Exception That Proves the Rule: Investigation of Privileged Stereochemistry in Designing Dopamine D₃R Bitopic Agonists

Francisco O. Battiti, Amy Hauck Newman, and Alessandro Bonifazi*

Cite This: *ACS Med. Chem. Lett.* 2020, 11, 1956–1964

Read Online

ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: In this study, starting from our selective D₃R agonist FOB02-04A (5), we investigated the chemical space around the linker portion of the molecule via insertion of a hydroxyl substituent and ring-expansion of the *trans*-cyclopropyl moiety into a *trans*-cyclohexyl scaffold. Moreover, to further elucidate the importance of the primary pharmacophore stereochemistry in the design of bitopic ligands, we investigated the chiral requirements of (+)-PD128907 ((+)-(4aR,10bR)-2)) by synthesizing and resolving bitopic analogues in all the *cis* and *trans* combinations of its 9-methoxy-3,4,4a,10b-tetrahydro-2H,5H-chromeno[4,3-*b*][1,4] oxazine scaffold. Despite the lack of success in obtaining new analogues with improved biological profiles, in comparison to our current leads, a “negative” result due to a poor or simply not improved biological profile is fundamental toward better understanding chemical space and optimal stereochemistry for target recognition. Herein, we identified essential structural information to understand the differences between orthosteric and bitopic ligand–receptor binding interactions, discriminate D₃R active and inactive states, and assist multitarget receptor recognition. Exploring stereochemical complexity and developing extended D₃R SAR from this new library complements previously described SAR and inspires future structural and computational biology investigation. Moreover, the expansion of chemical space characterization for D₃R agonism may be utilized in machine learning and artificial intelligence (AI)-based drug design, in the future.

KEYWORDS: Bitopic Ligands, D₃R Agonists, GPCRs, Stereochemistry

The dopamine D₃ receptor subtype (D₃R) is a target of great interest due to its role in locomotor behavior, motivation, and substance use disorders (SUDs).^{1–3} D₃R belongs to the dopamine D₂-like family which includes D₂R, D₃R, and D₄R.⁴ When activated by endogenous dopamine (DA), or synthetic agonists binding to the orthosteric binding site (OBS), a signaling cascade is triggered, mediated via G_{i/o} inhibitory protein (inhibition of adenylyl cyclase), or through the β-arrestins, a spatially and temporally independent pathway.⁴

D₃R are highly expressed in neural circuits responsible for emotional and cognitive functions,¹ and their involvement in SUDs has been reported.⁵ In addition, we recently demonstrated how highly selective D₃R antagonists or partial agonists show promise in attenuating opioid self-administration and reinstatement to drug seeking.^{6–9}

Despite D₃R selective agonists being long sought after due to their potential in treatment of Parkinson’s disease and other

locomotor associated disorders, the discovery of highly selective D₃R agonists remains a significant challenge.^{10–12}

Due to high colocalization with D₂R in brain regions mediating similar physiological processes (i.e., reward, motivation, and locomotion), the importance of achieving significant D₃R subtype selectivity over D₂R,¹³ to disentangle their relative functions, remains a challenge in drug development.

In the early stages of drug design, the main approach has been to target the OBS with compounds identified via

Special Issue: Medicinal Chemistry: From Targets to Therapies

Received: December 30, 2019

Accepted: February 28, 2020

Published: February 28, 2020

PRIMARY PHARMACOPHORE AND LINKER STEREOCHEMISTRY OPTIMIZATION FOR D₃R BINDING AND SELECTIVITY

Figure 1. Bitopic molecular approach combining functionalized PPs, linkers, and SPs.

screening of large libraries of compounds and comparing SAR. Advances in X-ray crystallography and cryomicroscopy have enabled the resolution of several GPCR structures in their active and inactive states, empowering a target-driven drug design approach.¹⁴ Crystal structures of D₂R,¹⁵ D₃R,¹⁶ and D₄R¹⁷ have highlighted the existence of extended and structurally less conserved secondary binding pockets (SBPs).^{18–20} Targeting this SBP, which often can function as an allosteric binding site (ABS), whose engagement can positively (positive allosteric modulator, PAM) or negatively (negative allosteric modulator, NAM) modulate the OBS functional response, has emerged as an attractive approach to selectively target GPCRs with small molecules to control

specific pharmacological behaviors. Several academic research laboratories have pioneered the development of allosteric modulators selectively targeting GPCR subtypes.^{21–27}

In light of the discovery of a distinct SBP within the dopamine D₂-like receptors, we adopted a bitopic molecular drug design to generate highly selective ligands capable of discriminating among dopamine receptor subtypes.^{8,28,29} Our approach combines a primary pharmacophore (PP)—targeting the OBS—with a secondary pharmacophore (SP)—targeting the SBP—connected via meticulously designed linkers.³⁰ We recently reviewed and detailed how this approach bridges orthosteric and allosteric pharmacology.³⁰ We posited that the PP primarily controls target recognition and functional

Scheme 1. Compound 10^a

^a(a) Di-*tert*-butyl dicarbonate, DMAP, DCM, RT; (b) mCPBA, DCM, from 0 °C to RT; (c) K₂CO₃, toluene, DMF, Δ, microwave 75 W, 120 °C, 275 psi set point; (d) HCl (2N–4N in Et₂O), DCM, from 0 °C to RT; (e) EDC·HCl, HOBt, DIPEA, indole-2-carboxylic acid, DCM, RT.

Scheme 2. Compound 11^a

^a(a) BH₃·(CH₃)₂S, THF, from 0 °C to RT; (b) HCl (2N–4N in Et₂O), DCM, from 0 °C to RT; (c) EDC·HCl, HOBt, DIPEA, indole-2-carboxylic acid, DCM, RT; (d) Dess-Martin periodinane, DCM, from 0 °C to RT; (e) cat. AcOH, Na(AcO)₃BH, DCE, RT.

signaling cascades, while the SP mediates potency, selectivity, bias signaling, or allosterism. The linker induces the optimal binding pose(s) and is involved in its own ligand–receptor interactions resulting in unique pharmacological responses, otherwise impossible to achieve with combinations of two separate molecules.

We demonstrated how canonical D₃R preferential agonists, such as pramipexole, PF592379 (**1**), and (+)-PD128907 ((+)-(4aR,10bR)-**2**), exhibit low selectivity in optimized binding assays using the agonist radioligand [³H]-(*R*)-(+)-7-OH-DPAT.²⁹ Alternatively, through our bitopic drug design strategy, we have been able to generate the most selective D₃R full agonist reported to date, FOB02-04A (**5**) (Figure 1).²⁹ A similar drug design has resulted in some of the most selective and potent D₂R G-protein biased agonists reported.²⁸ This synthon-based bitopic drug design provides a novel approach to selective D₂R and D₃R agonists, partial agonists, inverse agonists, and antagonists.^{30,31}

Starting from **5**, we further investigated the chemical space around the linker, via insertion of a hydroxyl substituent and ring-expansion into a *trans*-cyclohexyl scaffold (Figure 1). Inspired by previous observations,²⁹ we explored the chiral requirements of (+)-(4aR,10bR)-**2** (nonselective D₃R agonist) by synthesizing bitopic analogues in all the *cis* and *trans*

combinations of its 9-methoxy-3,4,4a,10b-tetrahydro-2H,5H-chromeno[4,3-*b*][1,4]oxazine scaffold (Figure 1).

Results. Drug Design and Chemistry. We previously demonstrated that **1** is a suitable PP to be used in bitopic molecule design.²⁹ We showed that to generate high affinity and selective bitopic ligands an inversion of configuration at its (2*R*) stereocenter was necessary. Indeed, when in a bitopic configuration with a *N*-butyl-1*H*-indole-2-carboxamide SP (**3** and **4**), the (2*S*,5*S*) conformation of the PP in **4** resulted in a privileged architecture with >1000-fold increased affinity, and consequent selectivity for the D₃R with respect to its (2*R*,5*S*) enantiomer.²⁹ Full resolution of the chiral centers in the linker enabled further analysis of stereochemistry on affinity and selectivity of the bitopic molecules **5** and **6**, identifying **5** as the current lead (Figure 1).²⁹

Inspired by our previous work on D₃R antagonists, we expanded the linker chemical space, utilizing molecular fragments known to induce D₃R recognition selectively. Both compounds SB269952 (**7**) and SB277011A (**8**) (Figure 1) present a *trans*-cyclohexyl moiety in the linker, both demonstrate selective D₃R binding, and interestingly **7** shows a unique noncompetitive antagonist profile at D₂R.^{32–34} Similarly, a hydroxyl substituent in the butyl linking chain provided the most D₃R selective antagonists hitherto reported, **9**,⁸ currently under investigation for treatment of opioid use

Scheme 3. Compound 12 with Overall Resolution of Diastereomers at the Final Step^a

^a(a) AcONa, NH₂OH·HCl, EtOH, H₂O; (b) *p*-TsCl, pyridine, from 0 °C to RT; (c) Na, EtOH, from 0 °C to RT, 2 N HCl in H₂O, RT; (d) 2-chloroacetyl chloride, DIPEA, EtOAc, RT; (e) NaBH₄, EtOH:THF (1:3), RT; (f) tBuOK, DCM, 2-PrOH, RT; (g) LiAlH₄, THF, from 0 °C to RT; (h) 48% HBr in H₂O, Δ; (i) propionaldehyde, cat. AcOH, Na(AcO)₃BH, DCE, RT; preparative chiral HPLC (Chiralpak AD-H); (j) *N*-(4-oxobutyl)-1*H*-indole-2-carboxamide,²⁸ cat. AcOH, Na(AcO)₃BH, DCE, RT; (k) preparative chiral HPLC (Chiralpak AD-H).

disorders. We posited that the presence of a hydroxyl substituent, and the additional chiral center it introduced, would allow supplementary study of the linker chirality. We have previously observed that a switch from (*R*)-OH to (*S*)-OH in similar structures changes the functional efficacies from antagonists to partial agonists.^{18,35}

Following this fragment-inspired approach, we pursued the modification of 4 and 5 to their corresponding hydroxylated analogue 10, and ring-expansion to the cyclohexyl linker, 11 (Figure 1). Compounds 10 and 11 were obtained as a mixture of diastereomers (d.r. 75:25, Figure S1), and relative trans enantiomer (ee% > 99%, Figure S2), respectively, shown in Schemes 1 and 2.

Previous observations show that the (4*aR*,10*bR*) absolute configuration of (+)-(4*aR*,10*bR*)-2 was privileged for OBS binding but not suitable to bitopic modifications.²⁹ Inspired by our success optimizing the stereochemistry of 1 to improve its suitability as a bitopic PP, we investigated all the possible *cis* and *trans* bitopic combinations of 2.

nor-29 was prepared optimizing the precedent literature.^{36,37} Previously, *cis* and *trans* mixtures of diastereomers were separated at intermediate 27; however, we decided to perform an overall resolution of diastereomers at the final step (Scheme 3). The ratio of diastereomers remained constant ~3:1 (*trans*:*cis*) throughout the additional steps yielding 29. The *O*-demethylation step to obtain **nor-29** required optimization, using 48% HBr (water solution) under reflux and quenching it after 1.5 h. Longer reaction times led to epimerization at the benzylic position and overall decrease in the 3:1 ratio of diastereomers. Both (+)-(4*aR*,10*bR*)-2 and (-)-(4*aS*,10*bS*)-2 were isolated via preparative chiral HPLC, their absolute configurations assigned by comparing optical rotations and the analytical chiral HPLC chromatograms of the synthesized (+)-(4*aR*,10*bR*)-2 with the commercially available (+)-PD128907 used as the analytical standard (Figures S3 and S4).

The (±)-*cis*-2 diastereoisomer, and its mixture of two enantiomers, was not separated as previous studies reported

Figure 2. Determination of relative stereochemistry for **rel-trans-12** (central panel), **rel-cis-12** (right panel), and direct comparison to (+)-PD128907 ((+)-**trans-2**; left panel) by ^1H NMR.

the loss of affinity for D_2R and D_3R for the *cis*-9-methoxy-3,4,4a,10b-tetrahydro-2*H*,5*H*-chromeno[4,3-*b*][1,4]oxazine scaffold of **2**.³⁷ The lack of affinity for the ($\pm$)-*cis-2* does not necessarily predict low affinity when in a bitopic configuration. The intermediate **29** was thus converted into the bitopic analogue **12** via reductive amination in the presence of **30**. The mixture of four enantiomers was analyzed by chiral HPLC, and the *trans* and *cis* diastereomers were separated by preparative HPLC, into the corresponding mixture of enantiomers **rel-trans-12** (er 65:35) and **rel-cis-12** which we then carried into biological screening (Figure S5). The relative enantiomer abundance was not determined for **rel-cis-12** because resolution could not be achieved. It is unknown whether the racemic mixture tested has an equal distribution of the two *cis* enantiomers, or it is unbalanced toward one; however, the lack of biological activity did not support additional efforts in resolving the tested racemate. *Cis* and *trans* diastereoisomers' conformations for (+)-**trans-2**, **rel-trans-12**, and **rel-cis-12** were readily identifiable by comparison with the precedent literature and by ^1H NMR spectroscopy analyzing the coupling constant (*J*) for the protons in positions 4a and 10b (Figure 2).

Radioligand Binding Studies. All new compounds were tested for their affinities at hD_2R and hD_3R , in competition with the agonist [^3H]-(*R*)-(+)-7-OH-DPAT (Table 1). As previously discussed,^{20,28,29} the ability to evaluate agonist affinities for the receptors of interest in their active states allows for a better estimation of subtype selectivity and better resembles the ligand–receptor interaction under physiological conditions where only a fraction of the total available receptors are in the active state, and the agonists have to compete with endogenous dopamine (DA) for the OBS. Moreover, the radiolabeled agonist simplifies the binding protocol for testing new agonists' affinity in cell membrane preparations and generates monophasic curves, with optimal fitting, representing the high-affinity agonist bound state (Figure S6).

Affinities for the orthosteric agonists **1**, (+)-(**4aR,10bR**)-**2**, (–)-(**4aS,10bS**)-**2**, ($\pm$)-*cis-2*,³⁷ and bitopic agonists **3**, **4**, **5**, and **6**²⁹ are reported in Table 1 for a useful comparison with the synthesized analogues. Both (+)-(**4aR,10bR**)-**2** and

(–)-(**4aS,10bS**)-**2** were tested alongside the new compounds for assay validation. We confirmed that (+)-(**4aR,10bR**)-**2** has the favored stereochemical configuration for the 9-methoxy-3,4,4a,10b-tetrahydro-2*H*,5*H*-chromeno[4,3-*b*][1,4]oxazine scaffold.²⁹ Inversion of configuration of the PP obtaining (–)-(**4aS,10bS**)-**2** is detrimental for D_3R binding, with a loss of affinity >3000-fold.³⁷ However, it is important to study the *cis* and *trans* PP stereochemistry for a complete investigation of the stereochemical space and suitability of **2** as a PP in a bitopic configuration. Both **rel-trans-12** and **rel-cis-12** complement extensive SAR observations, suggesting that (+)-(**4aR,10bR**)-**2** is not suitable for either O- or N-bitopic alkylation;²⁹ we further observed that this is not only due to regiochemistry but also stereochemistry dependent, since (**4aR,10bR**) appears to be the sole privileged absolute configuration for the bitopic analogues of **2**. Compounds **rel-trans-12** and **rel-cis-12** showed low micromolar affinity for D_3R , and an overall loss of subtype selectivity in comparison with the parent lead.

Compound **5**²⁹ is reported in Table 1 as reference due to its improved D_3R affinity and selectivity in comparison to its stereoisomer **6** and parent compound **1**. Herein, novel bitopic analogues were designed and synthesized using the 5-((2*S*,5*S*)-5-methylmorpholin-2-yl)pyridin-2-amine PP, and their SAR were directed toward the study of the linker's chemical space. Both butyl-hydroxy and *trans*-cyclohexyl linkers are known to enhance D_3R affinity and selectivity in D_3R antagonists and partial agonists (Figure 1).^{8,18,34} Despite a moderate sub-micromolar D_3R affinity, **10** presented decreased binding when compared to both **4** and lead **5**. Despite the loss of affinity and selectivity, the value of this observation resides in the fact that the linker hydroxyl substitution, though optimal for D_3R antagonism, is not directly transferable to agonism, probably due to different receptor active and inactive conformations and pose codependence between the PP and linker. Introduction of the *trans*-cyclohexyl moiety³² affords an intermediate flexibility between **4** and the more strained **5** and was better tolerated. Analogue **11** presented the highest D_3R affinity of this series, similar to antagonists **7** and **8**, in the same order of magnitude

Table 1. Radioligand Competition Binding Assays Performed on HEK293 Cells Stably Expressing hD₂R, hD₃R, hD₁R, and hμOR^c

Compounds	Structure	hD ₂ L ₁ R <i>K_i</i> ± SEM (μM) (n=5) ^a	hD ₃ R <i>K_i</i> ± SEM (μM) (n=8) ^a	hD ₁ R <i>K_i</i> ± SEM (μM) (n=3) ^a	hμOR <i>K_i</i> ± SEM (μM) (n=4) ^a
1 (d.r. 3:1)		1.74 ± 0.348 (n=5) ^a	0.185 ± 0.0205*** (n=8) ^a	>100 ^b	>100 ^b
(+)-(4aR,10bR)-2		0.0280 ± 0.00559 (n=4)	0.00167 ± 0.000257*** (n=3)	>100 ^b	36.6 ± 19.8 (n=4)
(-)-(4aS,10bS)-2		7.58 ± 2.62 (n=4)	5.62 ± 2.30 ^{ns} (n=3)	>100 ^b	77.9 ± 41.4 (n=5)
(±)-cis-2		>10 ^a	>10 ^a	NT	NT
3		5.22 ± 0.735 (n=3) ^a	6.47 ± 0.675 ^{ns} (n=3) ^a	NT	NT
4		0.134 ± 0.0216 (n=4) ^a	0.00596 ± 0.000477**** (n=4) ^a	NT	NT
5		0.0878 ± 0.00981 (n=3) ^a	0.00185 ± 0.000137**** (n=3) ^a	5.63 ± 2.05 (n=3)	16.9 ± 3.10 (n=3)
6		0.831 ± 0.0995 (n=3) ^a	0.282 ± 0.024*** (n=3) ^a	NT	NT
10		1.01 ± 0.162 (n=5)	0.423 ± 0.164* (n=5)	>100 ^b	0.739 ± 0.0701 (n=4)
11		0.104 ± 0.0123 (n=4)	0.0216 ± 0.00178**** (n=5)	>100 ^b	21.9 ± 7.92 (n=4)
rel-trans-12		5.88 ± 2.03 (n=4)	5.54 ± 1.43 ^{ns} (n=5)	>100 ^b	12.4 ± 6.36 (n=4)
rel-cis-12		9.42 ± 3.44 (n=4)	13.8 ± 3.13 ^{ns} (n=5)	>100 ^b	12.3 ± 2.88 (n=4)

^aData previously reported.^{29,37} Unpaired Welch's *t*-test performed to compare selectivity for D₃R over D₂R *K_i*. ns = *P* > 0.05; * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.001; **** = *P* < 0.0001. ^b<50% inhibition at 100 μM, from 2 to 5 independent triplicate experiments. NT = not tested. ^cEquilibrium dissociation constants (*K_i*) were derived from IC₅₀ values using the Cheng–Prusoff equation.⁴⁰ Each *K_i* value represents the arithmetic mean ± S.E.M; *n* = number of independent experiments, each performed in triplicate.

with agonist **5**, and ~10–15-fold improved with respect to **1**. Despite a retained high D₃R affinity, **11** yielded just a moderate selectivity over D₂R (6.5-fold). Structural and stereochemical requirements for both the PP and linker are unique in the design of antagonists or agonists; furthermore, for the optimal D₃R agonist affinity/subtype selectivity, (2*S*,5*S*)-2-(6-amino-pyridin-3-yl)-5-methylmorpholino PP, (1*R*,2*S*)-methyl-cyclo-propyl-ethyl linker, and 2-indoleamide SP seem to be the privileged synthons. Nevertheless, compounds **5** and **11** will be further evaluated in functional assays to assess whether their receptor-induced conformations will induce bias signaling or allosteric modulation, as well as in computational and molecular simulation studies to correlate the experimentally measured structure–functions to molecular interactions at the receptor. Similarly, compounds (–)-(4*aS*,10*bS*)-**2**, (±)-*cis*-**2**, *rel-trans*-**12**, and *rel-cis*-**12**, despite their limited biological profiles, are valuable tools for computational biology. For the first time we are observing at a molecular level how changes in each fragment of bitopic drugs affect the overall poses of the entire molecule, and how this translates into pharmacological profiles, with the potential to finally identify the structural

fingerprints discriminating D₃R agonism and antagonism, as well as D₃R and D₂R subtype selectivity.

Given the established poor D₄R affinity associated with these scaffolds,²⁹ a subset of compounds was selected for off-target binding screening investigating affinities at different GPCRs highly colocalized and/or functionally related to D₃R. We performed competition experiments on dopamine D₁ receptors (D₁R), known to form functional heteromers *in vivo* with D₃R,³⁸ and μ opioid receptors (μORs), which are located in brain regions associated with reward and motivation and where D₃R modulate opioid self-administration.³⁹ Experiments were conducted in the presence of [³H]-SCH23390 and [³H]-DAMGO, using HEK293 cell membranes stably expressing hD₁R and hμOR, respectively. Among all the compounds, **5** presented the highest affinity for D₁R, with a low micromolar *K_i*. This does not affect the overall pharmacological applicability of the compound, since it still maintains a very high selectivity for D₃R over D₁R and μOR. This observation highlights how structurally different GPCR OBS can potentially accommodate identical PPs; however, the linker negatively affects the binding affinity, as small changes in the

linker's substituents or rigidity result in complete inactivity for D₁R.

All the studied compounds were inactive or poor binders for μ OR, with the exception of **10**, whose hydroxyl substituted linker was detrimental for D₃R binding but appears to be an important structural feature to achieve μ OR binding. Indeed, **10** is equipotent at D₃R and μ OR, suggesting that both receptors can be targeted by identical structural scaffolds, with the linker being the main discriminant. Compound **10** might very well exemplify the first synthetic analogue probing the possibility of future SAR studies directed toward multitarget ligands for simultaneous comodulation of dopaminergic and opioid pharmacology.

Discussion. The current study led us to a series of important conclusions which can guide future drug design investigations focused on D₃R agonist–receptor interactions.

Our data suggest that, when switching from an orthosteric to bitopic binding mode, **1** and (+)-(4aR,10bR)-**2** diverge toward different binding modes, possibly due to different receptor conformations needed to accommodate them within the OBS and SBP simultaneously. We highlighted that inverting the stereochemistry of **1**, from (2R,5S) to (2S,5S), when designing bitopic ligands allows the enantiospecific recognition of D₃R.³⁹ However, independently from stereochemical combinations, (+)-(4aR,10bR)-**2** has a unique structural motif which binds the D₃R OBS and is unsuitable for modifications into bitopic analogues. This underscores the possible existence of multiple active states for the D₃R when engaged with agonists.

These “negative” observations can be essential to build computational models predicting how PPs can be modified in their core and stereochemistry to improve affinity and selectivities. A direct comparison between our compounds, covering a wide range of differences in binding affinities and selectivity, can feed extensive structural biology studies and molecular dynamic simulations. Further investigations are elucidating whether these different ligand-induced receptor conformations potentially affect recruitment of second messengers ultimately inducing bias signaling.

Our data also suggest that bitopic agonists and antagonists bind differently within the D₃R active and inactive states, respectively. Despite the presence of a hydroxy substituent or a *trans*-cyclohexyl group in the linker promoting high affinity and selectivity for various D₃R antagonists (i.e., **7**, **8**, and **9**), the same substitutions applied to our bitopic agonists reduce D₃R affinity and subtype selectivity. These exemplify how SAR directed to explore different linkers' chemical space and chirality might represent an important future direction in drug design of highly selective D₃R agonists.

Considering the limited information regarding D₂-like receptor active states,^{15–17,41} these extensive data sets, along with our previous studies, provide a large library of D₃R agonists to elucidate a greater understanding of ligand–D₃R engagement and clarify the differences ultimately underlying multiple active and inactive receptor conformations.

Moreover, the growing interest in drug design assisted by artificial intelligence (AI)–machine learning (ML), and the extensive efforts directed toward multiparameter physiochemical analyses, would greatly benefit from our D₃R agonists library.^{42,43} ML requires accurate learning data sets and model validation establishing recognizable structural pattern for a model's predictive validity. Active compounds used for training sets are often taken from known experimental observations; however, chemogenetic SAR libraries usually lack inactive or

poorly binding compounds with stereochemistry-dependent structural features.^{44–46} Our molecules, with multiple characterized chiral centers, and detailed *in vitro* pharmacology, can help ML training, provide structural information on fragment-based drug design, and expand drug prediction libraries based on chirality and PP suitability for bitopic modifications.

Linker chirality and chemical space investigation allowed us to identify pharmacological profiles depending predominantly on its substitutions and rigidity: (i) Cyclopropyl-containing linker (**5**) is privileged for high D₃R affinity and selectivity and is also an important synthon for D₁R recognition directed toward D₁R–D₃R multitarget drug design. (ii) Cyclohexyl-containing linker (**11**) is well-tolerated at D₃R with moderate subtype selectivity, despite a slight loss in overall affinity. (iii) Hydroxyl-substituted linker (**10**) causes a loss in D₃R affinity, maintains a moderate subtype selectivity over D₂R, and results in an increased μ OR affinity. This might inspire new SAR efforts to generate D₃R– μ OR dual-target chemical tools for studying dopaminergic and opioid pharmacology in pain, reward, and motivation. These data also suggest that identification of new leads and structural pharmacophores for multitarget GPCR studies can be identified via off-target binding screening based on receptor colocalization and functional similarities, more than just simple structural analogies between receptor proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00660>.

Experimental methods, compound purity and characterization, cell culture and radioligand binding protocols, HPLC chromatograms, and HRMS-MS/MS analyses (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Alessandro Bonifazi – Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse—Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224, United States; orcid.org/0000-0002-7306-0114; Phone: (443)-740-2897; Email: alessandro.bonifazi2@nih.gov; Fax: (443)-740-2111

Authors

Francisco O. Battiti – Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse—Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224, United States

Amy Hauck Newman – Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse—Intramural Research Program, National Institutes of Health, Baltimore, Maryland 21224, United States; orcid.org/0000-0001-9065-4072

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acsmedchemlett.9b00660>

Author Contributions

F.O.B., A.H.N., and A.B. designed the project, supervised and performed experiments, and wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This project was supported by the National Institute on Drug Abuse—Intramural Research Program Z1A DA000424 and Z1A DA000609. The authors thank Dr. Ludovic Muller from the Structural Biology Core at NIDA-IRP for high-resolution MS/MS analyses, Dr. Daryl Guthrie, and Dr. Anerbasha Shaik from NIDA-IRP.

■ ABBREVIATIONS

CNS, central nervous system; OBS, orthosteric binding site; SBP, secondary binding pocket; ABS, allosteric binding site; SUD, substance use disorder; ML, machine learning; AI, artificial intelligence

■ REFERENCES

- (1) Sokoloff, P.; Le Foll, B. The dopamine D3 receptor, a quarter century later. *Eur. J. Neurosci* **2017**, *45* (1), 2–19.
- (2) Keck, T. M.; John, W. S.; Czoty, P. W.; Nader, M. A.; Newman, A. H. Identifying Medication Targets for Psychostimulant Addiction: Unraveling the Dopamine D3 Receptor Hypothesis. *J. Med. Chem.* **2015**, *58* (14), 5361–80.
- (3) Heidbreder, C. A.; Newman, A. H. Current perspectives on selective dopamine D(3) receptor antagonists as pharmacotherapeutics for addictions and related disorders. *Ann. N. Y. Acad. Sci.* **2010**, *1187*, 4–34.
- (4) Beaulieu, J. M.; Gainetdinov, R. R. The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol. Rev.* **2011**, *63* (1), 182–217.
- (5) Newman, A. H.; Blaylock, B. L.; Nader, M. A.; Bergman, J.; Sibley, D. R.; Skolnick, P. Medication discovery for addiction: translating the dopamine D3 receptor hypothesis. *Biochem. Pharmacol.* **2012**, *84* (7), 882–90.
- (6) Keck, T. M.; Burzynski, C.; Shi, L.; Newman, A. H. Beyond small-molecule SAR: using the dopamine D3 receptor crystal structure to guide drug design. *Adv. Pharmacol.* **2014**, *69*, 267–300.
- (7) You, Z. B.; Gao, J. T.; Bi, G. H.; He, Y.; Boateng, C.; Cao, J.; Gardner, E. L.; Newman, A. H.; Xi, Z. X. The novel dopamine D3 receptor antagonists/partial agonists CAB2–015 and BAK4–54 inhibit oxycodone-taking and oxycodone-seeking behavior in rats. *Neuropharmacology* **2017**, *126*, 190–199.
- (8) Kumar, V.; Bonifazi, A.; Ellenberger, M. P.; Keck, T. M.; Pommier, E.; Rais, R.; Slusher, B. S.; Gardner, E.; You, Z. B.; Xi, Z. X.; Newman, A. H. Highly Selective Dopamine D3 Receptor (D3R) Antagonists and Partial Agonists Based on Eticlopride and the D3R Crystal Structure: New Leads for Opioid Dependence Treatment. *J. Med. Chem.* **2016**, *59* (16), 7634–50.
- (9) You, Z. B.; Bi, G. H.; Galaj, E.; Kumar, V.; Cao, J.; Gadiano, A.; Rais, R.; Slusher, B. S.; Gardner, E. L.; Xi, Z. X.; Newman, A. H. Dopamine D3R antagonist VK4–116 attenuates oxycodone self-administration and reinstatement without compromising its anti-nociceptive effects. *Neuropsychopharmacology* **2019**, *44* (8), 1415–1424.
- (10) Joyce, J. N.; Millan, M. J. Dopamine D3 receptor agonists for protection and repair in Parkinson's disease. *Curr. Opin. Pharmacol.* **2007**, *7* (1), 100–5.
- (11) Piercey, M. F. Pharmacology of pramipexole, a dopamine D3-preferring agonist useful in treating Parkinson's disease. *Clin Neuropharmacol* **1998**, *21* (3), 141–51.
- (12) Das, B.; Modi, G.; Dutta, A. Dopamine D3 agonists in the treatment of Parkinson's disease. *Curr. Top. Med. Chem.* **2015**, *15* (10), 908–26.
- (13) Michino, M.; Donthamsetti, P.; Beuming, T.; Banala, A.; Duan, L.; Roux, T.; Han, Y.; Trinquet, E.; Newman, A. H.; Javitch, J. A.; Shi, L. A single glycine in extracellular loop 1 is the critical determinant for pharmacological specificity of dopamine D2 and D3 receptors. *Mol. Pharmacol.* **2013**, *84* (6), 854–64.
- (14) Michino, M.; Beuming, T.; Donthamsetti, P.; Newman, A. H.; Javitch, J. A.; Shi, L. What can crystal structures of aminergic receptors tell us about designing subtype-selective ligands? *Pharmacol. Rev.* **2015**, *67* (1), 198–213.
- (15) Wang, S.; Che, T.; Levit, A.; Shoichet, B. K.; Wacker, D.; Roth, B. L. Structure of the D2 dopamine receptor bound to the atypical antipsychotic drug risperidone. *Nature* **2018**, *555* (7695), 269–273.
- (16) Chien, E. Y.; Liu, W.; Zhao, Q.; Katritch, V.; Han, G. W.; Hanson, M. A.; Shi, L.; Newman, A. H.; Javitch, J. A.; Cherezov, V.; Stevens, R. C. Structure of the human dopamine D3 receptor in complex with a D2/D3 selective antagonist. *Science* **2010**, *330* (6007), 1091–1095.
- (17) Wang, S.; Wacker, D.; Levit, A.; Che, T.; Betz, R. M.; McCorvy, J. D.; Venkatakrishnan, A. J.; Huang, X. P.; Dror, R. O.; Shoichet, B. K.; Roth, B. L. D4 dopamine receptor high-resolution structures enable the discovery of selective agonists. *Science* **2017**, *358* (6361), 381–386.
- (18) Michino, M.; Boateng, C. A.; Donthamsetti, P.; Yano, H.; Bakare, O. M.; Bonifazi, A.; Ellenberger, M. P.; Keck, T. M.; Kumar, V.; Zhu, C.; Verma, R.; Deschamps, J. R.; Javitch, J. A.; Newman, A. H.; Shi, L. Toward Understanding the Structural Basis of Partial Agonism at the Dopamine D3 Receptor. *J. Med. Chem.* **2017**, *60* (2), 580–593.
- (19) Lyu, J.; Wang, S.; Balias, T. E.; Singh, I.; Levit, A.; Moroz, Y. S.; O'Meara, M. J.; Che, T.; Alga, E.; Tolmachova, K.; Tolmachev, A. A.; Shoichet, B. K.; Roth, B. L.; Irwin, J. J. Ultra-large library docking for discovering new chemotypes. *Nature* **2019**, *566* (7743), 224–229.
- (20) Keck, T. M.; Free, R. B.; Day, M. M.; Brown, S. L.; Maddaluna, M. S.; Fountain, G.; Cooper, C.; Fallon, B.; Holmes, M.; Stang, C. T.; Burkhardt, R.; Bonifazi, A.; Ellenberger, M. P.; Newman, A. H.; Sibley, D. R.; Wu, C.; Boateng, C. A. Dopamine D4 Receptor-Selective Compounds Reveal Structure-Activity Relationships that Engender Agonist Efficacy. *J. Med. Chem.* **2019**, *62* (7), 3722–3740.
- (21) Xiang, Z.; Lv, X.; Maksymetz, J.; Stansley, B. J.; Ghoshal, A.; Gogliotti, R. G.; Niswender, C. M.; Lindsley, C. W.; Conn, P. J. mGlu5 Positive Allosteric Modulators Facilitate Long-Term Potentiation via Disinhibition Mediated by mGlu5-Endocannabinoid Signaling. *ACS Pharmacol Transl Sci.* **2019**, *2* (3), 198–209.
- (22) Butkiewicz, M.; Rodriguez, A. L.; Rainey, S. E.; Wieting, J.; Luscombe, V. B.; Stauffer, S. R.; Lindsley, C. W.; Conn, P. J.; Meiler, J. Identification of Novel Allosteric Modulators of Metabotropic Glutamate Receptor Subtype 5 Acting at Site Distinct from 2-Methyl-6-(phenylethynyl)-pyridine Binding. *ACS Chem. Neurosci.* **2019**, *10* (8), 3427–3436.
- (23) Poslusney, M. S.; Salovich, J. M.; Wood, M. R.; Melancon, B. J.; Bollinger, K. A.; Luscombe, V. B.; Rodriguez, A. L.; Engers, D. W.; Bridges, T. M.; Niswender, C. M.; Conn, P. J.; Lindsley, C. W. Novel M4 positive allosteric modulators derived from questioning the role and impact of a presumed intramolecular hydrogen-bonding motif in beta-amino carboxamide-harboring ligands. *Bioorg. Med. Chem. Lett.* **2019**, *29* (3), 362–366.
- (24) van der Westhuizen, E. T.; Spathis, A.; Khajehali, E.; Jorg, M.; Mistry, S. N.; Capuano, B.; Tobin, A. B.; Sexton, P. M.; Scammells, P. J.; Valant, C.; Christopoulos, A. Assessment of the Molecular Mechanisms of Action of Novel 4-Phenylpyridine-2-One and 6-Phenylpyrimidin-4-One Allosteric Modulators at the M1 Muscarinic Acetylcholine Receptors. *Mol. Pharmacol.* **2018**, *94* (1), 770–783.
- (25) Von Moo, E.; Sexton, P. M.; Christopoulos, A.; Valant, C. Utility of an "Allosteric Site-Impaired" M2 Muscarinic Acetylcholine Receptor as a Novel Construct for Validating Mechanisms of Action of Synthetic and Putative Endogenous Allosteric Modulators. *Mol. Pharmacol.* **2018**, *94* (5), 1298–1309.
- (26) Khajehali, E.; Valant, C.; Jorg, M.; Tobin, A. B.; Conn, P. J.; Lindsley, C. W.; Sexton, P. M.; Scammells, P. J.; Christopoulos, A. Probing the binding site of novel selective positive allosteric modulators at the M1 muscarinic acetylcholine receptor. *Biochem. Pharmacol.* **2018**, *154*, 243–254.

- (27) Llinas Del Torrent, C.; Casajuana-Martin, N.; Pardo, L.; Tresadern, G.; Perez-Benito, L. Mechanisms Underlying Allosteric Molecular Switches of Metabotropic Glutamate Receptor 5. *J. Chem. Inf. Model.* **2019**, *59* (5), 2456–2466.
- (28) Bonifazi, A.; Yano, H.; Guerrero, A. M.; Kumar, V.; Hoffman, A. F.; Lupica, C. R.; Shi, L.; Newman, A. H. Novel and Potent Dopamine D2 Receptor Go-Protein Biased Agonists. *ACS Pharmacol Transl Sci.* **2019**, *2* (1), 52–65.
- (29) Battiti, F. O.; Cemaj, S. L.; Guerrero, A. M.; Shaik, A. B.; Lam, J.; Rais, R.; Slusher, B. S.; Deschamps, J. R.; Imler, G. H.; Newman, A. H.; Bonifazi, A. The Significance of Chirality in Drug Design and Synthesis of Bitopic Ligands as D3 Receptor (D3R) Selective Agonists. *J. Med. Chem.* **2019**, *62* (13), 6287–6314.
- (30) Newman, A. H.; Battiti, F. O.; Bonifazi, A. 2016 Philip S. Portoghesi Medicinal Chemistry Lectureship: Designing Bivalent or Bitopic Molecules for G-protein Coupled Receptors - The Whole is Greater Than the Sum of its Parts. *J. Med. Chem.* **2019**, in press. DOI: 10.1021/acs.jmedchem.9b01105
- (31) Newman, A. H.; Beuming, T.; Banala, A. K.; Donthamsetti, P.; Pongetti, K.; LaBounty, A.; Levy, B.; Cao, J.; Michino, M.; Luedtke, R. R.; Javitch, J. A.; Shi, L. Molecular determinants of selectivity and efficacy at the dopamine D3 receptor. *J. Med. Chem.* **2012**, *55* (15), 6689–99.
- (32) Kumar, V.; Moritz, A. E.; Keck, T. M.; Bonifazi, A.; Ellenberger, M. P.; Sibley, C. D.; Free, R. B.; Shi, L.; Lane, J. R.; Sibley, D. R.; Newman, A. H. Synthesis and Pharmacological Characterization of Novel trans-Cyclopropylmethyl-Linked Bivalent Ligands That Exhibit Selectivity and Allosteric Pharmacology at the Dopamine D3 Receptor (D3R). *J. Med. Chem.* **2017**, *60* (4), 1478–1494.
- (33) Furman, C. A.; Roof, R. A.; Moritz, A. E.; Miller, B. N.; Doyle, T. B.; Free, R. B.; Banala, A. K.; Paul, N. M.; Kumar, V.; Sibley, C. D.; Newman, A. H.; Sibley, D. R. Investigation of the binding and functional properties of extended length D3 dopamine receptor-selective antagonists. *Eur. Neuropsychopharmacol.* **2015**, *25* (9), 1448–61.
- (34) Verma, R. K.; Abramyan, A. M.; Michino, M.; Free, R. B.; Sibley, D. R.; Javitch, J. A.; Lane, J. R.; Shi, L. The E2.65A mutation disrupts dynamic binding poses of SB269652 at the dopamine D2 and D3 receptors. *PLoS Comput. Biol.* **2018**, *14* (1), No. e1005948.
- (35) Lane, J. R.; Abramyan, A. M.; Verma, R. K.; Lim, H. D.; Javitch, J. A.; Shi, L. Distinct antagonist-bound inactive states underlie the divergence in the structures of the dopamine D2 and D3 receptors. 2019, bioRxiv 640870v1. bioRxiv Preprint Server. <https://www.biorxiv.org/content/10.1101/640870v1>.
- (36) Dijkstra, D.; Mulder, T. B.; Rollema, H.; Tepper, P. G.; Van der Weide, J.; Horn, A. S. Synthesis and pharmacology of trans-4-n-propyl-3,4,4a,10b-tetrahydro-2H,5H-1-benzopyrano[4,3-b]-1,4-oxazin-7- and -9-ols: the significance of nitrogen pKa values for central dopamine receptor activation. *J. Med. Chem.* **1988**, *31* (11), 2178–82.
- (37) van Vliet, L. A.; Rodenhuis, N.; Dijkstra, D.; Wikstrom, H.; Pugsley, T. A.; Serpa, K. A.; Meltzer, L. T.; Heffner, T. G.; Wise, L. D.; Lajiness, M. E.; Huff, R. M.; Svensson, K.; Sundell, S.; Lundmark, M. Synthesis and pharmacological evaluation of thiopyran analogues of the dopamine D3 receptor-selective agonist (4aR,10bR)-(+)-trans-3,4,4a,10b-tetrahydro-4-n-propyl-2H,5H [1]b benzopyrano[4,3-b]-1,4-oxazin-9-ol (PD 128907). *J. Med. Chem.* **2000**, *43* (15), 2871–82.
- (38) Guitart, X.; Moreno, E.; Rea, W.; Sanchez-Soto, M.; Cai, N. S.; Quiroz, C.; Kumar, V.; Bourque, L.; Cortes, A.; Canela, E. I.; Bishop, C.; Newman, A. H.; Casado, V.; Ferre, S. Biased G Protein-Independent Signaling of Dopamine D1-D3 Receptor Heteromers in the Nucleus Accumbens. *Mol. Neurobiol.* **2019**, *56* (10), 6756–6769.
- (39) Jordan, C. J.; Humburg, B.; Rice, M.; Bi, G. H.; You, Z. B.; Shaik, A. B.; Cao, J.; Bonifazi, A.; Gadiano, A.; Rais, R.; Slusher, B.; Newman, A. H.; Xi, Z. X. The highly selective dopamine D3R antagonist, R-VK4–40 attenuates oxycodone reward and augments analgesia in rodents. *Neuropharmacology* **2019**, *158*, 107597.
- (40) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I₅₀) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22* (23), 3099–3108.
- (41) Zhou, Y.; Cao, C.; He, L.; Wang, X.; Zhang, X. C. Crystal structure of dopamine receptor D4 bound to the subtype selective ligand, L745870. *eLife* **2019**, *8*, e48822.
- (42) Carpenter, K. A.; Cohen, D. S.; Jarrell, J. T.; Huang, X. Deep learning and virtual drug screening. *Future Med. Chem.* **2018**, *10* (21), 2557–2567.
- (43) Plante, A.; Shore, D. M.; Morra, G.; Khelashvili, G.; Weinstein, H. A Machine Learning Approach for the Discovery of Ligand-Specific Functional Mechanisms of GPCRs. *Molecules* **2019**, *24* (11), 2097.
- (44) Carpenter, K. A.; Huang, X. Machine Learning-based Virtual Screening and Its Applications to Alzheimer's Drug Discovery: A Review. *Curr. Pharm. Des.* **2018**, *24* (28), 3347–3358.
- (45) Polash, A. H.; Nakano, T.; Takeda, S.; Brown, J. B. Applicability Domain of Active Learning in Chemical Probe Identification: Convergence in Learning from Non-Specific Compounds and Decision Rule Clarification. *Molecules* **2019**, *24* (15), 2716.
- (46) Wallach, I.; Heifets, A. Most Ligand-Based Classification Benchmarks Reward Memorization Rather than Generalization. *J. Chem. Inf. Model.* **2018**, *58* (5), 916–932.