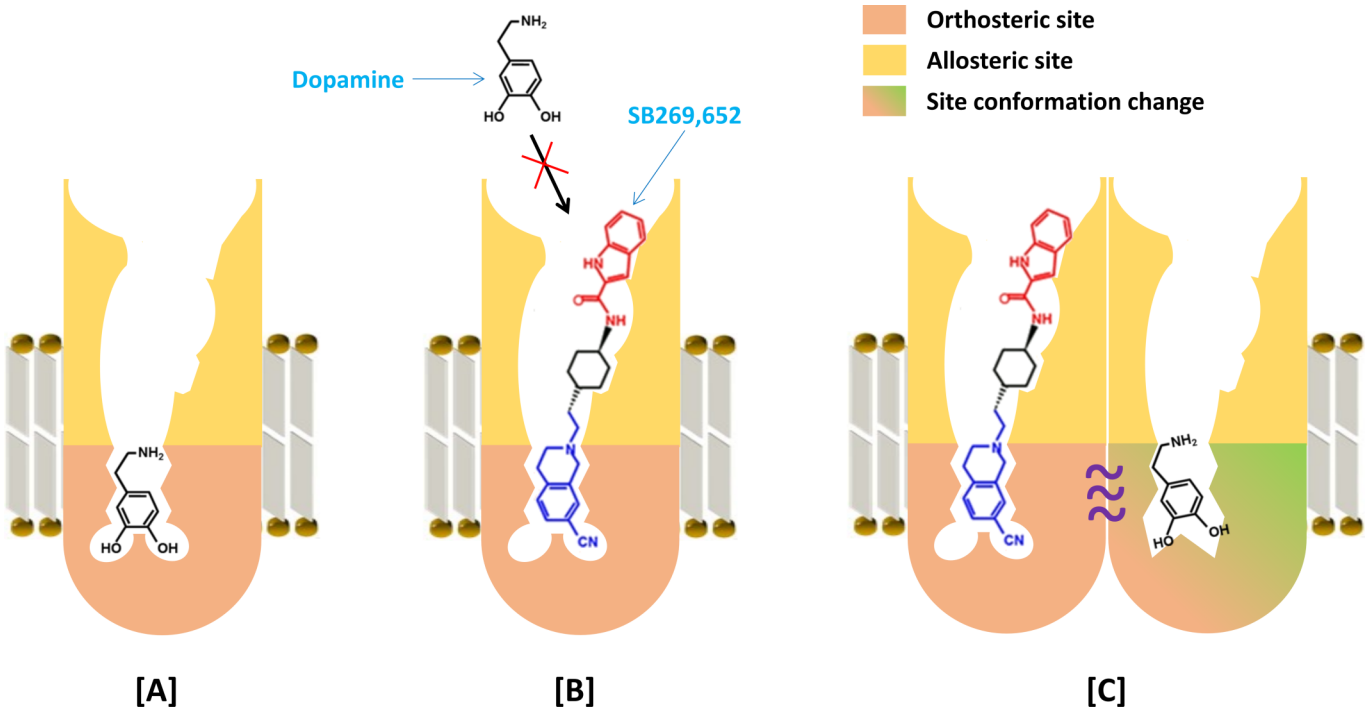

Fig. (2). Dualsteric binding mechanism of SB269,652 at dopamine D_2 receptor. [**A**] Binding of dopamine at the orthosteric site of D_2 monomer. [**B**] Binding of dualsteric compound SB269,652 at the orthosteric and allosteric sites of D_2 monomer. This prevents binding of dopamine at the orthosteric site through a competitive mechanism. [**C**] Binding of dualsteric compound SB269,652 at the orthosteric and allosteric sites of the D_2 monomer of a homodimeric complex. This binding induces a conformational change at the othosteric site on the other monomer (negative allosterism), thereby reducing the dopamine affinity.

of this second site for developing new allosteric compounds [43]. Thus, the SB269,652 molecule can be exploited in two different ways, either as a dualsteric compound as it is or eventually as a block from which new allosteric drugs can be generated based on its indole-2-carboxamide moiety.

In conclusion, dualsteric ligands such as SB269652, owing to their peculiar characteristics (allosteric/orthosteric dual action) can have different pharmacological properties in different areas of the brain based on the percentage of dimers with respect to their monomeric fraction. This evidence has important therapeutic implications while treating psychosis, such as schizophrenia or mania, where drugs acting as pure antagonists at D_2 receptors present relevant motor side effects. Conversely, in principle, a drug such as SB269,652 that in part is a negative allosteric at D_2 receptor may offer therapeutic advantages and better tolerability, not only in terms of motor side effects but also regarding anhedonia that may be associated with the use of D_2 antagonists.

4. THE REVERSIBLE NATURE OF DOPAMINE RECEPTOR DIMERS: DIMER FORMATION AT THE SINGLE-MOLECULE LEVEL

Recent studies have demonstrated that GPCR not only form dimers, but they are formed transiently through a very dynamic process. This remarkable evidence was obtained since the introduction of SMM that allows visualizing single proteins in their biological environment [44]. Notably, the existence of GPCR dimers is also evidenced by other techniques, such as resonance energy transfer (RET). However, the RET technique cannot quantify the amount of receptor dimerization, and also it cannot provide information about receptor dynamics at the single-molecule level [15].

Single-molecule techniques, such as single particle tracking (SPT), photo-activated localization microscopy (PALM) and SPT-PALM have been applied to study GPCR organization, dynamics and interactions [45]. In particular, the SPT has revealed the characteristics of dimer formation, where receptors show a monomer-dimer equilibrium characterized by rapid association and dissociation. At steady state, approximately 30-50% of the GPCRs that have been studied so far, including dopamine receptors, are part of dimeric complexes [16, 46, 47]. This evidence points out the complexity of GPCR dimerization process with consequences in relation to targeting pharmacologically receptor dimers. However, it also provides an opportunity to interfere in dimer formation with compounds, which could eventually influence the monomer/dimer equilibrium with a different outcome.

The first work was reported by Hern *et al*. on dimerization of M_1 muscarinic receptors labeled with the fluorescent antagonist Telenzepine using total internal reflection fluorescence (TIRF) microscopy [46]. Subsquently, Kasai *et al*. studied N-formyl peptide receptor (FPR) dimerization labeling the receptor with a fluorescent agonist [48]. They observed that FPR receptors display a monomer-dimer equilibrium characterized not only by fast association, but also by rapid dissociation. Similar was observed for β_1- and β_2-

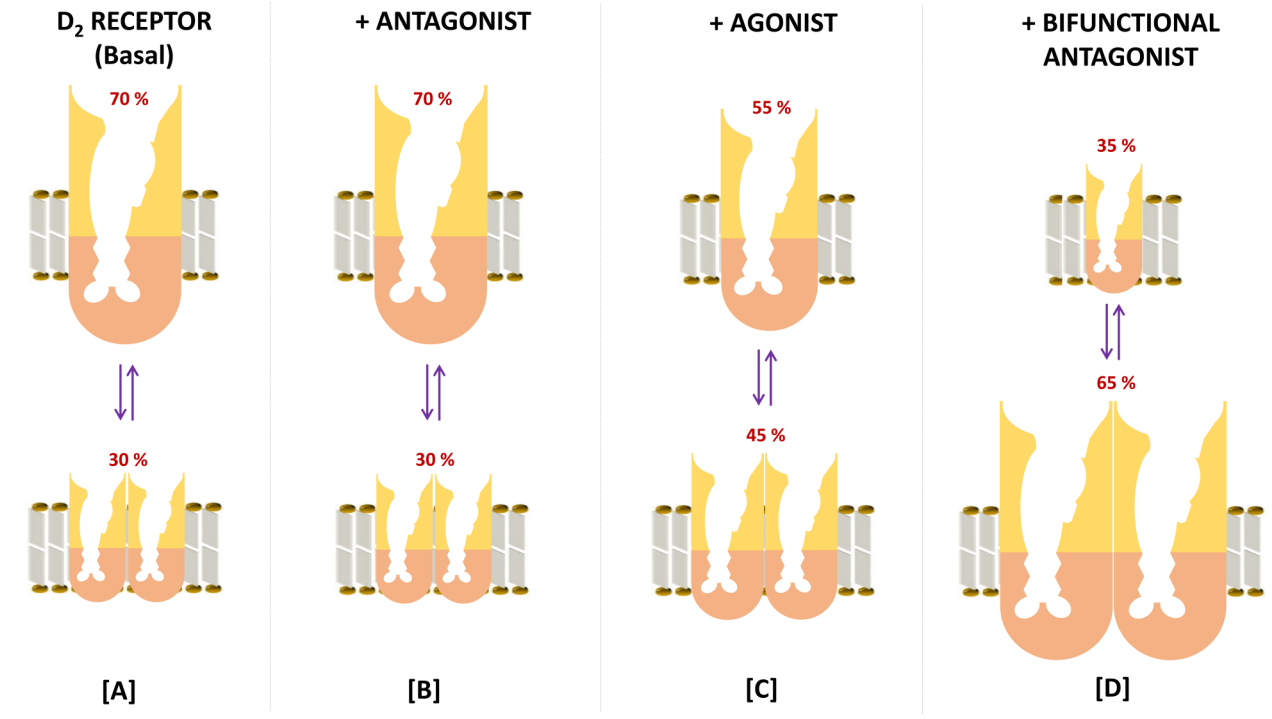

Fig. (3). Pharmacological modulation by different ligands of the monomer-dimer equilibrium percentage in D_2 receptor. [**A**] In absence of ligand (*basal condition*), the monomer-dimer equilibrium ratio is around 70% monomers to 30% dimers. [**B**] Under the presence of an *antagonist*, the monomer-dimer equilibrium ratio is around 70% monomers to 30% dimers. [**C**] Under the presence of an *agonist*, the monomer-dimer equilibrium ratio is around 55% monomers to 45% dimers. [**D**] Under the influence of a *bifunctional antagonist*, the monomer-dimer equilibrium ratio is around 35% monomers to 65% dimers.

monoaminergic receptors. The percentage of β_2 dimers was greater than β_1 dimers at equilibrium, demonstrating that the dimerization process can be different among receptor subtypes [49]. This evidence was substantiated by comparing these results with receptors that are known to be simply monomeric (*i.e*. CD86) or functional dimers (*i.e*. GABA-$_B$). Surprisingly, in these studies, the monomer-dimer equilibrium remained unchanged in the presence of agonists or different ligands. Although consistent from previous data, the role of agonist in relation to the increase or decrease of receptor dimerization is still controversial.

On the contrary, evidenced for the first time, the addition of different ligands clearly influenced dimer formation in dopamine D_2-like receptors. For example, the D_{2L} dimer percentage increased from 30% to 45% in the presence of agonist, while it increased up to 65% in the presence of a bifunctional antagonist (Fig. **3**). Tabor *et al*. used TIRF microscopy technique to individually visualize D_{2L}, D_{2S} and D_3 receptors with fluorescent labels on the membrane of living CHO cells using either SNAP-tag technology or selective fluorescent ligands [16]. With these settings, the spatial and temporal organization of the receptors at the single-molecule level were investigated ligand-free, and also in the presence of several compounds with different pharmacological properties. Importantly, the extent of dimerization among dopamine receptors obtained with fluorescent ligands was similar to that found with SNAP-tag technology. This straightforward result is relevant and it confirms that the tagged receptors can be visualized at very high percentage. Generally, the fluorescently labeled selective ligands provide the advantage to study receptor localization and dimerization in tissues with endogenous receptors and hence they are becoming an attractive tool to visualize GPCRs, also in physiological and pathological conditions.

Interestingly, Tabor *et al*. also observed that dopamine receptors exist as transient dimers with an average lifetime of 0.5s [16], similar to that previously observed in M_1 and FPR receptor dimer formation [48]. The duration of receptor dimer lifetime may be relevant for the activation of specific functions, either in the homodimer complexes and/or in the heterodimeric ones. Further research using single-molecule techniques should be able to clarify this issue. Dopamine receptors bound to bivalent ligands showed a longer dimer lifetime compared to unbound ones, up to 4s for the longest tracks. This increase may have functional and pharmacological consequences in relation to the therapeutic use of bivalent ligands. In addition, the higher extent of D_{2L} receptor dimerization also resulted in a decreased averaged diffusion coefficient of the receptor-ligand complexes. The existence of dopamine receptor dimers was also nicely confirmed by using high resolution cryogenic localization microscopy [16].

CONCLUSION

Last 15 years have witnessed an explosive amount of work aimed at generating bifunctional compounds as a novel strategy to target GPCR homo- and heterodimers, including dopamine receptors. Nevertheless, these drugs are far from being used as therapeutic agents in clinical practice. In fact, researchers have encountered many problems while developing these types of molecules such as their high molecular weights that make them problematic to reach the target *in-vivo* or their inability to bind the two monomers of the same dimer simultaneously. Nonetheless, these new compounds, at least, have been extremely useful to validate the existence of dimers in-animal models.

The bottom line on this subject is that we are still looking for a functional meaning for dimers, as the receptor monomer is capable of being active on its effectors. In particular, the scientific community is trying to answer fundamental questions about dimers, such as: whether dimer formation is physiologically relevant to better regulate the single monomer unit function; whether dimer activation is responsible for physiologically triggering specific molecular responses that are cell-type, organ-type specific. Hence, developing specific compounds that are able to target unambiguously receptor dimers may have a tremendous impact in GPCR field and neuroscience where dopamine receptors have a primary relevance. Indeed, dimeric receptor complexes in specific tissues makes them a favorable pharmacological target for "magic bullets", drugs with improved efficacy and/or less side effects. In addition, recent studies based on SMM have further complicated the picture by pointing out that dimer formation is a dynamic process where dimers dissociate and re-associate quickly, and this equilibrium can be pharmacologically modulated. Interestingly, the half-life of the dimeric complexes seem different among receptor families, and future research should further investigate the physiological consequences.

On this scenario, a new dualsteric compound named SB269,652 acting on dopamine D_2 receptors has paved the way for the development of a new generation of antipsychotic drugs. This bitopic ligand switches its antagonistic properties in favor of a milder negative allosterism in the presence of dopamine receptor dimers. This peculiar pharmacological profile may offer therapeutic advantages and better tolerability in comparison with pure antagonists at D_2 receptors, which are responsible for severe motor side effects while treating Schizophrenia and Mania. Taken together, data strongly suggest that future studies should further investigate the molecular mechanisms and the physiological or pathophysiological meanings of GPCR dimers in order to develop new therapies for the treatment of many debilitating diseases.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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M. Carli carried out in depth literature survey, covered technical aspects of this work and contributed in writing the manuscript. S. Kolachalam and S. Aringhieri analyzed the text, developed pictorial representations, finalised the draft and contributed in writing the manuscript. M. Rossi and R. Maggio contributed in writing the manuscript. L. Giovannini and R. Maggio revised the draft with critical inputs. M. Scarselli laid the foundation for this work with the final lay-out and wrote most of the manuscript.

All authors contributed to the completion of this manuscript.

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