

ORIGINAL ARTICLE

The atypical antidepressant and neurorestorative agent tianeptine is a μ -opioid receptor agonistMM Gassaway¹, M-L Rives^{2,3}, AC Kruegel¹, JA Javitch^{2,3,4} and D Sames¹

Current pharmacological treatments of depression and related disorders suffer from major problems, such as a low rate of response, slow onset of therapeutic effects, loss of efficacy over time and serious side effects. Therefore, there is an urgent need to explore new therapeutic approaches that address these issues. Interestingly, the atypical antidepressant tianeptine already meets in part these clinical goals. However, in spite of three decades of basic and clinical investigations, the molecular target of tianeptine, as well as its mechanism of action, remains elusive. Herein, we report the characterization of tianeptine as a μ -opioid receptor (MOR) agonist. Using radioligand binding and cell-based functional assays, including bioluminescence resonance energy transfer-based assays for G-protein activation and cAMP accumulation, we identified tianeptine as an efficacious MOR agonist (K_i Human of 383 ± 183 nM and EC_{50} Human of 194 ± 70 nM and EC_{50} Mouse of 641 ± 120 nM for G-protein activation). Tianeptine was also a full δ -opioid receptor (DOR) agonist, although with much lower potency (EC_{50} Human of 37.4 ± 11.2 μ M and EC_{50} Mouse of 14.5 ± 6.6 μ M for G-protein activation). In contrast, tianeptine was inactive at the κ -opioid receptor (KOR, both human and rat). On the basis of these pharmacological data, we propose that activation of MOR (or dual activation of MOR and DOR) could be the initial molecular event responsible for triggering many of the known acute and chronic effects of this agent, including its antidepressant and anxiolytic actions.

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INTRODUCTION

On the basis of shortcomings of existing therapies, the broad field of depression research has formulated several key goals for the development of novel therapeutics, including faster onset of antidepressant effects, efficacy in treatment-resistant subjects and minimization of side effects.¹ The atypical antidepressant tianeptine (Figure 1a), an agent with established clinical efficacy, already addresses several limitations of standard antidepressants.² Namely, it shows fast effects against some depressive symptoms (cognitive and anxiety symptoms), is effective in patients resistant to selective serotonin reuptake inhibitor therapy³ and shows a better side effect profile compared with selective serotonin reuptake inhibitors and tricyclic antidepressants.² Interestingly, unlike other tricyclic antidepressants, tianeptine does not inhibit biogenic amine transporters, and despite longstanding interest in this agent, its direct target, and thus its molecular mechanism of action, have remained elusive.²

In preclinical studies, tianeptine has beneficial effects in a number of behavioral models related to depression, anxiety and other stress-related disorders.^{2,4} Tianeptine has been shown to modulate glutamatergic transmission, and its effects on neuroplasticity have been extensively studied in the hippocampus and amygdala.² For example, tianeptine restores stress-induced reduction of dendritic arborization in hippocampal neurons. Moreover, tianeptine reverses stress-induced inhibition of long-term potentiation at excitatory synapses in the hippocampus and prefrontal cortex.² Although tianeptine has been shown to modulate N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-

5-methyl-4-isoxazolepropionic acid (AMPA) receptor function,⁵ several reports have shown that tianeptine has no measurable affinity for these receptors² and also does not bind appreciably to monoamine neurotransmitter receptors, kainate receptors or monoamine transporters.⁶

Driven by the impressive and wide-reaching biological effects of tianeptine, we set out to elucidate its primary molecular target. We report here that tianeptine is an efficacious μ -opioid receptor (MOR) and δ -opioid receptor (DOR) agonist, and we propose that MOR-agonism (or combined MOR/DOR-agonism) underlies the clinical, preclinical and *in vitro* effects of tianeptine.

MATERIALS AND METHODS

Materials

HEK-293T cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in a 5% CO₂ atmosphere at 37 °C in Dulbecco's Modified Eagle Medium (high glucose no. 11965; Life Technologies; Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Premium Select, Atlanta Biologicals; Atlanta, GA, USA) and 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin (no. 15140, Life Technologies). Tianeptine sodium salt was purchased from Selleck Chemicals (Houston, TX, USA); [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO) acetate salt, naltrexone hydrochloride and forskolin were purchased from Sigma-Aldrich (Saint Louis, MO, USA); [D-Pen(2,5)]Enkephalin (DPDPE) and nor-binaltorphimine dihydrochloride were purchased from Tocris Bioscience (Minneapolis, MN, USA); U-50,488 and TIPP[psi] were obtained from the National Institute on Drug Abuse Drug Supply Program; coelenterazine H was purchased from Dalton Pharma Services (Toronto,

¹Department of Chemistry, Columbia University, New York, NY, USA; ²Department of Psychiatry, Columbia University, New York, NY, USA; ³Division of Molecular Therapeutics, New York State Psychiatric Institute, New York, NY, USA and ⁴Department of Pharmacology, Columbia University, New York, NY, USA. Correspondence: Professor JA Javitch, Division of Molecular Therapeutics, New York State Psychiatric Institute, 1051 Riverside Drive, Unit 19, New York, NY 10032, USA and Professor D Sames, Department of Chemistry, Columbia University, 3000 Broadway, MC3101, New York, NY 10027, USA.

E-mail: jaj2@columbia.edu and ds584@columbia.edu

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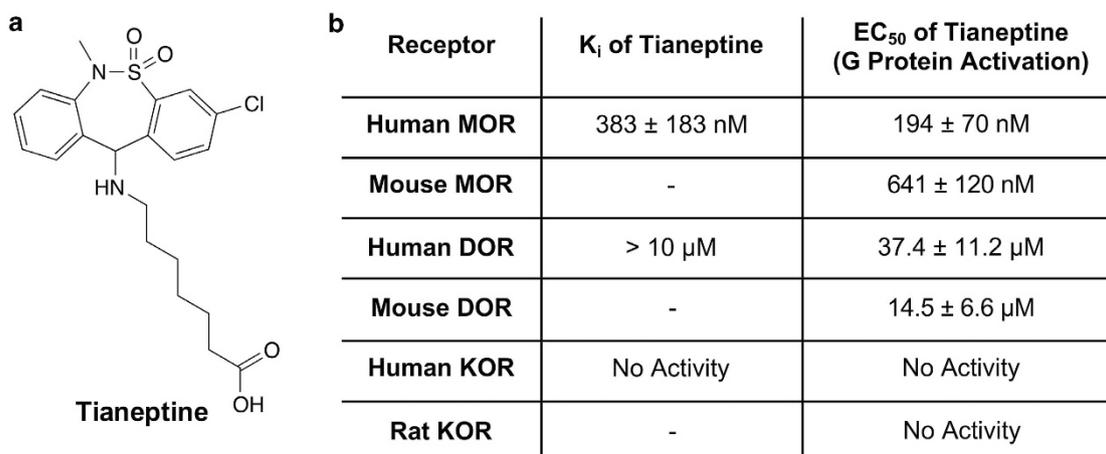


Figure 1. Summary of tianeptine's activity at the opioid receptors. (a) Chemical structure of tianeptine. (b) Radioligand displacement binding assay of tianeptine at μ -opioid receptor (MOR; $n = 3$), δ -opioid receptor (DOR; $n = 4$) and κ -opioid receptor (KOR; $n = 4$). Tianeptine's functional activity at MOR, DOR and KOR are also summarized. Data represent mean \pm s.e.m.

ON, Canada); polyethylenimine was purchased from Polysciences (Warrington, PA, USA).

Receptor screening and K_i determination

Receptor screening and K_i determination was generously performed by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract no. HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda, MD, USA. For experimental details, please refer to the PDSP website: <http://pdsp.med.unc.edu/>. Tianeptine was evaluated in a primary radioligand binding screen against a panel of 51 human central nervous system receptors and transporters, including serotonin receptors (5-HT1a,b,d,e; 5-HT2a,b,c; 5-HT3,5a,6,7); nicotinic acetylcholine receptors ($\alpha 2\beta 2$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 7$); adrenergic receptors ($\alpha 1A$, 1B, 1D, 2A, 2B, 2C; $\beta 1,2,3$); benzodiazepine receptor (BZP); cannabinoid receptors (CB1,2); dopamine receptors (D1–5); GABA_A; dopamine transporter (DAT); norepinephrine transporter (NET); serotonin transporter (SERT); DOR, κ -opioid receptor (KOR), MOR; histamine receptors (H1,3,4); muscarinic acetylcholine receptors (M1–5); peripheral benzodiazepine receptor (PBR); sigma 1,2. The primary screen was used to select ligands that showed >50% inhibition of binding of a radiolabeled reference compound at a single 10 μ M test concentration. This primary screen was followed by K_i determination when appropriate (>50% inhibition). Tianeptine was negative at all examined receptors (< 50% inhibition by 10 μ M tianeptine) except for MOR. K_i s were determined at MOR, as well as at DOR and KOR. The reported K_i values are the average of 4 (DOR), 3 (MOR) or 4 (KOR) independent experiments \pm s.e.m. Tianeptine also showed no agonist or antagonist activity at mGluR1a,2,4,5,6,8 receptors in primary functional assays at a 10 μ M test concentration (determined by PDSP).

DNA constructs

The mouse MOR (mMOR), the mouse DOR (mDOR) and the rat KOR (rKOR) were provided by Dr Lakshmi Devi at Mount Sinai Hospital. The human MOR (hMOR), human DOR (hDOR) and human KOR (hKOR) were obtained from the Missouri S&T Resource Center. The human G protein constructs used here have been previously described⁷ and were obtained from the Missouri S&T Resource Center unless otherwise noted. The G proteins used were: untagged $G_{\alpha_{OB}}$ ($G_{\alpha_{OB}}$); $G_{\alpha_{OB}}$ with *Renilla luciferase 8* (RLuc8) inserted at position 91 ($G_{\alpha_{OB}}$ -RLuc8, generously provided by C Galés^{7,8}); $G\beta_1$ (β_1); untagged $G\gamma_2$ (γ_2); $G\gamma_2$, which we fused to full-length mVenus at its N terminus via the amino acid linker GSAGT (mVenus- γ_2). YFP-Epac-RLuc (CAMYEL) was obtained from ATCC (no. MBA-277).⁹ All constructs were sequence confirmed.

Transfection

A total of 15 μ g of cDNA was transiently transfected into HEK-293T cells (5×10^6 cells per plate) in 10 cm dishes for G-protein activation (2.5 μ g

MOR/DOR/KOR, 0.125 μ g $G_{\alpha_{OB}}$ -RLuc8, 6.25 μ g β_1 , 6.25 μ g mVenus- γ_2) or cAMP inhibition assays (1.25 μ g MOR/DOR/KOR, 10 μ g CAMYEL, 1.25 μ g $G_{\alpha_{OB}}$, 1.25 μ g β_1 , 1.25 μ g γ_2) using polyethylenimine in a 1:1 ratio (diluted in Opti-MEM, Life Technologies). Cells were maintained in the HEK-293T media described above. After 24 h, the media was changed, and the experiment was performed 24 h later (48 h after transfection). Saturation curves using 3H-diprenorphine were performed to determine the expression of KOR, MOR and DOR. We estimated KOR density to be approximately five hundred thousand receptors per cell and the MOR and DOR cellular concentration to be approximately one million receptors per cell (data not shown).

Bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) experiments were performed as described previously.^{7,8,10} Briefly, transfected cells were dissociated and resuspended in phosphate-buffered saline. Approximately 200,000 cells per well were added to a black-framed, white well 96-well plate (no. 60050; Perkin Elmer; Waltham, MA, USA). The microplate was centrifuged and the cells were resuspended in either phosphate-buffered saline (G-protein activation) or phosphate-buffered saline containing 1 μ M forskolin (cAMP accumulation). After 5 min, 5 μ M solution of the luciferase substrate coelenterazine H was added to each well. After 5 min, ligands were added and the BRET signal was measured at 5 min on a PHERAstar FS plate reader (BMG Labtech, Cary, NC, USA). The BRET signal was calculated as the ratio of the light emitted by the energy acceptor, mVenus (510–540 nm), over the light emitted by the energy donor, RLuc8 (485 nm). After agonist stimulation, a decrease of the BRET signal was measured, which reflected a change of conformation or dissociation between the different subunits of the G protein. This drug-induced BRET signal was transformed (multiplied by -1) and normalized using the E_{max} of the full agonists DAMGO (MOR), DPDPE (DOR) or U-50,488 (KOR), which was defined as the 100% maximal response for G-protein activation or as 0% for the maximal inhibition of forskolin-stimulated cAMP accumulation in the cAMP inhibition assay. Dose–response curves were fit using a three-parameter logistic equation in GraphPad Prism 6 (Graphpad Software, La Jolla, CA, USA). All experiments were performed three to six times, and data represent mean \pm s.e.m. of those independent trials.

RESULTS

On the basis of *in vivo* pharmacological experiments, tianeptine has been proposed to act as an adenosine A1 receptor (A1R) agonist.¹¹ However, we found that tianeptine had no direct activity at A1R (Supplementary Information). Therefore, in an attempt to elucidate its primary molecular target, we screened tianeptine at a concentration of 10 μ M against a broad panel of human brain receptors (>50 receptors, Psychoactive Drug Screening Program, University of North Carolina). Tianeptine bound to

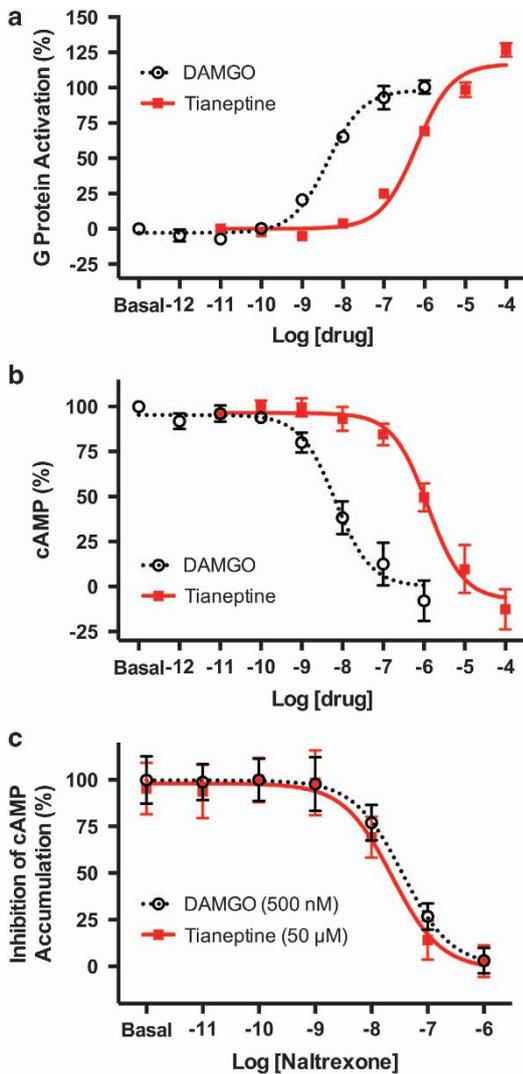


Figure 2. Tianeptine is a full agonist at μ -opioid receptors (MORs). (a) The mouse MOR was co-expressed with $G_{\alpha_{OB}}$ -RLuc8, β_1 , and mVenus- γ_2 to assay G-protein activation. MOR agonist [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO) was used as a positive control. (b) The mouse MOR was co-expressed with $G_{\alpha_{OB}}$, β_1 , γ_2 and the bioluminescence resonance energy transfer (BRET) sensor CAMYEL to assay inhibition of forskolin-stimulated cAMP accumulation. (c) The mouse MOR was co-expressed with $G_{\alpha_{OB}}$, β_1 , γ_2 and the BRET sensor CAMYEL to measure the inhibition of cAMP inhibition by tianeptine and the MOR agonist DAMGO. Naltrexone dose-dependently inhibits cAMP inhibition by both DAMGO and tianeptine with an IC_{50} of 35.3 ± 2.3 and 23.3 ± 3.9 nM, respectively. Data represent mean \pm s.e.m. of six independent experiments.

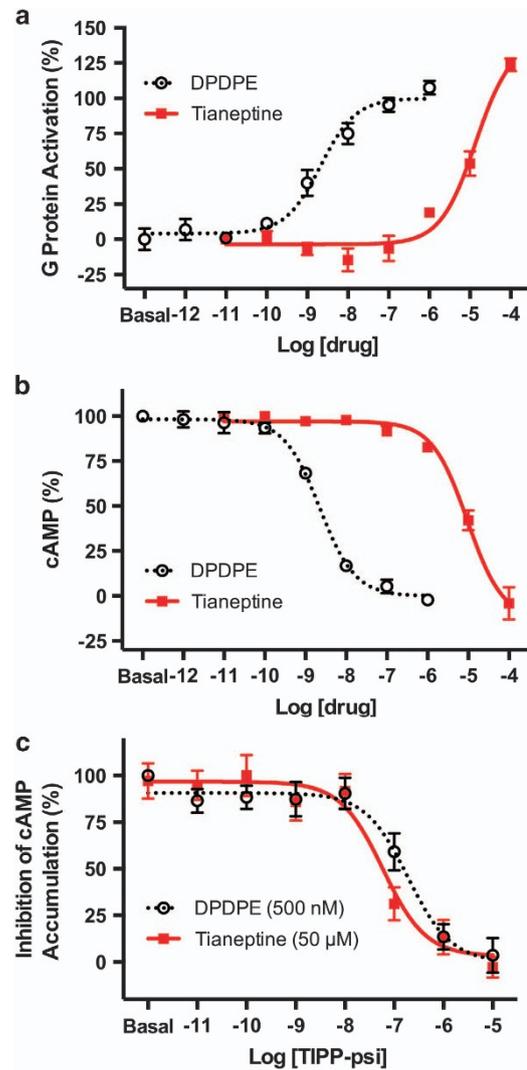


Figure 3. Tianeptine is a full agonist at δ -opioid receptors (DORs). (a) The mouse DOR was co-expressed with $G_{\alpha_{OB}}$ -RLuc8, β_1 and mVenus- γ_2 to assay G-protein activation. DOR agonist [D-Pen(2,5)] Enkephalin (DPDPE) was used as a positive control. (b) The mouse DOR was co-expressed with $G_{\alpha_{OB}}$, β_1 , γ_2 and the bioluminescence resonance energy transfer (BRET) sensor CAMYEL to assay inhibition of forskolin-stimulated cAMP accumulation. (c) The mouse DOR was co-expressed with $G_{\alpha_{OB}}$, β_1 , γ_2 and the BRET sensor CAMYEL to measure the inhibition of cAMP inhibition by tianeptine and the DOR agonist DPDPE. TIPP-psi dose-dependently inhibits the cAMP inhibition by both DPDPE and tianeptine with an IC_{50} of 232 ± 159 and 95.4 ± 76.6 nM, respectively. Data represent mean \pm s.e.m. of three independent experiments.

hMOR, the only hit identified in the entire panel, with a K_i of 383 ± 183 nM (Figure 1b). We therefore further investigated its activity at this receptor (both human and mouse isoforms), as well as at the related DOR and KOR, which also couple to $G_{i/o}$ proteins. Using previously reported BRET-based assays, tianeptine showed full agonism at mMOR for G-protein activation (Figure 2a, EC_{50} (mMOR, G Protein) = 641 ± 120 nM), as well as downstream inhibition of cAMP accumulation (Figure 2b, EC_{50} (mMOR, cAMP inhibition) = 1.03 ± 0.10 μ M).^{7–10} In addition, the tianeptine-induced activation of MOR was blocked by the opioid antagonist naltrexone in a dose-dependent manner (Figure 2c). The measured potency of tianeptine at hMOR was higher than that at mMOR in both G protein and cAMP inhibition (Figure 1b,

EC_{50} (hMOR, G Protein) = 194 ± 70 nM; EC_{50} (hMOR, cAMP Inhibition) = 151 ± 45 nM, Supplementary Information).

Next, we studied the activity of tianeptine at the DOR. We found that tianeptine binds to hDOR (Figure 1b, $K_i > 10$ μ M) and is also a full agonist at mouse DOR, although with an order of magnitude lower potency compared with MOR for both G-protein activation (Figure 3a, EC_{50} (mDOR, G Protein) = 14.5 ± 6.6 μ M) and inhibition of cAMP accumulation (Figure 3b, EC_{50} (mDOR, cAMP inhibition) = 9.46 ± 1.34 μ M). The DOR antagonist TIPP[psi]¹² dose-dependently blocked the tianeptine-induced activation of mDOR (Figure 3c). Interestingly, we also found a difference in the potency of tianeptine between the human and mouse DOR; in this case, tianeptine was less potent at the hDOR (EC_{50} (hDOR, G protein) =

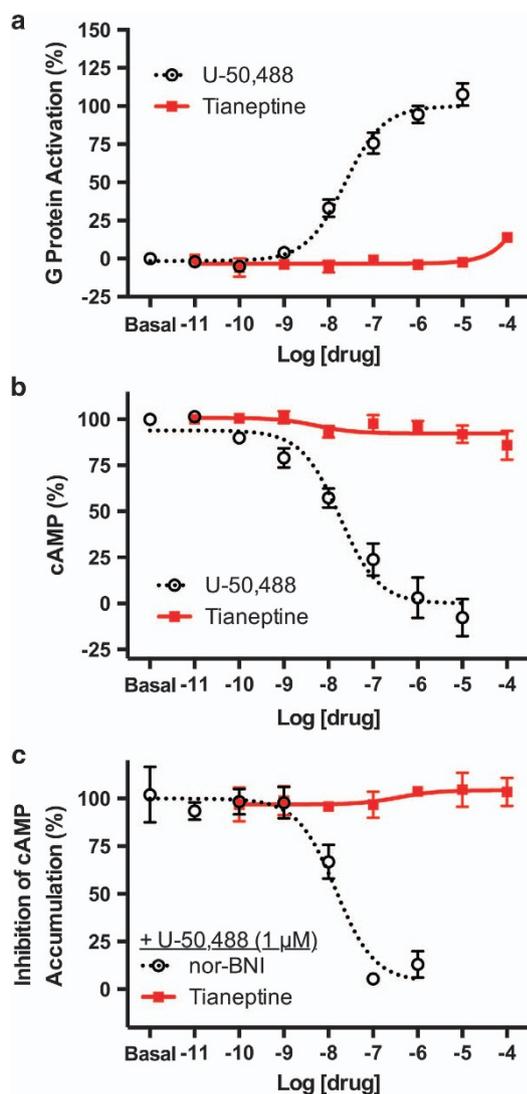


Figure 4. Tianeptine has no apparent activity at κ -opioid receptors (KORs). **(a)** The rat KOR was co-expressed with $G_{\alpha_{oB}}$ -RLuc8, β_1 and mVenus- γ_2 to assay G-protein activation. KOR agonist U-50,488 was used as a positive control. Tianeptine has no significant agonistic activity at KOR. **(b)** The rat KOR was co-expressed with $G_{\alpha_{oB}}$, β_1 , γ_2 and the bioluminescence resonance energy transfer (BRET) sensor CAMYEL to assay inhibition of forskolin-stimulated cAMP accumulation. In this assay, tianeptine also has no significant agonistic activity at KOR. **(c)** The rat KOR was co-expressed with $G_{\alpha_{oB}}$, β_1 , γ_2 and the BRET sensor CAMYEL to measure the inhibition of cAMP inhibition by the KOR agonist U-50,488. Tianeptine does not show any antagonistic activity at KOR. Nor-binaltorphimine (nor-BNI) showed an IC_{50} of 28.1 ± 27.2 nM. Data represent mean \pm s.e.m. of three independent experiments.

37.4 ± 11.2 μ M). Thus, while the selectivity of MOR over DOR is approximately 20-fold in mouse, it is nearly 200-fold in human.

In order to complete our characterization of tianeptine's functional activity at the opioid receptors, we assessed its activation of KOR. In both G-protein activation and cAMP inhibition assays, we found tianeptine to be inactive at rat KOR (Figure 4). Tianeptine was inactive at human KOR as well (Supplementary Information). Previous reports have highlighted the antidepressant effects of KOR antagonists.^{13–15} In order to eliminate the possibility that tianeptine's antidepressant effects may in fact result from activity as a KOR antagonist, we tested tianeptine's ability to dose-

dependently block the activation of KOR by control agonist U-50,488. In contrast to the known KOR antagonist, nor-binaltorphimine, tianeptine failed to inhibit the activation of KOR by U-50,488 (Figure 4c). Therefore, it is clear through both binding studies (Figure 1b) and functional assays that tianeptine has no activity at KOR.

DISCUSSION

We have described the first identification of specific molecular targets for the atypical antidepressant tianeptine, namely MOR and DOR. These are unexpected results as previous mechanistic studies have focused on tianeptine's modulation of aminergic and glutamatergic neurotransmission.² With regard to modulation of the glutamatergic system, previous reports have shown that tianeptine does not have appreciable affinity ($K_i > 10$ μ M) for NMDA, AMPA and kainate receptors.⁶ In the present work, we have demonstrated that tianeptine also has no agonist or antagonist activity at metabotropic glutamate receptors (including mGluR1a,2,4,5,6,8 receptors). It is therefore unlikely that tianeptine modulates glutamatergic neurons and synapses via a direct interaction with glutamate receptors, at least in the concentration range examined in the present study (< 10 μ M). In contrast, our evidence suggests that tianeptine's modulation of the glutamatergic system may occur indirectly, via activation of opioid receptor signaling.

In humans, a single dose of tianeptine (12.5 mg) results in ~ 1 μ M maximal concentration of the drug in the plasma, whereas in rodents, standard acute and chronic dosing (10 mg kg^{-1} per day) leads to plasma concentrations of ~ 10 μ M (*in vivo* brain concentrations have not been determined; an estimate in *ex vivo* tissue is low micromolar).^{16,17} Therefore, the *in vivo* concentration range appears sufficient for the activation of MOR ($EC_{50} \sim 0.2$ – 1 μ M, Figures 1 and 2), whereas activation of DOR ($EC_{50} \sim 12$ – 34 μ M) may only become relevant with higher dosing. Although it has been previously shown that DOR agonists have antidepressant-like effects *in vivo*,¹⁸ the evidence that MOR activation may have antidepressant/anxiety effects has only been suggestive.¹⁹

Taken together, these results lead us to hypothesize that activation of MOR (or possibly dual activation of MOR and DOR) is the initial molecular event responsible for tianeptine's modulation of the glutamatergic system and for triggering many of the known acute and chronic effects of this agent, including its antidepressant/anxiety actions. MORs are widely expressed in the hippocampus and are known to modulate glutamatergic neurons and synapses through a number of mechanisms. For example, MOR activation in dentate granule cells is known to decrease protein kinase A activity, which results in a decrease of NMDA receptor phosphorylation and activity.²⁰ This may underlie the corrective effect of tianeptine on increased NMDA receptor signaling in stressed animals.² In addition, activation of MORs (as well as DORs) in hippocampal inhibitory interneurons decreases their activity, thereby disinhibiting CA1 glutamatergic neurons,²¹ consistent with reports of tianeptine's enhancing effects on excitability and synaptic plasticity in CA1.² We note a striking similarity between these cellular and circuit-level effects of tianeptine and those exerted by direct NMDA receptor antagonists, which also show rapid onset of antidepressant effects.^{1,22}

Other circuit-level effects likely contribute to tianeptine's modulation of the glutamatergic system and antidepressant action. For example, tianeptine, like known MOR and DOR agonists, increases dopamine release in the nucleus accumbens,²³ and dopamine can in turn modulate glutamate release through action at presynaptic dopamine receptors on glutamatergic presynaptic terminals.

Taken together, the pharmacological results and mechanistic hypothesis presented here provide a new perspective for interpreting tianeptine's extensive clinical and preclinical data.

The proposed hypothesis suggests specific experiments to test its validity in different experimental paradigms (for example, MOR knockout animals and MOR pharmacological inhibition). Our data are also in agreement with growing evidence regarding the importance of the opioid system in depression, anxiety and stress-related disorders.^{18,19,24-26}

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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