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

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 seren-dipitous 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 intro-duced in clinical use were called atypical and had a better pharmacological profile, particularly in terms of motor extra-pyramidal side effects (Meltzer and Massey, 2011). In aripiprazole is a functionally selective antagonist for the D_2/b-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 schizo-phrenia 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 crystalli-zation 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 recep-tor versatility, homo- and hetero-dimerization have received general recognition as being responsible for tuning, diversify-ing, 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 antago-nists 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 dis-rupting 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 synthe-sized 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 $[^3H]$-nemanopride and $[^3H]$-spiperone radioligands at concentrations of 0.2 nM and 0.5 nM, respectively. Strikingly though, at concentrations of $[^3H]$-nemanopride and $[^3H]$-spiperone that were 10 times higher, the specific binding of both radioligands was only submax-imally inhibited, indicating an allosteric behavior of SB269652. In fact, if SB269652 was acting solely at the orthosteric site, increasing concentrations of this compound

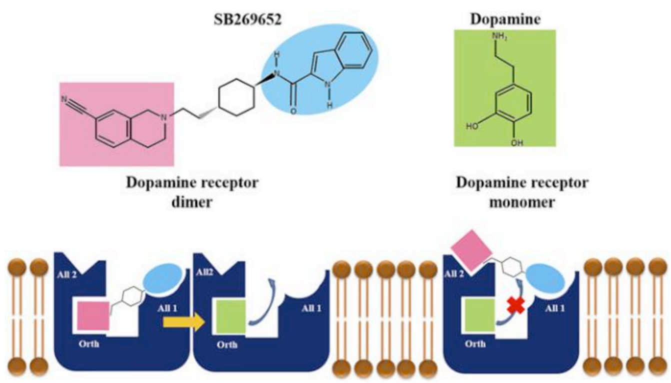

Fig. 1. Schematic representation of the allosteric binding modes of SB269652 to dopamine receptor dimer and monomer. SB269652 is represented with its three main parts, the 7CN-THIQ group (pink), the trans-cyclohexylene spacer in the middle, and the indole-2-carboxamide tail (sky blue). In the left part 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.

solely at the allosteric site and preventing its dissociation. If this is the case, the position of SB269652 in the allo-steric 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 $[^3H]$-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 impor-tant for maintaining the negative cooperativity of SB269652. Among several substitutions, the hydrogen substitution of

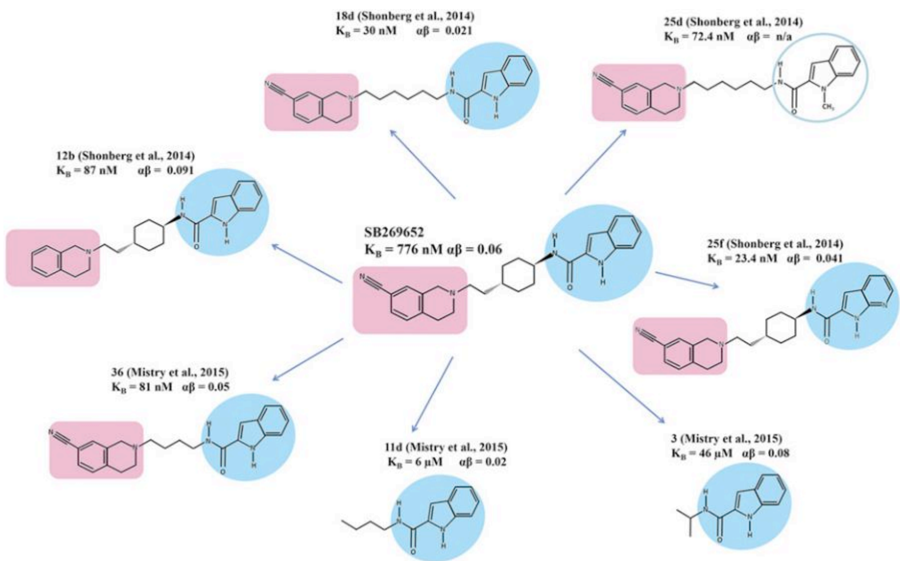

Fig. 2. Chemical structure of optimized SB269652 analogs. Value of KB (functional affinity) and ab (allosteric cooperativity with dopamine) were taken from Shonberg et al. (2015) and Mistry et al. (2015). The part of the molecule boxed in pink binds to the orthosteric site, whereas the part boxed in 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 im-proved “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 GTPgS recruit-ment 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 in-terestingly, depending on the functional assay, reduces dopa-mine 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 orthos-teric 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 carboxa-mide moiety. In particular, the mono-substituted deriva-tives 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 optimi-zations 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 alloste-ric 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 mecha-nism 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 com-pounds 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. Further-more, 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 modu-lator 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 re-ceptor 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 recep-tors, 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 allo-steric compounds (Wood et al., 2016).

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 dopa-mine 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 alloste-ric 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%, extrapy-ramidal 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 antipsy-chotics directly on patients to stay in such a narrow thera-peutic 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 dopa-mine receptor occupancy to be effective, are not extrapyra-midal 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 pro-pensity for extrapyramidal side effects. Even at low doses, aripiprazole reaches receptor occupancy of 85% without in-ducing 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 alterna-tive 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 demon-strated that treatment of rats with amphetamine, a drug that increases dopamine concentration in the synaptic cleft, in-creased 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 adeno-sine 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 alloste-ric effects depend on the integrity of the right quater-nary structure of the heterotetramer. This was shown in transfected mammalian cells and striatal tissue, by using heteromer-disrupting mutations and transmembrane pep-tides intercalating between receptors, respectively. The pecu-liarity 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 antipsy-chotic 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 hyperparathyroid-ism, and maraviroc, a negative allosteric modulator of chemo-kine 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 thera-peutic 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 li-gands, 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.