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MINIREVIEW

The First Negative Allosteric Modulator for Dopamine D_2 and D_3 Receptors, SB269652 May Lead to a New Generation of Antipsychotic Drugs

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ABSTRACT

D_2 and D_3 dopamine receptors belong to the largest family of cell surface proteins in eukaryotes, the G protein-coupled receptors (GPCRs). Considering their crucial physiologic functions and their relatively accessible cellular locations, GPCRs represent one of the most important classes of therapeutic targets. Until recently, the only strategy to develop drugs regulating GPCR activity was through the identification of compounds that directly acted on the orthosteric sites for endogenous ligands. However, many efforts have recently been made to identify small molecules that are able to interact with allosteric sites. These sites are less well-conserved, therefore allosteric ligands have greater selectivity on the specific receptor. Strikingly, the use of allosteric modulators can provide specific advantages, such as an increased selectivity for GPCR subunits and the ability to introduce specific beneficial therapeutic effects without disrupting the integrity of complex physiologically regulated networks. In 2010, our group unexpectedly found that *N*-[(1r,4r)-4-[2-(7-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-1H-indole-2-carboxamide (SB269652), a compound supposed to interact with the orthosteric binding site of dopamine receptors, was actually a negative allosteric modulator of D_2- and D_3-receptor dimers, thus identifying the first allosteric small molecule acting on these important therapeutic targets. This review addresses the progress in understanding the molecular mechanisms of interaction between the negative modulator SB269652 and D_2 and D_3 dopamine receptor monomers and dimers, and surveys the prospects for developing new dopamine receptor allosteric drugs with SB269652 as the leading compound.

Introduction

After more than half a century, dopamine receptors still remain the main target of antipsychotic drugs. First-generation antipsychotics began in 1952 with the serendipitous discovery that the antihistamine chlorpromazine reversed the symptoms of a severely agitated psychotic male in the military hospital in Paris (Hamon et al., 1952). This was the start of the neuropharmacological revolution (Ban, 2007).

At a later time, second-generation antipsychotics introduced in clinical use were called atypical and had a better pharmacological profile, particularly in terms of motor extrapyramidal side effects (Meltzer and Massey, 2011). In addition, they seemed to have had additional therapeutic properties, such as cognitive enhancement and an improvement in the negative symptoms of schizophrenia. In general, the mechanism of action of first-generation antipsychotics is the blockade of dopamine D_2 receptors, whereas for second-generation antipsychotics, other explanations have been proposed besides D_2 receptors, mostly involving 5-HT_{1a} and 5-HT_{2a} serotonin receptors, as well as muscarinic, adrenergic, glutamatergic, and histamine receptors (Meltzer and Massey, 2011). Among the second-generation antipsychotics, clozapine is considered the "gold standard" for treating schizophrenia (Meltzer, 2012).

In this class of second-generation antipsychotics, aripiprazole distinguishes itself from the others with its particular mechanism of action, such as being a partial agonist at D_2 receptor (Keck and McElroy, 2003). However, some authors have questioned this mechanism and have proposed that

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ABBREVIATIONS: 7CN-THIQ, 7-cyano-tetrahydroisoquinoline; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; GTPγS, guanosine 5′-O-(3-thiotriphosphate); SB269652, *N*-[(1r,4r)-4-[2-(7-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-1H-indole-2-carboxamide.

aripiprazole is a functionally selective antagonist for the D_2/β-arrestin-2 interaction and a partial agonist for D_2-induced G protein activation (Mailman and Murthy, 2010). In addition, interactions with 5-HT_{1a} and 5-HT_{2a} receptors may contribute to its antipsychotic activity (Stark et al., 2007). The synthesis of these two generations of antipsychotic drugs relied on targeting the orthosteric site of dopamine and other GPCRs.

Dopamine receptors belong to the monoaminergic G protein-coupled receptor (GPCR) family and represent an important pharmacological target for the treatment of schizophrenia and Parkinson disease (Missale et al., 1998). Five dopamine receptors have been cloned and classified as D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) receptors, each having distinct functions and distributions in the brain and in the periphery.

The dopamine receptor orthosteric site is located deep in the core structure of these receptors. In particular, dopamine binds, with its protonated amino group, to an aspartic acid in the transmembrane region III and with its catechol moiety to serine residues in the transmembrane region V. On this matter, GPCR crystallization has become a crucial step to a detailed understanding of the structure and dynamics of receptor binding and functioning. Consistent with this notion, the increased number of GPCR crystal structures resolved in recent years has allowed the characterization of mechanisms of receptor activation and led the way to the synthesis of more selective and potent drugs. For example, the recent crystallization of the dopamine D_3 receptor (Chien et al., 2010), which has 76% homology with the dopamine D_2 receptor, has opened the way to the structure-guided development of new dopamine receptor drugs (Keck et al., 2014), and to the characterization of antipsychotic inhibition mechanisms (Salmas et al., 2017).

Among the various mechanisms that could explain receptor versatility, homo- and hetero-dimerization have received general recognition as being responsible for tuning, diversifying, and amplifying GPCR signaling, which strongly suggests that very complex interactions take place between ligands and receptor quaternary structures (Maggio et al., 2007; Ferré et al., 2014). Indeed, it has been shown that agonists or antagonists bind to one protomer of a GPCR dimer, altering binding and the functional properties of agonists, or antagonists interacting with the other protomer, which suggests an allosteric type of interaction between the two protomers (Carrillo et al., 2003). Importantly, it has been shown that the three most abundant dopamine receptor subtypes, D_1, D_2, and D_3, form heteromeric complexes, and that, in functional assays, D_1–D_3, D_1–D_2, and D_2–D_3 heteromers have different signaling properties compared with the respective monomers (Scarselli et al., 2001; Lee et al., 2004; Fiorentini et al., 2008; Aloisi et al., 2011; Pou et al., 2012).

As a matter of fact, another way to target GPCRs is to use allosteric modulators, which are compounds that interact with binding sites that are topographically distinct from the orthosteric site recognized by the receptor's endogenous agonist and have not evolved to accommodate endogenous ligands (May et al., 2007; Rossi et al., 2009). The use of allosteric modulators has specific advantages, such as the increased selectivity for GPCR subunits and the ability to introduce specific beneficial therapeutic effects without disrupting the integrity of complex physiologically regulated networks. In particular, this review summarizes a new mechanism of allosteric regulation across dopamine receptor dimers and the development of new allosteric drugs for dopamine receptors.

SB269652 Is an Atypical Allosteric Modulator for D_2 and D_3 Dopamine Receptors

Recently, our group has discovered the first negative allosteric modulator for D_2 and D_3 dopamine receptors, *N*-((trans)-4-(2-(7cyano-3,4-dihydroisoquinolin-2(1H)-yl)ethyl) cyclohexyl)-1H-indole-2-carboxamide (SB269652) (Fig. 1) (Silvano et al., 2010). This compound was originally synthesized by SmithKline Beecham in its effort to find new selective dopamine D_3 antagonists binding to the orthosteric site of the receptor (Reavill et al., 2000; Stemp et al., 2000). Our group re-evaluated this compound in an effort to find ligands able to distinguish between D_2 and D_3 homo- and hetero-receptors. During this re-evaluation, SB269652 was confirmed to have a higher affinity for the D_3 compared with D_2 receptor, but importantly, a few properties of SB269652 were also identified that eventually led us to the conclusion that this compound was an atypical, negative, allosteric modulator for D_2 and D_3 receptors (Silvano et al., 2010). In particular, in binding assays with Chinese hamster ovary (CHO)–transfected cells, SB269652 potently abolished the specific binding of [^{3}H]-nemanopride and [^{3}H]-spiperone radioligands at concentrations of 0.2 nM and 0.5 nM, respectively. Strikingly though, at concentrations of [^{3}H]-nemanopride and [^{3}H]-spiperone that were 10 times higher, the specific binding of both radioligands was only submaximally inhibited, indicating an allosteric behavior of SB269652. In fact, if SB269652 was acting solely at the orthosteric site, increasing concentrations of this compound

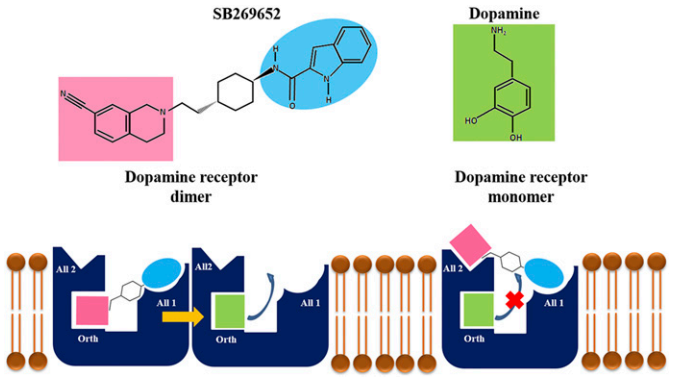

Fig. 1. Schematic representation of the allosteric binding modes of SB269652 to dopamine receptor dimer and monomer. SB269652 is represented with its three main parts, the 7CN-THIQ group (pink), the *trans*-cyclohexylene spacer in the middle, and the indole-2-carboxamide tail (sky blue). In the left part of the image, SB269652 is shown bind in a bitopic mode to one protomer of the dopamine dimer, the 7CN-THIQ group to the orthosteric site (Orth), and the indole-2-carboxamide group to the allosteric site (All1), and exert an allosteric effect across dimer on dopamine sitting on the orthosteric site of the other protomer (Lane et al., 2014). In the right part of the image, SB269652 is shown bind to a dopamine-occupied monomer and prevent the dissociation of dopamine from the same receptor. In this configuration, the indole-2-carboxamide group would bind to the allosteric site as shown for SB269652 in the bitopic pose (All1), and the 7CN-THIQ group would engage an additional site on the extracellular part of the receptor (All2). This second arrangement of SB269652 on the dopamine-occupied receptor would be unfavorable in respect to the bitopic binding mode and would occur only for high doses of the drug.

would have displaced [^{3}H]-nemanopride and [^{3}H]-spiperone completely, regardless of the concentration of the radioligands.

The orthosteric/allosteric nature of SB269652 for D_3 receptors was also confirmed in functional experiments in which, at low concentrations of dopamine (1 μM), SB269652 showed its orthosteric nature by potently blocking D_3 receptor-mediated activation of $G\alpha_{i3}$ and phosphorylation of the extracellular signal-regulated kinase (ERK)1/2, whereas it showed its negative allosteric nature at higher concentrations of dopamine (10 μM) by submaximally inhibiting the stimulatory effects of dopamine. The same kind of responses were also observed in functional assays with D_2 receptors, in which SB269652 only submaximally suppressed D_2 receptor-mediated stimulation of $G\alpha_{i3}$ and $G\alpha_{qi5}$ and phosphorylation of ERK1/2 and Akt at high concentrations of dopamine (Silvano et al., 2010).

The allosteric nature of this compound was also shown by using chimeric D_2/D_3 receptors in which the second extracellular loops were switched between the two dopamine receptors (Silvano et al., 2010). This loop has a pivotal role in binding SB269652 with high affinity, and crystallographic analysis has shown that, together with extracellular loop I and the junction of transmembrane helices I, II, and VII, loop II delimits an allosteric site that may interact with bitopic or pure allosteric ligands (Chien et al., 2010).

Because of the atypical allosteric nature of SB269652, this compound was denoted as an "atypical allosteric modulator" to indicate the strange behavior of SB269652 for D_3 and D_2 receptors in binding and functional experiments. Indeed, SB269652 behaves as an orthosteric ligand at low concentrations of the radioligands [^{3}H]-spiperone and [^{3}H]-nemonapride in binding assays, or at low concentrations of dopamine in functional assays; however, it acts as an allosteric compound at higher concentrations of the radioligands or dopamine. What causes this unusual behavior was later clarified by Arthur Christopoulos' group (Lane et al., 2014). In particular, they showed that SB269652 was a bitopic compound. Bitopic molecules are typically composed of two pharmacophores bridged by a spacer and characterized by the ability to simultaneously bind to the orthosteric and allosteric sites of the same protomer. The bitopic nature of SB269652 was revealed by fragmenting it. They generated progressively truncated fragments of the 7-cyano-tetrahydroisoquinoline (7CN-THIQ) moiety of SB269652 that contains the tertiary amine, the part of the molecule that is important for the interaction with the conserved aspartic acid of the amine receptors in the orthosteric site. Unlike whole SB269652, all 7CN-THIQ fragments were always able to inhibit dopamine action, in a competitive manner, regardless of its concentration, whereas the other active component of SB269652, the indole-2-carboxamide fragment, inhibited dopamine action in a noncompetitive manner. The properties of these fragments were tested in functional and radioligand binding experiments (Lane et al., 2014).

Nevertheless, bitopic ligands should not show allosteric properties because they bind to the orthosteric and allosteric sites simultaneously. In fact, allosteric interactions should change the orthosteric binding pocket environment and consequently affect the ability of orthosteric ligands to bind the orthosteric site of the receptor. For instance, extended-length 4-phenylpiperazine derivatives are known to dock to the orthosteric site with the 4-phenylpiperazine moiety and to an allosteric site with the extended aryl amide moiety, but their interaction at the secondary site does not allosterically modulate their binding to the orthosteric site (Furman et al., 2015). Lane et al. (2014) further extended the description of the mechanism of SB269652 binding, providing that the allosteric action of SB269652 was exerted across dopamine receptor dimers, as depicted in Fig. 1. To recapitulate, SB269652 binds in a bitopic mode to one protomer of a dopamine receptor dimer, the 7CN-THIQ part binds to the orthosteric site, and the indole-2-carboxamide part to the allosteric site. The allosteric effects are then the results of changes in the ability of ligands to bind the orthosteric binding pocket on the other promoter of the dimer. In other words, SB269652 behaves as competitive antagonist with receptor monomers and allosterically across receptor dimers.

This "binding" model clearly explains the atypical behavior of SB269652 in binding and functional experiments at low and high concentrations of radioligands or dopamine with D_2 and D_3 receptors. As concentrations of radioligands or dopamine increase, more dimers will be occupied and the allosteric effect of SB269652 will be more evident. Furthermore, as discussed below, agonists promote dopamine receptor dimer formation (Tabor et al., 2016), which might help to unveil the allosteric effects of SB269652 in the functional assays with dopamine.

SB269652 Influence on the Radioligand Dissociation Constant Reveals Additional Complexity

Negative and positive allosteric compounds alter ligand association and/or dissociation kinetics (Conn et al., 2009; Maggio et al., 2013). The affinity of a compound for its target is related to its association and dissociation rate constants and is quantified by $K_D = K_{off}/K_{on}$, where K_D represents the equilibrium dissociation constant and K_{off} and K_{on} represent the dissociation and association rate constants, respectively. Consistent with this notion, when an allosteric compound modifies the affinity of a ligand for the orthosteric site, its dissociation and association rate constants must change accordingly. Usually, negative allosteric compounds increase the dissociation rate constant and/or decrease the association rate constant. Strikingly, Silvano et al. (2010) demonstrated that SB269652 does not behave as a common negative allosteric modulator; in fact, it largely reduces both radioligand association and dissociation rate constants for D_2 and D_3 receptors (Silvano et al., 2010). A similar effect has been described for the antagonist methoctramine at muscarinic M_2 receptors (Jakubík et al., 2014). Methoctramine is a bitopic compound that competes with orthosteric ligands by simultaneously binding to both the orthosteric and the allosteric binding sites of the M_2 type muscarinic receptor. In addition, methoctramine is also able to bind, even though with a low affinity, to *N*-methylscopolamine-occupied receptors by interacting solely with the allosteric binding site of M_2. However, in this case, the interaction between methoctramine and the orthosteric ligand is allosteric. In particular, in dissociation binding experiments with *N*-[^{3}H]-methylscopolamine-prelabeled M_2 muscarinic receptors, Jakubík et al. (2014) found that the radioligand was trapped in the orthosteric site of M_2 by methoctramine. SB269652 could indeed be working in the same way: binding to the radioligand-occupied receptor

solely at the allosteric site and preventing its dissociation. If this is the case, the position of SB269652 in the allosteric site could be different between radioligand-occupied and -unoccupied receptors, and the 7CN-THIQ head group could be oriented to bind other regions of the dopamine receptor according to the receptor occupancy status. This was also suggested by our experiments with chimeric D_2/D_3 receptors, where it was demonstrated that the replacement of the extracellular loop II of D_2 with the same segment from D_3 greatly increased the inhibition potency of SB269652 against [^{3}H]-nemonapride, whereas the reverse chimera, D_3 with the second extracellular loop from D_2, highly reduced the affinity for SB269652 (Silvano et al., 2010). This second extracellular loop in the D_2 and D_3 receptor is divergent. For example, in D_3 a negatively charged aspartic acid at the end of the loop (D187) is replaced in D_2 by an alanine (A188). This aspartic acid residue could form a salt bridge with the basic tertiary amine of the 7CN-THIQ group, stabilizing the SB269652 molecule in an alternative pose. Interestingly, this aspartic acid residue of the D_3 receptor has been shown to play a major role in agonist-induced tolerance (Gil-Mast et al., 2013). It seems that after continuous agonist stimulation, D187 forms a salt bridge with histidine 354 in extracellular loop III, supporting the concept that the engagement of D187 by drugs could have a profound effect on receptor conformation and eventually an allosteric effect on orthosteric ligands.

Even though counterintuitive, an alternative explanation is that SB269652 could reduce the dissociation rate constant of the radioligand only across receptor dimers. Nevertheless, this seems improbable, as a biphasic dissociation curve of the radioligands should have been seen, inasmuch as only a fraction of the dopamine receptor is in the dimeric form (Tabor et al., 2016). The most parsimonious explanation is that SB269652 binds with high affinity in a bitopic mode to unoccupied receptors and exerts a predominant allosteric effect across dimers; furthermore, it could bind with low affinity solely at the allosteric site(s) of occupied receptors reducing ligand dissociation (Fig. 1). Clearly the ability of SB269652 to bind multiple allosteric sites depending on the D_2 and D_3 receptor occupancy status requires further investigations.

SB269652 as the Leading Compound That Led to the Development of New Antipsychotic Drugs

To develop new effective antipsychotic drugs, SB269652 could be exploited in two different ways: 1) by maintaining its bitopic effect and improving its affinity and allosteric effect across dimers at D_2 receptors, or 2) by designing pure and more potent allosteric drugs starting from its indole-2-carboxamide moiety.

By pursuing the first strategy, Shonberg et al. (2015) synthesized a series of compounds that bind to dopamine D_2 receptors in a bitopic manner but with higher affinity compared with the original ligand SB269652. To this purpose, they focused on modifying the three main parts of SB269652: the 7CN-THIQ and the indole-2-carboxamide moieties, and the *trans*-cyclohexylene linker. They found that the orthosteric "head" groups with small 7CN-THIQ substituents were important for maintaining the negative cooperativity of SB269652. Among several substitutions, the hydrogen substitution of the nitrile group (compound 12b in Shonberg et al., 2015, Fig. 2) resulted in a significant 9-fold increase in functional affinity (K_B = 87 nM and 776 nM, for 12b and SB269652, respectively) with no significant change in the allosteric cooperativity. Conversely, substitution of the 7CN-THIQ head group with chemical structures that are privileged scaffolds for dopamine D_2 receptors resulted in compounds with the highest increase in functional affinity but poor allosteric effect (Shonberg et al., 2015).

These results indicate that the 7CN-THIQ binding to the orthosteric site of D_2 receptor is important for the orientation and binding of the indolcarboxamide to the allosteric site. Even subtle modification of 7CN-THIQ can largely affect the functional affinity and negative allosteric cooperativity of the molecule.

Furthermore, linker length was critical for the allosteric effect of SB269652 analogs. The substitution of the *trans*-cyclohexylene spacer group with the one containing the 1,3-propylene or a 1,4-butylene resulted in an increase in binding affinity with the maintenance of negative allosteric cooperativity. On the contrary, incorporation of 1,5-pentylene spacer resulted in a compound with pure competitive activity, and further extending the length to 1,5-hexylene spacer restored allosteric pharmacology (compound 18d in Shonberg et al., 2015, Fig. 2). All of these results taken together suggest the important role of spacers in the reciprocal orientation of the 7CN-THIQ and indole-2-carboxamide groups for the binding to the orthosteric and allosteric sites. This is not surprising if it is considered that such cyclohexylene extension, even though relatively flexible, puts some constraints on the simultaneous binding of the two pharmacophores of SB269652 to the orthosteric and allosteric binding sites. The optimal engagement of the two sites requires a linear orientation of the indole-2-carboxamide moiety with the 7CN-THIQ head group (Lane et al., 2014).

Finally, Shonberg et al. (2015) analyzed the effect of modifications in the indole-2-carboxamide moiety of SB269652. As discussed previously, data from D_2 and D_3 chimeric receptors showed that SB269652 binding region extends from the orthosteric site of D_2 and D_3 dopamine receptors up to an allosteric site delimited by their extracellular loops I and II (Silvano et al., 2010). Successive experiments by Lane et al. (2014) supported this concept as they demonstrated that the cyclohexyl and indolic NH residues of SB269652 bind respectively to valine 91 and glutamic acid 95, located at the extracellular end of transmembrane region II of the D_2 dopamine receptor, conferring the allosteric property to the drug. These two residues are also conserved in the D_3 receptor and correspond to valine 86 and glutamic acid 95 in the transmembrane region II of D_3. As for the D_2 receptor, these residues play a crucial role in docking and conferring the allosteric properties to SB269652. Because the dopamine D_4 receptor has high homology with the D_2 receptor, and the glutamic acid residue (Glu95 in D_4) is conserved, whereas the hydrophobic valine residue is replaced by the aromatic amino acid phenylalanine, it would be interesting to test whether SB269652 loses its allosteric effect on D_4 receptors; in fact, this would shed some light on the importance of each of those amino acids in SB269652-mediated allosteric effects.

Supporting the concept that the indole-2-carboxamide moiety of SB269652 is important for its allosteric effect, SB269652 analogs whose indolic NH groups were replaced

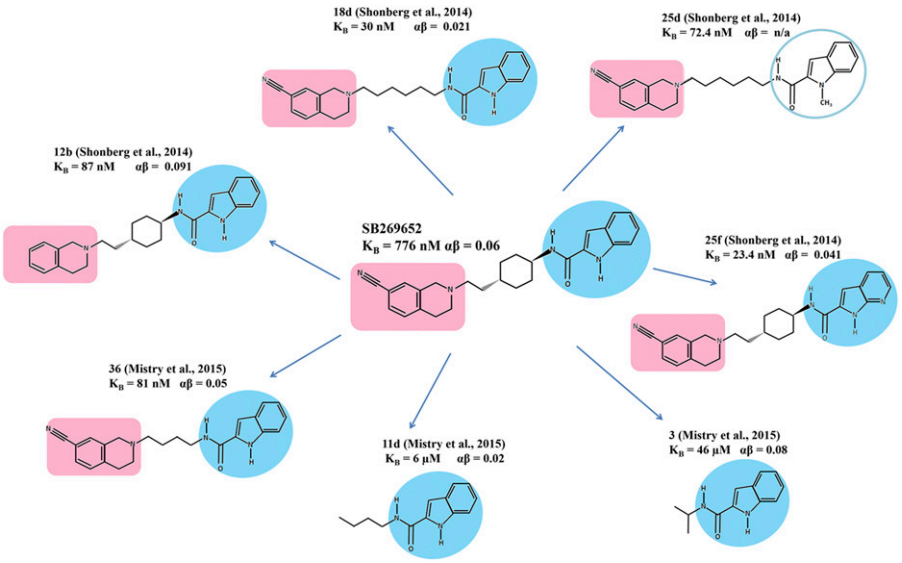

Fig. 2. Chemical structure of optimized SB269652 analogs. Value of KB (functional affinity) and $\alpha\beta$ (allosteric cooperativity with dopamine) were taken from Shonberg et al. (2015) and Mistry et al. (2015). The part of the molecule boxed in pink binds to the orthosteric site, whereas the part boxed in light blue binds to the allosteric site. For comparison we added a compound (25d) lacking an allosteric effect.

by others unable to generate hydrogen bonds (e.g., 1-methyl-1H-indole-2-carboxamide, 25d in Shonberg et al., 2015, Fig. 2) lost the ability to allosterically interact with the receptors even though maintaining and showing an increased functional affinity. In contrast, the replacement of the indole ring with an azaindole group increased its affinity 30-fold at the same time maintaining a negative cooperativity (compound 25f in Shonberg et al., 2015, Fig. 2).

The second approach to generating analogs with an improved "allosterism" was to break down SB269652 into small molecules containing the indole-2-carboxamide group (Mistry et al., 2015). Strikingly, Mistry et al. (2015) showed that, in a series of binding and functional assays, the 1H-indole-2-carboxamide moiety (compound 3 in Mistry et al., 2015, Fig. 2) behaved as a pure allosteric drug for D_2 receptors, even though its affinity and negative cooperativity were weaker than SB269652 (Mistry et al., 2015). Furthermore, Mistry et al. (2015) showed that this molecule had similar affinity and negative allosteric effects for D_3 dopamine receptors compared with the D_2 receptors, which strongly suggested that the difference in SB269652 affinity for D_2 and D_3 receptors resulted from the binding of the 7CN-THIQ moiety to the orthosteric site of the two receptors (Silvano et al., 2010). Interestingly, Mistry et al. (2015) also found that the 1H-indole-2-carboxamide moiety in ERK1/2 functional assays reduced not only dopamine potency but also its maximal responses, whereas in dopamine-mediated GTPγS recruitment and cAMP production assays, 1H-indole-2-carboxamide showed only a reduction in dopamine potency (Mistry et al., 2015). This indicates that this ligand is able to modulate dopamine binding to the orthosteric site of the receptor in such a way that it not only reduces dopamine potency but interestingly, depending on the functional assay, reduces dopamine efficacy. Furthermore, they confirmed that the residues valine 91 and glutamic acid 95 on the second extracellular loop of the D_2 receptor were crucial for maintaining the allosteric properties of both the leading compound SB269652 and the 1H-indole-2-carboxamide derivative.

The next step taken by Mistry et al. (2015) was to extend the carboxamide group of the 1H-indole-2-carboxamide moiety by adding a fragment derived from SB269652 that extended away from the indole-2-carboxamide moiety and included a tertiary amine group, *N*-((*trans*)-4-(2-(dimethylamino)ethyl) cyclohexyl)-1H-indole-2-carboxamide (compound 9a in Mistry et al., 2015). Interestingly, the modification did not alter the negative allosteric effect of the molecule in functional assays with dopamine; however, in spiperone radioligand-binding assays, the compound behaved as a competitive ligand. This suggests that the protrusion of the molecule into the orthosteric binding site is not sufficient to alter dopamine binding and action but is sufficient to alter the binding of spiperone, which has a bulkier structure. Moreover, a further extension of the 1H-indole-2-carboxamide moiety transformed these analogs into pure competitive ligands.

Subsequently, Mistry et al. (2015) generated analogs from the 1H-indole-2-carboxamide moiety with bicyclic heteroaromatic ring at the carboxamide N-substituent of the molecule. A key determinant of affinity for these analogs was the degree of N-substitution of the carboxamide moiety. In particular, the mono-substituted derivatives retained their activity, whereas the di-substituted derivatives mainly lost it (Mistry et al., 2015). Notably, the linear increased in the size of alkyl substituents (from N-ethyl to N-propyl and to N-butyl) improved both affinity and negative cooperativity, progressively. The N-butyl substituent (compound 11d in Mistry et al., 2015, Fig. 2) was the compound with the most pronounced profile, which suggests that the alkyl substituents bind to an hydrophobic part of the receptor core. Consistent with this, polar substituents were not active.

Mistry et al. (2015) decided then to test the importance of the indole core of the 1H-indole-2-carboxamide moiety by synthesizing a set of analogs bearing alternative bicyclic heterocyclic cores. Strikingly, compounds unable to form hydrogen bonds did not show any allosteric property, further supporting the concept that the NH group of the indole core binds to the glutamic acid 95 of the D_2 receptor.

Mistry et al. (2015) completed their work by generating a new bitopic ligand that contained all the chemical optimizations that greatly improved the activities of the SB269652 analogs. In particular, compound 11d was incorporated in the bitopic pharmacophore of SB269652 by anchoring it to

the 7CN-THIQ group; this resulted in an improved affinity (81 nM) for D_2 receptors and a similar negative allosteric cooperativity compared with its leading compound SB269652 (compound 36 in Mistry et al., 2015, Fig. 2). It is worth noting that compound 36 is identical to compound 18b from the study by Shonberg et al. (2015), and, therefore, two independent approaches led to the same optimized compound.

One important aspect of the work of Mistry et al. (2015) that needs to be addressed concerns the allosteric mechanism through which SB269652 analogs operate across D_2 or D_3 receptor dimers. As previously discussed, the allosteric properties of SB269652 might lie in the ability to modify the affinity for orthosteric ligands across D_2 or D_3 receptor dimers. However, as discussed by Mistry et al. (2015), the 1H-indole-2-carboxamide moiety and its derivatives do not require an allosterism across dimer types of interaction to function as allosteric modulators. In fact, these compounds do not have an orthosteric pharmacophore and are small enough to interact solely with the allosteric site of the receptor and, therefore, to exert allosteric effects within the same receptor protomer (Mistry et al., 2015). Moreover, adding fragments of SB269652 and thus extending the carboxamide group away from the 1H-indole-2-carboxamide moiety led to a critical extension: the synthesis of the peculiar compound 9a (see above), which remained allosteric only for dopamine but not for spiperone. These two distinctive behaviors of 9a for the two orthosteric ligands strongly suggest that the chemical extension was interfering with the binding of spiperone at the orthosteric site of the same promoter. Otherwise, if 9a allosteric effects were exerted across dimers, the compound would have shown an allosteric effect for spiperone as well. Furthermore, there is evidence that supports the concept that many allosteric modulators modify the binding properties of orthosteric ligands at the promoter level instead of across dimers. In particular, Kruse et al. (2013) crystallized the ternary complex structure formed by the allosteric modulator LY2119620 and the orthosteric agonist iperoxo, which were simultaneously bound to the muscarinic M_2 receptor (Kruse et al., 2013). The crystal structure showed that LY2119620 binds to a largely preformed binding site in the extracellular vestibule of the iperoxo-muscarinic M_2 receptor complex and induces slight contraction of the outer binding pocket of the receptor that profoundly modifies the binding properties of the orthosteric site of the M_2 receptor.

Finally, it is worth noting that the indole-2-carboaxamide chemical structure of SB269652 is very similar to a recently identified positive allosteric modulator for D_2 and D_3 receptors, suggesting that understanding the binding mechanism of SB269652 to these receptors could lead to the development of both D_2 and D_3 receptor-specific positive and negative allosteric compounds (Wood et al., 2016).

Dopamine Receptor Dimerization and Allosteric Effect of SB269652

Dopamine receptors are distributed in several areas of the brain and are pharmacologically targeted to treat psychosis by decreasing their activity, or Parkinson disease by increasing their activity. Like most GPCRs, they have been shown to behave like homodimers and heterodimers (Maggio et al., 2008, 2015). As previously explained, SB269652 and its derivatives exert their preeminent allosteric effect on dopamine receptors across dimers. Given that their allosteric effect is strictly dependent on the fraction of receptor dimers in proportion to the fraction of monomers, these compounds would exert a negative modulatory effect only on receptor dimers, and a competitive antagonism only on receptor monomers. Regarding GPCR dimerization, mounting evidence indicates that it is a dynamic process whereby receptors show a monomer-dimer equilibrium characterized by rapid association and dissociation processes (Scarselli et al., 2013, 2016). In a recent work, Tabor et al. (2016) have shown that the percentage of interacting dopamine D_2 and D_3 receptors is strictly dependent on their concentrations on the plasma membrane and that agonists but not antagonists increase D_2 or D_3 receptor dimerization. In particular, they calculated that, at expression levels comparable to those detected in the caudate and putamen, in vivo (Boyson et al., 1986), the percentage of dopamine D_2 receptor dimers compared with monomers was about 20%. Therefore, bitopic compounds would act as competitive antagonists on 80% of receptors and as allosteric modulators on the remaining 20%. In comparison, if it is considered that in other parts of the brain, like the limbic system (ventral striatum) and the cortex, dopamine receptor levels are considerably lower (Hall et al., 1994), and so also the percentage of receptor dimers, the chances of an allosteric type of behavior for these bitopic compounds would dramatically decrease in comparison with the caudate/putamen. As also explained in the review of Conn et al. (2009), one of the advantages of allosteric modulators with limited negative cooperativity is that they would impose a "ceiling" on the magnitude of their pharmacological effects. In fact, this property would allow a high degree of titratability for these compounds, which could be administered at large doses with minor side effects compared with large administered doses of orthosteric ligands.

Moreover, because it is commonly accepted that blocking D_2 receptor activity in the ventral striatum is responsible for the therapeutic antipsychotic effects and blocking the same receptors in the caudate/putamen is responsible for unwanted motor side effects (Jones and Pilowsky, 2002), drugs that are able to target these areas of the brain differently might lead to the development of novel pharmacological tools for the treatment of psychosis. We might then speculate that bitopic drugs acting as allosteric modulators across dimers may be able to discriminate between regions of the brain containing different percentages of dopamine receptor dimers. As a consequence, allosteric modulators in the caudate/putamen, with a ceiling set on the magnitude of their pharmacological effects for this area, could result in a reduction of their motor side effects and thus an increase in their tolerance.

Importantly, as demonstrated by Tabor et al. (2016), agonists promote dopamine receptor dimer formation. Therefore, increases in dopamine levels would result in increases in the percentage of dopamine receptor dimers, which would make bitopic drugs with a negative modulatory effect across dimers even safer.

Consistent with this concept, the [^{35}S]GTPγS recruitment data in Silvano's paper could be explained by the inability of 1 μM dopamine and the ability of 10 μM dopamine to generate enough dopamine receptor dimers on which SB269652 could direct its allosteric effects (Silvano et al., 2010).

To this purpose, it is interesting to mention that the only in vivo data available with SB269625 were published in an abstract form long before the compound was recognized to be allosteric (Taylor et al., 1999). SB269652 was shown to have no effect on the basal release of dopamine in the striatum and nucleus accumbens, but it prevented the inhibition of dopamine release induced by the D_2/D_3 agonist quinelorane in the nucleus accumbens but not in the striatum. These data clearly indicate that the activity of SB269652 has a brain regional selectivity. This regional selectivity cannot be attributed, as was assumed originally, to the preferential antagonism of this compound at D_3 receptor, as D_3 receptors have a marginal role in regulating dopamine release compared to D_2 receptor (Joseph et al., 2002). Furthermore, SB269652 showed no effect on amphetamine-induced hyperactivity, and, in contrast to the orthosteric antagonist haloperidol, it did not induce catalepsy, suggesting that this compound may be devoid of side effects normally associated with orthosteric dopamine receptor antagonists (Taylor et al., 1999).

In contrast to bitopic compounds that exert their negative allosteric effect across dimers, allosteric compounds that exert negative modulatory effect on the monomer would not be influenced by receptor dimerization. In this case, differences in receptor concentration among areas of the brain should not influence their allosteric properties Even though pure allosteric modulators would behave in the same way, regardless of tissue-specific receptor dimerization, they would be good drugs anyway, because of the physiologic ceiling effects imposed by their intrinsic nature.

Allosteric Drugs as New Antipsychotic Agents

One of the main disadvantages in the use of first-generation antipsychotics is that when occupancy of the dopamine D_2 receptors in the caudate/putamen reaches 75–80%, extrapyramidal side effects start to appear. Nevertheless, a critical point of receptor occupancy of 60–80% should be reached in other areas of the brain, such as the limbic system and the cortex, to obtain a therapeutic effect (Kasper et al., 1999; Remington and Kapur, 1999; Kapur et al., 2000). As a consequence, clinicians have to titrate the dosage of antipsychotics directly on patients to stay in such a narrow therapeutic range of receptor occupancy, a "gamble" that often leads to poor adherence to antipsychotic prescriptions (Haddad et al., 2014).

Unfortunately, even the second generation of antipsychotic drugs, which have additional effects on serotonin receptors (mostly $5HT_{2A}$ type) and therefore need a reduced D_2 dopamine receptor occupancy to be effective, are not extrapyramidal side effect–free (Rummel-Kluge et al., 2010). In particular, the severity of extrapyramidal side effects varies in relation to the particular second-generation agent used (with clozapine having the lowest risk and risperidone the highest) and its dosage. The higher the dose, the more intense are the extrapyramidal side effects, which indicates that the safety of these second-generation drugs depends on the level of receptor occupancy (Divac et al., 2014).

The antipsychotic aripiprazole seems to have lower propensity for extrapyramidal side effects. Even at low doses, aripiprazole reaches receptor occupancy of 85% without inducing dystonia and parkinsonism, which are conversely observed for higher striatal occupancy (>90%) (Mamo et al., 2007). The lack of dystonia and parkinsonism is attributed to its partial agonist properties that prevent complete receptor inactivation, but, as described above, other mechanisms are probably responsible for it (Mailman and Murthy, 2010). Nevertheless, akathisia is one of aripiprazole's most frequent and troublesome extrapyramidal side effects, being present in approximately 15–25% of patients with schizophrenia and bipolar mania taking the drug (Fleischhacker, 2005; Kinghorn and McEvoy, 2005; Poyurovsky, 2010).

Given the arguments above, the need for new antipsychotic drugs showing minor or no extrapyramidal side effects is evident. Since targeting dopamine D_2 receptors still remains the main strategy to reduce psychotic symptoms, an alternative way to target these receptors could be the use of allosteric compounds. As discussed above, allosteric drugs with modest negative cooperativity have a ceiling effect that should allow physiologic adaptation of dopamine neurotransmission to overcome their block and prevent the onset of extrapyramidal side effects.

Furthermore, the hypothetical higher dopamine receptor dimerization in caudate/putamen in respect to other areas of the brain should further improve the benefit/risk profile of compounds with bitopic properties that exert the allosteric effect only on receptor dimers. As a matter of fact, Wang et al. (2010) found a significantly enhanced expression of dopamine D_2 receptor dimers and decreased expression of D_2 receptor monomers in the postmortem striatal tissue of schizophrenic patients (Wang et al., 2010). Furthermore, they demonstrated that treatment of rats with amphetamine, a drug that increases dopamine concentration in the synaptic cleft, increased D_2 dopamine receptor dimerization in the striatum, whereas the antipsychotic haloperidol did not alter D_2 dimer levels (Wang et al., 2010). These data are in good agreement with the study in vitro of Tabor et al. (2016) and they support the concept that bitopic drugs with allosteric effect across dimers could have a profound impact on the development of novel and more safe antipsychotic therapies.

Until now, SB269652 remains unique in its mechanism of action, as no other bitopic ligands have been reported to have an allosteric effect across dimers. Nevertheless, allostery across dimers has been reported for ligands that bind to the orthosteric site of GPCR homo- and heteromers (Smith and Milligan, 2010). Recently, Bonaventura et al. (2015) described a novel unsuspected allosteric mechanism within the adenosine A_{2A}-dopamine D_2 receptor heteromer by which either orthosteric A_{2A} agonists or antagonists decrease the affinity and intrinsic efficacy of dopamine D_2 agonists and the affinity of D_2 antagonists. They explained these data by a model that considers A_{2A}-D_2 heteromers as heterotetramers constituted of A_{2A} and D_2 homodimers, and demonstrated that allosteric effects depend on the integrity of the right quaternary structure of the heterotetramer. This was shown in transfected mammalian cells and striatal tissue, by using heteromer-disrupting mutations and transmembrane peptides intercalating between receptors, respectively. The peculiarity of this study was to uncover the negative allosteric modulation of orthosteric A_{2A} antagonists on D_2 receptors, challenging the traditional view that antagonists are inactive ligands. This new reported mechanism of allostery across adenosine A_{2A}-dopamine D_2 receptor heteromers offers a new target to be investigated in developing drugs with antipsychotic effect, which, as stated in this review many times,

should show a drastic reduction of side effect symptoms because of their allosteric nature.

Concluding Remarks

The last ten years have witnessed an explosion in the amount of work done on allosteric modulation of GPCRs. Muscarinic (Jakubík and El-Fakahany, 2010), glutamate (Lundström et al., 2016), and adenosine (Romagnoli et al., 2015) are only some of the receptors that have been widely explored to find allosteric drugs. At the moment, only two allosteric compounds for GPCRs have been approved for marketing: cinacalcet, a positive allosteric modulator of the calcium-sensing receptor, which is used in hyperparathyroidism, and maraviroc, a negative allosteric modulator of chemokine receptor 5, which is used in the treatment of HIV infections. The experience in using these drugs is still somewhat limited; nevertheless they are a proof of concept that allosteric compounds for GPCRs can be used as therapeutic agents in clinic.

Schizophrenia is a chronic disease and treatment with the currently available drugs is often troublesome because of difficulty in obtaining a good therapeutic effect without serious collateral side effects. For these reasons, allosteric drugs are good candidates to overcome these problems, at least in part. Indeed, SB269652 is the first negative allosteric modulator of dopamine D_2 and D_3 receptors, and the search for optimized analogs has led to more potent bitopic modulators and pure allosteric drugs. Although it is difficult to predict whether allosteric drugs at dopamine receptors will have the same efficacy as do classic orthosteric drugs, their therapeutic potential stands on a solid preclinical background, and it is worth the effort to design optimized derivatives of SB269652 to explore their efficacy in clinic.

In conclusion, compared with the orthosteric-targeted ligands, allosteric molecules show increased specificity for particular GPCR subunits and reduced side effects. Although difficult to develop, allosteric modulators represent one of the most valuable pharmaceutical tools for the development of potent and more selective therapeutic strategies for the treatment of a variety of pathologies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Rossi, Fasciani, Marampon, Maggio, Scarselli.

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