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Brief Article

Subtle modifications to the indole-2-carboxamide motif of the negative allosteric modulator *N*-((*trans*)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-1*H*-indole-2-carboxamide (SB269652) yield dramatic changes in pharmacological activity at the dopamine D receptor

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Subtle modifications to the indole-2-carboxamide motif of the negative allosteric modulator *N*-((*trans*)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-1*H*-indole-2-carboxamide (SB269652) yield dramatic changes in pharmacological activity at the dopamine D_2 receptor

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ABSTRACT: SB269652 (**1**) is a negative allosteric modulator of the dopamine D_2 receptor. Herein, we present the design, synthesis and pharmacological evaluation of 'second generation' analogues of **1** whereby subtle modifications to the indole-2-carboxamide motif confer dramatic changes in functional affinity (5000-fold increase), cooperativity (100-fold increase), and a novel action to modulate dopamine efficacy. Thus structural changes to this region of **1** allows the generation of a novel set of analogues with distinct pharmacological properties.

INTRODUCTION

The dopamine D_2 receptor (D_2R) is a member of the class A G protein-coupled receptor (GPCR) family.[1] To date, drug discovery at this target has primarily focused on the development of ligands that compete for the orthosteric site of the D_2R where dopamine binds. Such drugs are used in the clinic to treat disorders including schizophrenia (antagonists) and Parkinson's disease (agonists). Although orthosteric D_2R antipsychotics are effective at treating the positive symptoms of schizophrenia, use of these drugs is associated with significant side-effects.[2] First generation or typical antipsychotics (e.g. haloperidol and chlorpromazine), are associated with extrapyramidal side-effects (EPS). In contrast, second generation antipsychotics (exemplified by clozapine) display a lower risk of EPS, but are known to cause hematological (agranulocytosis), metabolic and cardiovascular side-effects.[2,3]

More recently, GPCR research has extended towards the development of allosteric modulators that interact with a topographically distinct binding site to that occupied by the endogenous ligand. Allosteric modulators may provide improved receptor sub-type selectivity compared to orthosteric drugs. In addition, unlike orthosteric ligands, because allosteric modulators can allow the endogenous neurotransmitter to bind the receptor, they may maintain the spatio-temporal profile of physiological signaling and instead modulate the magnitude of this signal. Finally, allosteric modulators have a saturable effect determined by their cooperativity with the endogenous ligands, thus a negative allosteric modulator (NAM) that displays moderate cooperativity with dopamine may act to partially antagonize the D_2R, relieving the positive symptoms of the disease without causing complete receptor blockade which is associated with the EPS of typical antipsychotics.[4]

SB269652 (**1**) was recently identified as the first small molecule NAM of the D_2R displaying an affinity of 776 nM and moderate negative cooperativity with dopamine ($\alpha\beta$ = 0.06, conferring a maximal 17-fold decrease in dopamine potency).[5,6] **1** was shown to bind the receptor in a bitopic manner whereby the 1,2,3,4-tetrahydroisoquinoline-7-carbonitrile (7CN-THIQ) "head" group occupies the orthosteric site and the cyclohexylene "linker" and the indole-2-carboxamide "tail" extend into a secondary pocket formed by residues from the extracellular ends of transmembrane helices 1, 2 and 7.[5,6] Further SAR investigations of **1** were subsequently conducted to determine the structural determinants of affinity and negative cooperativity at the D_2R. Modifications to the 7CN-THIQ head, the linker and the indole-2-carboxamide tail were examined for their impact upon affinity and negative cooperativity at the D_2R (Figure 1).[7] The results of this study showed that head groups with small substituents at the 7-position were necessary to maintain the moderate negative cooperativity of **1**, whilst replacement of the cyclohexylene linker with polymethylene linkers of varying lengths was observed to cause linker length dependent changes to allosteric modulation. Alterations to the indole-2-carboxamide tail, however, proved a key determinant of negative cooperativity. Replacement of the bicyclic indole with smaller aromatic and non-aromatic heterocyclic moieties (i.e. pyrrole and proline, respectively) led to a change in pharmacology from the weak negative cooperativity to apparently competitive pharmacology. Additionally, a derivative of **1** that lacked the indolic NH as a hydrogen bond donor displayed apparently competitive behavior, suggesting that this group is necessary for NAM action. Indeed, mutagenesis and molecular modeling studies, in addition to the above SAR study provided evidence of a hydrogen bond interaction between the indolic NH and $Glu95^{2.65}$ (where superscript numbering refers to Ballesteros-

Weinstein nomenclature)[8] and that this interaction was an important determinant of the affinity and negative cooperativity of these compounds.[6] Of particular note, substitution of the indole-2-carboxamide with a 7-azaindole-2-carboxamide (**2**, Figure 1) led to the highest affinity analogue of **1** to date with 30-fold higher affinity (K_B = 23.4 nM) and a similar level of negative cooperativity ($\alpha\beta$ = 0.038).[7]

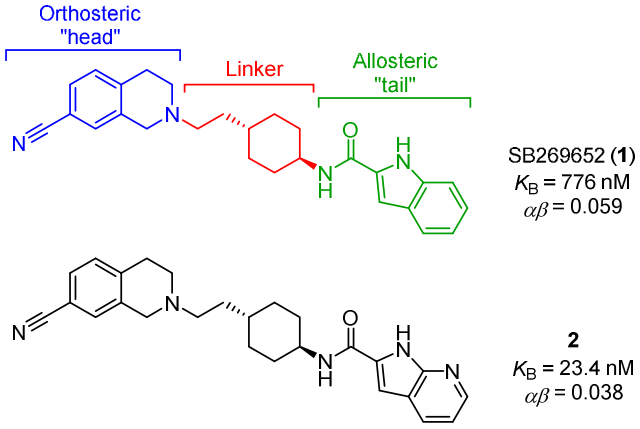


Figure 1. The chemical structure of SB269652 (**1**) highlighting its orthosteric head, linker and allosteric tail sections, and the chemical structure of the 7-azaindole-2-carboxamide analogue (**2**) that resulted from our initial SAR investigation, which exhibited a relative 30-fold increase in functional affinity whilst maintaining robust negative cooperativity with dopamine.

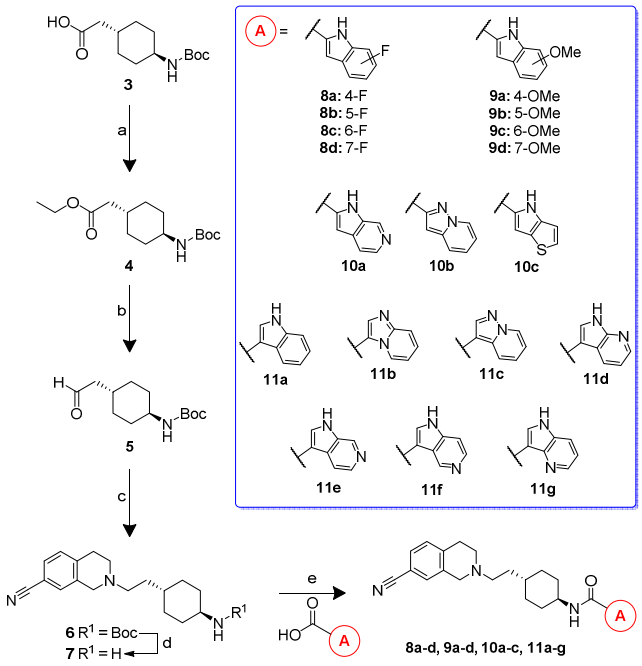
Given that our initial SAR investigation determined the indole-2-carboxamide motif to be an integral structural determinant in maintaining the NAM properties of **1** and the magnitude of this allosteric effect, a further SAR study focused primarily on the effects of modifications to the indole-2-carboxamide motif of SB269652 was conducted. In making modifications to this specific region of **1**, the aim was to develop a suite of analogues that displayed varying degrees of negative cooperativity in order to further our understanding of allosterism at the D_2R. The SAR study began with a preliminary investigation into the effects of electron-donating and electron-withdrawing substituents on negative cooperativity through attachment of either a methoxy or fluorine substituent, respectively, at positions 4-7 of the indole ring. To further explore various azaindole-2-carboxamide tail motifs, a nitrogen walk around the benzo component of the indole ring of **1** was planned since **2** showed a 30-fold enhancement in affinity and maintained negative cooperativity at the D_2R. Additionally, various azaindole-3-carboxylic acids were investigated to complement the azaindole-2-carboxamide series and enabled us to determine the effect of repositioning the carboxylic acid functionality (and therefore resulting carboxamide) from the 2- to the 3-position. Finally, a thienopyrrole analogue was also synthesized representing an isosteric replacement for the indole ring system of **1**.

RESULTS AND DISCUSSION

Chemistry. Scheme 1 depicts the synthetic route employed to furnish analogues of **1** containing differing "tail" moieties. The synthesis of all compounds generally followed previously established methods devised for the synthesis of **1** and associated analogues from our initial SAR investigations.[6,7] The versatile primary amine intermediate (**7**) and its precursors were synthesized following the procedures outlined in Lane et al.[6] Following Boc de-protection, two differing amide coupling reagents 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, HATU or (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate, BOP) were used in order to attach the required "tail" moieties since some carboxylic acids were unreactive in the presence of a variety of amide coupling reagents (e.g. HCTU, HBTU, EDC). HATU in the presence of DIPEA and dry DMF, however, facilitated the majority of amide couplings. The fluoro- (**8a-d**) and methoxy-substituted (**9a-d**) derivatives of SB269652 were successfully synthesized using HATU and BOP, respectively, in good yields (30-57% & 47-74%). Given the improved functional affinity of **2** in our previous SAR investigation, a nitrogen walk comprising of the various azaindole-2-carboxylic acids, and the corresponding pyrazolo- and imidazo-pyridine-2-carboxylic acids was undertaken. Upon attempting these couplings however, only **10a-c** were successfully synthesized. The remaining 2-substituted carboxylic acids generally favored self-dimerisation over coupling to the primary amine (**7**) (see supporting information), and in some cases, no reaction was observed. To enable a more thorough investigation of the biological impact of isosteric replacement of the CH for N, the corresponding 3-carboxylic acid substituted derivatives of indole, azaindoles, pyrazolo-pyridine and imidazo-pyridine (**11a-g**) were successfully synthesized in respectable yields (12-51%).

Pharmacology. In order to determine the effect of the synthesized compounds upon the action of the neurotransmitter dopamine, all compounds were tested in an assay measuring phosphorylation of ERK1/2 (pERK1/2) through activation of the long isoform of the D_2R ($D_{2L}R$) expressed in FlpIn CHO cells. All final compounds (**8a -11g**) were assessed for their ability to antagonize the action of increasing concentrations of dopamine.An example of these data is presented in Figure 2. The data was fitted with a derivation of the operational model of allosterism to derive values of functional affinity (K_B) and cooperativity with dopamine ($\alpha\beta$), where α is negative cooperativity with dopamine binding (that manifests in a limited concentration-dependent rightward shift in curves), and β is the modulation of dopamine efficacy (that, depending upon the level of receptor reserve, may manifest in both a limited a concentration-dependent decrease in potency and/or a depression in E_{max}).[6,9] The data were also fit to a Gaddum-Schild model of competitive antagonism to derive values of K_B and a Schild slope.[10] The data for each compound were analyzed using both models and the best fit was determined by an F-test. Note that, as such, the description of compounds being either allosteric or competitive within this study is an operational rather than mechanistic one. Thus, using this approach we cannot distinguish between competitive behavior and a ligand that displays very high negative cooperativity such that a saturable effect is not observed within the concentration range of the ligand that was used. These data reported (Table 1 and Figure 2) are presented as logarithms to allow for statistical comparison.[11] Values of Log $\alpha\beta$ < 0 signify negative cooperativity.

Scheme 1. Synthesis of the varying indole/azaindole-carboxamide derivatives of 1 (8a-d, 9a-d, 10a-c, 11a-g)[a]



[a]Reagents and conditions: (a) EtI, K2CO3, ACN, overnight 50 °C, quantitative; (b) 1 M DIBALH in toluene, -78 °C, 1 h, quantitative; (c) $NaBH(OAc)_3$, 1,2-DCE, rt, 16−24 h, 80%; (d) TFA, DCM, base extraction, 84%-quantitative; (e) HATU or BOP, DCM or dry DMF, DIPEA, rt, 3-24 h, 5-74%.

Initially, we were interested in evaluating the impact of electronic effects arising from simple aryl substituents given that replacement of the indole-2-carboxamide tail of **1** with the electron-deficient 7-azaindole-2-carboxamide (**2**) resulted in the most potent analogue of our initial SAR investigation. We synthesized a series of compounds containing fluorine-substituted and methoxy-substituted indole-2-carboxamide tails (**8a-8d** and **9a-9d**, respectively; Table 1) to test the effects of electron-withdrawing and electron-donating substituents on affinity and negative cooperativity. Upon pharmacological evaluation, it was evident that the electron-withdrawing fluorine substituent was beneficial for NAM activity at the D_2R. **8a** (4-F, K_B = 47 nM, $\alpha\beta$ = 0.03, 32-fold attenuation of dopamine potency) was observed to have virtually identical pharmacological characteristics as **2**, with a 30-fold improvement in functional affinity relative to **1**. **8b** (5-F, K_B = 6.8 nM, $\alpha\beta$ = 0.016) and **8d** (7-F, K_B = 4.5 nM, $\alpha\beta$ = 0.016) were observed to have significant improvements in functional affinity (110- and 194-fold, respectively) and a moderate increase in negative cooperativity (3-fold) compared to **1**. In contrast, **8c** (6-F, K_B = 219 nM, $\alpha\beta$ = 0.065) maintained functional affinity and negative cooperativity similar to that of **1**. This suggests that whilst incorporation of a fluorine substituent at the 4, 5 and 7 position of the indole-2-carboxamide tail conferred increases in affinity, fluorine substitution at the 6 position had no effect compared to **1** (Table 1). It is also interesting to compare these results with those of a previous study conducted on a novel class of indole-2-carboxamide small molecule allosteric modulators for the D_2R.[12] Within this study the *N*-isopropyl-1*H*-indole-2-carboxamide compounds with a fluorine substituent at the 4- or 7-position demonstrated negative cooperativity whilst analogues containing a fluorine substituent at the 5- and 6- position displayed apparently competitive pharmacology. In contrast, we observed NAM profiles for **8b** and **8c** despite the fact that their small fragment counterparts displayed competitive pharmacology. These results suggest that the SAR of these extended compounds does not match that of the isopropyl-1*H*-indole-2-carboxamide fragments in terms of apparent allosteric versus competitive behavior. As suggested by our previous study, such differences may relate to the role of the head and linker moieties conferring a distinct orientation of this motif within the allosteric binding site and/or additional roles for the head and tail group in dictating an allosteric versus an apparently competitive profile.[12] In contrast to the fluorinated derivatives, the methoxy derivatives (**9b-d**) demonstrated that a ring activating substituent conferred behavior best fit by a competitive model (Table 1) with significant improvements in functional affinity (**9b**; 111-fold, **9c**; 31-fold and **9d**; 15-fold), although in all three cases the Schild slope was below the value of unity expected for a competitive antagonist. However, the exception to this rule, compound **9a** (4-OMe, K_B = 617 nM, $\alpha\beta$ = 0.30) appeared to act as a NAM with weak negative cooperativity (5-fold less than **1**) and similar functional affinity to **1**. We speculate that the methoxy substituent at the 4 position (**9a)** may confer a distinct orientation of the indole motif within the allosteric pocket as compared to substitutions at the other positions. This distinct orientation may in turn lead to the distinct pharmacology observed. Based on this series of compounds, it is apparent that electronic effects may influence NAM activity at the D_2R, whereby the electron-withdrawing fluorine substituent appeared more favorable for NAM pharmacology whilst the electron-donating methoxy-containing derivatives appeared to confer competitive pharmacology. Another interesting observation from this series of analogues was that the addition of either substituent caused significant increases in functional affinity for the majority of the analogues. This finding is also significant as it suggests that the tail moieties of this series make a significant contribution to the affinity of these compounds. While this is in contrast to the findings from our initial findings with **1**, whereby the orthosteric head group was shown to be the predominant driver of affinity, it extends the observations of our previous manuscript in which relatively subtle changes to this motif conferred significant increases in affinity as exemplified by **2**.[8,12]

Based on the marked improvement in functional affinity observed for **2** (30-fold) compared to **1**, we undertook a nitrogen walk around the six-membered ring of the indole tail. This type of scaffold-hopping is a commonly applied medicinal chemistry strategy for molecular backbone replacements (in our instance, replacing the carbon at various positions of the benzo portion of the indole ring with nitrogen). It is a drug design strategy that is often utilised in the development of novel compounds with increased potency and altered physicochemical characteristics (Table 1).[13] Due to the synthetic intractability of the imidazopyridine- and a number of the azaindole-2-carboxylic acids, only **10a-c** were able to be synthesized in appreciable quantities. Nonetheless, we observed dramatic increases in functional affinity and negative cooperativity upon moving the nitrogen from the 7-position (**2**) to the 6-position (**10a**) of the azaindole motif. **10a** (K_B = 2.7 nM, $\alpha\beta$ = 0.0006) was observed to have a functional affinity that was 260- and 8-fold greater than **1** and **2**, respectively. Further-

more, **10a** is the NAM with the highest detectable negative cooperativity that we have identified to date (causing a 1800-fold maximal decrease in dopamine potency), 100-fold and 67-fold greater than that of **1** and **2,** respectively. We must acknowledge, however, that the action of a ligand with such high negative cooperativity may be indistinguishable compared to that of a competitive antagonist in an *in vivo* setting. Indeed, it is also possible that other derivatives that we have classified as competitive in our in vitro experiments may instead display even higher negative cooperativity such that they appear competitive. The pyrazolopyridine-2-carboxamide derivative, **10b** (K_B = 165 nM, $\alpha\beta$ = 0.03) displayed a 5-fold improvement in functional affinity compared to **1**, but maintained negative cooperativity similar to that of **2** despite a comparative 16-fold loss in functional affinity. In contrast to this, the thienopyrrole-2-carboxamide derivative, **10c** (K_B = 87 nM) displayed apparently competitive antagonism at the $D_{2L}R$, suggesting that isosteric replacement of the benzene ring of the indole motif with thiophene may be detrimental to the moderate NAM activity displayed by **1** (Table 1).

The attachment point to the scaffold was altered from the 2-position to the 3-position in order to conduct a more comprehensive investigation into the effects of a nitrogen-containing scaffold hop around the six-membered ring of the indole tail. Alteration of the substitution point of the carboxylic acid functionality resolved the incidence of self-dimerisation that was observed with some of the aryl-2- carboxylic acids. Consequently, the pyrazolopyridine-, imidazopyridine- and regioisomeric azaindole-3-carboxamides were successfully synthesized (**11a-g**, Table 1 and Figure 2). The indole-3-carboxamide variant of **1** (**11a**, K_B = 63 nM), was best fit by a model of competitive antagonist pharmacology but displayed a 12-fold improvement in functional affinity compared to **1**. **11c** (K_B = 40 nM), unlike its 2-carboxamide analogue (**10b**), displayed a 4-fold improvement in functional affinity compared to **1** but acted in an apparently competitive manner. The 7-azaindole-3-carboxamide derivative, **11d** (K_B = 34 nM, $\alpha\beta$ = 0.03), maintained similar values of negative cooperativity and functional affinity as compared to **2**. Compound **11e** (K_B = 10 nM, $\alpha\beta$ = 0.003) displayed an 8-fold loss in functional affinity and a 6-fold loss in negative cooperativity compared to its 2-carboxamide derivative, **10a**. The 5-azaindole-3-carboxamide variant, **11f** (K_B = 3.5 nM) (Figure 2B), displayed a functional affinity similar to that of **10a** but was best fit by a competitive mode of action (Table 1). Finally, the imidazopyridine-3-carboxamide analogue, despite lacking an indolic NH (**11b**; K_B = 24 nM, $\alpha\beta$ = 0.005), displayed NAM activity similar to **11e**.

Of particular note, the 4-azaindole-3-carboxamide derivative (**11g**) displayed a novel pharmacological profile compared to the other analogues in this series. Not only was **11g** observed to cause a limited dextral shift in dopamine potency in a similar fashion to our previous analogues, but it effected a significant limited depression in the E_{max} of dopamine (Figure 2). **11g** (K_B = 0.148 nM, α = 0.05, β = 0.16) is, to our knowledge, the first drug-like D_2R NAM to display an apparent sub-nanomolar functional affinity for the $D_{2L}R$ (~5000-fold improvement relative to **1**) and acts to modulate both dopamine potency (21-fold maximal decrease) as observed for **1**, but also exerts an additional effect upon dopamine efficacy (6-fold maximal decrease). A similar profile was observed for SB269652 analogues at the dopamine D_3 receptor.[14] Furthermore, a series of D_2R NAMs based upon the indole-2-carboxamide scaffold but lacking the 7CN-THIQ motif displayed a similar effect upon dopamine affinity and efficacy but displayed much lower affinity for the D_2R.[12] Thus, this finding expands the allosteric behavior of bitopic SB269652 derivatives at the D_2R. However, it should be noted that observations of decreases in E_{max} are not, on their own, conclusive evidence of an allosteric mode of action. Such effects can also be caused by a competitive antagonist with a slow rate of dissociation in conditions of hemi-equilibrium.[15] As such further experimental evidence is required to definitively describe the pharmacology of this intriguing ligand.

Finally, we observed that moderate negative cooperativity was maintained despite some analogues (e.g. **10b** and **11b**) lacking the indolic NH. This is in contrast to our previous SAR study in which we found that all derivatives of **1** that lacked this motif as a hydrogen bond donor displayed apparently competitive pharmacology with dopamine.[7] Furthermore, our mutagenesis studies and molecular modeling studies led us to propose that the indolic NH formed a hydrogen bond interaction with $E95^{2.65}$ residue at the top of TM2. Thus this study suggests that such a hydrogen bond is not necessary for allosteric pharmacology. This conclusion is in agreement with our mutagenesis studies that revealed that mutation of $E95^{2.65}$ to alanine did not result in a competitive mode of action for **1** but instead decreased both the affinity and the degree of negative cooperativity.[7]

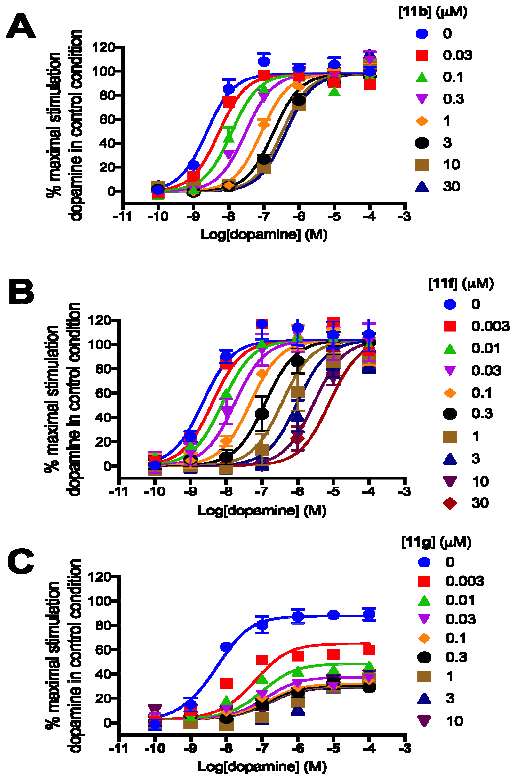
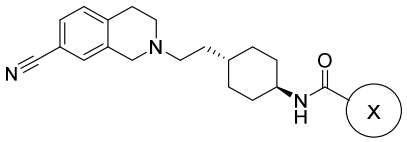
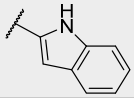
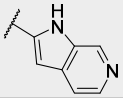
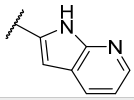
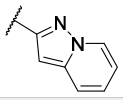
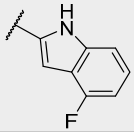
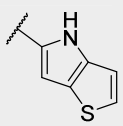
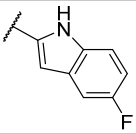
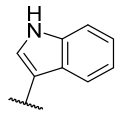
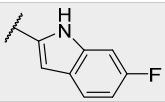
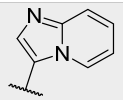
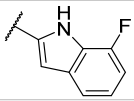
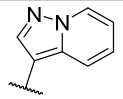
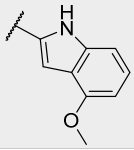
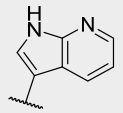
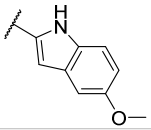
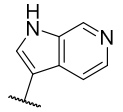
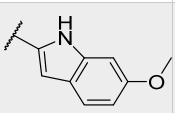
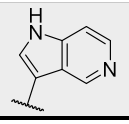
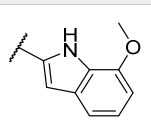
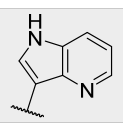


Figure 2: A scaffold hop strategy, replacing an aromatic carbon with nitrogen at different positions of the indole-3-carboxamide motif yielded analogues with distinct pharmacological profiles in an assay measuring ERK1/2 phosphorylation as a readout of D_2LR activation. Compound **11b** (**A**) was best fit by an allosteric model and causes a limited dextral shift of dopamine potency. This is distinct from the action of **11f** which causes a limitless shift and was best fit by a competitive model (**B**). (**C**) Compound **11g** causes a decrease in dopamine potency and E_{max} indicative of an additional modulatory effect upon dopamine efficacy.

Table 1. Pharmacological evaluation of the second generation SB269652 analogues through modifications of the indole-2-carboxamide tail



#	X	pK_B ± SEM[a] (K_B, nM)	Log$\alpha\beta$ ± SEM[b] ($\alpha\beta$) [Fold-shift][f]	Schild Slope ± SEM[c]	#	X	pK_B ± SEM[a] (K_B, nM)	Log$\alpha\beta$ ± SEM[b] ($\alpha\beta$) [Fold-shift][f]	Schild Slope ± SEM[c]
1		6.11±0.02 (776)	-1.23 ± 0.14 (0.059) [17×]	n/a[e]	**10a**		8.57 ± 0.09 (2.7)	-3.26 ± 0.09 (0.00055)* [1800×]	n/a[e]
2		7.63 ± 0.10 (23)*	-1.42 ± 0.09 (0.038)* [26×]	n/a[e]	**10b**		6.78 ± 0.12 (165)	-1.52 ± 0.09 (0.030) [33×]	n/a[e]
8a		7.33 ± 0.24 (47)*	-1.50 ± 0.19 (0.032) [32×]	n/a[e]	**10c**		7.06 ± 0.10 (87)*	n/a[b]	1.04 ± 0.04
8b		8.17 ± 0.29 (6.8)*	-1.80 ± 0.22 (0.016)* [63×]	n/a[e]	**11a**		7.20 ± 0.15 (63)*	n/a[b]	1.01 ± 0.06
8c		6.66 ± 0.39 (219)	-1.19 ± 0.29 (0.065) [15×]	n/a[e]	**11b**		7.62 ± 0.12 (24)	-2.31 ± 0.12 (0.0048) [210×]	n/a[e]
8d		8.35 ± 0.29 (4.5)*	-1.79 ± 0.18 (0.016)* [62×]	n/a[e]	**11c**		7.39 ± 0.21 (40)	n/a[b]	0.71 ± 0.07
9a		6.21 ± 0.06 (617)	-0.52 ± 0.11 (0.30)* [3×]	n/a[e]	**11d**		7.47 ± 0.11 (34)*	-1.50 ± 0.09 (0.032) [32×]	n/a[e]
9b		8.70 ± 0.32 (2.0)*	n/a[d]	0.54 ± 0.05	**11e**		8.00 ± 0.11 (10)	-2.44 ± 0.15 (0.0036) [275×]	n/a[e]
9c		7.60 ± 0.17 (25)*	n/a[d]	0.69 ± 0.04	**11f**		8.46 ± 0.21 (3.5)	n/a[b]	0.90 ± 0.04
9d		7.29± 0.13 (51)*	n/a[d]	1.28 ± 0.09					

#	X	pK_B ± SEM (K_B, nM)	Logα ± SEM (α) [Fold-shift][f]	Logβ ± SEM (β) [Fold-shift][f]
11g		9.83 ± 0.16 (0.148)	-1.32 ± 0.14 (0.048) [21×]	-0.80 ± 0.08 (0.16) [6×]

[a]Estimate of the negative logarithm of the equilibrium dissociation constant, [b]the logarithm of the net cooperativity factor with dopamine and [c]Schild slope, determined in an ERK1/2 phosphorylation assay measuring dopamine activation of the $D_{2L}R$ expressed in FlpIn CHO cells. Data represents the mean ± SEM of three experiments conducted in duplicate; [d]n/a: data were best fit by a competitive model of action therefore no cooperativity factor was derived. [e]n/a: data were best fit by an allosteric model therefore a Schild slope was not determined; [f]Fold-shift denotes the maximal fold attenuation of dopamine potency in the presence of the test compound. (*) Statistically different from the corresponding parameters for **1** ($p < 0.05$, one way ANOVA with Dunnett's post-hoc test.

More recent studies have revealed that derivatives of SB269652 that display apparently competitive behavior are also sensitive to this mutation.[16] Thus a hydrogen bond interaction between the indolic NH of **1** and the side chain of $E95^{2.65}$ appears to determine the degree of cooperativity but not whether **1** and its derivatives display allosteric versus competitive pharmacology. Furthermore, molecular dynamics simulations have provided evidence that the $E95^{2.65}$ to alanine mutation changes the shape of the D_2R secondary pocket and that this can modulate the allosteric properties of SB269652 that does not require a direct interaction with the ligand.[17] However, we should also note that even in the above derivatives, the amide NH of **1** and its derivatives may also be able to form an interaction with $E95^{2.65}$. Future studies using information from the recently determined crystal structure of the D_2R will provide further insight into such interaction.[18]

CONCLUSIONS

In our previous SAR investigation of 1, we determined the indole-2-carboxamide tail to be a key structural determinant of NAM activity at the D_2R. In particular, incorporation of a second nitrogen atom within the indole-2-carboxamide tail of **1** in our initial SAR investigation led to the discovery of our highest affinity analogue (**2**). Based on these findings, in the present study we paid particular attention to the influence of electronic effects upon affinity and cooperativity. A series of derivatives substituted with either a fluorine or methoxy substituent at the various positions around the benzo portion of the indole tail were synthesized. The fluoro-substituted derivatives (**8a-d**) demonstrated significant increases in functional affinity and modulated cooperativity based on the positioning of the fluorine. All fluoro-substituted compounds displayed moderate negative cooperativity from a level that was equivalent to 1 up to a 4-fold increase. Electron-donating methoxy-substituted derivatives (**9a-d**), in contrast, displayed both an increase in affinity relative to **1** and apparent competitive pharmacology at the D_2R with the exception of **9a** which displayed similar functional affinity and reduced negative cooperativity (5-fold). The positioning of the second nitrogen atom within the tail as well as the attachment point of the bicyclic aryl motif to the linker unit (either 2- or 3-substituted) influenced both affinity and, in particular, negative cooperativity The effects of incorporating a second nitrogen atom at different positions within the indole tail were studied via a scaffold hop. The introduction of a second nitrogen was observed to increase the functional affinity of all compounds within the 2-substituted series relative to **1**. It was particularly interesting to note that the level of negative cooperativity was markedly modulated by the position of the nitrogen within the 6-membered ring of the indole tail in a similar fashion to the fluorine-substituted analogues. For example, moving the nitrogen from the 7-(**2**) to the 6-position (**10a**) caused a dramatic (70-fold) increase in negative cooperativity. The repositioning of the attachment point of the indole-carboxamide tail from the 2- position to the 3-position yielded a compound that displayed 10-fold higher affinity than **1** but with apparently competitive pharmacology with dopamine. Interestingly, a similar scaffold-hop strategy to incorporate a second nitrogen at different positions yielded derivatives that displayed a range of different properties; from those that acted in an apparently competitive manner with dopamine to those that acted as NAMs. Of particular note **11g** was found to have subnanomolar affinity for the D_2R and acted to decrease the potency and maximal effect (E_{max}) of dopamine. Such an action may be consistent with a NAM that acts to modulate both the affinity and efficacy of dopamine. Thus this compound displays a distinct action at the D_2R compared to other NAM derivatives and therefore warrants further investigation. In general, we find that modifications to the tail motif of **1** provides an approach to modulate both affinity and negative cooperativity, and that relatively subtle changes to this motif result in quite dramatic differences in pharmacology.

EXPERIMENTAL

Refer to supporting information for details pertaining to the synthesis, purification, structural characterization and pharmacological evaluation of target compounds **8a-d**, **9a-d**, **10a-c** and **11a-g**. Representatives from the two coupling strategies employed to afford the target compounds are presented.

2-(2-((*trans*)-4-(1*H*-Pyrrolo[3,2-b]pyridine-3-carboxamido)cyclohexyl)ethyl)-7-cyano-1,2,3,4-tetrahydroisoquinolin-2-ium 2,2,2-trifluoroacetate (11g) (Method A). 2-(2-((*trans*)-4-Aminocyclohexyl)ethyl)-1,2,3,4-tetrahydroisoquinoline-7-carbonitrile (**7**, 114 mg, 402 μmol, 1 eq.), 1*H*-pyrrolo[3,2-*b*]pyridine-3-carboxylic acid (78.3 mg, 483 μmol, 1.2 eq.) and HATU (184 mg, 483 μmol, 1.2 eq.) were stirred in a minimal volume of anhydrous DMF (3-4 mL). To this was added an excess of DIPEA (140 μL, 804 μmol, 2 eq.) and the reaction was left to stir overnight and ceased upon complete consumption of the amine (LCMS). A mixture of sat. $NaHCO_3$ and water (1:1, 30-40 mL) was added to the reaction and stirring continued for a further 30 min. The resulting precipitate was filtered, washed then purified via preparative HPLC to yield the product as an off-white solid (30.0 mg, 14%). ^{1}H NMR (TFA salt) (CD_3OD) δ 8.70 – 8.56 (m, 3H), 7.76 (dd, J = 8.2, 5.9 Hz, 1H), 7.69 - 7.60 (m, 2H), 7.50 - 7.43 (m, 1H), 4.68 – 4.32 (m, 2H), 3.94 (m, 1H), 3.60 (m, 2H), 3.43 – 3.32 (m, 2H), 3.30 – 3.23 (m, 1H), 2.13 - 2.14 (m, 2H), 1.98 - 1.90 (m, 2H), 1.86 – 1.75 (m, 2H), 1.53 – 1.40 (m, 4H), 1.34 – 1.14 (m, 2H). ^{13}C NMR (CD_3OD) δ 162.4 (C), 136.8 (C), 135.9 (CH), 135.8 (C), 135.6 (CH), 132.9 (C), 131.3 (CH), 130.5 (CH), 129.7 (CH), 129.6 (C), 128.7 (CH), 117.8 (CH), 117.7 (C), 110.8 (C), 106.9 (C), 54.7 (CH_2), 52.0 (CH_2), 49.2 (CH_2), 48.3 (CH), 34.6 (CH), 32.0 (CH_2), 31.3 (CH_2), 30.6 (CH_2), 25.4 (CH_2). HPLC: t_R 4.14 min, >95% purity (214 and 254 nm). HRMS (*m/z*): $C_{26}H_{30}N_5O$ requires $[M+H]^+$ 428.2445; found 428.2449.

***N*-((*trans*)-4-(2-(7-Cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-4-methoxy-1*H*-indole-2-carboxamide (9a) (Method B).** To a solution of 4-methoxy-1*H*-indole-2-carboxylic acid (35 mg, 0.18 mmol, 1 eq.) in DMF (5 mL) was added DIPEA (1.2 eq.) under N_2 at rt. BOP (1.1 eq.) was then added, and the reaction left to stir for 5-10 min. The amine **7** (1.2 eq.) was then added slowly, and after 16 h the solvent was removed in vacuo and the resulting residue partitioned between DCM (20 mL) and sat. $NaHCO_3$ (30 mL). The organic phase was removed and the aqueous phase was further extracted with 3 × 10 mL portions of DCM. The organic layers were pooled, washed with water (30 mL), brine (30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to give the crude product. To remove excess HMPA, the crude product was dissolved in EtOAc and washed with 3 × 20 mL portions of 2 M brine solution. The EtOAc layer was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo and the resulting residue was purified by column chromatography (2:98 MeOH:$CHCl_3$) to give the title compound as a beige solid (47 mg, 56%). ^{1}H NMR (d_6-DMSO) δ 11.50 (d, J = 1.7 Hz, 1H) 8.13 (d, J = 8.1 Hz, 1H), 7.57 – 7.56 (m, 2H), 7.31 (d, J = 8.4 Hz, 1H), 7.24 (m, 1H), 7.09 – 6.99 (m, 2H), 6.49 (d, J = 7.4

Hz, 1H), 3.87 (s, 3H), 3.74 (m, 1H), 3.58 (s, 2H), 2.88 (m, 2H), 2.67 (m, 2H), 2.48 (m, 2H), 1.88 – 1.79 (m, 4H), 1.45 (dd, J = 14.4, 6.8 Hz, 2H), 1.34 – 1.28 (m, 3H), 1.06 (m, 2H). ^{13}C NMR (d_6-DMSO) δ 160.1 (C), 153.6 (C), 140.6 (C), 137.7 (C), 136.7 (C), 130.6 (C), 130.4 (CH), 129.7 (CH), 129.5 (CH), 124.1 (CH), 119.1 (C), 118.1 (C), 108.2 (C), 105.4 (CH), 100.1 (CH), 99.2 (CH), 55.3 (CH_2), 55.0 (CH_3), 54.8 (CH_2), 49.8 (CH_2), 48.2 (CH), 34.7 (CH), 33.6 (CH_2), 32.3 (CH_2), 31.7 (CH_2), 28.9 (CH_2). HPLC: t_R 6.32 min, >99% purity. HRMS (*m/z*): $C_{28}H_{33}N_4O_2$ requires $[M+H]^+$ 457.2604; found 457.2614.

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ABBREVIATIONS USED

allosteric cooperativity, $\alpha\beta$; functional affinity, K_B; phosphorylated extracellular signal-regulated kinase 1/2, pERK1/2.

ASSOCIATED CONTENT

Supporting Information. Synthesis and characterization for all final compounds including Molecular Formula Strings. This material is available free of charge via the Internet at http://pubs.acs.org.

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TOC Graphic

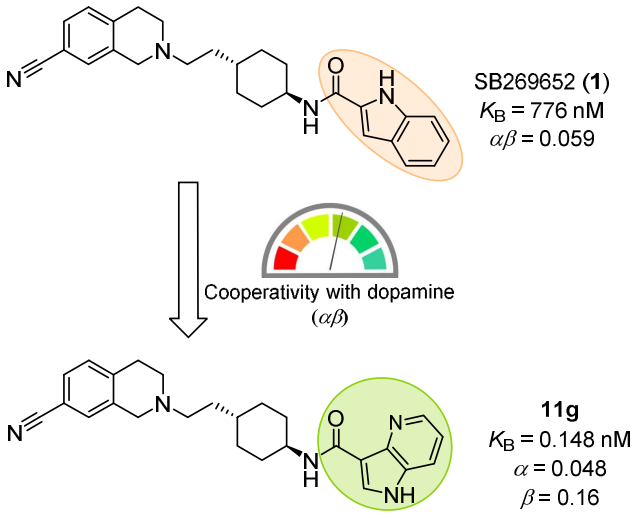

Table of Contents Graphic

Subtle modifications to the indole-2-carboxamide motif of the negative allosteric modulator *N*-((*trans*)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl)cyclohexyl)-1*H*-indole-2-carboxamide (SB269652) yield dramatic changes in pharmacological activity at the dopamine D_2 receptor