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PNU-99194A: A Preferential Dopamine D_3 Receptor Antagonist

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INTRODUCTION

The neurotransmitter dopamine plays a central role in central nervous system (CNS)-related disorders such as schizophrenia and Parkinson's disease. Dopamine research has lately focused on the discovery of various receptor subtypes (42). At present, the dopamine family of G-protein-coupled receptors is divided into five subtypes. The D_1 family consists of the D_1 and D_5 receptors, while the D_2 family includes the D_2, D_3, and D_4 subtypes. Sokoloff and co-workers found that the D_3 receptor mRNA has a high abundance in limbic brain areas associated with cognitive and emotional functions (44). This is in contrast to the D_2 receptor subtype, which shows a high density both in limbic and striatal areas of the brain. It has been suggested that antagonists with selectivity for the D_3 receptors may offer some advantages as antipsychotics by means of increased efficacy, especially against the deficit aspects of schizophrenia (including negative symptoms and cognitive impairment) and fewer side effects (45,30).

A series of 2-aminoindans was synthesized with the aim of developing a selective D_3-receptor antagonist as a pharmacological tool and a template for drug discovery. This report will review the *in vitro* profile and *in vivo* pharmacology of the preferential D_3 antagonist PNU-99194A and its relevance towards understanding the functional role of the dopamine D_3 receptor.

CHEMISTRY

In order to identify new lead structures as dopamine D_3 receptor ligands, a variety of dopaminergic compounds were screened in an *in vitro* binding assay. It was found

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that the 5-OH indan analog **1** displayed a slight preference for the D_3 site (K_i ratio $D_2/D_3 = 3$), whereas the corresponding 4-OH analog **2** displayed a preference for the D_2 site (K_i ratio $D_2/D_3 = 0.08$) (Table 1, Fig. 1). Compound **2** was reported as a mixed $5\text{-}HT_{1A}$ and D_2 agonist, whereas compound **1** was inactive (20). It was hypothesized that disubstitution on the aromatic ring in the C5 and C6 positions may increase the preference for the D_3 receptor. In order to test this, compound PNU-99194A (3) was synthesized using slight modifications of the published route (9). This compound did indeed possess a 20-fold preference for the D_3 receptor site *in vitro*. PNU-99194A (5,6-dimethoxy-2-[dipropylamino]indan monohydrochloride) is a white, crystalline solid with a molecular weight of 313.18 ($C_{17}H_{27}NO_2 \cdot HCl$) and is highly soluble in aqueous solutions.

Fig. 1. Chemical structures.

PHARMACOLOGY

In Vitro Assays

Cannon and co-workers described the *in vitro* binding profile of PNU-99194A (JPC-211) in 1982 (9). They found that the compound displaces [^{3}H]spiperone from dopamine receptor sites (presumably D_2) in the calf striatum with an IC_{50} of 10.4 μM. When the agonist [^{3}H]ADTN (6,7-dihydroxy-2-aminotetralin) was used as a ligand, the IC_{50} was found to be 147 mM. PNU-99194A displaces the dopamine agonist [^{3}H]PNU-86170 from cloned D_2 (rat) receptors expressed in Chinese hamster ovary (CHO) cells with a K_i of 1572 nM (Table 1) (54). About 20-fold higher potency was observed for displacement of [^{3}H]spiperone from cloned D_3 receptors (rat) in stably

transfected CHO cells (K_i = 78 nM, Table 1). PNU-99194A was evaluated in a battery of binding assays at 1 μM concentration and was found to be inactive at acetylcholine, histamine, and other monoaminergic receptors and uptake sites.

PNU-99194A was inactive in various assays for intrinsic efficacy *in vitro*, e.g., assays for mitogenesis and extracellular acidification in CHO cells stably transfected with cloned D_3 receptors. On the other hand, PNU-99194A was found to antagonize the increase in extracellular acidification induced by dopamine (48,54). These data provide *in vitro* evidence for full D_3 receptor blockade by PNU-99194A. Interestingly, Ekman observed a small but significant GTP-sensitive binding in cloned D_3 cells, indicating intrinsic efficacy at these sites (12). This is in contrast to the other *in vitro* mitogenesis studies which suggested that this compound lacked intrinsic activity at D_3 receptors.

The *in vitro* D_3 selectivity for PNU-99194A is an improvement over originally identified D_3-preferring antagonists. For example, (+)-UH232 and close structural analogs show a 4- to 14-fold D_3 preference (19,44). Other D_3 preferring antagonists include nafadotride (39), S-(+)-14297 (16,33), GR-218231 (35), GR 103691 (34), PD 58491 (55) and the partial D_3 agonist PD-151328 (56). These compounds display D_3 vs. D_2 selectivities of 10- to 100-fold. It is important to note that these compounds still have quite high affinity for the D_2 receptor, despite the high selectivity ratios.

In Vivo Assays

Effects in Behavioral Models

Locomotor activity: Arneric and colleagues reported in 1982 that PNU-99194A failed to affect spontaneous locomotion at a dose of 12 μmol/kg in rats (2,6). In line with this, Waters et al. observed only a moderate elevation of exploratory behavior at 50 μmol/kg in rats to about 50% above control. On the other hand, when the rats were habituated to their environment for 60 min, PNU-99194A increased motor activity to approximately 600% of control over a wide dose range (54). A similar locomotor stimulation, including increased rearing, sniffing, licking, and eating was reported by Clifford and Waddington (10). In this case, the animals were habituated for three

TABLE 1. In vitro *binding (K_i, nM)*[a]

hours to the observation cages. In both studies, the activation was blocked by a dopamine antagonist. Also, other D_3 receptor antagonists, such as nafadotride (39), S-(+)-14297 (33), and a newly discovered series of dimeric benzimidazoles (56), appear to share these behavioral stimulatory effects. Clifford and Waddington failed to observe behavioral stimulation with nafadotride and GR 103691 (10). In the case of nafadotride, this may be explained by a lower D_3 selectivity as compared to PNU-99194A. PNU-99194A blocked the hypolocomotion induced by the D_3-preferring agonist pramipexole in rats, which indicates that the behavioral effects of PNU-99194A are mediated via D_3 receptors (52).

A weak motor activation was observed in non-habituated mice at lower doses of PNU-99194A while a decrease was observed at higher doses (15). While PNU-99194A failed to alter motor activity in the elevated plus maze, it increased flight reactivity in the social reactivity paradigm in a dose-dependent manner, suggesting an anxiogenic-like effect. This is in contrast to a recent report from Steiner et al. indicating that mice devoid of DA D_3 receptors (as a result of transgenic knock-out) display reduced anxiety (47). In addition, PNU-99194A was shown to be inactive in the ultrasonic vocalization assay in rats, suggesting a lack of potential anxiolytic activity (5).

The behavioral activation produced by PNU-99194A is likely mediated via a direct action in the CNS since local injections of the drug in the cerebral ventricles or the nucleus accumbens of the rat produced a locomotor stimulation (26). The effect is likely mediated via postsynaptic dopamine D_3 receptors since local injections of the drug into the cell body region (ventral tegmental area, A10) had no effect on motor activity (26). Furthermore, the locomotor stimulation induced by PNU-99194A is likely to be mediated via dopaminergic mechanisms since it could be blocked by reserpine or a low dose of haloperidol or raclopride (10,54). This suggests that an intact D_2 tone is necessary for the locomotor stimulatory effects of PNU-99194A.

Our interest in thoroughly characterizing the spectrum of activity of D_3 receptor blockade led to a speculation about possible interactions of D_1 agonists and antagonists with D_3 receptor antagonists. Data from recent experiments with habituated rats showed that the D_1 receptor agonist SKF38393 and the D_3 receptor antagonist PNU-99194A acted synergistically to produce locomotor stimulation at doses that, by themselves, failed to affect locomotor activity. Also, low doses of the dopamine D_1-antagonist SCH23390 did not change locomotor activity in habituated rats but blocked the hyperactivity induced by PNU-99194A. Thus, our preliminary behavioral data also suggest that D_1 and D_3 receptors may act in opposition regarding psychomotor activity (48). The inhibitory effects of D_3 receptors on psychomotor behavior is supported by observations in transgenic knock-out mice (1,27,57). Xu et al. observed that synergistic effects of D_1 and D_2 agonists on locomotor activity were more pronounced in D_3-receptor knock-out mice.

Interaction with dopamine agonists in behavioral assays: PNU-99194A failed to consistently block dopamine-agonist-induced hyperactivity in rodents. Thus, a biphasic interaction was observed when co-administered with d-amphetamine with a potentiation at lower doses and blockade at higher doses (54,52). A similar potentiation was observed when PNU-99194A was combined with the dopamine agonist 5,6-DiPr-ADTN (53). Interestingly, in a recent study in 1-methyl-4-phenyl-1,2,3,6-tetrahy-

dropyridine (MPTP)-lesioned cynomolgus monkeys, PNU-99194A was found to antagonize both, the increase in motor activity and the dyskinesia induced by the mixed dopamine agonist apomorphine and the D_3 preferring agonist PD 128907 (7). The development of supersensitive dopamine receptors as a result of MPTP-induced lesions may explain the profound agonist blocking properties of PNU-99194A in this assay.

Disruption of prepulse inhibition (PPi) or sensorimotor gating by psychostimulants in rodents is regarded to be a model of psychosis, since schizophrenic patients also suffer from a disrupted sensorimotor gating. Nichols et al. (36) reported that PNU-99194A failed to antagonize the hyperactivity or disruption of PPi induced by the NMDA-receptor antagonist, (+)-MK-801. This is in contrast to other dopamine antagonists, including olanzapine. Virden et al. also showed that PNU-99194A failed to block d-amphetamine induced disruption of pre-pulse inhibition (50).

Effects on positive reinforcement/drug discrimination in rodents: Although PNU-99194A produces a relatively weak stimulation of locomotor activity, it does establish conditioned place preference in the rat (25). In addition, Kling-Petersen (24) reported that PNU-99194A by itself failed to facilitate intracranial self-stimulation behavior in the rat while it potentiated the facilitation induced by a low dose (0.25 mg /kg) but not a high dose (1.0 mg/kg) of subcutaneously (s.c.) administered d-amphetamine. These observations are of interest since a number of studies have indicated a role for dopamine D_3 receptors in the reinforcing effects of cocaine (8). Baker et al. (4) reported that PNU-99194A was able to establish and maintain discriminative stimulus control in rats. Furthermore, the behavioral effects of PNU-99194A are dissimilar from those of the psychomotor stimulants cocaine, d-amphetamine, and caffeine, since PNU-99194A failed to substitute for these agents in drug discrimination studies in the rat (4). Thus, given the unique behavioral profile of D_3-receptor antagonists, i.e., a weak psychomotor activation with a lack of strong reinforcing properties, our data indicate the potential use of these agents as adjunctive treatments in the rehabilitation of drug addicts.

In a recent study, rats were trained to discriminate PNU-99194A from vehicle in a two-choice discrimination procedure under a FR 10 schedule of food reinforcement. In a substitution test, only the D_3 receptor-preferring locomotor stimulants, (–)-DS121 and (+)-AJ76, produced complete substitution (14). A series of other nonselective dopamine antagonists (haloperidol, amisulpiride, and sulpiride) as well as various dopamine agonists (d-amphetamine, apomorphine, pramipexole, a D_2-selective agonist PNU-95666, and the D_1-selective agonist SKF38393) all produced insignificant amounts of drug-appropriate responding and in several cases reduced response rates. It was concluded that PNU-99194A produces a distinct, subjective cue that is based on D_3-receptor antagonism. This drug discrimination paradigm appears to be well suited for verifying the *in vivo* activity of other D_3 receptor antagonists.

Effects on Brain Neurochemistry

PNU-99194A has only minor effects on the turnover of brain monoamines. Thus, the release of dopamine, measured by means of microdialysis in the rat striatum or the nucleus accumbens, was significantly elevated only at the highest dose tested (54).

Also, in a separate experiment, dopamine levels were not significantly changed when PNU-99194A was infused directly into the nucleus accumbens via the dialysis probe (52). It was found, however, that (DOPAC) and homovanillic acid (HVA) levels were increased at the highest concentration of PNU-99194A administered. In addition, unlike other dopamine (D_2)-receptor antagonists such as haloperidol, PNU-99194A failed to synergistically elevate striatal or accumbal levels of dopamine when combined with the dopamine reuptake inhibitor GBR 12909 (53). The disappearance rate of striatal or limbic dopamine in rats pretreated with α-methyl-para-tyrosine was also not affected by PNU-99194A (54). In the γ-butyro-lactone (GBL) model, which is designed to study interaction with synthesis regulating dopamine autoreceptors, a high dose of PNU-99194A was not only inactive but also failed to antagonize the decrease of dopamine synthesis rate induced by apomorphine (54). These results are in clear contrast to the effects of compounds with more potent D_2-blocking properties, including haloperidol, and they suggest that the D_3-receptor lacks direct release- or synthesis-regulating properties. Data from D_3 knock-out mice also support the suggestion that the D_3 receptors lack autoregulatory properties, (see below; 27).

In line with its weak dopamine D_2 receptor blocking properties, a high dose of PNU-99194A produced only a modest 30% displacement of the *in vivo* D_2 agonist ligand, 5,6-DiPr-ADTN, at the highest dose tested (200 μmol/kg s.c.). As a comparison, the classical antagonists haloperidol and raclopride produced approximately 80% maximal displacement of 5,6-DiPr-ADTN at striatal binding sites *in vivo* (53).

PNU-99194A antagonized the amphetamine-induced increase in brain energy metabolism (2-deoxyglucose utilization; 2-DG) in cortical brain areas whereas the elevation of 2-DG in the basal ganglia, including the striatum, remained essentially unchanged (51). McMichael et al. found that PNU-99194A blocked phencyclidine (PCP)-induced increase in brain energy metabolism, especially in the cortical brain areas (31). Only partial antagonism of PCP was observed in the extrapyramidal system. This would indicate potential antipsychotic properties.

Interestingly, in an assay studying the expression of the immediate early gene *fos*, PNU-99194A and clozapine both increased this endpoint in the infralimbic cortex (32). This is of particular interest since classical neuroleptics increase *c-fos* primarily in the caudate, while atypical agents are known to be more active in the cortical areas. In this same study, the effects on neurotensin gene expression were investigated. There was no significant effect by PNU-99194A in the accumbal shell while both remoxipride and clozapine were. active in this assay. The cortical activity of PNU-99194A in this assay is in agreement with the data on amphetamine antagonism in the 2-DG assay (51).

The relatively weak activity of PNU-99194A in the striatum was confirmed in an assay where brain levels of acetylcholine were measured in the rat. The dopamine agonists quinpirole and 7-OH-DPAT both elevated striatal acetylcholine levels measured postmortem. A high dose of PNU-99194A (30 μmol/kg) produced only a weak reduction of striatal acetylcholine levels and it completely failed to reverse the increase produced by 7-OH-DPAT in this assay. In contrast, both raclopride and haloperidol effectively blocked 7-OH-DPAT-induced elevation of striatal acetylcholine levels (43).

Effects on Physiological Parameters

Arneric et al. showed that PNU-99194A 12.8 μmol/kg s.c. did not inhibit food intake in male rats (2). PNU-99194A also failed to produce smooth muscle contraction the dog in an *in vitro* preparation, indicating a lack of sympatomimetic effects (3). Along the same line, it was observed that intravenous (i.v.) PNU-99194A (3 μmol/kg) failed to affect the activity of the cat cardioaccelerator nerve (9). This is in contrast to 4,5-dihydroxy, *N*,*N*-dipropyl-2 aminoindan, which inhibited the positive chronotropic effect of stimulation of the cardioaccelerator nerve in the anesthetized cat (an index of presynaptic dopamine receptor activity).

Kujacic showed that a high dose of PNU-99194A (30 mg/kg) blocked the increase of adrenal dopamine levels induced by dopamine agonists, including PNU-91356A, quinpirole, pramipexole, and 7-OH-DPAT (28). A higher dose of PNU-99194A (64 mg/kg) produced a weak increase (50%) in brain DOPAC levels, while it failed to affect adrenal levels of dopamine by itself (54). This is in contrast to the D_2 antagonist domperidone, which reduces adrenal dopamine levels (29). From these studies, it was concluded that D_3 receptors are not involved in the release of adrenal dopamine.

PNU-99194A was comparatively weak in antagonizing the decrease in substantia nigra pars compacta (SNPC) neuronal firing rate in the rat brain induced by a dopamine D_2 agonist PNU-91356A (21,22,38). PNU-99194A was more potent in antagonizing the inhibition of substantia nigra pars reticulata (SNPR) firing induced by the D_3 preferring agonist pramipexole, however, as compared to its ability to block PNU-91356-induced activation of these neurons (22). This observation is consistent with PNU-99194A's D_3 receptor preference. In contrast, haloperidol was more potent in blocking PNU-91356A-induced stimulation of SNPR firing rate. These findings provide additional support for an inhibitory postsynaptic D_3 receptor.

The dopamine D_3-receptor-preferring agonists 7-OH-DPAT and PD 128907 were both found to alter the firing rate (increases or decreases) of the ventral palladum neurons in the rat brain (23). Both PNU-99194A and (+)-AJ76 antagonized these effects in 73% and 70% of the neurons, respectively. In contrast, (+)-AJ76 was clearly weaker in blocking the responses of PNU-91356A (a D_2 receptor agonist) in the ventral pallidal neurons. These electrophysiological studies clearly support an important functional role of the D_3 receptor subtype in the regulation of basal forebrain neuronal activity.

Hypothesis on the functional role of dopamine D_3 receptors

In order to explain the behavioral stimulation induced by the D_3 receptor antagonist PNU-99194A, we proposed a hypothesis stating that the D_3 receptor appears to serve a behavioral inhibitory function at the postsynaptic level (54). In this way the postsynaptic D_2 and D_3 receptors would have opposing roles. We assumed that the activity of PNU-99194A is dependent on an intact dopaminergic tone, since the locomotor stimulation is blocked by means of monoamine depletion with reserpine or dopamine receptor blockade (haloperidol or raclopride). The postsynaptic action of PNU-

99194A is supported by the fact that the compound by itself is inactive in reserpinized animals, while it is able to potentiate the weak stimulatory effects of a low dose of apomorphine. In further support of a postsynaptic inhibitory D_3 receptor, Thorn et al. showed that the dopamine D_3-preferring agonist quinelorane can block d-amphetamine-induced hyperactivity at doses that do not reduce dopamine release (49). The possibility of a postsynaptic inhibitory dopamine receptor was actually proposed by Scheel-Krüger (40), before the dopamine D_3 receptor was discovered (see also 46).

The hypothesis of opposing roles for D_2, D_1, and D_3 receptors is also strengthened by the observation that the behavioral activation produced by the combined treatment with D_1 and D_2 agonists is more pronounced in transgenic D_3-receptor knock-out mice than in their corresponding wild types (57).

There are also other, nonbehavioral, studies in support of the suggestion that D_2 and D_3 receptors may act in opposition to each other. This is exemplified by the neuroleptic-induced increase or decrease in neurotensin mRNA in various brain regions of the rat (11,17). Furthermore, a recent study proposed that the signal transduction pathway of the D_3 receptor can involve both opposing and synergistic interactions with cyclic AMP (18). Flores and co-workers observed that animals with neonatal lesions of the ventral hippocampus display postpubertal super-sensitivity to dopamine agonists. In addition, they noted decreased D_3 receptor binding in several limbic brain areas while D_2 binding remained unchanged and D_1 receptor binding slightly increased (13). This strongly indicates a role for D_3 receptors in the behavioral changes induced by neonatal ventral hippocampal lesions. In light of this, it is interesting to note that the mRNA for the D_3 receptor is reported to be reduced in the parietal and motor cortices of patients with schizophrenia (41). The importance of D_3 receptors for cortical activity was highlighted in a recent 2-DG study. A high dose of PNU-99194A was shown to antagonize the effects of d-amphetamine on 2-DG in the rat brain and the antagonism occurred essentially in cortical areas, not in the striatum. This result indicates that D_3 receptor antagonists differ from classical antipsychotics (51).

Studies with D_3 Knock-Out Mice

In support of our hypothesis of a postsynaptic D_3 receptor inhibitory on psychomotor function, transgenic D_3 knock-out mice (D_3 KO) display weak but significant behavioral stimulation when exposed to a novel environment (1,57), an effect not associated with an anxiety state (47,57). Furthermore the synergistic increase of motor activity produced by combined D_2 and D_1 agonist treatment was more profound in D_3 KO mice than wild types (57). The authors proposed that a possible functional role of the D_3 receptor is to modulate behaviors by inhibiting the cooperative effects of postsynaptic D_1 receptors with other D_2 class sites. These findings are in line with our own observation that PNU-99194A needs an intact D_1 and D_2 tone to exert its behavioral effects. Somewhat surprisingly, White and colleagues (27) presented data suggesting that PNU-99194A and nafadotride induce behavioral stimulation in both the wild type and the D_3 KO mice. This observation suggests either that these compounds

may produce behavioral activation via a mechanism other than D_3 antagonism, or that compensatory changes may have developed in the transgenic knock-out animals.

Potential Clinical Utility of a D_3 Selective Antagonist

An important question that arises is whether a dopamine D_3-selective antagonist is optimal for the treatment of schizophrenia (including relief of both positive and negative symptoms). PNU-99194A potentiated the effects of d-amphetamine, probably via a blockade of postsynaptic D_3 receptors; although very high doses tended to antagonize d-amphetamine (52,54). PNU-99194A failed to show a robust antagonism of the disruption of pre-pulse inhibition induced by d-amphetamine or MK-801, or the locomotor hyperactivity induced by this latter stimulant. Thus, it is reasonable to suggest that efficacy in many of these traditional locomotor activity-based assays may reflect antagonism of dopamine D_2 receptors. On the other hand, PNU-99194A was shown to block the increase in brain energy caused by d-amphetamine or PCP, especially in the prefrontal cortex. In line with this, PNU-99194A-induced selective elevation of *c-fos* mRNA in the cortical areas is thought to be of importance for cognitive impairments in schizophrenia. Taken together with the fact that PNU-99194A produces a robust behavioral activation in habituated rats, it is likely that D_3 antagonists with this profile may be particularly effective against the deficit aspects of schizophrenia, perhaps ameliorating cognitive deficits and social withdrawal. In addition, the potential use of D_3 receptor ligands in the rehabilitation of drug addicts is supported by a number of studies (4,30).

SUMMARY

We have described the medicinal chemistry leading to the discovery of PNU-99194A, a preferential dopamine D_3 receptor antagonist. In addition, we have presented the *in vitro* and *in vivo* characterization of this unique compound. PNU-99194A binds with nanomolar potency to the D_3 receptor and displays a 20-fold lower potency at the D_2 receptor *in vitro*. *In vivo* studies suggest that this compound produces behavioral activation over a wide dose range, but no sedation or catalepsy. Although PNU-99194A produces behavioral activation, it does not possess strong positive reinforcing properties. Thus, the profile of this compound is very different from that of the D_2-preferring antagonists such as haloperidol (Table 2). These findings, in combination with data on *c-fos* expression and 2-DG utilization in the brain, indicate that compounds with this profile may prove to be antipsychotic with particular efficacy against negative symptoms and cognitive deficits, and are devoid of extrapyramidal side effects. Finally, PNU-99194A has proven to be a valuable tool for the investigation of the functional role of the D_3 receptor and has led to the hypothesis that the functional D_3 receptor is located postsynaptically and exerts an inhibitory role on psychomotor function.

TABLE 2. Comparison of some in vivo effects in the rat of the D_3-preferring antagonist PNU-99194A and the D_2-preferring antagonist haloperidol

Assay	PNU-99194A	Haloperidol
Effects on locomotor activity:		
Spontaneously exploring	weak increase	decrease
Habituated	increase	decrease
d-Amphetamine hyperactivity	potentiation	blockade
***In vivo* brain neurochemical effects:**		
Striatal DA metabolism/release	weak increase	strong increase
c-fos induction	med. PFCx	dorsal striatum
Striatal ACh	inactive	strong decrease
Electrophysiology:		
Antagonism of a DA D_2 agonist in the SNPC	weak	strong
Antagonism of a DA D_3 agonist in the SNPR	strong	weak

Abbreviations: DA, dopamine; Ach, acetylcholine; SNPC, substantia nigra pars compacta; SNPR, substantia nigra pars reticulata; med. PFCx, medial prefrontal cortex.

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