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Occupancy of Brain Dopamine D_3 Receptors and Drug Craving: A Translational Approach

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Selective dopamine D_3 receptor (D_3R) antagonists prevent reinstatement of drug-seeking behavior and decrease the rewarding effects of contextual cues associated with drug intake preclinically, suggesting that they may reduce drug craving in humans. GSK598809 is a selective D_3R antagonist recently progressed in Phase I trials. The aim of this study was to establish a model, based on the determination of the occupancy of brain D_3Rs (O^{D_3R}) across species, to predict the ability of GSK598809 to reduce nicotine-seeking behavior in humans, here assessed as cigarette craving in smokers. Using *ex vivo* [^{125}I](*R*)-*trans*-7-hydroxy-2-[*N*-propyl-*N*-(3′-iodo-2′-propenyl)amino] tetralin ([^{125}I]7OH-PIPAT) autoradiography and [^{11}C]PHNO positron emission tomography, we demonstrated a dose-dependent occupancy of the D_3Rs by GSK598809 in rat, baboon, and human brains. We also showed a direct relationship between O^{D_3R} and pharmacokinetic exposure, and potencies in line with the *in vitro* binding affinity. Likewise, GSK598809 dose dependently reduced the expression of nicotine-induced conditioned place preference (CPP) in rats, with an effect proportional to the exposure and O^{D_3R} at every time point, and 100% effect at O^{D_3R} values $\geqslant$72%. In humans, a single dose of GSK598809, giving submaximal levels (72–89%) of O^{D_3R}, transiently alleviated craving in smokers after overnight abstinence. These data suggest that either higher O^{D_3R} is required for a full effect in humans or that nicotine-seeking behavior in CPP rats only partially translates into craving for cigarettes in short-term abstinent smokers. In addition, they provide the first clinical evidence of potential efficacy of a selective D_3R antagonist for the treatment of substance-use disorders.

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INTRODUCTION

The dopamine (DA) D_3 receptor (D_3R) is selectively expressed in important areas of the brain implicated in addiction, including the ventral striatum (VST), midbrain, and pallidum (Bouthenet *et al*, 1991; Staley and Mash, 1996). These findings, together with data demonstrating plasticity changes in drug-dependent subjects, such as the increase in D_3R density in cocaine addicts and metamphetamine polydrug users (Staley and Mash, 1996; Boileau *et al*, 2012), have generated interest in developing selective D_3R antagonists as potential pharmacotherapeutics for addiction (Heidbreder and Newman, 2010). Although selective D_3R antagonists fail to alter significantly drug self-administration (S/A) under low fixed-ratio (FR) schedules of reinforcement in rodents, they are very potent in decreasing drug S/A when the unit dose of the drug is decreased, when the work demand for the drug is increased (eg, from an FR1 to FR10 schedule or in progressive-ratio schedules of reinforcement), or when drug-seeking behavior is maintained by a drug-associated conditioned reinforcer (see Heidbreder and Newman, 2010). Selective D_3R antagonists are also potent in preventing cue-, drug-, or stress-induced reinstatement of drug-seeking behavior, and reducing the acquisition and/or expression of drug-induced conditioned place preference (CPP; Heidbreder and Newman, 2010). Taken together, these observations suggest that D_3R antagonists do not alter the primary reinforcing properties of drugs of abuse. Instead, they appear to (i) regulate motivation to self-administer drugs under schedules of

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reinforcement that require an increase in work demand and (ii) disrupt responsiveness to stimuli that have an important role in reinstatement of drug-seeking behavior. The latter may include re-exposure to the drug itself, exposure to environmental cues previously associated with drug-taking behavior, and stress.

In humans, drug craving (the desire or urge to take the drug in response to conscious or unconscious stimuli associated with drug taking) is an important factor of the stage of the addiction cycle in which the individual reinstates drug-seeking behavior after abstinence (the preoccupation/anticipation stage; Koob and Volkow, 2010). Assuming that craving is a fundamental drive of relapse in humans, and knowing that conditioned-drug effects have a prominent role in cue-elicited craving (O'Brien *et al*, 1998; Bedi *et al*, 2011), it is reasonable to think that selective D_3R antagonists may decrease craving and, therefore, act as relapse prevention medications. The hypothesis that selective D_3R antagonists may reduce drug craving in humans, however, has not been tested previously because of the lack of selective D_3R antagonists with appropriate profiles for clinical investigation. Moreover, the complexity, long duration, and high cost of relapse prevention trials may delay the possibility to perform Phase II studies. Small, exploratory efficacy studies, in which dosing is limited to avoid exposing human subjects to potentially unsafe concentrations, are an alternative approach to determine if selective D_3R antagonists warrant further investigation as potential medications to prevent relapse.

Previous research has shown that GSK598809 (derivative **74** in Micheli *et al*, 2010) is a selective and potent competitive D_3R antagonist with good physicochemical, pharmacokinetic (PK), and safety properties, which dose dependently decreased the expression of nicotine-induced CPP in rats. The first objective of this work was to evaluate the relationship between the ability of GSK598809 to reduce nicotine-seeking behavior in rats and decrease cigarette craving in short-term abstinent smokers, based on the occupancy of brain D_3Rs (O^{D_3R}) as a marker of efficacy for translation across species. The secondary aim of this study was to provide information to define the doses to be tested in further, more extensive Phase II studies. The O^{D_3R} was determined by *ex vivo* [^{125}I]7OH-PIPAT autoradiography in rat and by [^{11}C]PHNO positron emission tomography (PET) in baboon (to have a preliminary evaluation of the technique) and human.

An introductory overview of this work has been presented at the American College of Neuropsychopharmacology (ACNP) 50th Annual Meeting (Merlo Pich, 2011). Some of the data reported in this work have been published previously (Micheli *et al*, 2010; Searle *et al*, 2010).

MATERIALS AND METHODS

Study 1: *In Vitro* Pharmacological Profile

[^{35}S]Guanosine-5′-(γ-thio)triphosphate ([^{35}S]GTPγS) binding was performed as described by Micheli *et al* (2010), with the following changes: membranes were from a Chinese Hamster Ovary (CHO) cell line expressing human D_3Rs (hD_3Rs) after induction with mifrepistone (hD_3R-CHO/inducible) and the buffer solution contained 0.02% pluronic F-127, 30 μg/ml saponin, and 10 μM GDP. [^{3}H]-(+)-2*H*-naphth[1,2-*b*]-1,4-oxazin-9-ol, 3,4,4a,5,6,10b-hexahydro-4-propyl-([^{3}H]PHNO) binding was carried out on membranes from CHO cell lines stably expressing hD_3Rs (hD_3-CHO/stable) or the long variant of human D_2Rs (hD_{2L}-CHO/stable). To mimic the physiological conditions of PET-[^{11}C]PHNO experiments, incubation was performed at 37 °C (for 60 min) in artificial cerebral spinal fluid: 124 mM NaCl, 1.9 mM KCl, 2 mM $MgSO_4$, 1.3 mM KH_2PO_4, 11 mM glucose, 25 mM $NaHCO_3$, 1.2 mM $CaCl_2$ (pH 7.4). [^{125}I]7OH-PIPAT binding was carried out as described by Micheli *et al* (2010), with the following changes: membranes were from a CHO cell line stably expressing rat D_3Rs (rD_3-CHO/stable) and incubation was performed at room temperature (RT), as in the experiments aimed at determining the O^{D_3R} by means of *ex vivo* autoradiography. GSK598809 pK_i and pA_2 values were determined using the method of Arunlakshana and Schild (1959), and K_{off} and K_{on} with that of Motulsky and Mahan (1984).

In Vivo Studies

All *in vivo* studies were pre-reviewed and approved by a local animal care committee in accordance with the guidelines of the 'Principles of Laboratory Animal Care' (NIH publication no. 86-23, revised 1985) and with a project license that was obtained according to the Italian law (Art. 7, Legislative Decree no. 116, 27 January 1992), which acknowledges European Directive 86/609/EEC on the care and welfare of laboratory animals.

A preliminary investigation of GSK598809 PKs following intraperitoneal administration in rat showed that the total GSK598809 concentration in blood ($C^{t,blood}$) reached the peak concentration ($C^{t,blood}_{max}$) after 20 min (T_{max}), and was then relatively steady for 40 min ($C^{t,blood}$ at 1 h = 84 ± 5% $C^{t,blood}_{max}$; mean ± SD, $n = 3$), halved at 2 h ($C^{t,blood}$ at 2 h = 47 ± 16% $C^{t,blood}_{max}$), and was almost undetectable after 24 h ($C^{t,blood}$ at 24 h = 1 ± 0.3% $C^{t,blood}_{max}$).

Study 2: D_3R Occupancy in Rat

In the first experiment (dose–response), rats received an intraperitoneal administration of vehicle (saline) or GSK598809 (range 0.01–3 mg/kg) and were then terminated by decapitation after 1 h; in the second experiment (time–course), rats received vehicle or GSK598809 (1 mg/kg) and were terminated at different times between 0.75 and 24 h. Blood sampling, for GSK598809 quantification, was performed from the tail vein immediately before decapitation. After discarding the cerebellum, the brain was divided along the longitudinal fissure: one hemisphere was used for GSK598809 quantification in the brain tissue, and the other was frozen in isopentane, and subsequently cut into 14-μm-thick coronal hemisections at +2.70, +2.20, and +1.60 mm from bregma. [^{125}I]7OH-PIPAT autoradiography was performed with a protocol derived from Burris *et al* (1994) and Stanwood *et al* (2000), with some modifications. Briefly, sections were rinsed (5 min, RT) in a solution consisting of 50 mM Tris buffer (pH = 7.4), 40 mM NaCl, and 300 μM guanosine 5′-triphosphate (GTP). Incubation (15 min, RT) with 0.4 nM [^{125}I]7OH-PIPAT was performed in a solution consisting of 50 mM Tris buffer (pH = 7.0),

40 mM NaCl, 300 μM GTP, and 5 μM 1,3-di(2-tolyl)guanidine. Nonspecific binding was determined in the presence of 10 μM DA. The reaction was stopped by washing (90 min, 4 °C) the slides in 40 mM NaCl/50 mM Tris buffer (pH = 7.4). After a dip in water (4 °C), sections were dried and put in contact to Fuji Imaging Plates BAS-SR2025 overnight. The plates were read at the Bio-image Analyzer BAS5000 (Fuji Photo Film, Japan). Preliminary experiments (not reported) were performed to assess the specificity of [^{125}I]7OH-PIPAT binding to D_3Rs in these conditions and reduce GSK598809 dissociation from the receptor to minimize the risk of underestimating occupancy (Li *et al*, 2006).

Quantitative analysis was performed by computer-assisted microdensitometry (MCID basic; Imaging Research, Canada). Photostimulated luminescence per mm^2 values were converted to the corresponding radioligand concentration (fmol/mg) by referring to iodine standards (Microscales; Amersham Biosciences) on the same plate. [^{125}I]7OH-PIPAT binding was measured in the shell of the nucleus accumbens (AcbSh), a subregion of the VST containing high levels of D_3Rs. The O^{D_3R}, numerically equivalent to the occupancy of the [^{125}I]7OH-PIPAT binding sites (O^{PIPAT}) in these binding conditions, was calculated with the following equation:

$$O^{D_3R}(\%) = O^{PIPAT}(\%) = 100 \times \left(\frac{\overline{SB}^{Vehicle} - SB^{GSK598809}}{\overline{SB}^{Vehicle}} \right)$$

where $\overline{SB}^{Vehicle}$ is the specific binding (SB) in the vehicle-treated animals (mean value) and $SB^{GSK598809}$ the SB in the GSK598809-treated animal. Occupancy data were fit by nonlinear regression analysis using the GraphPad Prism 4.0 (GraphPad Software) with the following equations:

$$O^{D_3R}(\%) = \frac{O^{D_3R}_{max} \times (C^{t,blood})^{\gamma}}{\left(OC^{t,blood}_{50}\right)^{\gamma} + (C^{t,blood})^{\gamma}}$$

and

$$O^{D_3R}(\%) = \frac{O^{D_3R}_{max} \times (C^{t,brain})^{\gamma}}{\left(OC^{t,brain}_{50}\right)^{\gamma} + (C^{t,brain})^{\gamma}}$$

where $C^{t,blood}$ and $C^{t,brain}$ are the total concentration in the blood and brain, respectively, $OC^{t,blood}_{50}$ and $OC^{t,brain}_{50}$ are the total concentration, giving 50% of maximal occupancy ($O^{D_3R}_{max}$), in the blood and brain, respectively, and γ the Hill coefficient of this function.

Study 3: Nicotine CPP in Rat

Similar to the RO study, two different CPP experiments were performed (dose–response and time–course). Animal husbandry, the apparatus, and the first three phases (acclimation, handling, and conditioning) of the nicotine CPP procedure were exactly as described in Micheli *et al* (2010). In the last phase (testing), rats from the dose–response experiment received vehicle or GSK598809 (range 0.05–3 mg/kg, intraperitoneally) 30 min before they were placed in the apparatus. In the time–course experiment, rats received vehicle or GSK598809 (range 0.3–3.0 mg/kg, intraperitoneally) 4 or 8 h before they were placed in the apparatus. In both experiments, the animals were allowed to remain in the apparatus for 15 min, and the time spent in each chamber was automatically recorded (see Micheli *et al*, 2010).

The conditioning properties of nicotine were quantified as preference index (PI), defined as the time spent in the nicotine-paired chamber minus the time spent in the unpaired chamber. The ability of the antagonist (GSK598809) to reduce nicotine preference, or inhibitory effect (E), was calculated with the following equation:

$$E(\%) = 100 \times \left(\frac{\overline{PI}^{Vehicle} - PI^{GSK598809}}{\overline{PI}^{Vehicle}} \right)$$

in which $\overline{PI}^{Vehicle}$ is the PI in the vehicle-treated animals (mean value) and $PI^{GSK598809}$ the PI in the GSK598809-treated animal. Data were fit using a nonlinear regression analysis (GraphPad Prism 4.0) as follows:

$$E(\%) = \frac{E_{max} \times \left(\overline{C}^{t,blood}\right)^{\gamma}}{\left(EC^{t,blood}_{50}\right)^{\gamma} + \left(\overline{C}^{t,blood}\right)^{\gamma}}$$

and

$$E(\%) = \frac{E_{max} \times \left(\overline{C}^{t,brain}\right)^{\gamma}}{\left(EC^{t,brain}_{50}\right)^{\gamma} + \left(\overline{C}^{t,brain}\right)^{\gamma}}$$

where $\overline{C}^{t,blood}$ and $\overline{C}^{t,brain}$ are the total concentration (in the blood or brain, respectively) of the animals receiving the same dose of GSK598809 in the satellite RO experiment (median value), and $EC^{t,blood}_{50}$ and $EC^{t,brain}_{50}$ the total concentration, giving 50% of maximal effect (E_{max}), in the blood or brain, respectively, and γ the Hill coefficient.

Prediction of Human Values

GSK598809 concentrations in human blood were estimated from the rat experimental data with the following equation (assuming similar brain penetration across species):

$$(XC_{50})_{human} = (XC_{50})_{rat} \times \frac{10^{-(pK_i)_{human}}}{10^{-(pK_i)_{rat}}} \times \frac{(f_u)_{rat}}{(f_u)_{human}}$$

in which $(XC_{50})_{human}$ and $(XC_{50})_{rat}$ are the concentration giving 50% of O^{D_3R}or E in human and rat, respectively, $(K_i)_{human}$ and $(K_i)_{rat}$ the affinity of GSK598809 for hD_3R and rD_3R, respectively, and $(f_u)_{human}$ and $(f_u)_{rat}$ the fraction unbound (ie, 100 minus the percentage of tissue binding) in human and rat blood, respectively.

Study 4: D_3R Occupancy in Baboon and Human

The PET studies in baboon (*Papio anubis*) and human have been described previously (Searle *et al*, 2010). In brief, each subject received a baseline [^{11}C]PHNO scan, followed by 60-min intravenous infusion of 0.1–1 mg/kg GSK598809 (baboon) or oral administration of 5–175 mg GSK598809 (human). After 15 min from the end of the infusion (baboon) or 2–3 h from the oral administration (human), the subjects received the second scan. Blood samples (for determination of GSK598809 concentration in the plasma) were taken at regular intervals. Image analysis was performed as described

by Rabiner *et al* (2009; baboon) and Searle *et al* (2010; human). Quantitative analysis was performed using the simplified reference tissue model with cerebellum as reference region to derive regional estimates of [^{11}C]PHNO binding potential relative to the non-displaceable compartment (BP_{ND}). The occupancy of [^{11}C]PHNO binding sites by GSK598809 (O^{PHNO}) was defined with the following equation:

$$O^{\mathrm{PHNO}}(\%) = 100 \times \left(\frac{BP_{ND}^{\mathrm{Baseline}} - BP_{ND}^{\mathrm{GSK598809}}}{BP_{ND}^{\mathrm{Baseline}}} \right)$$

where $BP_{ND}^{\mathrm{Baseline}}$ is the BP_{ND} at the baseline scan and $BP_{ND}^{\mathrm{GSK598809}}$ the BP_{ND} measured in the second scan (ie, in the presence of GSK598809).

As previous analysis revealed that it is reasonable to assume that GSK598809 binding to D_2Rs was negligible in the current set of data (Searle *et al*, 2010), we adopted a simplified procedure to estimate the potency of GSK598809 in occupying D_3Rs. We applied a population PK/pharmacodynamic (PD) modeling approach to model individual data using a nonlinear mixed-effect method, as implemented in NONMEM VI (Globomax, Hanover, MD, USA). Data were fit with the following equation:

$$O^{\mathrm{PHNO}}(\%) = \frac{O_{\max}^{\mathrm{PHNO}} \times \left(C^{\mathrm{t,plasma}}\right)^{\gamma}}{\left(OC_{50}^{\mathrm{t,plasma}}\right)^{\gamma} + \left(C^{\mathrm{t,plasma}}\right)^{\gamma}}$$

in which $C^{\mathrm{t,plasma}}$ is the total GSK598809 concentration in the plasma at the beginning of the scan, $OC_{50}^{\mathrm{t,plasma}}$ the total GV598809 concentration, in the plasma, giving 50% of maximal occupancy of PHNO binding sites ($O_{\max}^{\mathrm{PHNO}}$), and γ the Hill coefficient. In each region, the $O_{\max}^{\mathrm{PHNO}}$ value paralleled the fraction of D_3Rs in the $BP_{ND}^{\mathrm{Baseline}}$ ($f_{\mathrm{PHNO}}^{D_3R}$) in that region (Searle *et al*, 2010), although subtle differences were observed (see Results). The parameter $OC_{50}^{\mathrm{t,plasma}}$, theoretically equivalent to the total GV598809 concentration, in the plasma, giving 50% of maximal occupancy of D_3Rs ($O_{\max}^{D_3R}$), was shared between the VST, the globus pallidus (GB), and the substantia nigra (SN), three regions having both good $f_{\mathrm{PHNO}}^{D_3R}$ value and BP_{ND} values. The proportion of O^{PHNO} corresponding to occupancy of D_3Rs (O^{D_3R}) was calculated, in each point, with the following equation:

$$O^{D_3R}(\%) = \frac{O^{\mathrm{PHNO}}}{O_{\max}^{\mathrm{PHNO}}}$$

Dose Selection for the Human Efficacy Study

The dose of GSK598809 to be tested in the human efficacy study (Study 5) was selected by translating the results of the GSK598809 efficacy experiment in rat to human, using O^{D_3R} as a marker of efficacy: the dose selected was designed to ensure, in the time frame during which the efficacy on craving was studied in human, a minimal occupancy level similar to that reached by the lowest dose giving maximal (100%) effect in the rat efficacy study.

The O^{D_3R} value corresponding to the lowest dose of GSK598809 giving maximal effect in the rat nicotine CPP model (Study 3) was calculated from the *PK/O* relationship generated in the satellite *ex vivo* [^{125}I]7OH-PIPAT autoradiography experiment (Study 2). The dose for the human study was selected on the basis of the *PK/O* relationship generated in the human PET-[^{11}C]PHNO/GSK598809 occupancy experiment (Study 4) and the plasma concentration time profiles of previous single-dose PK studies.

Study 5: Efficacy in Human

For the efficacy study in human, a total of 48 treatment-seeking (ie, interested in quitting smoking), healthy male and female adult current smokers were recruited from one center, the Transdisciplinary Tobacco Use Research Centre (TTURC), Philadelphia (USA). Candidates with medical or psychiatric contraindications were excluded. Written informed consent was obtained from each subject before the performance of any study-specific procedures. A total of 40 subjects completed the study (24 males and 16 females; see Table 1 for participants' characteristics). The study was sponsored by GlaxoSmithKline (GSK study number DAN106593) and approved by the University of Pennsylvania Institutional Review Board. The protocol was posted on http://clinicaltrial.gov (identifier NCT00605241) before study initiation.

The study was a randomized, double-blind, placebo-controlled, balanced two-way crossover design. Following screening, subjects participated in two dosing sessions (with a washout period of at least 7 days), in which they received a single oral dose of 75 mg GSK598809 or placebo. Each session was conducted in the clinic in the morning. Subjects were required to abstain from smoking for 14 h before attending the clinic. In each session visit, medical and safety assessments, blood sampling, recording of adverse events, and efficacy assessments were performed. Blood samples were collected at regular intervals for the analysis of total GSK598809 concentration in the plasma. Abstinence was confirmed by measuring exhaled carbon monoxide level and nicotine, cotinine, and *trans*-3-hydroxycotinine plasma levels at pre-dose.

Three different behavioral tasks were carried out to assess PD response to GSK598809, at three different times post-dose. Task 1, a modified version of the classic Stroop task (Waters *et al*, 2003), was carried out 30 min post-dose and lasted approximately 60 min. The difference in average response times to color-name words related to cigarette smoking (smoking reaction time, RT) and neutral control words (neutral RT) was quantified and considered an index of the degree to which smoking-related content disrupts ongoing cognitive processes. Task 2, a Behavioral Economic task, adapted from Perkins *et al* (2002), was carried out 90 min post-dose and lasted approximately 60 min. The level of effort participants were willing to expend for cigarette puffs or money was measured (as breakpoint in a progressive-ratio schedule) and considered an index of the relative reinforcing value of smoking. Task 3, a Cigarette Choice task (Blendy *et al*, 2005), was carried out to measure the relative reinforcing value of nicotine between 6.5 and 8.5 h post-dose. The dependent variable was the number of times a nicotine-containing cigarette was selected.

Participants also completed the following self-report questionnaires (aimed at determining the extent of

self-reported craving) at 0.5, 1.25, 2.75, 5, 8, and 10 h post-dose: the Tiffany Smoking Urges-Brief (QSU-B; Cox *et al*, 2001) and the Minnesota Nicotine Withdrawal Scale (MNWS; Hughes, 2007). Finally, cigarette consumption in the natural environment subsequent to the experimental session (8.5–10.5 h post-dose) was also determined.

RESULTS

Study 1: *In Vitro* Pharmacological Profile

Without changing basal [^{35}S]GTPγS levels in hD$_3$R/inducible membranes, GSK598809 caused a parallel right-ward shift of the quinelorane-induced increase of [^{35}S]GTPγS, with a potency value (A_2) of 0.5 nM ($pA_2 = 9.3 \pm 0.1$, $n = 3$; Figure 1a). GSK598809 displaced [^{3}H]PHNO binding to hD$_3$R/stable and hD$_{2L}$R/stable membranes, with K_i values of 6.2 nM ($pK_i = 8.21 \pm 0.09$, $n = 3$) and 740 nM ($pK_i = 6.13 \pm 0.02$, $n = 3$), respectively (not shown).

GSK598809 inhibited [^{125}I]7OH-PIPAT binding to rD$_3$R/stable membranes, with a K_i of 1.5 nM ($pK_i = 8.83 \pm 0.004$, $n = 3$). Kinetic experiments showed that GSK598809 bound to rD$_3$Rs with an association rate constant (K_{on}) value of 0.0508 ± 0.0081/nM/min ($n = 3$) and a dissociation rate constant (K_{off}) value of 0.2601 ± 0.0616/min ($n = 3$), respectively (see Figure 1b), from which it was calculated a kinetically derived dissociation constant value of 5.1 nM ($pK_D = 8.29$). Half-time ($t_{1/2}$) values of few minutes for association and 3 min for dissociation were calculated from such rate constant values. GSK598809 did not change the dissociation kinetics of the radioligand, confirming the competitive interaction with the receptor (see Figure 1c).

Study 2: D$_3$R Occupancy and *PK/O* Relationship in Rat

Specific [^{125}I]7OH-PIPAT binding was higher in the brain of vehicle-treated rats than that of GSK598809-treated animals (Figure 2a). Data fitting revealed that GSK598809 occupied D$_3$Rs (Figure 2b) with an $OC_{50}^{t,blood}$ of 15 ng/ml and an $OC_{50}^{t,brain}$ of 47 ng/g, with Hill coefficient values not significantly different from 1, and $O_{max}^{D_3R}$ values not significantly different from 100%. Accounting for tissue binding, it was calculated that the free (unbound) concentration giving 50% of occupancy (OC_{50}^{u}) was 0.6 ng/ml (≈ 1.2 nM; $pOC_{50}^{u,blood} = 8.91$) and 1.1 ng/g ($\approx 2.3$ nM; $pOC_{50}^{u,brain} = 8.64$) in the blood and brain, respectively (see Table 2). The O^{D_3R} decreased with time after GSK598809 administration, in parallel to $C^{t,blood}$ and $C^{t,brain}$ (see Figure 2c).

Table 1 Participants' Characteristics

Total number of subjects (*n*)	40
Sex	
Male (*n*)	24
Female (*n*)	16
Race	
Black or African American (*n*)	20
White (*n*)	16
More than one race (*n*)	4
Age at phone screen (years; mean ± SD)	42.00 ± 10.687
Number of cigarettes smoked on a typical day (mean ± SD)	16.92 ± 5.828
Fagerström nicotine dependence summary score	5.55 ± 1.694

Study 3: Efficacy in Nicotine CPP and *PK/PD* Relationship in Rat

Micheli *et al* (2010) reported previously that GSK598809 dose dependently reduced the expression of nicotine-conditioned preference (see also Figure 3a). Data fitting *vs* compound exposure (as from the animals treated with the same doses in the previous PK/O experiment) indicated that

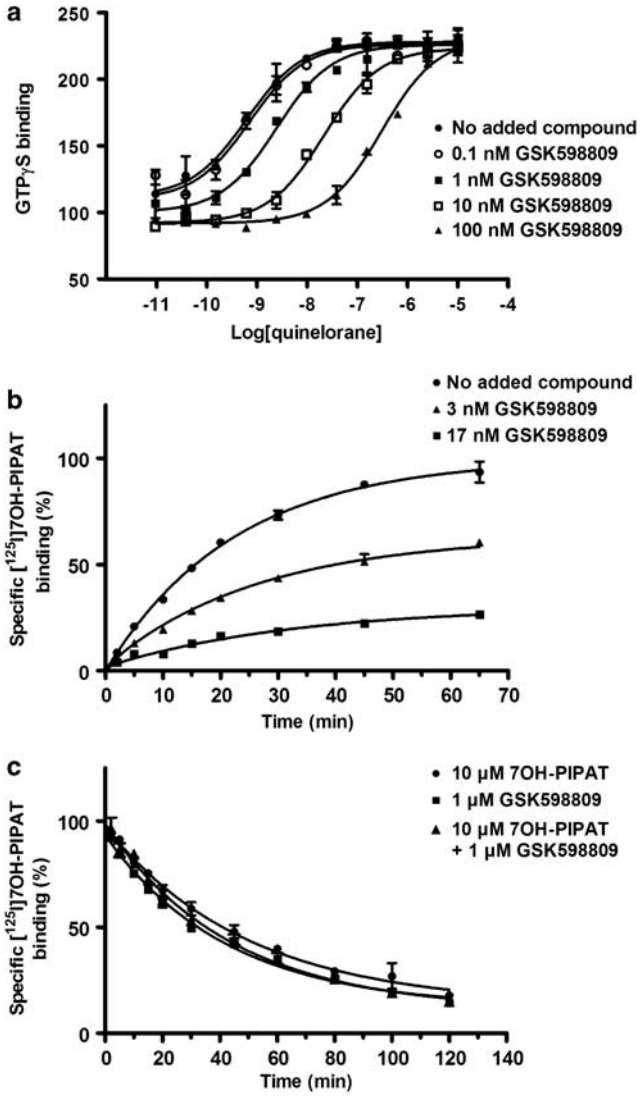

Figure 1 (a) Increase of basal [^{35}S]guanosine-5′-(γ-thio)triphosphate ([^{35}S]GTPγS) binding to (hD$_3$R)/inducible cell membranes by the agonist quinelorane, alone or in the presence of increasing concentrations of GSK598809. (b) Association of [^{125}I](*R*)-*trans*-7-hydroxy-2-[*N*-propyl-*N*-(3′-iodo-2′-propenyl)amino] tetralin ([^{125}I]7OH-PIPAT) binding to rD$_3$Rs/stable cell membranes, alone or in the presence of increasing concentrations of GSK598809. (c) Dissociation of [^{125}I]7OH-PIPAT binding to rD$_3$Rs/stable cell membranes induced by the addition of an excess of unlabeled ligand(s).

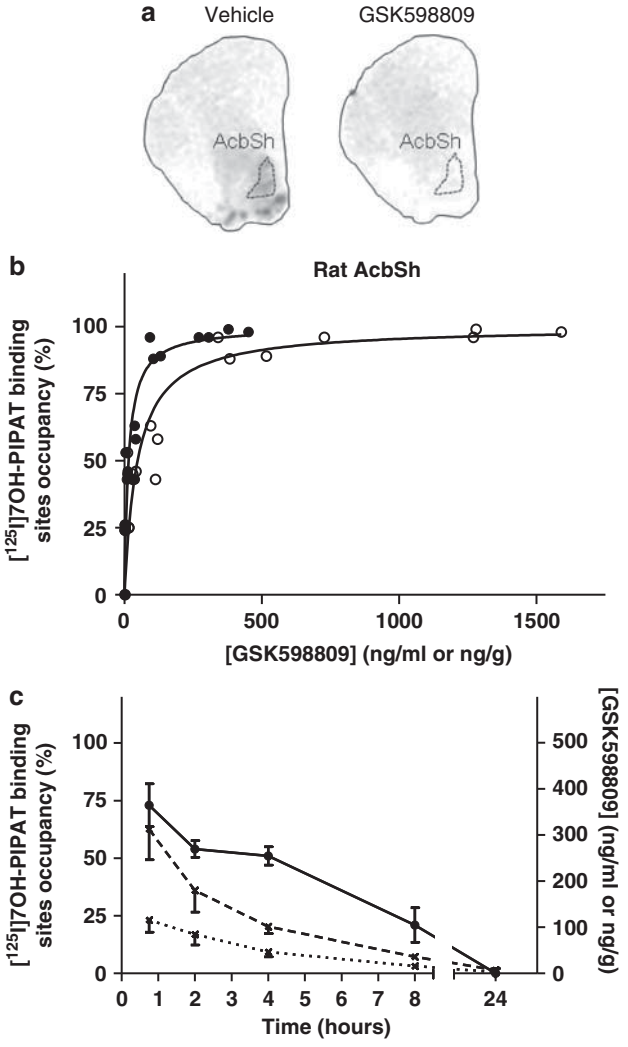

Figure 2 (a) *Ex vivo* [^{125}I](*R*)-*trans*-7-hydroxy-2-[*N*-propyl-*N*-(3′-iodo-2′-propenyl)amino] tetralin ([^{125}I]7OH-PIPAT) binding to representative, coronal, one-hemisphere brain sections from rats treated with vehicle or GSK598809 (3 mg/kg, intraperitoneally (i.p.)). (b) Occupancy of [^{125}I]7OH-PIPAT binding sites ($=O^{D_3R}$) in the rat shell of the nucleus accumbens (AcbSh) at increasing blood (●; ng/ml) and brain (○; ng/g) GSK598809 concentrations, 1 h after administration of GSK598809 (0.05–3 mg/kg intraperitoneally). (c) Time–course of the O^{D_3R} in the rat AcbSh (●) after a single administration of GSK598809 (1 mg/kg intraperitoneally); the figure also shows GSK598809 concentration in the blood (dotted line; ng/ml) and brain (dashed line; ng/g).

GSK598809 reduced the expression of nicotine CPP with $EC_{50}^{t,blood}$ and $EC_{50}^{t,brain}$ values of 7.2 ng/ml and 21.4 ng/g, respectively, γ values of 1.7 and 2.1, respectively, and E_{max} values not significantly different from 100 (not shown). Considering tissue binding, it was calculated that the free concentration giving 50% of effect (EC_{50}^{u}) was 0.3 ng/ml ($\approx$0.6 nM; $pEC_{50}^{u,blood}=9.23$) and 0.5 ng/ml ($\approx$1.0 nM; $pEC_{50}^{u,brain}=8.98$) in the blood and brain, respectively.

The effect was lower when GSK598809 was administered 4 h before the test, and significantly different from control only at 1 and 3 mg/kg (Figure 3b). The same doses of GSK598809 had no effect when administered 8 h before the test.

Prediction of Human Values

From the rat $OC_{50}^{t,blood}$ determined in Study 2, it was possible to predict that the $OC_{50}^{t,blood}$ in human was 54 ng/ml (see Materials and Methods). By adjusting for the blood/plasma ratio in human, it was calculated that the predicted total concentration, in the plasma, to obtain 50% of occupancy in human ($OC_{50}^{t,plasma}$) was 87 ng/ml (see Table 2). In the same manner, from the rat $EC_{50}^{t,blood}$ determined in Study 3, it was possible to predict that the $EC_{50}^{t,blood}$ in human was 26 ng/ml and that the predicted total concentration, in the plasma, to obtain 50% of efficacy in human ($EC_{50}^{t,plasma}$) was 42 ng/ml.

Study 4: D_3R Occupancy and *PK/O* Relationship in Baboon and Human

Data fitting revealed that GSK598809 occupied baboon brain D_3Rs (see Figure 4) with an $OC_{50}^{t,plasma}$ of 36 ng/ml (see Table 2). These results showed that the potency of GSK598809 in occupying brain D_3Rs in baboon was not far from that predicted for the human ($OC_{50}^{t,plasma}=87$ ng/ml) from the O^{D_3R}studies in rat, encouraging further studies in human.

GSK598809 occupied human brain D_3Rs (see Figure 4) with an $OC_{50}^{t,plasma}$ of 66 ng/ml. Using blood/plasma ratio and blood tissue binding in human (see Table 2), it was estimated that the blood free concentration giving 50% of occupancy in human ($OC_{50}^{u,blood}$) was 1.9 ng/ml ($\approx$3.9 nM; $pOC_{50}^{u,blood}=8.41$).

In each region, the O_{max}^{PHNO} value was in line with the $f_{PHNO}^{D_3R}$ value estimated by Searle *et al* (2010). In the SN, $O_{max}^{PHNO}$was 77% (95% confidence interval (CI): 70–84%), slightly lower than the 100% (95% CI: 79–100%) value reported by Searle *et al* (2010).

GSK598809 Dose Selection for the Human Efficacy Study

The lowest dose of GSK598809 giving maximal effect in the rodent nicotine CPP model (Study 3) was 0.3 mg/kg (see Figures 3a). Rats from the same group (0.3 mg/kg) of the satellite occupancy experiment (Study 2) had a median total GSK598809 blood concentration of 37.7 ng/ml, which corresponded to an O^{D_3R} value of 72% in the rat *PK/O* curve. From the *PK/O* relationship generated by the human PET-[^{11}C]PHNO/GSK598809 occupancy experiment (in which an $OC_{50}^{t,plasma}$ of 66 ng/ml was determined), it was calculated that a similar occupancy value ($O^{D_3R}=72\%$) was reached at a plasma concentration of 175 ng/ml. Previous single-dose PK studies in healthy volunteers and smokers had shown that comparable (or higher) plasma concentrations were obtained with a 75 mg oral dose in the time window between 0.5 and 10 h after administration. Therefore, the human single oral dose of 75 mg GSK598809 was chosen to yield, at the time of behavioral testing, a slightly higher O^{D_3R} to the lowest dose of GSK598809 giving maximal effect in the rat nicotine CPP experiment.

Study 5: Efficacy in Human

Following administration of an oral dose of 75 mg, GSK598809 was absorbed with a median $T_{max}$of 1.5 h (range from 0.5 to 8.2 h) and a mean $t_{1/2}$ value of 17 h. The

Table 2 Potency of GSK598809 in Occupying Brain D_3Rs in Rat, Baboon, and Human (comparison with GSK598809 affinity for D_3Rs)

	Potency as total concentration			Potency as free concentration		Affinity for D_3R
	$OC_{50}^{t,plasma}$ (ng/ml)	$OC_{50}^{t,blood}$ (ng/ml)	$OC_{50}^{t,brain}$ (ng/g)	$OC_{50}^{u,blood}$ (nM)	$OC_{50}^{u,brain}$ (nM)	K_i (nM)
Rat	23[a]	15	47	1.2[b]	2.3[c]	1.5
Baboon	36	ND	ND	ND	ND	ND
Human	66	41[d]	ND	3.9[e]	ND	6.2
Human (predicted)	87[f]	54[g]	NA	NA	NA	NA

Abbreviations: NA, not applicable; ND, not determined.
As explained in Materials and Methods, $OC_{50}^{t,blood}$ and $OC_{50}^{t,brain}$ values in rat were determined by fitting the occupancy measured in the AcbSh *vs* the total concentration in the blood or brain, respectively; in human and baboon, the $OC_{50}^{t,plasma}$ values were determined by simultaneous fitting of the occupancy measured in VST, GB ,and SN *vs* the total concentration in the plasma, during which the $OC_{50}^{t,plasma}$ was considered a shared parameter.
[a]Calculated from the rat $OC_{50}^{t,blood}$, adjusting for the blood/plasma ratio in rat (0.65).
[b]Calculated from the rat $OC_{50}^{t,blood}$, adjusting for the f_u in rat blood (3.93) and the molecular weight (MW; 481.52 g/mol).
[c]Calculated from the rat $OC_{50}^{t,brain}$, adjusting for the f_u in rat brain (2.33) and the MW.
[d]Calculated from the human $OC_{50}^{t,plasma}$, adjusting for the blood/plasma ratio in human (0.62).
[e]Calculated from the human $OC_{50}^{t,blood}$, adjusting for the f_u in human blood (4.55) and the MW.
[f]Calculated from the human $OC_{50}^{t,blood}$ (predicted), adjusting for the blood/plasma ratio in human (0.62).
[g]Predicted from rat experimental data as explained in Materials and Methods.

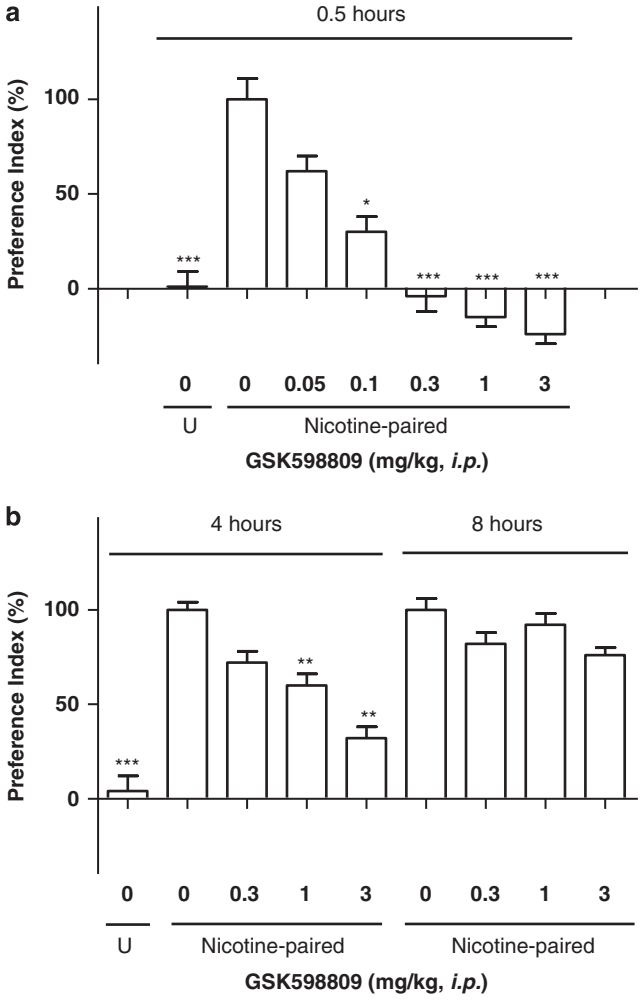

Figure 3 Expression of nicotine-induced conditioned place preference (CPP) in rat and dose-dependent inhibition by GSK598809 at different pre-treatment times. (a) Pre-treatment time was 30 min (preference index (PI) values were calculated from data already reported in Micheli *et al* (2010)). (b) Pre-treatment time was 4 or 8 h, as shown in the figure. *, **, and ***$P<0.01$, 0.001, and 0.0001, respectively; U, unpaired rats.

maximum concentration in the plasma ($C_{max}^{t,plasma}$) was 520 ng/ml. From these values of actual concentration in the plasma and the *PK/O* relationship established with the PET studies, it was estimated that the O^{D_3R} passed from zero at pre-dose to 89% at the T_{max} (1.5 h) and then decreased slowly to 80% at 17 h.

In the smoking Stroop test (carried out between 0.5 and 1.5 h post-dose), the difference between the smoking RT and the neutral RT was 47.52 ms in the GSK598809 treatment condition, lower (−20 ms; 95% CI: −65, 25) than the placebo treatment condition (67.52 ms). One subject (Subject 29, treated with GSK598809) who had clear difficulties in performing the task, as demonstrated by the very low percentage (<20%) of correct scores in the incongruent word group and the longest reaction time across all the other word groups (congruent, neutral, smoking) compared with other subjects, was considered an outlier and excluded from the analysis.

There were no significant treatment-related effects of GSK598809 on performance of the Behavioral Economic task (total number of presses made for puffs of a cigarette, or a trend across trials for switching behavior) and the Cigarette Choice paradigm (number of puffs from nicotinized cigarette).

There was a significant effect of GSK598809 on responses to MNWS-R item 4 ('desire or craving to smoke') at 30 min post-dose: mean (SD) = 2.30 (1.07) for placebo, 1.82 (1.28) for drug, t (39 d.f.) = 2.21, $P=0.033$ (paired t-test). The comparison of responses to MNWS-R item 4 at 30 min post-dose was repeated using a nonparametric test (Wilcoxon's matched-pairs signed-rank test) and the effect remained significant ($Z=-2.25$, $P=0.024$). There was no significant GSK598809 effect on summary scores for the MNWS-R. A significant effect of GSK598809 on response to the QSU-brief, factor 1 ('desire and intention to smoke, with smoking anticipated as pleasurable') scores was found at 30 min post-dose: mean (SD) = 27.22 (7.09) for placebo, 25.35 (7.92) for GSK598809; t (39 d.f.) = 2.06, $P=0.046$ (paired t-test). There was no effect of GSK598809 on factor 2 ('anticipation of relief from negative affect and nicotine withdrawal, with an urgent

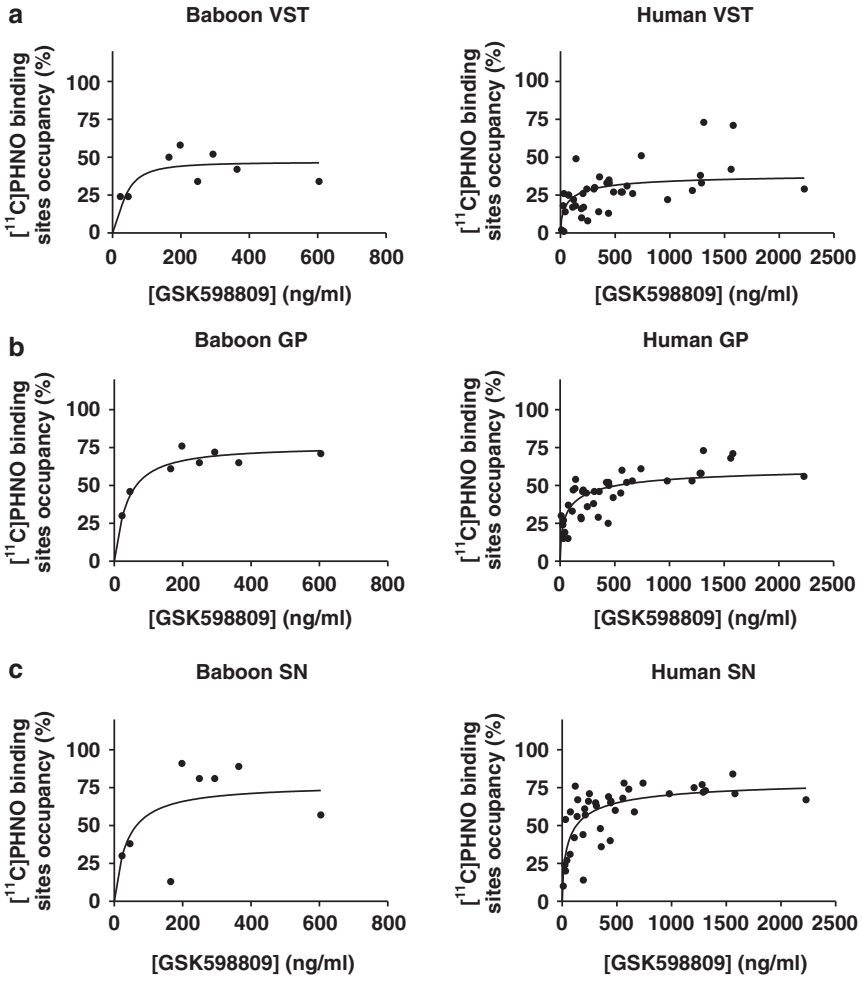

Figure 4 Occupancy of *in vivo* [^{11}C]-(+)-2*H*-naphth[1,2-*b*]-1,4-oxazin-9-ol, 3,4,4a,5,6,10b-hexahydro-4-propyl-([^{11}C]PHNO) binding sites (O^{PHNO}) in the baboon and human ventral striatum (VST) (a), globus pallidus (GP) (b), and substantia nigra (SN) (c) at increasing plasma GSK598809 concentrations (at the beginning of the scan), as revealed by positron emission tomography (PET) methodology (original values were partially already reported in Searle *et al* (2010)).

desire to smoke') of the QSU-brief, or on any craving measures at the later assessment points.

Finally, 8.5–10.5 h after smoking, subjects took, on average, 4.05 total puffs more during *ad lib* smoking when on GSK598809 compared with placebo (95% CI: 1.67–6.43). Similarly, subjects smoked 0.35 cigarettes more during *ad lib* smoking when on GSK598809 compared with placebo (95% CI: 0.12–0.59).

DISCUSSION

In this work we show, for the first time, that a selective D_3R antagonist modifies cigarette craving in short-term abstinent smokers, partially validating the D_3R as a target for the treatment of substance-use disorders. In addition, we establish a relationship between the ability of GSK598809 to reduce nicotine-seeking behavior preclinically and decrease craving in humans, based on the occupancy of brain D_3Rs (O^{D_3R}) and provide a model to early predict the right doses of novel D_3R antagonists for the treatment of addiction in larger Phase II clinical trials.

Historically, technical issues related to the low density of D_3Rs, binding occlusion by endogenous DA, and low specific activity of radioligands, have hampered the determination of O^{D_3R} after systemic administration of dopaminergic ligands (Schotte *et al*, 1996; Langlois *et al*, 2005). In this work, we have first confirmed that GSK598809 is a high-affinity, competitive antagonist at D_3Rs, with more than 100-fold selectivity over $D_{2L}Rs$ and fast association and dissociation kinetics. Then, we have developed an *ex vivo* [^{125}I]7OH-PIPAT autoradiography protocol for the measurement of O^{D_3R} and showed that GSK598809 occupies brain D_3Rs with a direct (time-independent) relationship between exposure and occupancy, and with a potency, expressed as a function of the bio-phase concentration ($OC_{50}^{u,brain} = 2.3$ nM), in agreement with the binding affinity ($K_i = 1.5$ nM for rD_3R). Likewise, we showed that GSK598809 dose dependently reduces the expression of nicotine-induced CPP, with an effect directly related to the exposure and with a similar potency ($EC_{50}^{u,brain} = 0.6$ nM).

Various studies have shown that selective D_3R antagonists reduce the expression of heroin-, cocaine-, metamphetamine-, and nicotine-induced CPP and prevent reinstatement

to alcohol-, cocaine-, metamphetamine-, and nicotine-seeking behavior (Heidbreder and Newman, 2010), but correlation with the O^{D_3R} has never been reported. Here we show a strong relationship between the exposure of GSK598809, the O^{D_3R} in an important area of the addiction circuit (the AcbSh, a main neuroanatomical target of addictive drugs) and the efficacy in an animal model of drug-seeking behavior (the nicotine CPP).

By fitting GSK598809 plasma concentration and the D_3Rs component of [^{11}C]PHNO occupancy from our previous PET study (Searle *et al*, 2010), it was possible to show that GSK598809 occupied human brain D_3Rs with an $OC_{50}^{t,plasma}$ of 66 ng/ml, in line with the value predicted from the rat RO studies ($OC_{50}^{t,plasma} = 87$ ng/ml). In addition, we found an $OC_{50}^{u,blood}$ value of 3.9 nM, in line with GSK598809 affinity for hD_3Rs ($K_i = 6.2$ nM), suggesting that the free drug hypothesis (for a freely diffusible molecule that passively permeates through the blood–brain barrier and is not excluded or uptaken by carrier-mediated systems) is valid for GSK598809 and that the $C^{u,blood}$ can be reasonably considered as a surrogate for the bio-phase concentration (ie, the $C^{u,brain}$; Read and Braggio, 2010). Overall, these results showed that the *PK/O* relationship was consistent between the rat, baboon, and human species, and that O^{D_3R} was a biomarker with good translational value.

As a limitation of our approach, however, prediction of GSK598809 potency in human was based on K_i values determined with different radioligands (namely [^{125}I]7OH-PIPAT for rD_3R and [^{3}H]PHNO for hD_3R) and different binding conditions. Even if these radioligands present similar properties (in that they are both high-affinity, dual D_3R/D_2R agonists), more accurate predictions might be obtained by measuring the affinity of GSK598809 for the rD_3R and hD_3R with exactly the same binding assay. In addition, our method of estimating the potency in occupying D_3Rs in human, based on the simultaneous, one-site fitting of the data from the regions containing consistent amount of D_3Rs, produced slightly different results from those of Searle *et al* (2010), who performed a simultaneous, two-site fitting of all data (including the data from the regions containing a substantial amount of D_2Rs, or very low amounts of [^{11}C]PHNO binding). Interestingly, we found that the maximal occupancy of [^{11}C]PHNO sites by GSK598809 in the SN was slightly lower than the 100% value reported previously (Searle *et al*, 2010), suggesting the presence of D_2Rs also in this region, as reported in human *post-mortem* studies (Murray *et al*, 1994; Gurevich and Joyce, 1999).

In the light of the results obtained in the occupancy studies, we selected the lowest GSK598809 dose that would reach O^{D_3R} values giving maximal effects in the rat nicotine CPP model (ie, $O^{D_3R} \geqslant 72\%$), and tested its effect in human, to make a parallel between this preclinical paradigm of nicotine-seeking behavior and commonly used human behavioral investigations to measure craving and reward.

In the smoking Stroop test, abstinent smokers took significantly longer to color-name words related to cigarette smoking than to color-name neutral control words, confirming previous findings that abstinence decreases the ability to ignore the meaning of smoking-related information (Gross *et al*, 1993). This attentional bias is considered a mark of craving during abstinence. It is worth noting that the difference between the smoking and neutral RT was lower (although not significantly) in the group treated with a single oral dose of 75 mg GSK598809, suggesting that GSK598809 partially reversed the attentional bias of abstinent smokers. In addition, GSK598809 significantly decreased subjective craving (MNWS-R item 4 and QSU-brief factor 1) shortly after administration, but such effects were not maintained at later time points. Overall, these findings were in agreement with the studies reporting an association of the smoking Stroop effects and self-reported craving (Mogg and Bradley, 2002; Mogg *et al*, 2003; Zack *et al*, 2001), and suggest that GSK598809 was effective in decreasing craving. Yet, the same findings partially disagree with the effects observed in the rat CPP model, in which the level of O^{D_3R} reasonably paralleled the degree of efficacy in blocking the rewarding properties of nicotine-associated cues at any time point. In the first place, this difference may suggest that possibly higher O^{D_3R} levels (ie, higher than the 72–89% range estimated in this study), maintained over time, are needed to obtain a robust effect on craving in shortly abstinent smokers. Alternatively, it is worth to consider that nicotine CPP in rat is only a clearcut measure of a process (incentive motivation) in which place-related cues (conditioned stimuli) previously associated to nicotine intake (unconditioned stimuli) allow the animal to seek out and anticipate the reward related to nicotine intake in that specific environment. In smokers, many other factors may affect the intensity of craving, from the variety of cues previously associated with their individual smoking habit, to the length of their smoking history and/or severity of nicotine dependence, which might explain the lower efficacy of GSK598809 in blocking craving in human. Finally, it should be noted that, in contrast to the expression of CPP model, where rats were abstinent from 24 h and in a completely nicotine-free condition, the smokers in this study were abstinent only overnight (14 h) and self-administered nicotine during the Cigarette Choice paradigm, which they knew they would be doing later in the morning. Craving in the subjects of this study, therefore, might be different from the craving of smokers who have been abstinent for a longer time and/or are completely free of nicotine.

The mechanisms by which selective D_3R antagonists partially reverse the attentional bias of abstinent smokers in the Stroop test are unknown. Recent preclinical studies suggest that blockade of D_3Rs facilitates cholinergic transmission in the frontal cortex and have pro-cognitive effects (Lacroix *et al*, 2003; Millan *et al*, 2007; Glickstein *et al*, 2005; Laszy *et al*, 2005; Loiseau and Millan, 2009), which may explain the improved performance of GSK598809-treated patients in the Stroop test. Interestingly, not only acetylcholine but also DA was reported to increase in the frontal cortex following D_3R antagonist administration (Lacroix *et al*, 2003). Considering that lower DA levels are observed after a prolonged period of drug dependence (Volkow *et al*, 2009), it can be hypothesized that D_3Rs antagonists may restore DA levels to normal or, interfering with the cortical glutamatergic signaling to the Acb and ventral tegmental area (Surmeier *et al*, 2007; Kalivas *et al*, 2009), decrease the salience of smoke-associated cues in the Stroop test.

No differences were detected between a single oral dose of 75 mg GSK598809 and placebo when measuring the relative

reinforcing value of smoking and nicotine with the Behavioral Economic task and Cigarette Choice paradigm. Nevertheless, GSK598809-treated subjects, as compared with placebo, slightly increased cigarette consumption and puffs/cigarette when subjects were allowed to smoke freely in the natural environment subsequent to the experimental session, suggesting less satisfaction from the same number of puffs and compensatory increases.

Overall, these results suggest that when a single dose of GSK598809 is administered to smokers, it does not affect the rewarding properties of nicotine *per se* (in line with preclinical findings showing that selective D_3R antagonists do not interfere with the primary reinforcing properties of drugs of abuse), while reducing self-reported craving and slightly increasing smoking, probably as a compensatory mechanism. Further experiments, possibly in different types of smokers (interested and not interested in quitting smoking) are needed to understand if D_3R antagonists may decrease the rewarding properties of nicotine in specific circumstances in humans.

In conclusion, we found that GSK598809 PK exposure is predictive of brain O^{D_3R} at any time point, in both rat and human species. In rats repeatedly exposed to nicotine and abstinent for 24 h, the level of O^{D_3R}produced by a single dose of GSK598809 reasonably paralleled the degree of efficacy in blocking nicotine-seeking behavior driven by associated cues. In humans, a single oral dose of 75 mg GSK598809 estimated to reach submaximal levels of O^{D_3R}only partially alleviated craving in smokers abstinent for 14 h. These data suggested that higher and persistent O^{D_3R} by a selective DA D_3R antagonist or additional effects on other biological mechanisms are needed to more efficiently block craving for cigarettes in smokers under transient (hours) abstinence in humans. Studies with long-term abstinent smokers motivated to quit, with repeated dose treatment designed to achieve higher and more sustained levels of O^{D_3R}, and incorporating tests that better reflect the cue associative nature of the preclinical CPP test, are probably needed to further investigate the role of GSK598809 or other DA D_3R antagonists in human craving.

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DISCLOSURE

This study was sponsored by GlaxoSmithKline. All authors were full-time employees of GlaxoSmithKline at the time the study was conducted. EMP declares that during the past 3 years he was full-time employee of GlaxoSmithKline in the period 2010–2011 and since 2012 he has been a full-time employee of F Hoffmann-La Roche, Basel. JB has been full-time employee of Maccine Pte Ltd (Singapore) in the period November 2010–March 2012 and is full-time employee of Abbott Laboratories (Greater Chicago Area, USA) since April 2012. The other authors declare no conflict of interest.

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