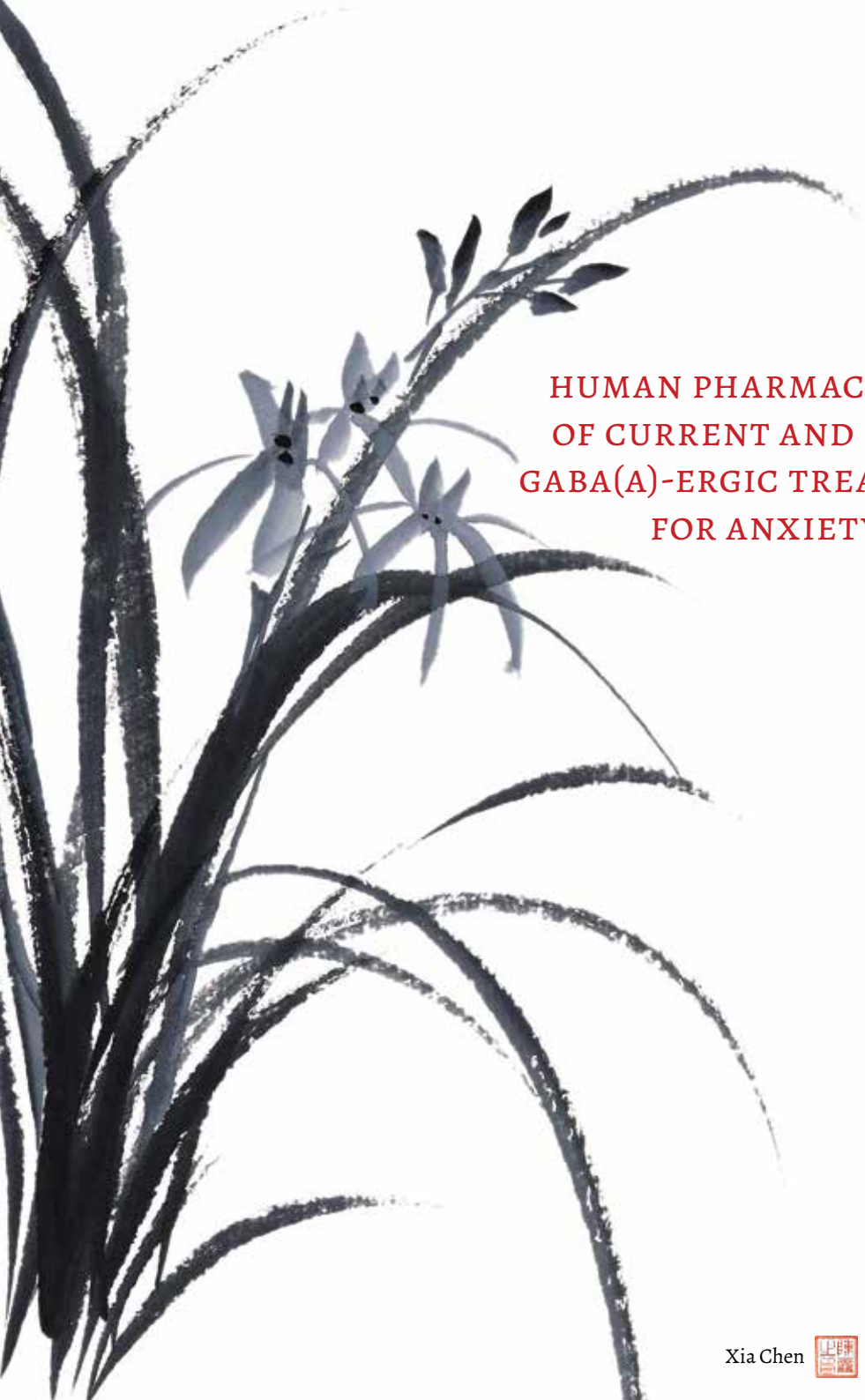
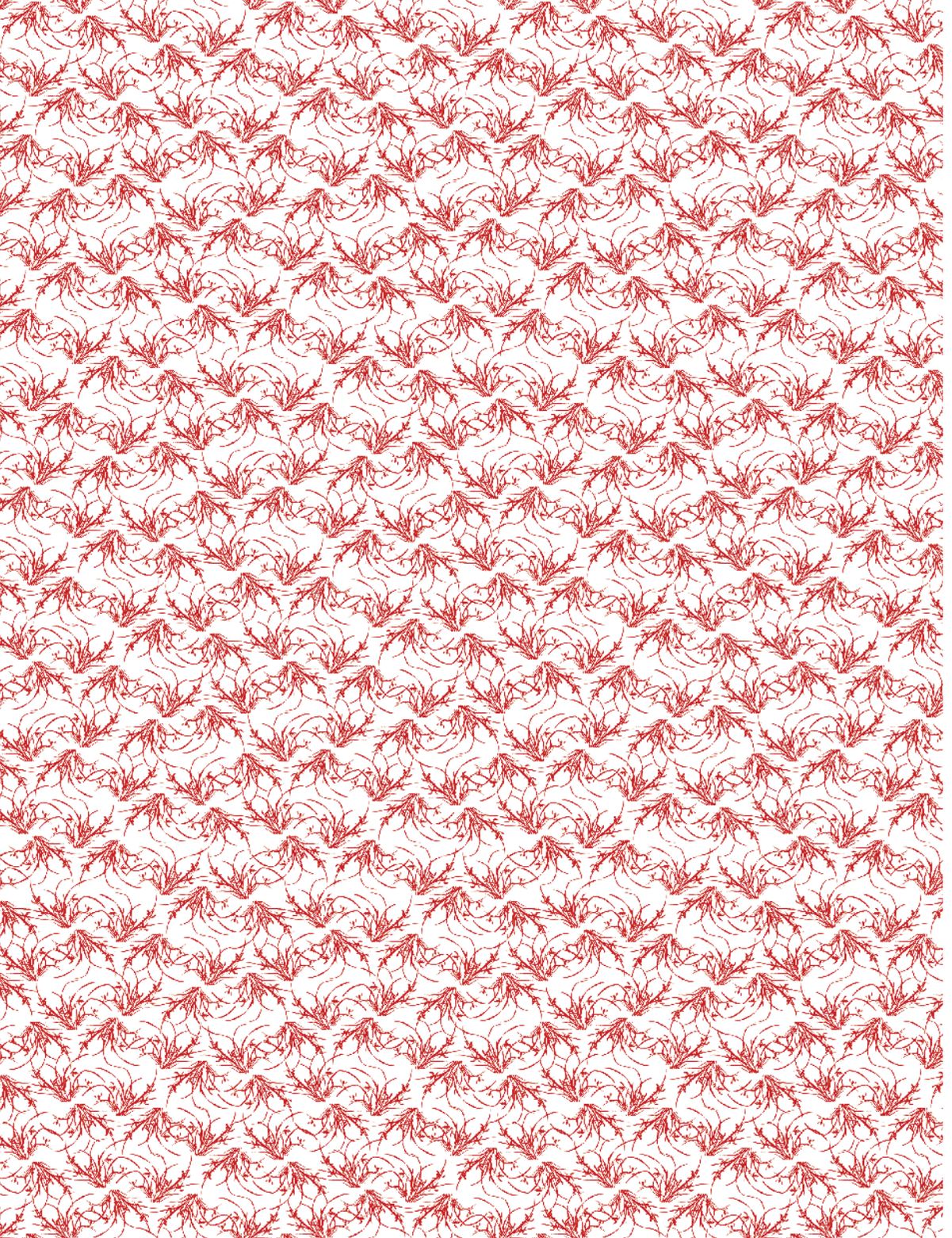


HUMAN PHARMACOLOGY OF CURRENT AND NOVEL GABA(A)-ERGIC TREATMENTS FOR ANXIETY

Xia Chen



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DOOR
Xia Chen,
geboren te Beijing, China
in 1976

PROMOTORES
Prof. dr. J.M.A. van Gerven
Prof. dr. A. Cohen

CO-PROMOTOR
Dr. G. Jacobs

LEDEN PROMOTIECOMMISSIE
Dr. D. Cath, psychiater, Universitair Medisch Centrum Groningen
Prof. Dr. Wim Riedel, experimental psychopharmacology, Maastricht University
Prof. dr. N. vd Wee

DESIGN
Caroline de Lint (caro@delint.nl)

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I
INTRODUCTION

ANXIETY: DEFINITION, DIAGNOSIS, EPIDEMIOLOGY, AND CURRENT TREATMENT STATUS

Anxiety is a commonly occurring negative human emotional state and is characterized by subjective feelings of worry and fear. By definition, worry or apprehension refers to thoughts and expectations about future events while fear is an acute reaction to perceived imminent danger. Subjective phenomena are usually accompanied by physical symptoms such as increased heart rate, shakiness, fatigue, and muscle tension, as well as cognitive, and behavioral manifestations. Anxiety can be adaptive that occurs in response to a threat and prepares to cope with the environment. However, anxiety becomes pathological when it causes significant personal distress and impairs everyday functioning. In order to be diagnosed with an anxiety disorder, individuals have to experience a certain number of symptoms that are disproportionate to either actual or imagined environmental threat for at least six months [1,2].

Anxiety disorders are chronic, disabling conditions that impose enormous costs both on individuals and on society [3-6]. These disorders are prevalent in Western countries. According to a recent 3-year multi-method study covering 30 European countries, 14% of the total population (i.e., 514 million people) were suffering from anxiety disorders [4]. In the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (*DSM 5) [1], seven anxiety syndromes are classified, including panic disorder, agoraphobia, social anxiety disorder (SAD), generalized anxiety disorder (GAD), specific phobias, separation anxiety disorder and selective mutism. The etiology of anxiety disorders is multifactorial and includes genetic liability to a certain extent for some syndromes. In addition, drug withdrawal, substance/medication (e.g. alcohol, caffeine, and benzodiazepines) abuse and dependence, occupational exposure to organic solvents, and life stresses have been related to the etiology of anxiety disordersm while psychiatric complications of endocrine disorders like pheochromocytoma and hyperthyroidism have been demonstrated to mimic anxiety disorders. Taken together, the phenomenologically-based diagnostic classification and the multifactorial nature of anxiety disorders are expected to affected efficacy of anxiolytic CNS active drugs that have been discovered in the past decades.

Current treatment modalities for anxiety disorders can be categorized into psychological treatments (e.g., exposure therapy, cognitive therapy and cognitive behavioral therapy) and pharmacological interventions [2]. The pharmacological interventions can be further divided to chronic or maintenance treatments and short-term treatments inducing acute anxiolysis. Monoamine modulating drugs such as the selective serotonin reuptake inhibitors (SSRIs) and serotonin-noradrenaline reuptake inhibitors (SNRIs) are considered the first-line drugs for anxiety disorders. This is mainly due to their 'broad spectrum' anxiolytic efficacy in both short-term and long-term therapy and the relatively good tolerability in terms of side effects and treatment adherence [2]. However, since it is not unusual for treatment response to be reached only after 12 weeks of treatment at a therapeutic dose, the delayed onset of action of SSRIs and SNRIs remains a major disadvantage. In addition, when patients do not respond to or are intolerant of SSRI/SNRI treatment, alternative classes of psychotropic drugs, such as other antidepressant drugs (e.g., tricyclic antidepressants [TCAs], irreversible monoamine oxidase inhibitor [MAOI] phenelzine), anticonvulsant drug pregabalin, antipsychotic drugs (e.g., quetiapine), and anti-histamine drug hydroxyzine, are considered. Nonetheless, even after treatment with multiple anxiolytic drugs, up to 40% of patients with anxiety disorders do not respond to such drugs at all or only respond partially [7]. Given the rapid-onset effectiveness of benzodiazepines (BZDs) in many patients with anxiety disorders, especially in panic disorder, GAD and SAD patients, these drugs are generally reserved for the treatment of patients who have failed to respond to at least three previous treatments (such as after non-response to an SSRI, an SNRI and a psychological intervention). The use of BZDs should however be minimalized and preferably be reserved for short-term treatments to mitigate the risks of troublesome sedation, cognitive impairment and discontinuation symptoms after abrupt withdrawal [8] in both short-term and long-term treatment, and to avoid development of tolerance and dependence with prolonged use. Taken together, an obvious unmet clinical need in the pharmacological treatment of anxiety disorders opens an opportunity for novel pharmacological approaches that demonstrate rapid anxiolytic efficacy that is superior to existing treatments and lacks tolerance induction, abuse liability and withdrawal symptoms.

THE BRAIN CIRCUITRY INVOLVED IN ANXIETY AND THE ROLE OF GAMMA-AMINOBUTYRIC ACID A (GABA) IN THE AMYGDALA

On a neurobiological level, anxiety disorders arise from disruption of the highly interconnected circuits normally serving to process the stream of potentially threatening stimuli detected by the human brain from the outside world. Perturbations anywhere in these circuits cause imbalance in the entire system, resulting in a fundamental misinterpretation of neural sensory information as threatening and leading to the inappropriate emotional- and thereby behavioral-responses seen in anxiety disorders [9].

Briefly speaking, anxiety is linked to compromised interactions between the amygdala and the dorsal and ventral medial prefrontal cortex (MPFC). Tract-tracing studies in rats show that axons originating in the infra-limbic cortex of the MPFC terminate most densely in the ventromedial lateral nucleus, the rostral part of the

accessory basal amygdala, lateral capsular subdivision of the central nucleus and the superficial nuclei (lateral olfactory tract, periamygdaloid cortex and cortical nuclei) [10-12]. Neurons in the more caudal areas of the infra-limbic sub-region also project to the medial and intermediate subdivisions of the central nucleus [11,13]. The pre-limbic cortex of the mPFC is located dorsally adjacent to the infra-limbic sub-region and it has a different pattern of connectivity with the amygdala. Pre-limbic cortex neurons target the basal nucleus of the amygdala (BA), primarily the dorso-medial part [11,14], while caudal pre-limbic cortex neurons concentrate inputs in the medial parvicellular basal nucleus [15].

Fear extinction is defined as a decline in conditioned fear responses following repeated exposure to a feared conditioned stimulus (e.g., a tone in both animals and humans) in the absence of the unconditioned stimulus (usually a footshock in animals) with which it was previously paired [16]. Extincted fear can be recovered with time or change of the experimental context, suggesting that fear extinction reflects a learning process. The fear reduction is associated with inhibition rather than erasure of the original fear memory. Given that fear extinction has a close therapeutic analogue in the form of exposure therapy for patients with anxiety disorders, it has been implicated in many preclinical studies to investigate drugs acting as adjuncts to strengthen extinction and reduce intrusive fear memories in PTSD and specific phobias [17]. The acquisition, consolidation and retrieval of extinction therefore are separable processes that are controlled by different brain regions and neural systems [18].

In both experimental animal and human functional imaging studies, the amygdala and the mPFC has been demonstrated to be associated with the regulation of negative emotion, such as anxiety or worry and apprehension. Neuroimaging studies consistently show that higher levels of anxiety are associated with both attenuated ventral medial prefrontal cortex (vmPFC) activity and exaggerated dorsal medial prefrontal cortex (dmPFC) activity [19,20] in the presence of threatening stimuli. In the absence of threatening stimuli (i.e., at rest) Kim and colleagues [21] reported that the negative connectivity normally seen between the amygdala and the dmPFC at rest was attenuated in high anxious subjects, whereas the positive connectivity normally observed between the amygdala and vmPFC at rest, manifested as negative connectivity in high anxious subjects. Interestingly, the mPFC-amygdala coupling is inversely correlated with self-reported measures of anxiety or anxious temperament, indicating that the mPFC functions to actively regulate the amygdala and impaired connection between the two neural structures may lead to inadequate response to threatening stimuli. On the other hand, the amygdala – nuclei situated in the median temporal lobes – appears to play a crucial role in the regulation of negative affect and therefore anxiety-related symptomatology.

Emerging evidence from functional magnetic resonance imaging supports that amygdala is the key brain region of activity in response to negative emotional stimuli in healthy volunteers [22-24]. Besides, patients with anxiety disorders are prone to amygdala activation in response to a given threatening stimulus more than the non-anxious controls [25]. Moreover, successful treatment of anxiety disorders with cognitive behavioral therapy leads to extinction of this hyperactivation in the amygdala [26]. Taken together, mPFC functions to regulate amygdala function by actively suppressing activity, and so deficiency in the top-down regulation of mPFC and hyperactivation of the amegdela have been implicated in the pathophysiology of anxiety-related disorders.

In the amygdela, two groups of nuclei should be noted, namely the basolateral amygdala complex (BLA) and the centromedial amygdala complex, in particular the central nucleus (CeA) [27,28]. The BLA receives afferent information on potentially negative emotional signals from the thalamus and the sensory association cortex. The BLA activates the CeA either directly through an excitatory glutamatergic pathway or indirectly by activating a relay of inhibitory GABAergic interneurons that lie between the BLA and the CeA and exert an inhibitory influence upon the latter [29,30]. The CeA is the principal efferent pathway from the amygdala. Inhibitory GABAergic neurons project from the CeA to the hypothalamus and brainstem; the activation of these neurons leads to the somatic manifestations of anxiety [31]. Projections to other basal forebrain nuclei such as the ventrotegmental area and the locus ceruleus may be involved in the subjective effects that are related to anxiety, such as apprehension and dysphoria [32]. In addition, neurons from the BLA also activate cells in the adjacent bed nucleus of the stria terminalis, which project to the same areas as the CeA and apparently play a similar role [28,32].

The knowledge about the neurobiology underlining anxiety disorders serves as the basis for the search of novel anxiolytic agents. Compounds that manipulate this potential pathway may provide new options for the treatment of anxiety disorders. Moreover, neuroimaging and neurophysiological measurements that address the corresponding processes may be used to assess human responses to drug-mediated target modulation.

THE INVOLVEMENT OF GABA SYSTEM IN THE PATHOPHYSIOLOGY OFANXIETY AND ANXIETY DISORDERS

Mounting evidence has suggested the pathogenesis of human anxiety disorders is related to a dysfunction of central top-down inhibitory mechanisms. By providing the major source of inhibitory neurotransmission in the mPFC and amygdala, GABA exerts a powerful influence on a range of fear- and anxiety-related behaviours,

including fear extinction [33-37]. Temporary inactivation induced by GABA(A) receptor agonists has been implicated to establish necessary contribution of the infralimbic subregion or basolateral amygdala (BLA) (but not prelimbic cortex) to fear extinction [38,39]. Infusions of GABA or GABA receptor agonists into the amygdala were found reducing measures of fear and anxiety (possibly related to effects on memory reconsolidation) in several animal species [40,41]. On the other hand, infusion of the GABA antagonist bicuculline was found to block chlordiazepoxide-induced anxiolytic-like activity in rats, whereas injecting bicuculline methiodide to the anterior basolateral amygdala of rats elicited anxiogenic-like effects in both the social interaction paradigm and the conflict paradigm. Microinjection of bicuculline methiodide into the central nucleus of the amygdala elicited no change in experimental anxiety [42].

In humans, administration of benzodiazepines is translated to anxiolytic effect by attenuating amygdala activation in response to negative emotional stimuli [43,44]. To the contrary, Nutt et al. [45] performed an interesting study, in which they injected the benzodiazepine-antagonist flumazenil to 10 patients with panic disorder and 10 control subjects. Subjective anxiety responses after flumazenil infusion were significantly higher in patients with panic disorder than in the controls, and panic attacks were successfully induced in eight patients with panic disorder but no panic attack occurred in the controls. Although such findings have not been replicated [46], they are regarded as a potential signal for the possible shift of the "receptor set-point" [45]. Nikolaus et al reviewed 14 nuclear neuroimaging (Positron emission tomography [PET] and Single-Photon Emission Computed Tomography [SPECT]) studies conducted in patients with anxiety disorders (160 patients [mostly GAD patients] vs. 172 healthy controls). They identified a widespread decline of GABA(A) receptor binding sites and reduced binding extent in the whole mesolimbocortical system in patients suffering from anxiety disorders, suggesting attenuation of physiological central depression. The disturbances of the downstream dopaminergic and serotonergic neurotransmission are thought to, at least partly, result from the diminished tone of GABAergic neurotransmission [47]. A decrease of cortical GABA neurons and reduction of GABA levels were reported in patients with major depressive disorder (MDD) using proton magnetic resonance spectroscopy [48]. Considering the frequent comorbidity of MDD with anxiety states, a shared underlying pathology that emphasizes the causal contribution of GABAergic deficit is proposed for both anxiety disorders and depression [49-51]. Similar GABA(A) receptors reduction is also seen in patients with panic anxiety or post-trauma stress disorder (PTSD). Noteworthy, the extent of GABA(A) receptor deficit is significantly correlated to the clinical severity of these two disorders [52-56], suggesting an 'exposure'-response relationship and hence reinforcing the contribution of GABAergic deficit to anxiety status.

In summary, all aforementioned research findings suggest GABAergic neurotransmission in the mPFC-amygdala coupling is a promising target for modulation of anxiety-related responses.

GABA(A) RECEPTOR STRUCTURE, FUNCTION, AND ITS IMPLICATION IN THE PHARMACOTHERAPY OF ANXIETY DISORDERS

The discovery of the GABA(A) receptor in the 1970s, originally called benzodiazepine receptor, was essential for elaborating the mechanism of action of benzodiazepines, it was the recognition of benzodiazepine-sensitive GABA(A) receptor subtypes that opened up a new GABA pharmacology [57].

GABA(A) receptors belong to the class of ligand-gated ion channels [58]. The GABA(A) receptors are hetero-pentamers traversing the neuronal membrane. To date, a large number of GABA(A) receptor subtypes have been identified: α 1-6, ß 1-3, γ 1-3, Δ, δ 1-3, θ, π [59]. The majority of GABA(A) receptors in the brain are comprised of two α subunits, two ß subunits, and a γ sub-unit. These subunits construct a cylinder. Activation of the receptor by GABA leads to a conformational change in the protein subunits and results in transient opening of a pore along the axis of the cylinder, allowing the flow of chloride ions from one side of the membrane to another [60]. The pharmacological interaction between benzodiazepines and GABA(A) receptors occurs at a different site independent from the GABA binding site on the GABA(A) receptor. GABA binds within the two interfaces between the α and ß subunits on the GABA(A) receptor. Benzodiazepines bind within the interface between the α and γ sub-units, thereby potentiating GABA-related activation of the chloride conductance through allosteric modulation [61]. Nevertheless, such benzodiazepine recognition site does not exist in all α and γ2 subunit combinations. Therefore, although GABA(A) receptors containing ß, γ2 plus either α_1, α_2, α_3, or α_5 subunits possess a binding site for classical benzodiazepines, analogous receptors containing α4 or α6 subunits do not. The research by Seeburg et al has attributed the benzodiazepine-sensitivity of α_1, α_2, α_3, and α_5 subunits to the histidine residue in a homologous position in their N-terminal extracellular region, which switches to an arginine residue in the benzodiazepine-insensitive α4 and α6 subunits [62].
Given the evolutional preservation of the GABA(A)/Gly receptor-like (GRL) gene sequences in the vertebrates [63], the function of each GABA(A) receptor subunit was initially investigated through a gene knock-out approach. Thanks to the gained experience in gene targeting techniques that enables introduction of specific point mutations, and the recognition that a single amino acid residue in the α subunit determines the sensitivity of a GABA(A) receptor to diazepam, point mutation of the histidine to an arginine in the α_1, α_2, α_3, and α_5 subunits was employed in *in vivo* animal studies to convey the interaction between benzodiazepines and the

$\alpha_{1,2,3,5}$-containing GABA(A) receptors from agonism to inverse agonism [64]. This knock-in approach was used to investigate the underlying pharmacological action of the manipulated receptor subunit.

Based on various experimental knock-in and knock-out mice models, α_1-containing GABA(A) receptors are linked to sedative effect [65-68], while spinal α_2/α_3 GABA(A) receptors are found to mediate analgesia [69-71] and α_5-containing GABA(A) receptors, which relatively specifically express in the hippocampus (the central domain for learning and memory), are associated with cognition [72-77]. The GABA(A) subtype responsible for the anxiolytic effects of benzodiazepines are less clear. The involvement of α_2 GABA(A) receptors in anxiolysis is anticipated given their high expression in human amygdala-prefrontal circuitry [78,79]. Most studies suggest that the α_2 rather than the α_3 subtype is related to the benzodiazepines-induced anxiolysis [80,81], while pharmacological studies using either an α_3-selective inverse agonist [82] or an α_3-selective agonist [83] implicates the α_3 subtype. Despite of the controversies, the affinity and efficacy of current investigational compounds acting at the α_2 and α_3 subtypes are mostly similar at the α_2- and α_3- subunits containing GABA(A) receptors [84].

NOVEL $\alpha_{2,3}$-SUBTYPE SELECTIVE COMPOUNDS FOR ANXIOLYSIS

In contrast to other areas of pharmacology, in the field of GABAergic receptor modulator, it has been particularly difficult for medicinal chemists to develop subtype-selective ligands [85], mainly because the high flexibility of GABA(A) receptors and the existence of multiple drug-binding sites. In addition, the distinct subunit composition among the GABA(A) receptor subtypes, the contribution of distinct subunit sequences to binding sites of different receptor subtypes, as well as the fact that even subunits not directly connected to a binding site are able to influence affinity and efficacy of drugs, contribute to a unique pharmacology of each GABA(A) receptor subtype [86].

The binding and efficacy profiles of candidate $\alpha_{2,3}$ subtype-selective drugs can be classified to either binding-selectivity or efficacy-selectivity. A compound with binding-selectivity is expected to have higher affinity for α_2 and/or α_3 subtypes in vitro and hence specific receptor occupancy and CNS distribution in vivo. Even though the compound may have comparable efficacy at the four benzodiazepine-sensitive GABA(A) receptor subtypes, its pharmacological selectivity is determined in vivo by preferential occupancy. As for efficacy-selectivity, an ideal compound should have opposite pharmacological interactions at different subtypes. In other words, it should exert agonism at the $\alpha_{2,3}$ subtypes whereas present antagonism or inverse agonsim at the α_1 and α_5 subtypes. Between these two extreme conditions, there could be multiple permutations, including a compound behaves as a full agonist or a relatively high partial agonist at α_2 and/or α_3 subtypes but has weak or none activity at the α_1 and α_5 subtypes.

Based on these principles, a number of conceptually GABA(A) $\alpha_{2,3}$ subtype-selective compounds have been identified through *in vitro* studies using recombinant human GABA(A) receptors and carried forward into clinical development. Because of their pharmacological selectivity, these compounds are expected to have favorable therapeutic effect with less sedating or cognition impairing effect. Table 1 listed the *in vitro* pharmacological properties of these novel GABAergic compounds.

Table 1 · In vitro pharmacological properties of the GABAergic compounds

	α_1		α_2		α_3		α_5	
Compound	**K_i[1] (nM)**	**Efficacy[2] (%)**	**K_i (nM)**	**Efficacy (%)**	**K_i (nM)**	**Efficacy (%)**	**K_i (nM)**	**Efficacy (%)**
TPA023[3]	0.27	0#	0.31	11	0.19	21	0.41	5
MK-0343[3]	0.22	18	0.40	23	0.21	45	0.23	18
SL65.1498[4]	17	45	73	115	80	83	215	48
Zolpidem	20	75[5]	400 (d)	78[5]	400 (d)	80[5]	5000(d)	9[5]
AZD7325[6]	0.5	0	0.3	18	1.3	15	230	8
AZD6280[7]	0.5	0	21	32	31	34	1680	7
NS11821[8]	1.6	4	9.7	17	3.8	40	2.5	41

1. Ki = constant of receptor-subtype binding / 2. Relative efficacy is defined as the extent of the potentiation of GABA(A) EC_{20}-equivalent current produced by the compound compared to that produced by a nonselective full agonist (chlordiazepoxide/diazepam) / 3. Mean values of 3 experiments in Xenopus oocytes with human recombinant αß3γ2 receptors; efficacy relative to chlordiazepoxide [86,89] / 4. Mean values of 3 experiments in HEK293 cells with recombinant rat receptors αß2γ2; efficacy relative to chlordiazepoxide [97] / 5. Mean values of 3 experiments in Xenopus oocytes with human recombinant αß2γ2 receptor; efficacy relative to diazepam [98,99] / 6. Data adapted from [100] / 7. Data adapted from [101] / 8. Data adapted from [102].

EVALUATION OF HUMAN PHARMACOLOGY

BZDs exert their CNS actions in a concentration-dependent manner [87]. The anxiolytic, hypnotic, muscle relaxant, and amnesic effects of benzodiazepines generally appear concomitantly, and the onset and duration of action correlate closely with the pharmacokinetic profiles of these compounds. Based on non-clinical investigations using *in vitro* assays and animal models of anxiety, the human pharmacology of novel GABAergic agents is approached through clinical pharmacology studies investigating pharmacokinetics, receptor occupancy, and pharmacodynamics (PD) in healthy volunteers. Direct links have been proposed between plasma drug concentration and GABA receptor occupancy [84], as well as between plasma drug concentration and the pharmacodynamic measurements

[88-91]. Such pharmacokinetic/pharmacodynamic (PK/PD) relationships warrant the use of surrogate biomarkers in healthy volunteers treated with single-dose administration of selective novel GABAergic compound(s).

More than 170 pharmacodynamic tests or test variants have been developed to assess the CNS effects of benzodiazepines. De Visser et al. [87] analyzed the inter-study consistency, sensitivity, and pharmacological specificity of the frequently used biomarkers. Saccadic peak velocity (SPV) and visual analogue scale of alertness ($VAS_{alertness}$) were identified as the most sensitive parameters for benzodiazepines. Both measurements showed consistently dose-dependent responses to a variety of benzodiazepines. Based on these finding, the Centre for Human Drug Research (CHDR) has established a selection of computerized CNS-pharmacodynamic tests called the Neurocart battery [92]. The components of this battery target a variety of neurophysiological and/or neuropsychological domains (Table 2).

Table 2 · Component tests of the Neurocart battery and the related CNS domains

Neurocart test	Targeted function	Related CNS domains
Saccadic eye movement	Neurophysiologic function	Superior colliculus, substantia nigra, amygdala
Smooth pursuit	Neurophysiologic function	Midbrain
Adaptive tracking	Visuo-motor coordination	Neocortex, basal nuclei, brain stem, cerebellum
Body sway	Balance	Cerebellum, brain stem
Visual verbal learning test (VVLT)	Memory	Hippocampus
VAS Bond and Lader	Alertness, mood, calmness	Cortex, prefrontal cortex
VAS Bowdle	Feeling high, internal and external perception	Cortex, prefrontal cortex, amygdala

Of this battery, adaptive tracking, saccadic eye movements, and body sway were proven sensitive to the sedating effects of sleep deprivation [93], as well as to the effects of benzodiazepines and other GABAergic hypnotic drugs [89,91]. In the recent years, the Neurocart battery was used in a series of phase I studies to assess CNS pharmacodynamics of partial $\alpha_{2,3}$ subtype selective GABA(A) agonists. Both nonselective and/or selective GABA(A) agonists were administered as single oral dose to healthy volunteers. Clear distinctions were observed between the effect profile of non-subtype-selective full GABA(A) agonist and that of selective partial GABA(A) agonist in these trials [88-90], probably because the subtype specificity of the pharmacodynamic measurements for the pharmacological modulation of GABA(A)-ergic compounds. Unfortunately, none of the novel receptor subtype-selective compounds have reached the market: the development of GABA(A) receptor α_2 and α_3 subunit agonist SL65.1498 [90], was discontinued owing to unexpected amnestic effects, while the phase 2 studies of another compound of this drug class, TPA023, were terminated prematurally due to preclinical toxicity (cataract formation) in long-term dosing studies [94], despite exhibiting anxioselective activity in GAD; MK-0343 also displayed an anxioselective profile in animal models but produced sedation in humans at low levels of receptor occupancy (<10%) [95].

In summary, these reports indicate that the human pharmacodynamic approach with sensitive and CNS-domain specific neuropsychological and neurophysiological measures is useful in predicting the drug's clinical effect on the central nervous system. Inter-species difference is also noted between human and rodents or primates: although a low *in vitro* efficacy at the α_1-containing GABA(A) receptors may not lead to an overtly sedative effect in the experimental animals, it apparently causes sedation in humans at comparable exposure levels. The following questions remain to be answered: 1) is reduction of saccadic peak velocity a promising surrogate marker for clinical anxiolysis? 2) can we also differentiate partial agonism from the full agonism of benzodiazepines via this pharmacodynamic package? 3) is such selective CNS-pharmacodynamic effect profile characteristic for the family of GABA(A) $\alpha_{2,3}$-subtype receptor agonists?

CONCLUSION AND AIM OF THESIS

Anxiety disorders are highly prevalent psychiatric disorders and have high personal and societal costs. The transition from "normal" negative affect or anxiety to an anxiety disorder is implemented by the interplay between psychosocial stressors and a wide array of neurobiological alterations which lead to subjective suffering and functional impairment. Monoamine modulating treatments are widely applied to treat anxiety disorders but are not effective in a large proportion of patients. As the predominant inhibitory neurotransmitter system in the human brain, the GABAergic system in general and its $\alpha_{2,3}$ subunit-containing GABA(A) receptor subtypes in particular, have been implicated in the pathophysiology of anxiety disorders. Novel pharmacological treatments selectively targeting the anxiolysis-mediating GABA(A) receptor subtypes are currently emerging. These range from affinity-selective agents to efficacy-selective agents and represent potentially useful future pharmacological treatments for anxiety disorders [95].

In this thesis, we report several human pharmacology studies that were performed to identify the pharmacologically active doses/exposure levels of several novel compounds with potential anxiolytic effects (Chapter 2, 3, 4). Because of their pharmacological selectivity at the $\alpha_{2,3}$ GABA(A) receptor subtypes, the novel drugs were expected to elicit clinical anxiolysis and less sedating effects. An overview of the performance of the selected and validated pharmacodynamic measurements is composed to summarize the utility of these neurophysiological and neuropsychological biomarkers in early clinical development of novel anxiolytic drugs

(Chapter 5). However, the difficulty of evaluating therapeutic anxiolytic drug effects in healthy volunteers has led to further explorations on the neuroendocrine biomarkers (Chapter 6) and the integration of a stress-challenging procedure into the evaluations (Chapter 7).

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II

THE CENTRAL NERVOUS SYSTEM EFFECTS OF THE PARTIAL GABA-A α2,3-SELECTIVE RECEPTOR MODULATOR AZD7325 IN COMPARISON WITH LORAZEPAM IN HEALTHY MALES

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Xia Chen[1,2], Gabriel Jacobs[2], Marieke de Kam[2], Judith Jaeger[3,4], Jaakko Lappalainen[3], Paul Maruff[4], Mark A. Smith[3,5], Alan J. Cross[3], Adam Cohen[2], Joop van Gerven[2]

1. Phase I Unit, Clinical Pharmacology Research Center (CPRC), Peking Union Medical College Hospital, Beijing, China | 2. Centre for Human Drug Research, Leiden, The Netherlands | 3. AstraZeneca, R&D, Wilmington Delaware and Cambridge MA | 4. Cogstate, Melbourne Australia and New Haven CT, USA | 5. Shire Pharmaceuticals, PA, USA

ABSTRACT

Aims: AZD7325 is a novel $\alpha_{2,3}$-subtype-selective partial GABA-A-receptor modulator. This study investigated the pharmacodynamics of single-oral-dose AZD7325 2 mg and 10 mg on the central nervous system (CNS) compared to placebo and lorazepam 2 mg. **Methods:** This double-blind, randomized, 4-way-crossover study enrolled sixteen healthy males and administered two validated CNS-test-batteries to measure drug effects on cognitive, neurophysiologic and psychomotor function, and subjective feelings. The pharmacological selectivity of AZD7325 was compared to lorazepam by plotting saccadic peak velocity change from baseline (ΔSPV) against body sway (Δsway) and visual analogue scale for alertness ($\Delta VAS_{alertness}$). This analysis has previously been used to identify $\alpha_{2,3}$-subtype-selectivity. **Results:** In contrast with the robust impairment caused by lorazepam (all $p<0.05$ *vs.* placebo), neither dose of AZD7325 induced statistically significant effects on any pharmacodynamic measurements. Lorazepam-induced SPV-reduction was linearly related to changes in other neurophysiologic biomarkers. In contrast, the slopes of the regression lines were flatter for AZD7325, particularly for the ΔLog(Sway)-ΔSPV relation (estimate slope, AZD7325 10 mg vs. lorazepam, difference [95% confidence interval], p-value: -0.00036 vs. -0.00206, 0.001704 [0.000639, 0.002768], p=0.0018) and the $\Delta VAS_{alertness}$-ΔSPV relationship (0.01855 vs. 0.08216,-0.06360 [-0.1046, -0.02257]; p=0.0024). AZD7325 10 mg and lorazepam induced different response patterns on VAS 'feeling high' and electro-encephalography. **Conclusion:** The characteristic ΔSPV-relative effect profiles of AZD7325 versus lorazepam suggests anxio-selectivity related to $\alpha_{2,3}$-selective GABA-A agonism. However, exploration of higher doses may be warranted. The paucity of effects on most CNS-PD parameters also indicates a mitigated side-effect pattern, with potentially lower cognitive and neurophysiological side-effect burden than non-selective benzodiazepines.

INTRODUCTION

Benzodiazepines (BZDs) are widely used in the treatment of anxiety disorders and for symptomatic relief of various anxiety states related to diverse psychiatric disorders, including mood-, psychotic- and personality disorders. However, concerns have been raised regarding the untoward effects of these drugs, which include movement/balance disorders, cognitive impairment, as well as problems with tolerance and abuse liability. All these facts limit the usefulness of BZDs as a long-term therapy in vulnerable patient populations.

Benzodiazepines elicit their pharmacological effects through allosteric modulation of GABA-A receptors. These compounds have non-selective binding affinities and present full *in vitro* efficacy at the GABA-A receptors that contain subunits α_1, α_2, α_3 or α_5. A collection of loss-of-function studies were performed to compare the BZD-mediated behavior between wild-type animals and knock-in animals [1] and the pharmacological role of each GABA-A subtype. These studies in experimental animals have suggested that the GABA α_1 subtype is associated with sedation [2,3]; α_2/α_3 receptors are responsible for the anxiolytic properties of BZDs [4,5], where α_2 is found more correlated to anxiolysis than α_3 [1]; and the α_5 subunit is related to modification of memory and cognition [6,7]. Based on these findings, the adverse effects of benzodiazepines are attributed to the pharmacological effects of these compounds on GABA-A receptors other than the $\alpha_{2,3}$ subtype. Compounds with relatively high efficacy at the $\alpha_{2,3}$ subunits but reduced efficacy at the α_1 and/or α_5 subunits are classified as subtype-selective GABAergic compounds and expected to be potential anxioselective treatments with reduced sedation and no impact on cognition and psychomotor performance.

AZD7325, 4-amino-8-(2-fluoro-6-methoxy-phenyl)-N-propyl-cinnoline-3-carboxamide [8], is a novel partial subtype-selective GABA-A $\alpha_{2,3}$ receptor modulator, which is in development for anxiety disorders. *In vitro* AZD7325 demonstrated functional specificity for the GABA-A α_2 and GABA-A α_3 receptor subtypes. AZD7325 exerts neutral antagonism at the α_1-subunit and partial efficacy at the $\alpha_{2,3}$-subunits over the α_5-subunit ($\alpha2\sim\alpha_3$ vs. α_5: 18%~15% vs. 8%, percentage compared to maximal diazepam response). Meanwhile, the compound has much higher binding affinity (mean Ki [nm], $\alpha_1\sim\alpha_2\sim\alpha_3$ vs. α_5: 0.3~1.3 vs. 230) and larger relative efficacy at the $\alpha_{2,3}$-subunits over the α_5-subunit (AstraZeneca data on file). Selective *in vitro* properties have also been confirmed in preclinical biomarker studies using EEG and PET imaging in rodents and primates, which revealed that exposures that result in 50% occupancy produce robust anxiolytic effects without benzodiazepine-like side effects (AstraZeneca data on file). However, translation of the effects of GABA(A) $\alpha_1$modulation from pre-clinical studies into human has been unpredictable, with some weak partial GABA(A) α_1 modulator showing persistence of sedative properties [9], whereas some non-subtype selective GABA-A-ergic compounds, such

as ocinaplon [10] and alpidem [11,12], were found anxiolytic but less sedating or less psychomotor- and cognition-impairing in the clinic [13]. In addition, the ideal degree of modulation at each of the two preferred subtypes is not known since the behavior of non-sedating benzodiazepines has not been extensively investigated in clinical settings. Since preclinical data that have been obtained on the pharmacology, pharmacokinetics and toxicology of AZD7325 support the conduct of clinical studies in humans, the current phase I trial was designed to provide an initial assessment of the side-effect profile of AZD7325.

The present study aimed to investigate the pharmacodynamic (PD) effects and evaluate the safety and tolerability of single oral doses of AZD7325 in healthy subjects, in comparison with placebo and lorazepam. Lorazepam is clinically effective as an anxiolytic at a dose of 2 mg. Single oral doses of lorazepam 2 mg have demonstrated robust effects on saccadic peak velocity (SPV), smooth pursuit, body sway, and visual analogue scale (VAS) of alertness in healthy volunteers [14,15,16]. These effects reflect the typical effect profile of benzodiazepines on different central nervous system (CNS) functions [17]. More importantly, SPV is very sensitive to the effect of BZDs, and the drug-induced changes of SPV seem to reflect the anxiolytic potency of different anxiolytic compounds [17]. For this study, the doses of AZD7325 were determined at 2 mg and 10 mg. A previous single-ascending-dose (SAD) study in healthy volunteers indicated that AZD7325 has an acceptable safety profile in oral doses up to 100 mg (AstraZeneca data on file). PET study using [^{11}C]-flumazenil suggested that AZD7325 2 mg is associated with approximately 50% occupancy of the GABA-A receptors and 10 mg causes maximal (>80%) displacement of flumazenil at peak concentration of the compound in occipital cortex (AstraZeneca data on file). Compared to the low receptor occupancy of lorazepam 1 mg [18] as well as the *in vitro* $\alpha_{2,3}$-subtype modulation of AZD7325, AZD7325 2 mg and 10 mg are expected to fall within the anticipated clinical therapeutic window of this compound.

METHODS

DESIGN

The trial was designed as a randomized, double-blind, double-dummy, placebo- and comparator- controlled study in sixteen male healthy volunteers, where the positive control was used to benchmark the effects of the investigational product.

SUBJECTS

Following the approval of the Medical Ethics Review Board of Leiden University Medical Centre (LUMC), subjects who provided written informed consents received medical screening at the Centre for Human Drug Research (CHDR). The eligibilities of sixteen healthy male subjects were confirmed before their entry into the trial. These subjects should be aged between 18 and 55 years, with a body mass index (BMI) of 18 to 30 kg/m^2. All subjects were required to refrain from alcoholic beverages, smoking and caffeine-containing products during study days. A normal diurnal rhythm was advised from minimally two weeks before the first study day until the last visit.

SAMPLE SIZE DETERMINATION

Based on power calculations using data from previous studies [14], a sample size of 16 was determined to have equal to or greater than 80% power to detect the mean differences of 1.24 mm in $VAS_{alertness}$ and 20.6 degree/second in SPV, respectively, assuming standard deviations of 1.66 mm (VAS alertness) and 27.4 degree/second (SPV) between placebo and lorazepam 2 mg using a paired t-test with a 0.050 two-sided significance level.

TREATMENTS

The study treatments were randomly allocated based on a 4×4 William's Latin Square. The treatment sequence was unique for each subject. Each subject received 1) AZD7325 2 mg (two capsules of AZD7325 1 mg and two tablets of lorazepam placebo), 2) AZD7325 10 mg (one capsule of AZD7325 10 mg, one capsule of AZD7325 placebo and two tablets of lorazepam placebo), 3) lorazepam 2 mg (two capsules of AZD7325 placebo and two tablets of lorazepam 1 mg), or 4) placebo (two capsules of AZD7325 placebo and two tablets of lorazepam placebo) on the morning of each study day. A washout period of at least 7 days was arranged between treatments.

PHARMACODYNAMIC MEASUREMENTS

A standard Neurocart battery of neurophysiologic and neuropsychological tests included the following validated pharmacodynamic assessments: body sway, visual analogue scale (VAS) of Bond & Lader, VAS Bowdle, saccadic eye movements, smooth pursuit eye movements, adaptive tracking and electro-encephalograms (EEG). The repeatable measurements were presented to the subjects during a pre-dose visit in order to familiarize subjects with the CNS tests and prevent potential learning effects during the post-dose measurements. In each study period, the Neurocart battery was performed twice at baseline and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 8, and 12 hours post-dose. In the meantime, the CogState Early Phase Battery (described below) was carried out thrice pre-dose and four times (i.e. at 1.25, 2.25, 3.25, and 4.25 hours) post-dose. Moreover, subjects completed the Cogstate International Shopping List task, which required them to memorize a shopping

list of 16 words at 1.75 hours post-dose and recall these items both immediately and at 21 hours post-dose without being read the words again. All assessments were performed by one subject at a time, in a quiet room with subdued ambient illumination.

NEUROCART BATTERY

SACCADIC EYE MOVEMENTS

Saccadic eye movements were evaluated using a computer-based system composed of 1) stimulus display and signal collection (Nihon Kohden Corporation, Tokyo, Japan), 2) signal amplification (Grass-Telefactor, Astro-Med, Inc., Braintree, USA), 3) data recording (Cambridge Electronics Design, Cambridge, UK), 4) disposable silver-silver chloride electrodes (Medicotest N-00-S, Olstykke, Denmark), as well as 5) the sampling and analysis scripts developed by CHDR (Leiden, the Netherlands). The parameters of this test were the average values of saccadic peak velocity (SPV, degree/sec), latency (i.e. reaction time, sec) and inaccuracy (%) of all artefact-free saccades that were calculated on each session. Saccadic peak velocity appears to be the most sensitive measure for the sedative effect of benzodiazepines [17], which has been found to be related to the anxiolytic component of benzodiazepines [17] and to be selectively affected by some newly developed GABAergic compounds with potential anxiolytic effects [14,15,16].

BODY SWAY

Body sway was measured with an apparatus similar to the Wright ataxia meter, which integrates the amplitude of unidirectional body sway. Two-minute measurements were made in the antero-posterior direction with eyes closed. The subject was asked to stand comfortably on a stable floor with his/her feet slightly apart. Body sway measures postural (in)stability. It has demonstrated considerable sensitivity to the effect of benzodiazepines [19].

VISUAL ANALOGUE SCALES (VAS) OF BOND & LADER AND BOWDLE

Visual analogue scales as originally described by Norris have often been used previously to quantify subjective effects of a variety of sedative agents [20]. Dutch versions of the scales have been frequently employed at the CHDR, for a variety of sedative agents [21] and circumstances [22]. During the test, the subject indicated (with a mouse click on the computer screen) on horizontal visual analogue scales how he/she feels. From the sixteen measurements of VAS Bond & Lader, three main factors are the calculated [23] for subjective alertness, contentedness, and calmness.

The Bowdle Psychotomimetic Effects Scores have been developed to quantify the psychotomimetic effects of ketamine [24]. A translated Dutch version of the original scales has been computerized and used at the CHDR to study the effects of cannabinoids [25] and zolpidem [26], among others. This scale has thirteen 10 cm visual analogue lines ranging from 0 ('not at all) to 100 mm ('extremely') [27], addressing various abnormal states of mind. From the thirteen measurements of VAS Bowdle, three distinct total sum scores are calculated: internal perception (reflects inner feelings that do not correspond with reality, including mistrustful feelings), external perception (reflects a misperception of an external stimulus or a change in the awareness of the subject's surroundings) and feeling high [28].

SMOOTH PURSUIT EYE MOVEMENTS

The same system as used for saccadic eye movements was also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moved sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, by steps of 0.1 Hz. The amplitude of target displacement corresponded to 22.5 degrees eyeball rotation to both sides. Four cycles were recorded for each stimulus frequency. The method has been validated at the CHDR by van Steveninck *et al.* [21,28] based on the work of Bittencourt *et al.* [29] and the original description of Baloh *et al.* [30]. The time in which the eyes were in smooth pursuit of the target were calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies were used as the test parameter. Smooth pursuit is a measure of neurophysiological function and has been shown sensitivity to the effects of BZDs [16], zolpidem [31], and some $\alpha_{2,3}$-subtype selective GABA-A receptor modulators [16].

ADAPTIVE TRACKING

Adaptive tracking is a pursuit-tracking task that measures drug effect on visuo-motor coordination. The adaptive tracking test was performed as originally described by Borland and Nicholson [32], using customised equipment and software. After a 0.5-minute run-in time without data-recording, the average performance over 3.0 minutes was scored and was used as the test parameter. The subject was required to operate a joystick and try to keep a dot inside a circle moving randomly on the computer screen. If he/she succeeded, the speed of the moving circle increased, or vice versa.

EEG

Pharmaco-electroencephalography (Pharmaco-EEG) was used to monitor any drug effects, which can be interpreted as evidence of penetration and activity in the brain [33]. EEG recordings were made using gold electrodes, fixed with EC2 paste (Astromed) and using standard pharmaco-EEG lead placement, with the same common ground electrode as for the eye movement registration (international 10/20 system). The electrode resistances were kept below 5 kOhm. The signals were

amplified with a Grass 15LT series Amplifier Systems, using a time constant of 0.3 seconds and a low pass filter at 100 Hz. Data collection and analysis were performed using customized CED and Spike2 for Windows software (Cambridge Electronics Design, Cambridge, UK). Per session eight consecutive blocks of eight seconds were recorded. The signal was AD-converted using a CED 1401 Power (Cambridge Electronics Design, Cambridge, UK) and stored on hard disk for subsequent analysis. Data blocks containing artifacts were identified by visual inspection and these were excluded from analysis. For each lead, fast Fourier transform analysis was performed to obtain the sum of amplitudes in the delta- (0.5-3.5 Hz), theta- (3.5-7.5 Hz), alpha- (7.5-11.5 Hz), beta- (11.5-30 Hz) frequency ranges. Frequency band above 30 Hz was also recorded for exploratory pharmaco-EEG analyses, in order to test whether the findings of an effect of AZD7325 on gamma-frequency band (> 30 Hz) in animals translate to humans.

COGSTATE BATTERY

The CogState Early Phase Battery is a computer administered cognitive test battery that takes about 12 minutes to perform. It is designed to provide objective information regarding possible drug effects on cognitive function [34]. The CogState Early Phase Battery consists of the following tests that address pharmacodynamic effects on different cognitive domains [35]. The tests were presented in the order listed below. In addition, the 16-word CogState International Shopping List Task and its delayed recall session were presented once per dosing period, respectively [36].

GROTON MAZE LEARNING TASK (GMLT)

A 28-step pathway was hidden among the 100 possible locations of a 10 x 10 grid of tiles showing on a computer touch screen. Subjects were instructed to move from the start location (top left), one tile at a time, toward the end (bottom right). The entire 28-step pathway could be figured out based on the computer's feedbacks. Once completed, subjects returned to the start location and repeated the task for 4 more times. Twenty well-matched alternate forms of this task were cycled among measurements so that subjects would not take a same trial during one dosing period. The GMLT is a measure of executive functioning. During a 'timed chase' part of the test, the subject was asked to quickly follow a moving tile around in a 10 x 10 grid of tiles on a computer touch screen with a stylus pen for 30 seconds. This test measures attention and psychomotor function.

DETECTION TASK

During the test, a playing card was presented in the center of the screen. Subjects were required to press the 'Yes' key whenever the card flipped over and faced up.

Subjects were encouraged to work as quickly and accurately as they could, but try not to press the 'Yes' key before a card flips over. If subjects did this or did not respond to a card that had flipped over, they would hear an error sound. After a short period for practice, the real test began. The test measures attention and psychomotor function.

IDENTIFICATION TASK

A playing card was presented in the center of the screen and flipped over from time to time. When the card faced up, the subject should press 'Yes' for a red card but 'No' for a non-red one. An error sound would appear when the subject pressed a key before a card flipped over or made a mistake. The real test began after practice. The test measures speed of mental processing and attention.

ONE CARD LEARNING TASK

Subjects were asked to identify whether the playing card presented on the screen had been shown during the current test trial. They responded by pressing the 'Yes' or 'No' key. An error noise would appear when there was an incorrect or missing response. The real test began after practice. The test measures spatial learning.

INTERNATIONAL SHOPPING LIST TASK (ISLT)

At 1.75 h post-dose, the test supervisor read a shopping list of 16 words forthe subject as they appear on the computer screen at a rate of one word every two seconds. Subjects were instructed to memorize and recall as many words as possible, while the test supervisor clicked / touched the appropriate button on the screen with the stylus or mouse. As such, the entire word list reading session (in the same order) and the immediate recall session were repeated for another two times. The task measures verbal learning ability. At 21 h post-dose, subjects were required to recall the previous shopping list without being read them again, while the test supervisor clicked / touched the appropriate button on the screen with the stylus or mouse. Performance on this test reflects long term storage, memory consolidation and retrieval.

SAFETY

Safety and tolerability were evaluated using clinical laboratory tests, 12-lead electrocardiograms (ECGs), and records of adverse events and vital signs. Twelve-Lead ECG recordings were assessed with Cardiofax V (Nihon Kohden, Tokyo, Japan) or Marquette 5000/5500. After a 5-minute rest in supine position, blood pressures and pulses were taken with a semi-automatic blood pressure recording device (a Nihon-Kohden BSM-1101K monitor or a Colin Pressmate BP 8800 or a Dash 4000) at supine and, 2 minutes later, at standing position. The Central Clinical Chemistry

and Haematology Laboratories of LUMC were responsible for the safety laboratory assays on blood or urine samples.

PHARMACOKINETIC MEASUREMENTS

In order to acquire the plasma concentrations of AZD7325 or lorazepam, venous blood samples (6 mL) were collected at pre-dose and 0.5, 1, 1.25, 1.5, 2, 2.5, 3.25, 4, 4.5, 6, 8, 12, and 21 hours post-dose into ethylenediamine tetra-acetic acid (EDTA K2) spray-dried tubes. These tubes were immediately ice-bathed and centrifuged for 10 minutes at 2°C to 8°C at a relative centrifugal force of 2000g within 30 minutes from collection. Thereafter, the plasma was transferred to two 2 mL Sarstedt tubes and immediately frozen upright at or below -70°C within 15 minutes of plasma preparation and stored at this condition until bioassay.

STATISTICAL ANALYSIS

The pharmacodynamic parameters (short names are written in parentheses) of the Neurocart consist of amplitude of body sway (Sway), saccadic peak velocity (SPV), percentage of smooth pursuit (Smooth), performance of adaptive tracking (Tracking), visual analogue scale for alertness ($VAS_{alertness}$), feeling high (VAS_{high}), internal perception ($VAS_{internal}$), and external perception ($VAS_{external}$), power of various EEG bands (delta, theta, alpha, beta, gamma bands in the frontal-central [Fz-Cz] and the parietal-occipital [Pz-Oz] areas, respectively). EEG parameters, body sway and VAS Bowdle sub-scales were log-transformed prior to analysis and thus corrected for the expected log-normal distribution of the data. Safety variables were frequency and incidence of adverse events (AEs) and related information, vital signs (blood pressure, heart rate, respiratory rate and auricular temperature), laboratory parameters, and ECG outputs. All statistical analyses were performed with SAS (version 9.1).

ANALYSIS OF THE SPV CHANGE FROM BASELINE (ΔSPV)-RELATIVE EFFECT PROFILES

Previous studies suggested good sensitivity of SPV to the effect of BZDs [17] and $\alpha_{2,3}$ subtype-selective GABA-A receptor modulators [14,15,16]. Based on these results, SPV is hypothesized as a biomarker indicative of clinical anxiolysis associated with GABA $\alpha_{2,3}$ activation, and the predictability of SPV was supported by early clinical findings with TPA023 [6]. BZDs also affected body sway and $VAS_{alertness}$, suggesting balance impairment and subjective sedation, respectively [14,15,16,17]. Given the clinical relevance of these pharmacodynamic parameters, the SPV-relative effect profiles of both body sway and $VAS_{alertness}$ have been shown to differentiate the pharmacological selectivity of $\alpha_{2,3}$ subtype-selective GABA-A receptor modulators from the non-selective GABA-A agonism with BZDs [37]. As such, we performed a regression analysis to demonstrate the relationship of individual changes from baseline on body sway (Δsway) and VAS alertness ($\Delta VAS_{alertness}$) against the change from baseline of SPV (ΔSPV). The slopes of these regression lines are thought to correspond with the relation between off-target sedating effects and anxiolysis [37]. A mixed effect model was used, where the fixed factors were treatment and treatment by SPV, whereas the random factors were subject, slope and intercept. The estimates of the slopes of the regression lines of these ΔSPV-relative effect profiles were compared between each dose of AZD7325 and lorazepam.

Repeated pharmacodynamic measurements were also compared with a mixed model analysis of variance with fixed factors of treatment, period, time and treatment by time, and random factors subject, subject by treatment and subject by time, and the average pre-value (average over all measurements at or before time=0) as covariate. The least square means (LSMs) of the measurements up to 8h post-dose were calculated within the statistical model. Contrasts of placebo *vs* each active treatment and between each two active treatments were reported along with 95% confidence intervals. The log-transformed parameters were back-transformed after analysis, where the results were interpreted as percentage change.

Moreover, the ΔSPV-relative effect profiles of adaptive tracking (ΔTracking) and smooth pursuit (ΔSmooth) were explored to gain further insights about the pharmacological selectivity of AZD7325.

The pharmacokinetic analysis was performed at Clinical Pharmacology, AstraZeneca Wilmington, DE, USA using the WinNonlin program (Pharsight Corporation, MountainView, California, USA) using non-compartmental analysis. The resultant PK parameters were summarized with descriptive statistics by treatment. The frequency and incidence of adverse events were summarized based on preferred terms by system organ class (SOC) and treatment. Parameters of vital signs, 12-lead ECGs and safety laboratories, along with their changes from baseline, were summarized using descriptive statistics by treatment.

RESULTS

SUBJECTS

Eighteen healthy male subjects, aged 24.6±7.6 years, were eligible for the trial. Two subjects withdrew their informed consents for personal reasons after completion of the first treatment period and were replaced by another two male subjects who received the same sequences of treatments. Sixteen subjects completed the study

per protocol. The mean (standard deviation, SD) body weight and body mass index (BMI) of the completers were 74.3 (7.2) kg and 22.6 (2.4) kg/m^2, respectively. Safety analyses were performed on data from all treated subjects. Valid data from subjects who completed at least one dosing period per protocol were included into the pharmacokinetic and pharmacodynamic analyses.

PHARMACODYNAMIC (PD) RESULTS

The profiles of various pharmacodynamic parameters were obtained with each study treatment and graphically presented in Figure 1-4. In general, the maximal effect of lorazepam 2 mg appeared around 3 hours post-dose, which was slightly behind the time to peak plasma concentration, whereas the EEG effects of lorazepam and AZD7325 reached their peak level around T_{max}.

An overview of the regression analyses for the slopes of effects relative to SPV is plotted in Figure 5, in combination with the calculated population regression lines (Table 1). In figure 5, each dot represents the average change from baseline of the y-axis PD parameter versus that of the x-axis PD parameter (i.e. ΔSPV) of 17 subjects at a certain time point. There are in total 12 dots per treatment arm in each graph panel. Each dot refers to one post-dose time-point pre-scheduled in the study. The connecting lines represent the time line, which suggest there was no obvious time-shift between the effect on SPV and any of the other CNS-PD effects. The straight lines indicate the regression lines for the ΔPD-ΔSPV relations. These regression lines are not based on the average dots but on the underlying individual values that are not shown in the graphs.

As is can be seen the figure, the slopes of the regression lines are generally flatter for either dose of AZD7325 than for lorazepam. 10 mg demonstrated statistically significant difference from lorazepam 2 mg in most ΔSPV-relative relations, except the ΔSmooth-ΔSPV relation. The effects of AZD7325 2mg were too small for a reliable determination of effect slopes.

The effects of AZD7325 10 mg also failed to reach statistical significance for $VAS_{alertness}$, SPV, body sway, smooth pursuit, or adaptive tracking. In contrast, lorazepam 2 mg induced robust and significantly larger effects on these pharmacodynamic parameters compared to either dose of AZD7325 or placebo.

There was a trend towards a short-lasting small increase in VAS_{high} after AZD7325 10 mg, without significant alteration in either internal ($VAS_{internal}$) or external ($VAS_{external}$) perceptions. The only statistically significant effect of AZD7325 10 mg was an EEG power reduction in the delta (2-4 Hz) and theta (4-7.5 Hz) bands of the frontal-central area. These EEG profiles of AZD7325 differed from the characteristic benzodiazepine EEG signature induced by lorazepam, which was associated with increased power in delta, beta (13.5-35 Hz) and gamma (35-48 Hz) bands, as well as reductions in theta and alpha (7.5-13.5 Hz). The pharmacodynamic (PD) effects of each active treatment and the results of statistical comparisons are summarized and tabulated in Table 2 and Table 3.

Results of the CogState Early Phase Battery are presented in Figure 4. As expected, repeated exposure to the test paradigms resulted in no significant learning effects in the placebo group. Neither dose of AZD7325 showed statistically significant effects on any individual CogState variable. In contrast, lorazepam induced statistically significant impairments on the following cognitive parameters compared to placebo (lorazepam *vs.* placebo, [unit], *p*-value): reaction time of correct responses in the detection task (2.59 *vs.* 2.52 [log(msec)], p<0.0001), reaction time of correct responses in the identification task (2.78 *vs.* 2.70 [log(msec)], p<0.0001), response accuracy in the one card learning task (0.70 *vs.* 0.86 [arc(%)], p<0.0001), moves per second (mps) in the chase test (1.58 vs. 1.84 [mps], p<0.0001), and the sum of errors in GMLT (62.0 vs. 33.1, p=0.0003), as well as reduced the number of words recalled in both the ISLT and the ISL delayed-recall task.

SAFETY

Single oral dose of AZD7325 2 mg, AZD7325 10 mg or lorazepam 2 mg were generally safe and well-tolerated in the eighteen selected healthy male participants. A majority of subjects reported adverse events after administration of lorazepam 2 mg (AE frequency, incidence%: 14, 87.5%), whereas the high dose of AZD7325 was associated with relatively fewer adverse events (12, 70.6%), and even lower incidences of AEs were observed after AZD7325 2 mg (4, 23.5%) and placebo (9, 56.3%). As was observed with lorazepam 2 mg (14, 87.5%), most AEs that occurred in subjects receiving AZD7325 10 mg (11, 64.7%) were classified as 'nervous system disorders', but fewer subjective somnolence (AZD723510 mg: 7, 41.2%; AZD7325 2 mg: 2, 11.8%) and dizziness (AZD723510 mg: 3, 17.6%; AZD7325 2 mg: 1, 5.9%) were reported with either dose of AZD7325 than with lorazepam (somnolence: 9, 56.3%; dizziness: 5, 31.3%). On the other hand, the incidence of somnolence and dizziness was higher with AZD732510 mg than with placebo (3, 18.8%). The frequency of gastrointestinal events was also less with AZD732510 mg (2, 11.8%) than with lorazepam (6, 37.5%). No changes or individual abnormalities of vital signs or laboratory or ECG results were judged clinically important by the investigator.

PHARMACOKINETIC (PK) RESULTS

Both AZD7325 2 mg and AZD7325 10 mg were quickly absorbed after oral administration, with a short lag time of maximally 0.5 hours. Mean (SD) peak plasma concentration (C_{max}) arrived at 14.2 (5.36) ng/ml between 1 hours and 3.25 hours after AZD7325 2 mg, and at 67.4 (33.5) ng/ml between 0.5 hours and 3.25 hours after AZD7325 10 mg, with a median time to C_{max} (T_{max}) of 1.75 hours and 2 hours,

respectively. The area under the concentration-time curve from zero to the last detectable concentration (AUC_{0-t}) was 51.9 (18.9) h·ng/mL for AZD7325 2 mg and 259 (77.6) h·ng/ml for AZD7325 10 mg. As is shown in Figure 6, drug elimination seemed to exhibit roughly three phases after T_{max}. The mean elimination half-life was 8.5 to 9.0 hours for both doses of AZD7325 (ranging from 5.09 to 15.4 hr). The apparent oral clearance (CL/F) of AZD7325 was 38.3 L/hr on average (ranging from 9.87 to 89.9 L/hr). No statistically significant differences were found between AZD7325 2 mg and AZD7325 10 mg with respect to T_{max}, $T_{1/2}$, or CL/F ($p>0.05$). In comparison, lorazepam reached a mean (SD) C_{max} of 20.7 (4.86) ng/ml in a longer median T_{max} of 2.50 hr (range 0.50-6.00 hr) and was eliminated with a mean $T_{1/2}$ of 14.6 hr (range 8.31-25.1 hr). The AUC_{0-t} for lorazepam was 233 (35.8) h·ng/mL. The average levels of AUC and C_{max} increased in a dose proportional manner with similar dose-normalized values of C_{max} and AUC_{0-t} between AZD7325 2 mg and AZD7325 10 mg.

DISCUSSION

In vitro, AZD7325 exhibits relatively potent positive modulation at the $\alpha_{2,3}$ subunits together with neutral α_1-antagonism and weak α_5-affinity. Based on these properties, the compound was expected to have a rapid onset of anxiolysis with less untoward effects at its therapeutic dose(s) in healthy volunteers. Prior to initiating phase II trials, the present study aimed to provide support for the pharmacological selectivity of AZD7325 in healthy volunteers by comparing its pharmacodynamic profile to the non-selective GABA-A receptor modulator, lorazepam.

Compared with lorazepam, both doses of AZD7325 demonstrated smaller absolute slopes of the regression lines in the ΔSPV-ΔLog(Sway) relation and the $\Delta SPV\text{-}\Delta VAS_{alertness}$ relation. Thus, AZD7325 is associated with a ΔSPV-dominant response profile, whereas the pharmacodynamic responses to lorazepam are more comparable and balanced among the same set of CNS parameters. This has also been observed with other subtype-selective GABA-A $\alpha_{2,3}$ receptor modulators [14,16]. Since SPV was found sensitive [17] and functionally specific to the effect of anxiolytic drugs acting on the GABAergic system [37], the distinction between AZD7325 and lorazepam suggests that the $\alpha_{2,3}$-selective agonist may cause less sedation than the benzodiazepine, at doses with a similar anxiolytic effect.

An alternative explanation for the non-significant SPV effects of AZD7235 could be that the doses of this compound may have been too low to be pharmacologically equipotent to lorazepam 2 mg. In the human PET study with [^{11}C]flumazenil, a 50% receptor occupancy was linked to a free plasma concentration of approximately 4 ng/ml [AstraZeneca data on file] which corresponded to estimated maximal concentrations achieved after AZD7325 1.3 mg orally. Doses greater than 5 mg were linked with high levels of occupancy (> 70%). In the present study, the 10 mg dose

resulted in average peak plasma concentration of 67.4 ng/ml and average plasma concentration of 12.3 ng/mL over 21 hours, which are expected to produce GABA-A occupancy levels accounting for 80-90% and 60-70%, respectively, of the maximal occupancy level. It remains unknown whether higher doses of AZD7325 would have more profound effects on SPV. As another member of the family of GABA-A $\alpha_{2,3}$ subtype-selective partial agonist, TPA023 also produced average receptor occupancies over 70% at a dose of either 3 mg in immediate-release (IM) formulation or 8-12 mg in a controlled release (CEM) formulation [1]. However, a relatively small single dose of TPA023 (IM 1.5 mg) is required to produce comparable SPV reduction as lorazepam 2 mg in healthy volunteers [14]. The development of TPA023 was discontinued due to toxicity findings in rodents following long-term administration. Nevertheless, limited clinical efficacy data that were accumulated before termination suggest an anxiolytic-like effect of TPA023 with flexible-doses (1.5-4.5mg b.i.d. or 3-8mg b.i.d.) of the extended-release (CEM) formulation of TPA023 [1]. *In vitro*, TPA023 exerts 11% and 21% modulation at the α_2 and α_3 subunits relative to chlordiazepoxide [38], whereas the α_2- and α_3-agonism of AZD7325 are equivalent to 18% and 15% of the full efficacy of diazepam, respectively. The combination of these information suggests that relative to the doses of AZD7235 used in this study, either stronger partial agonism at the α_2 or α_3 subunits or higher exposure (with larger or repeated dosing) are necessary for clinical anxiolysis.

Subsequent to the current study, two double-blind placebo-controlled proof-of-concept studies were performed in patients with GAD. AZD7325 doses 2 mg BID, 5 mg BID, or 10 mg QD were investigated in study NCT00808249 (register identifier in ClinicalTrial.gov) [39] and doses 5 mg BID or 15 mg BID and lorazepam 2 mg BID were investigated in study NCT00807937 [40]. Both studies were of 28 days duration. These studies were designed when the results of this study were available. Given that the AZD7325 10 mg dose was well tolerated in the present study and the SPV-effects of AZD7325 10 mg were not equipotent with the lorazepam effects, it was decided that a higher dose of AZ7325 could be tested in the GAD study (i.e. 15 mg BID). Since the incidence of CNS side-effects appeared to be dose-dependent in the current study and the use of SPV as a benchmark for anxiolytic efficacy is still experimental, the dose of AZD7325 15 mg BID was selected as the highest dose to be tested with predicted positive benefit to risk ratio. Although AZD7325 demonstrated some anxiolytic activity in selected secondary end-points in these two studies, none of the AZD7325 doses met the statistical significance for the primary end-point of improvement in Hamilton Rating scale for Anxiety (HAM-A) at 4 weeks. Lorazepam 2mg BID was shown to be marginally anxiolytic at 4 weeks based on the improvement in HAM-A (mean change from baseline ± standard error: -10.8 ± 0.88 vs. -9.5 ± 0.88 [with placebo] vs. -10.4 ± 0.89 [with AZD7325 15mg BID]) [40]. In line with our observations described here, lower incidence of 'fatigue', 'somnolence' and 'sedation' occurred in the AZD7325-treated groups compared with lorazepam 2 mg BID.

AZD7325 up to doses of 15 mg BID was generally well tolerated in GAD patients. The most common adverse event associated with AZD7325 was dizziness. In addition, more adverse events of euphoric mood were seen with AZD7325 in comparison to placebo.

The high dose of AZD7325 elicited a transient increase of VAS_{high}, with the maximal back-transformed amplitude (2.01 mm) similar to that after lorazepam (1.71 mm). In contrast to AZD7325, however, lorazepam also caused concomitant enhancement of VAS internal ($VAS_{internal}$) and external ($VAS_{external}$) perception. This is in line with the findings in the phase-II studies, in which adverse event 'euphoric mood' was more frequently reported by GAD patients dosed with AZD7325 than those taking placebo [39,40] or lorazepam [40].

The main pharmacodynamic effects of AZD7325 were on EEG parameters, which were distinct from the EEG effects of lorazepam. The decrease in delta-activity contrasts with the increase in delta-power seen with lorazepam and is consistent with the lack of effect of AZD7325 on measures of sedation and alertness. A reduction of theta activity was seen with both AZD7325 and lorazepam and may relate to a common effect independent of sedation. Whatever their physiological or clinical meaning, these findings demonstrate a central pharmacodynamic effect of AZD7325. The EEG effects exhibited dose-response relationships and a close temporal link to the plasma concentrations. T_{max} was short and associated with a rapid peak effect, which may reflect a potential of quick-onset clinical effect after AZD7325.

In conclusion, the pharmacodynamic profile of AZD7325 differed from that of a typical benzodiazepine. At doses up to 10 mg, AZD7235's SPV-effects were non-significant by themselves, but showed preference over other CNS-effects. Since the doses of AZD7325 were not equivalent to lorazepam 2 mg, the lack of effects on subjective alertness, visuo-motor coordination, postural balance, and psychomotor and cognitive functions cannot be directly extrapolated as reduced clinical side-effects. Therefore, further clinical evaluations with higher doses are warranted, but the dose-dependent side-effects on the central nervous system should be considered to balance dose selection. The effects of AZD7325 10 mg on EEG spectrum and VAS_{high} suggest entry of the compound into the central nervous system and a rapid onset of pharmacodynamic effect.

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Table 1 · Results of the linear model for the Δlog(Sway)-ΔSPV, $\Delta VAS_{alertness}$-ΔSPV, ΔTracking-ΔSPV, and Δsmooth-ΔSPV relations

Slope	AZD7325 2 mg	AZD7325 10 mg	Lora 2mg	AZD-2mg vs. Lorazepam Estimate of difference [95% CI]	*p*-Value	AZD-10mg vs. Lorazepam Estimate of difference [95% CI]	*p*-Value
ΔLog(Sway)-ΔSPV	-0.00124	-0.00036	-0.00206	0.000826 [-0.00024, 0.001892]	0.1286	0.001704 [0.000639, 0.002768]	0.0018
$\Delta VAS_{alertness}$-ΔSPV	0.003046	0.01855	0.08216	-0.07911 [-0.1209, -0.03735]	0.0002	-0.06360 [-0.1046, -0.02257]	0.0024
ΔTracking-ΔSPV	0.04789	0.01547	0.04791	-0.00001 [-0.02190, 0.02187]	0.999	-0.03244 [-0.05390, -0.01098]	0.0031
ΔSmooth-ΔSPV	0.000958	0.03297	0.06075	-0.5979 [-0.1040, -0.01557]	0.0081	-0.02778 [-0.07115, 0.01559]	0.2087

CI=confidence interval

Table 2 · Summary of pharmacodynamic (PD) effect of single doses of lorazepam 2 mg, AZD7325 10 mg, and AZD7325 2 mg, compared to placebo as estimated difference (95% confidence interval)

PD Parameter	Lorazepam-2mg vs. Placebo	AZD7325-2mg	AZD7325-10mg
$VAS_{alertness}$ (mm)	-7.9(-11.0, -4.7)	-1.6(-4.8, 1.6)	-1.6(-4.7, 1.6)
	p<0.0001	p=0.3111	p=0.3225
$VAS_{calmness}$ (mm)	3.5 (0.6, 6.4)	-1.7 (-4.6, 1.2)	1.1 (-1.8, 4.0)
	p=0.0195	p=0.2417	p=0.4452
VAS_{mood} (mm)	0.2 (-2.9, 3.2)	-2.0 (-5.0, 1.1)	-1.4 (-4.5, 1.7)
	p=0.9146	p=0.2012	p=0.3593
Sway (%)	89.13(60.99,122.2)	1.03(-13.9,18.50)	-10.1(-23.4,5.52)
	p<0.0001	p=0.8968	p=0.1869
SPV (deg/sec)	-40.4 (-58.6, -22.1)	-14.1 (-32.2, 4.1)	-15.2 (-33.3, 2.9)
	p<0.0001	p=0.1240	p=0.0974
SacInacc (%)	1.1 (0.5, 1.6)	0.3 (-0.3, 0.8)	-0.1 (-0.6, 0.4)
	p=0.0002	p=0.3385	p=0.6511
Sac RT (sec)	0.014 (0.001,0.026)	-0.009 (-0.021,0.003)	-0.009 (-0.021,0.003)
	p=0.0305	p=0.1337	p=0.1389
Smooth (%)	-10.5 (-14.3, -6.7)	-3.2 (-7.0, 0.6)	-1.9 (-5.7, 1.9)
	p<0.0001	p=0.0997	p=0.3094
Tracking (%)	-7.26 (-8.98, -5.54)	-0.59 (-2.32, 1.13)	-0.15 (-1.91, 1.61)
	p<0.0001	p=0.4890	p=0.8648
$VAS_{external}$ log(mm)	0.13 (0.06, 0.19)	0.01 (-0.05, 0.08)	0.04 (-0.03, 0.10)
	p=0.0003	p=0.6941	p=0.2242
$VAS_{internal}$ log(mm)	0.06 (0.03, 0.10)	0.00 (-0.04, 0.04)	0.02 (-0.02, 0.06)
	p=0.0009	p=0.9964	p=0.2613
VAS_{high} log(mm)	0.25 (0.09, 0.41)	0.00 (-0.16, 0.16)	0.16 (-0.00, 0.32)
	p=0.0028	p=0.9889	p=0.0570

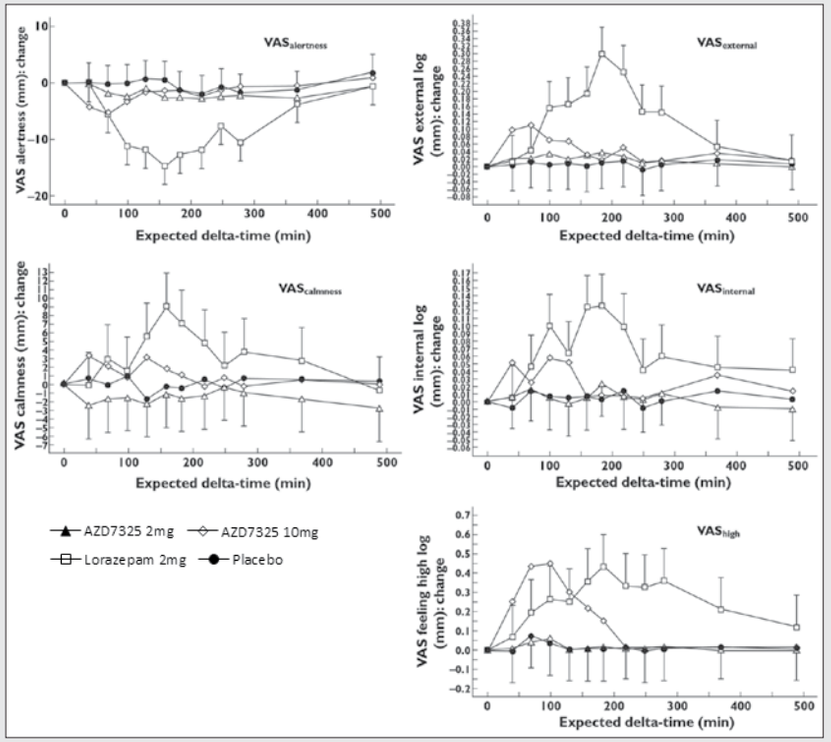
VAS=visual analogue scale; Smooth=Smooth pursuit; Tracking=Adaptive Tracking; SPV=saccadic peak velocity; SacRT=saccadic reaction time; SacInacc=saccadic inaccuracy

Table 3 · Summary of electroencephalogram (EEG) effect of single doses of lorazepam 2 mg, AZD7325 10 mg, and AZD7325 2 mg, compared to placebo as estimated difference (95% confidence interval)

EEG Parameters(%)	Lorazepan-2mg	AZD-2mg	AZD-10mg
	vs. Placebo		
Alpha Fz-Cz	-19.5(-27.2,-11.0)	2.89(-6.96,13.80)	0.19(-9.40,10.80)
	p<0.0001	p=0.5700	p=0.9696
Alpha Pz-Oz	-41.4(-51.4,-29.4)	0.31(-16.8,20.97)	10.33(-8.58,33.15)
	p<0.0001	p=0.9732	p=0.2937
Beta Fz-Cz	12.15(3.94,21.00)	-0.84(-8.11,7.00)	2.14(-5.32,10.19)
	p=0.0040	p=0.8240	p=0.5760
Beta Pz-Oz	-12.9(-22.3,-2.34)	-4.26(-14.7,7.43)	3.09(-8.21,15.79)
	p=0.0194	p=0.4487	p=0.5987
Delta Fz-Cz	10.21(2.42,18.58)	-3.66(-10.5,3.67)	-18.7(-24.5,-12.4)
	p=0.0108	p=0.3087	p<0.0001
Delta Pz-Oz	7.64(-2.54,18.88)	1.16(-8.39,11.70)	-15.9(-23.8,-7.11)
	p=0.1421	p=0.8157	p=0.0011
Gamma Fz-Cz	8.57(2.39,15.13)	-0.68(-6.52,5.52)	1.73(-4.09,7.90)
	p=0.0073	p=0.8207	p=0.5593
Gamma Pz-Oz	1.02(-12.6,16.75)	-8.20(-20.8,6.34)	-1.80(-15.2,13.65)
	p=0.8885	p=0.2469	p=0.8033
Theta Fz-Cz	-7.75(-13.8,-1.33)	-2.45(-8.81,4.36)	-13.5(-19.1,-7.43)
	p=0.0200	p=0.4624	p<0.0001
Theta Pz-Oz	-15.0(-24.2,-4.80)	1.55(-9.43,13.86)	-10.3(-19.9,0.53)
	p=0.0062	p=0.7874	p=0.0610

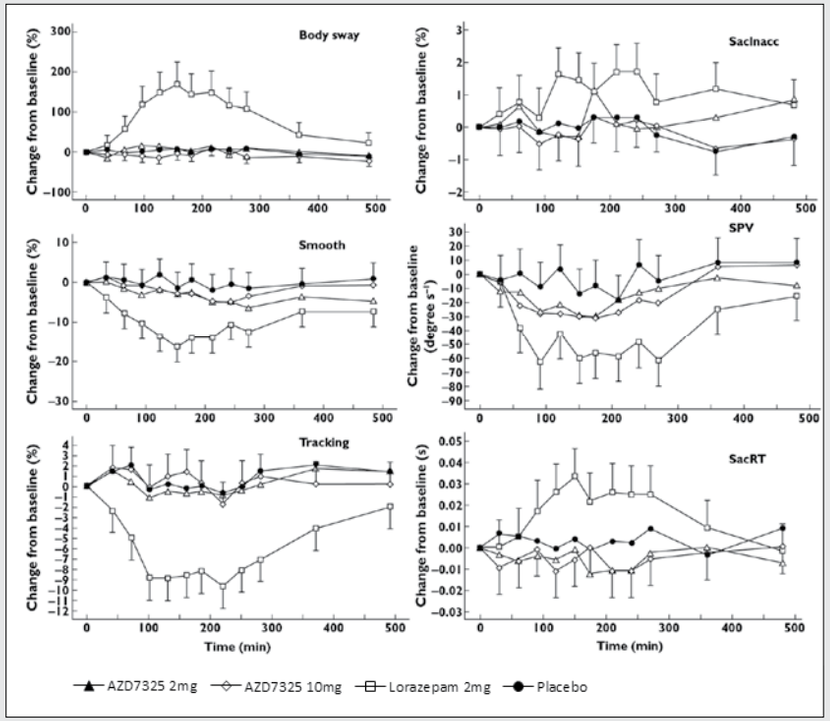
Fz-Cz=Frontal-central area; Pz-Oz=Parietal-occipital area.

Figure 1 · Least square means of change-from-baseline profiles of subjective pharmacodynamic paramters (i.e. visual analogue sub-scales) after the treatments of placebo, lorazepam 2 mg, AZD7325 2 mg, and AZD7325 10 mg



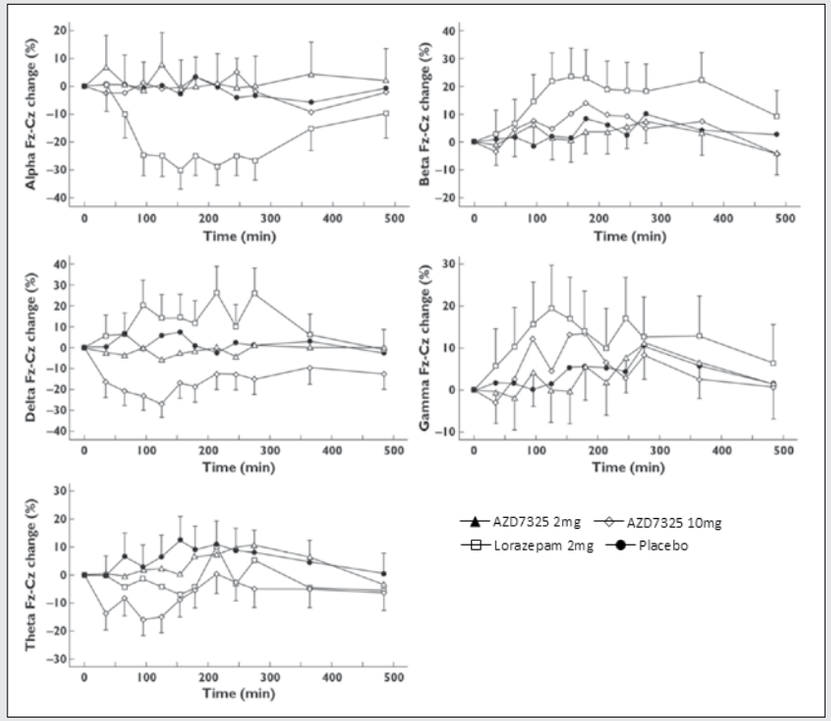
With 95% CI error bars; VAS=visual analogue scale

Figure 2 · Least square means of change-from-baseline profiles of objective pharmacodynamic parameters after the treatments of placebo, lorazepam 2 mg, AZD7325 2 mg, and AZD7325 10 mg



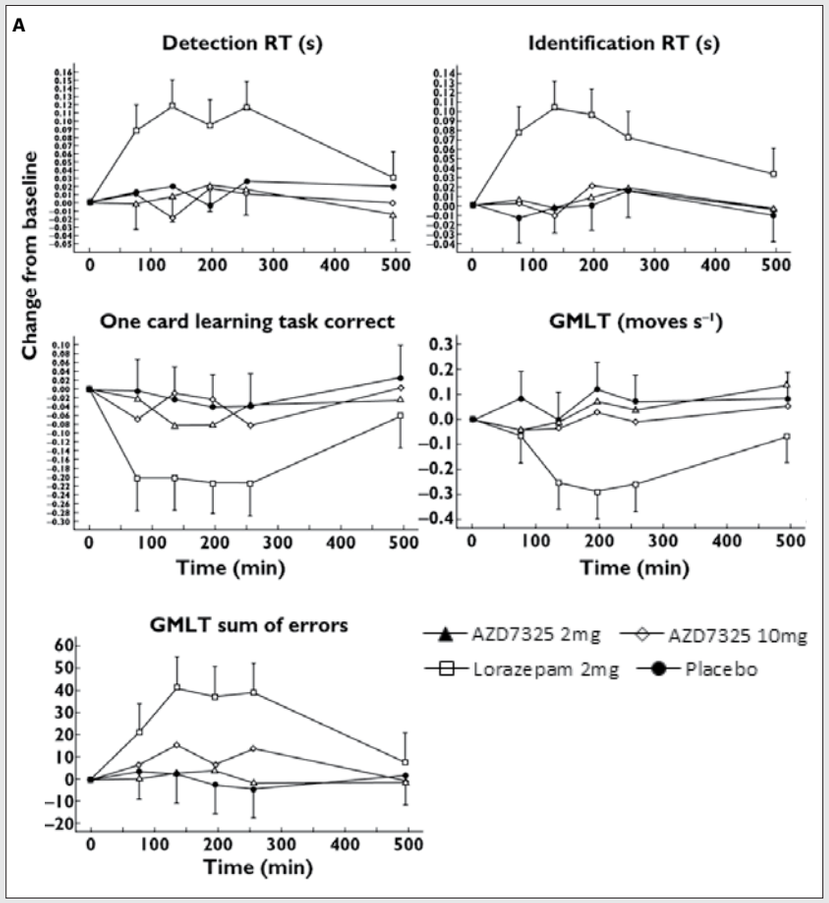
With 95% CI error bars ; Smooth=Smooth pursuit; Tracking=Adaptive Tracking; Sac Inacc=Saccadic Inaccuracy;SacRT=Saccadic Reaction Time; SPV=Saccadic Peak Velocity.

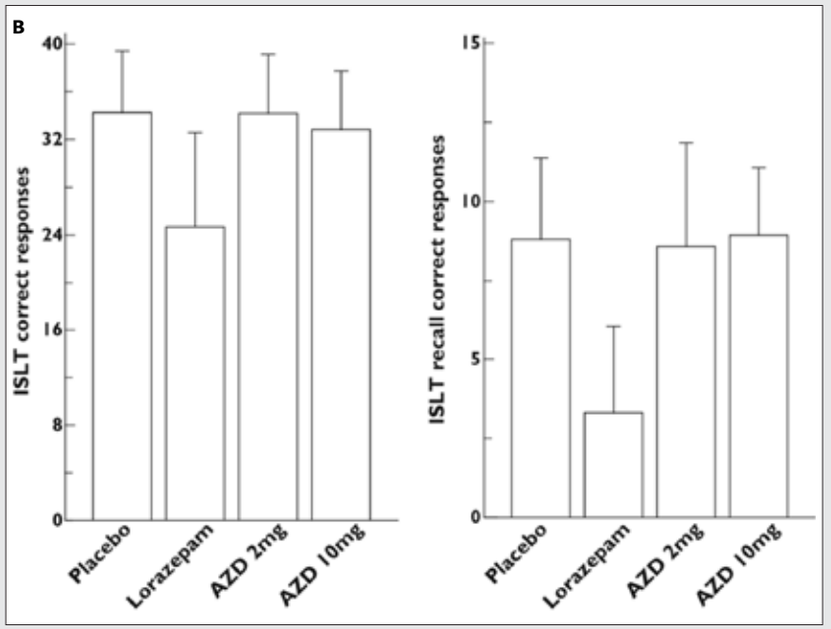
Figure 3 · Least square means of change-from-baseline profiles of electroencephalogram parameters after the treatments of placebo, lorazepam 2 mg, AZD7325 2 mg, and AZD7325 10 mg



With 95% CI error bars

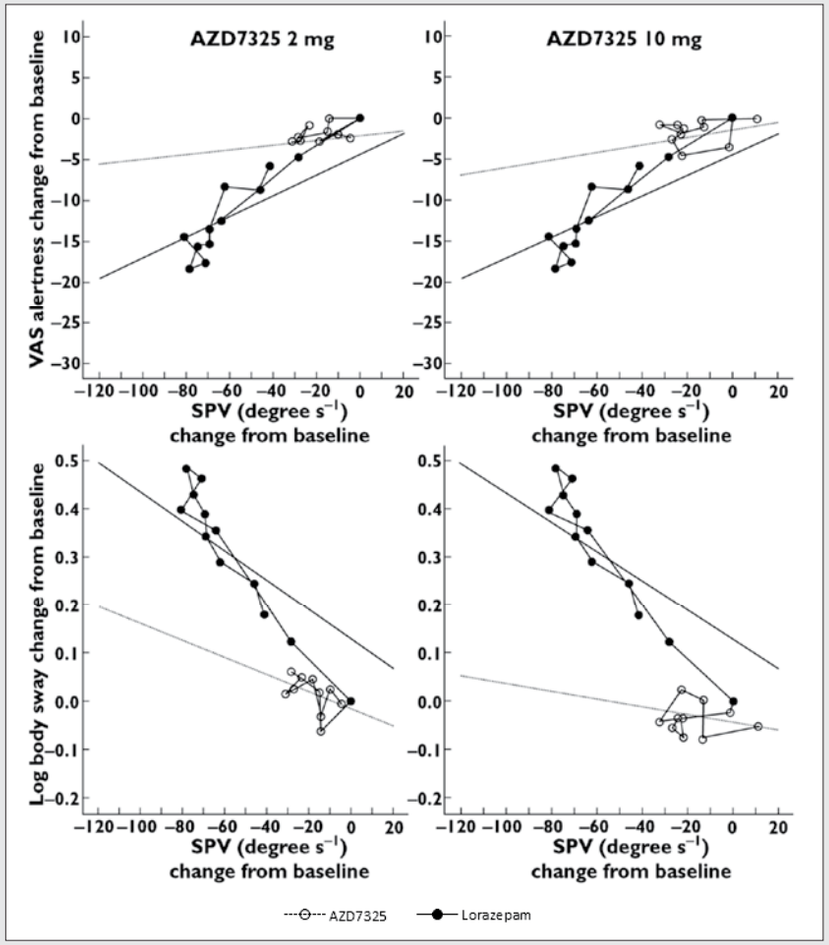
Figure 4 · Least square means of change-from-baseline profiles of CogState parameters after the treatments of placebo, lorazepam 2 mg, AZD7325 2 mg, and AZD7325 10 mg (Panel A); mean number of correct responses in the International Shopping List Test (ISLT) and the delayed recall ISLT (Panel B).

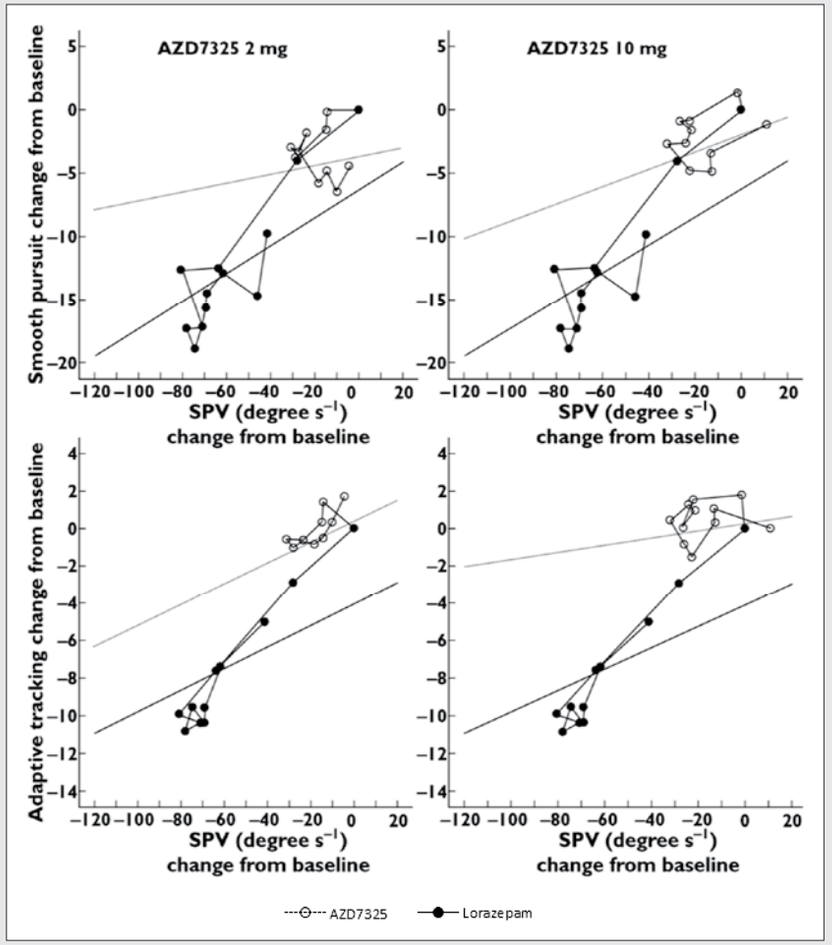




With 95% CI error bars ; RT=Reaction Time; GMLT= Groton Maze Learning Task; ISLT=International Shopping List Task.

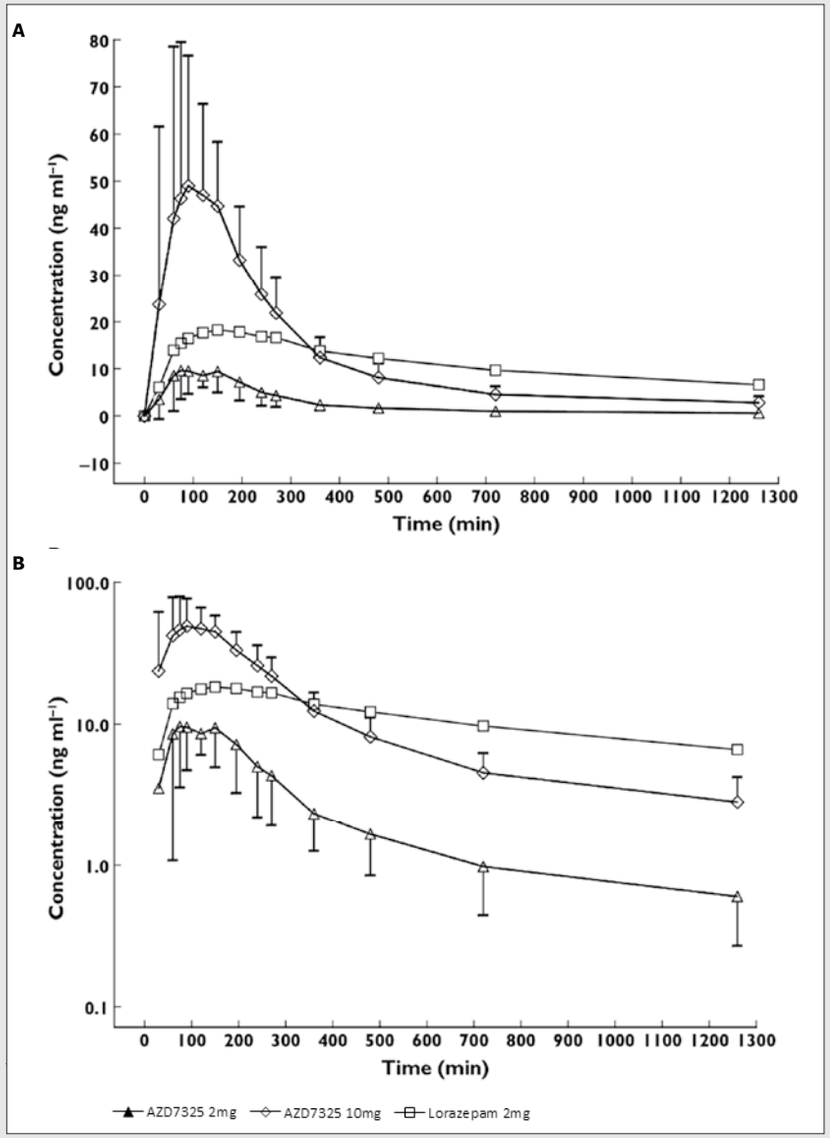
Figure 5 · The ΔVAS$_{alertness}$ (mm)-Δspv (deg/sec), Δsway(logmm)-Δspv (deg/sec), Δsmooth(%)-Δspv (deg/sec), and Δtracking(%)-Δspv(deg/sec) relation profiles of AZD7325 2 mg and AZD7325 10 mg vs. lorazepam 2 mg, respectively.





The AZD7325 2 mg at time=30 min does not have an error bar down, because the value of the average - error bar reaches a below zero value (AVG=3.53, SD=4.169) and cannot be shown on a log based axis.

Figure 6 · Mean concentration-time profiles of AZD7325 2 mg, AZD7325 10 mg and lorazepam with standard deviation as error bars linear (Panel A) and semi-logarithmic coordinates (Panel B)



III

AZD6280, A NOVEL PARTIAL γ-AMINOBUTYRIC ACID A RECEPTOR MODULATOR, DEMONSTRATES A PHARMACODYNAMICALLY SELECTIVE EFFECT PROFILE IN HEALTHY MALE VOLUNTEERS

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Chen X[1,2], Jacobs G[2,3], de Kam ML[2], Jaeger J[1,2], Lappalainen J[1,2], Maruff P[1,2], Smith MA[1,2], Cross AJ[4], Cohen A[2], van Gerven J[2]

1. Phase I Unit, Clinical Pharmacology Research Center (CPRC), Peking Union Medical College Hospital, Beijing, China | 2. Centre for Human Drug Research, Leiden | 3. VU University Medical Centre, Amsterdam, the Netherlands | 4. AstraZeneca, R&D, Wilmington, DE | 5. Cogstate, Melbourne, Australia | 6. Teva Pharmaceuticals, Frazer, PA

ABSTRACT

Objective: AZD6280 is a novel GABA-A receptor modulator with higher *in vitro* efficacy at the $\alpha_{2,3}$-subtypes as compared to the α_1- and α_5-subtypes. This study compared the pharmacodynamic effects of single-oral-dose AZD6280 10 mg and 40 mg on the central nervous system (CNS) with lorazepam 2 mg. **Methods:** Sixteen healthy males were enrolled into the double-blind, randomized, 4-way crossover study. Two validated CNS test-batteries, Neurocart and CogState, were administered to measure drug effects on cognition, neurophysiologic function, psychomotor and subjective feelings. Statistical analysis was performed using mixed model analysis of variance, with fixed factors of treatment, period, time and treatment by time, and random factors of subject, subject by treatment and subject by time and the average pre-value as covariate. **Results:** Most pharmacodynamic parameters were affected by lorazepam. AZD6280 induced dose-dependent smaller-than-lorazepam effects on saccadic peak velocity (SPV) (AZD6280 10 mg vs. AZD6280 40 mg vs. lorazepam [degree/second, deg/sec]: -22.6 vs. -50.0 vs. -62.9, $p<0.001$), while the impacts on adaptive-tracking, body-sway, smooth-pursuit and the one-card-learning tests were significant but much smaller than lorazepam. Thus the slopes of regression lines for the ΔLog(Sway)-ΔSPV, ΔTracking-ΔSPV, and ΔSmooth-ΔSPV relations were flatter with AZD6280 than with lorazepam. AZD6280 caused a distinct electro-encephalography signature from that of lorazepam. **Conclusion:** The SPV responses to AZD6280 suggest potential concentration-related anxiolytic effects, while the smaller SPV-normalized effects of AZD6280 on various non-SPV pharmacodynamic parameters suggest a more favorable side-effect profile compared to lorazepam. Overall, the pharmacodynamic profile of AZD6280 matches the pharmacological specificity and selectivity of this compound at the $\alpha_{2,3}$ GABA-A receptor subtypes.

INTRODUCTION

The different anxiety disorders together constitute one of the most prevalent groups of psychiatric disorders [1]. Evidence supports the use of the selective serotonin reuptake inhibitors (SSRIs) and the tricyclic antidepressant drugs (TCAs) in the pharmacological treatment of virtually all anxiety disorders. In addition, the non-selective GABA-A receptor potentiating benzodiazepines (BZDs) are particularly effective in the management of acute forms of anxiety due to their robust anxiolytic effects and rapid onset of action. However, patients frequently discontinue SSRIs and TCAs prematurely due to a delayed onset of action and unacceptable side effects, and the widespread application of BZDs is restricted by untoward effects such as day-time sedation, fatigue, deleterious effects on cognition, memory impairment, tolerance and concerns regarding dependence liability [2,3]. These limitations of existing anxiolytic drugs underlie the pressing need for the development of efficacious innovative anxiolytic agents with more favorable side-effect profiles.

It is well-established that BZDs act through modulation of GABA-A receptors. A range of GABA-A receptor subtypes, defined by their subunit composition, mediate these effects. The use of knock-out and knock-in techniques in rodents has helped to characterize the physiological role of various GABA-A receptor subtypes as candidates for mediating the clinical effects of BZDs [4]: GABA-A receptor subtypes that contain GABA-A α_2 and α_3 subunits may mediate anxiolytic effects [5,6], while GABA-A α_1 and α_5 subunits account for sedation and cognitive impairment [4,7,8,9,10], respectively.

AZD6280 (4-Amino-8-(2,5-dimethoxyphenyl)-*N*-propylcinnoline-3-carboxamide) [11,12] is a novel, subtype-selective GABA-A receptor modulator, which in contrast to BZDs, exerts minimal efficacy at α_1-subunit containing GABA-A receptors. Although AZD6280 has relatively high (±standard deviation, SD) binding affinity to the α_1 (Ki=0.5±0.2 nM), α_2 (Ki=21±5 nM), and α_3 (Ki=31±17 nM) GABA-A subunits, its affinity for the α_5 subunit is much lower (Ki=1680±650 nM). On the other hand, the *in vitro* efficacy of AZD6280 at the GABA-A α_2 (32%) or α_3 (34%) receptor subtypes is 4-5-fold higher than that at the GABA-A α_1 (8%) or α_5 (7%) receptor subtypes relative to the corresponding maximal responses to diazepam. This profile is distinct from previously characterized $\alpha_{2,3}$ preferring compounds TPA-023 and AZD7325 in that AZD6280 has greater intrinsic activity at $\alpha_{2,3}$ subunits. Clinical relevance of these pharmacological characteristics has been tested in several pre-clinical animal models, where the compound demonstrated potential anxiolysis with reduced motor and cognitive side effects.

The objectives of this study were to investigate the pharmacodynamic (PD) effects of single oral doses of AZD6280 on the central nervous system (CNS), and compare those effects to lorazepam, a commonly used BZD.

AZD6280 10 mg and AZD6280 40 mg were selected as the investigational doses. In the AZD6280-ascending-dose study single doses up to 60 mg were tested. This dose was associated with increased rate of sedation and one event of transient depersonalization. The 10 mg and 40 mg doses were predicted to lead to peak plasma concentrations above minimally efficacious concentrations in animal models of anxiety, and provide GABA-A receptor occupancy levels 50% or higher of the maximal displaceable binding as determined by [^{11}C]flumazenil. These data demonstrate that AZD6280 crosses the blood brain barrier, interacts with the target, and has the potential to produce anxiolytic activity in humans.

For the current study, the Neurocart CNS test battery [13] and the CogState cognitive test battery were used. Components of these two batteries provide biomarkers for CNS function(s) that have been shown to be sensitive to the effects of BZDs and/or $\alpha_{2,3}$-selective GABA-A agonists [13,14,15,16]. Recent studies have suggested that partial selective $\alpha_{2,3}$ GABA-A agonists exhibit distinct effect profile in the central nervous system, which is characterized by a preserved effect on the saccadic peak velocity (SPV) but relatively reduced impairment of subjective alertness, postural balance and memory, compared to BZDs [13,14,15,16].

METHODS

DESIGN

This was a single-center, four-way crossover, randomized, double blind, double-dummy, placebo-controlled study in 16 healthy male volunteers.

SUBJECTS

Healthy male volunteers, aged 18 to 55 years, with a body mass index (BMI) between 18 and 30 kg/m^2, were medically screened after provision of written inform consent. Eligible subjects were advised not to use alcoholic beverages from 24 hours preceding each study day and refrain from smoking and using caffeine-containing products from 22:00 prior to each study day. Keeping a normal diurnal pattern was also required from two weeks before the first study day until the last study day.

TREATMENTS

All subjects arrived in the research unit at around 08:00hr on the dosing day of each study period. Study medication was administered orally between 09:00 and 11:00hr in the morning, when all pre-dose assessments were completed. Either capsules containing AZD6280 or placebo, or tablets containing lorazepam (identical to the clinically available formulation of lorazepam) or placebo were orally administered.

On each study day, subjects received one of the four treatments according to a randomly allocated treatment schedule: AZD6280 10 mg, AZD6280 40 mg, lorazepam 2 mg, and placebo. The study days were separated by washout periods of 7 days minimum. The order of the treatments was defined by a Williams Latin Square design that led to sixteen completely different sequences of four treatments.

SAFETY

Safety and tolerability were assessed by the incidence and severity of adverse events, abnormalities in vital sign assessments, clinical laboratory parameters, and electrocardiograms (ECG). Twelve-Lead ECG recordings were made, using Cardiofax V equipped with ECAPS12 analysis program (Nihon Kohden, Tokyo, Japan) or Marquette 5000/5500. Supine blood pressure and pulse were measured using a semi-automatic blood pressure recording device (a Nihon-Kohden BSM-1101K monitor or a Colin Pressmate BP 8800 or a Dash 4000). Subjects were required to rest in a supine position for at least 5 minutes prior to these measurements. Safety laboratory tests on blood or urine samples were assayed in the Central Clinical Laboratories of Leiden University Medical Centre.

PHARMACOKINETIC MEASUREMENTS

Venous blood samples (6 mL) for determination of AZD6280 or lorazepam in plasma were collected at pre-dose and 0.5, 1, 1.25, 1.5, 2, 2.5, 3.25, 4, 4.5, 6, 8, 12, and 21 hours post-dose. The plasma concentrations of AZD6280 and lorazepam were measured using two validated methods at Bioanalytical Systems, Inc., West Lafayette, IN, USA. Plasma concentrations of AZD6280 was determined with a solid-phase extraction / liquid chromatography coupled with tandem mass spectrometry [(SPE)/LC-MS/MS] method and lorazepam was measured using a liquid/liquid extraction/LC-MS/MS method. In brief, the plasma samples of AZD6280 were pre-purified by solid-phase extraction (SPE) and analyzed using gradient chromatographic separation on an Atlantis T3 column with a gradient mobile phase, while the plasma samples of lorazepam were pre-purified by liquid/liquid extraction and analyzed using gradient chromatographic separation on an XBridge C18 column with a formic acid/acetonitrile/water mobile phase. The bioassay method for AZD6280 was validated over the concentration range of 0.150 to 120 ng/mL. The lower limit of quantification (LLOQ) was 0.150 ng/mL, utilizing a 50.0 µL sample aliquot with a validated dilution of 50-fold with human plasma. The bioassay method for lorazepam was validated over the concentration range of 0.300 to 100 ng/mL. The lower limit of quantification (LLOQ) was 0.300 ng/mL, utilizing a 150 µL sample aliquot with a validated dilution of 20-fold with human plasma. Inter- and intra- batch precision of both methods were less than 15% and the accuracy was within 85-115%. A matrix-effect

test indicated that the determination was not affected by the matrix. In addition, AZD6280 and lorazepam in plasma were proven to be stable under the storage condition of -80 °C for at least 205 days and 144 days, respectively.

PHARMACODYNAMIC MEASUREMENTS

A collection of computerized neurophysiologic and neuropsychological tests was performed during the study. Most of these assessments were given during the CNS training session to familiarize subjects with the tests and reduce learning effects. The Neurocart battery was performed in the following chronological order: body sway, visual analogue scale (VAS) Bond & Lader, VAS Bowdle, saccadic eye movements, smooth pursuit eye movements, adaptive tracking and electro-encephalogram (EEG). In each treatment period, this battery was assessed at pre-dose (twice) and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 6, 8, and 12 hours post-dose. The CogState early phase battery was carried out before dosing (three times) and at 1.25, 2.25, 3.25, and 4.25 hours post-dose. This cognitive test battery contains the Groton Maze Learning Task (GMLT), Detection task (DET), Identification Task, and One Card Learning Task [17,18]. Each abovementioned Neurocart or Cogstate test lasted 1 to 5 minutes, thus the total duration of these pharmacodynamic assessments was 15-30 minutes at different time points. At 1.75 hours post-dose subjects also completed the International Shopping List Task, a word verbal list learning test presented three times, each time followed by an immediate recall trial. Delayed recall was tested 21 hours later. At each of these assessments, one subject at a time was tested in a quiet room with ambient illumination.

NEUROCART

BODY SWAY

Body sway was measured with an apparatus similar to the Wright ataxiameter [19], which integrates the amplitude of unidirectional body sway. Two-minute measurements were made in the anterio-posterior direction with eyes closed. The subject was asked to stand comfortably on a floor with his/her feet slightly apart. Body sway measures postural (in)stability. It has demonstrated considerable sensitivity to the effect of benzodiazepines [20].

VISUAL ANALOGUE SCALES OF BOND & LADER (VAS B&L) AND BOWDLE

Visual analogue scales as originally described by Norris have often been used previously to quantify subjective effects of a variety of sedative agents [21,22]. Dutch versions of the scales have been frequently employed at the Centre for Human Drug Research (CHDR), for a variety of sedative agents [23] and circumstances [24]. During the test, the subjects indicated (with a mouse click on the computer screen) on horizontal visual analogue scales how he/she feels. From the sixteen measurements of VAS Bond & Lader, three main factors are the calculated [25] for subjective alertness, contentedness, and calmness.

The Bowdle Psychotomimetic Effects Scores have been used to quantify the psychotomimimetic effects of ketamine [26]. A translated Dutch version of the scale originally developed by Bowdle et al. has been computerized and used at the CHDR to study glutamatergic drug effects. This scale has thirteen 10 cm visual analogue lines ranging from 0 ('not at all') to 100 mm ('extremely') [27], addressing various abnormal states of mind.

SACCADIC EYE MOVEMENTS

Saccadic eye movements were evaluated using a computer-based system composed of 1) stimulus display and signal collection (Nihon Kohden Corporation, Tokyo, Japan), 2) signal amplification (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA), 3) data recording (Cambridge Electronics Design, Cambridge, UK), 4) disposable silver-silver chloride electrodes (Medicotest N-00-S, Olstykke, Denmark), as well as 5) the sampling and analysis scripts developed by CHDR (Leiden, the Netherlands). The parameters of this test were the average values of saccadic peak velocity (SPV, deg/sec), latency (i.e. reaction time, msec) and inaccuracy (%) of all artefact-free saccades that were calculated on each session. Saccadic peak velocity appears to be the most sensitive measure for the effect of benzodiazepines [22] and has been found to be closely related to the anxiolytic component of benzodiazepines and some newly developed compounds with potential anxiolytic effect [14,15,16].

SMOOTH PURSUIT EYE MOVEMENTS

The same system as used for saccadic eye movements was also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moved sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, by step of 0.1 Hz. The amplitude of target displacement corresponded to 22.5 degrees eyeball rotation to both sides. Four cycles were recorded for each stimulus frequency. The method has been validated in healthy volunteers dosed with benzodiazepines at the CHDR by van Steveninck *et al.* [23] based on the work of Bittencourt *et al.* [28] and the original description of Baloh *et al.* [29]. The time in which the eyes were in smooth pursuit of the target were calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies were used as the parameter.

ADAPTIVE TRACKING

The adaptive tracking test was performed as originally described by Borland and Nicholson [30], using customised equipment and software. After a 0.5-minute

run-in time without data-recording, the average performance over the rest 3.0 minutes was scored and was used as the test parameter. Adaptive tracking is a pursuit-tracking task. The subject was required to operate a joystick and try to keep a dot inside a circle moving randomly on the computer screen. If he/she succeeded, the speed of the moving circle increases, and vice versa.

ELECTROENCEPHALOGRAPHY

Pharmaco-electroencephalography (pharmaco-EEG) was used to monitor any drug effects, which can be interpreted as evidence of penetration across the blood brain barrier and changes in the activity of the brain [31,32]. EEG provides non-specific measures of CNS functions. EEG recordings were made using gold electrodes, fixed with EC2 paste (Astromed) and using standard pharmacoEEG lead placement, with the same common ground electrode as for the eye movement registration (international 10/20 system for EEG electrode placement [33]). The electrode resistances were kept below 5 kOhm. The signals were amplified by use of a Grass 15LT series Amplifier Systems with a time constant of 0.3 seconds and a low pass filter at 100 Hz. Data collection and analysis were performed using customized CED and Spike2 for Windows software (Cambridge Electronics Design, Cambridge, UK). Per session eight consecutive blocks of eight seconds were recorded. The signal was AD-converted using a CED 1401 Power (Cambridge Electronics Design, Cambridge, UK) and stored on hard disk for subsequent analysis. Data blocks containing artifacts were identified by visual inspection and these were excluded from analysis. For each lead, fast Fourier transform analysis was performed to obtain the sum of amplitudes in the delta- (0.5-3.5 Hz), theta (3.5-7.5 Hz), alpha- (7.5-11.5 Hz), beta- (11.5-30 Hz) frequency ranges. Frequency band above 30 Hz was also recorded for exploratory pharmacoEEG analyses in order to test whether the findings of an effect of AZD6280 on gamma-frequency band (>30 Hz) in animals translate to humans.

COGNITION MEASURES

The computerized CogState Early phase Battery, consists of four tasks with demonstrated sensitivity to cognitive change associated with drug effects [34]. These tests are listed below, in order of administration. In addition Cogstate's International Shopping List Task immediate and 21 hour delayed recall were administered once per dosing period.

GROTON MAZE LEARNING TASK (GMLT)

This is an executive problem solving and spatial learning task which requires the subject to find a 28-step pathway that is hidden under a 10 x 10 grid of tiles displayed on a computer touch screen. Subjects were instructed to move from the start location (top left), one tile at a time, toward the end (bottom right) while adhering to several rules (no diagnonals, no skipping, no retracing movements). Once completed, subjects returned to the start location and repeated the task for 4 more times. Twenty well-matched alternate forms of this task were cycled among measurements so that subjects would not take a same trial during one dosing period. During a 'timed chase' part of the test, the subject was asked to quickly follow a moving tile around in a 10 x 10 grid of tiles on a computer touch screen with a stylus pen for 30 seconds. This aspect of the task measures attention and psychomotor function.

DETECTION TASK

During the test, a playing card was presented in the center of the screen. Subjects were required to press the 'Yes' key whenever the card flipped over and faced up. Subjects were encouraged to work as quickly and accurately as possible. If subjects responded before the card flipped over or did not respond to a card that had flipped over, an error sound was emitted. After a brief practice test, the real test began. The test measures attention and psychomotor function.

IDENTIFICATION TASK

A playing card was presented face down in the center of the screen and flipped over from time to time. When the card faced up, the subject should press 'Yes' for a red card but 'No' for a non-red (black) one. An error sound would appear when the subject pressed a key before a card flipped over or made a mistake. The real test began after practice. The test measures speed of mental processing and attention.

ONE CARD LEARNING TASK

Subjects were asked to identify whether the playing card presented on the screen had been shown during the current test trial. They responded by pressing the 'Yes' or 'No' key. An error noise would appear when there was an incorrect or missing response. The real test began after practice. The test measures working memory and learning ability.

INTERNATIONAL SHOPPING LIST TASK (ISLT)

At 1.75h post-dose, the test supervisor read a shopping list of 16 words to the subject as they appeared on the computer screen at a rate of one word every two seconds. Subjects were instructed to memorize and recall as many words as possible, while the test supervisor clicked / touched the appropriate button on the screen with the stylus or mouse. The list was read (in the same order) and the immediate recall session were repeated two more times for a total of three trials. The task measures verbal learning ability. At 21h post-dose, subjects were required to recall the shopping list without being read them again, while the test supervisor clicked / touched the appropriate button on the screen with the stylus or mouse. This part of the ISLT task measures long term memory and retrieval (delayed recall).

STATISTICAL ANALYSIS

Statistical analysis was carried out using SAS (version 9.1.3). The primary variables for pharmacodynamic evaluation were outcome parameters from VAS assessments for alertness, CogState battery and tests about other CNS functions. The secondary variables for safety and tolerability evaluation were adverse events (AEs), vital signs assessments (blood pressure, heart rate, respiratory rate and [auricular] temperature), laboratory parameters, and ECGs. The variables for pharmacokinetic evaluation were C_{max}, T_{max}, AUC_{0-t}, $T_{1/2\lambda z}$, and CL/F.

The pharmacokinetic analyses were performed by Clinical Pharmacology, AstraZeneca Wilmington, DE, USA using the WinNonlin program (Pharsight Corporation, MountainView, California, USA) and descriptive statistics of the PK parameters were summarized by treatment.

In total, twenty-nine pharmacodynamic (PD) parameters and their change from baseline were analyzed by mixed model analyses of variance (using SAS PROC MIXED) with treatment, period, time and treatment by time as fixed effects, with subject, subject by time and subject by treatment as random effects, and with the baseline value as covariate, where baseline was defined as the average of the available values obtained prior to dosing. Treatment effects were reported as the contrasts of each active treatment vs. placebo, each dose of AZD6280 vs. lorazepam, as well as AZD6280 40 mg vs. AZD6280 10 mg. No multiplicity adjustment was used for this study. The least square means (LSMs) of the measurements up to 8h post-dose were calculated within the statistical model. Contrasts were reported along with 95% confidence intervals. The EEG, body sway, and VAS Bowdle subscales were analyzed after log-transformation, while the other parameters were analyzed without transformation. Log-transformed parameters were back-transformed after analysis where the results were interpreted as percentage change.

Adverse events were listed and summarized by system organ class and treatment. Vital sign assessments, parameters of the 12-lead ECG recordings and safety laboratories, along with their changes from baseline, were summarized using descriptive statistics for each scheduled measurement by treatment.

Previous studies demonstrated good sensitivity of SPV to the effect of BZDs [13,22] and $\alpha_{2,3}$ subtype-selective GABA-A agonists [13,14,15,16]. As $\alpha_{2,3}$ subunits are the common pharmacological targets shared by these compounds, SPV is hypothesized to be a biomarker for GABA-A $\alpha_{2,3}$ subunit modulation. Moreover, early clinical findings with TPA023 [4] also link the pharmacodynamics effect on SPV to therapeutic anxiolysis. Body sway, tracking, $VAS_{alertness}$, and smooth pursuit, on the other hand, are thought to reflect the sedative and adverse properties of GABA-ergic compounds. As such, we performed a regression analysis to explore the relationship of individual changes from baseline in body sway (Δsway), tracking (ΔTracking), VAS alertness ($\Delta VAS_{alertness}$), and smooth pursuit (Δsmooth), relative to the changes from baseline in SPV (ΔSPV). The slopes of these regression lines are thought to reflect the relations between drug-induced anxiolysis and CNS-depression. A mixed effect model was used, where the fixed factors were treatment and treatment by SPV, whereas the random factors were subject and subject by SPV. The estimate of the slopes of the regression lines of these ΔSPV-relative effect profiles were compared between each dose of AZD6280 and lorazepam.

The pharmacodynamic effects (i.e., the changes from baseline) on SPV were listed with plasma drug concentrations obtained at the same post-dose time points with AZD6280 and lorazepam, respectively. Based on the effect profile of SPV (Figure 2), the median effect size was summarized from all negative values, that is, the same direction of effect as the maximal effect of lorazepam and AZD6280. Plasma drug concentrations of AZD6280 and lorazepam that correspond to 40% to 60% percentile of the overall PD effect size were summarized. The two resultant geometric mean concentrations, termed as 'pharmacodynamically equivalent concentrations' in this case, were used to normalize the actual concentration measurements of each compound. Subsequently, the post-dose PD effect values were plotted with the normalized drug concentrations.

RESULTS

SUBJECTS

A total of seventeen male healthy volunteers participated in the study. Sixteen subjects completed the study. One subject was withdrawn from the study due to positive THC result in urine drug screen test on his 2nd treatment period. This drop-out was replaced by a subject receiving the same order of study treatments. Subjects had an average age of 31.7±12.6 years, and BMI of 23.4±2.0 kg/m². Data from all treated subjects were used in the analyses of safety and pharmacokinetics. Subjects who completed the study per protocol were included in the pharmacodynamic analysis.

PHARMACOKINETICS (PK)

As demonstrated in Figure 1, both AZD6280 10 mg and AZD6280 40 mg were rapidly absorbed after oral administration with no absorption lag time. The median time to maximum plasma-concentrations (T_{max}) was 1.50 hr (range 0.50 hr-3.23 hr) with AZD6280 10 mg and 1.38 hr (range 0.50 hr-4.00 hr) with AZD6280 40 mg. The mean termination half-life ($T_{1/2}$) was comparable between the two dose levels of AZD6280 (7.10 hr for 40 mg and 6.65 hr for 10 mg) Lorazepam 2 mg had similar T_{max} (median 1.50 hr, range 1.00 hr-4.50 hr) but longer $T_{1/2}$ (mean 12.9 hr, range 8.28 hr-18.4 hr) when compared to AZD6280. The dose-normalized PK parameters appeared to be independent from the dose of AZD6280 (see Table 1).

PHARMACODYNAMICS

Table 2 summarizes the pharmacodynamic (PD) effects of each active treatment compared to placebo. $VAS_{alertness}$, was not significantly impaired by either AZD6280 10 mg or AZD6280 40 mg compared to placebo (Table 2). However, alertness was significantly decreased after lorazepam 2 mg. Sedation caused by lorazepam was significantly larger than that of AZD6280 10 mg (mean change from baseline: -5.9 mm *vs.* -0.8 mm, p=0.0051), but was marginally distinguishable from the effect of AZD6280 40 mg (-5.9 mm *vs.* -3.1 mm, p=0.1055).

Saccadic peak velocity (SPV) was significantly reduced by the three active treatments (Table 2), respectively, compared to placebo (Figure 2). SPV reductions differed between lorazepam and AZD6280 [estimated difference in SPV, p-value] (lorazepam vs. AZD6280 10 mg: -40.3 deg/sec, p<0.0001; lorazepam vs. AZD6280 40 mg: -12.9 deg/sec, p=0.0367) and were dose-dependent (AZD6280 40 mg vs. AZD6280 10 mg: -27.3 deg/sec, p<0.0001).

Body sway, smooth pursuit, tracking and VAS 'feeling high' were significantly affected by lorazepam 2 mg and AZD6280 40 mg, compared to both placebo and AZD6280 10mg. The effects of lorazepam 2 mg on body sway, smooth pursuit, and tracking were significantly larger than those of AZD6280 40 mg (Sway: 89.04% vs. 21.12%, p<0.0001; Smooth: -10.8% vs. -4.0%, p=0.0003; Tracking: -9.53% vs. -2.65%, p<0.0001). VAS 'feeling high' [estimated difference between two treatments, 95% CIs, p-value] was significantly increased by AZD6280 40 mg and lorazepam 2 mg (Table 2). Although the effect size of AZD6280 40 mg was comparable to that of lorazepam 2 mg (0.02 [log(mm)], [0.08, -0.12], p=0.6282), the effect lasted considerably shorter with AZD6280 40 mg (Figure 3). Moreover, lorazepam also significantly distorted internal and external perceptions compared to placebo, but the effects of AZD6280 did not differ from placebo (Table 2).

The three active treatments had a different spectrum of pharmaco-EEG effects. All frequencies of EEG bands were statistically significantly affected by lorazepam 2 mg. In comparison, AZD6280 40 mg induced lower effects on alpha bands, no effect on gamma band, opposite effects on delta band (estimated difference versus placebo, 95% confidence interval, p-value: AZD6280 40 mg: -6.7% (-12.3%, -0.6%), p=0.0325; lorazepam 2 mg: 8.6% (1.9%, 15.8%), p=0.0129) and affected only the theta and beta bands in the Fz-Cz (i.e. frontal-central) leads. AZD6280 10 mg only induced changes in theta and alpha bands and the extent and distribution of these effects were similar to those of AZD6280 40 mg.

Results of the CogState early battery are presented in Figure 4. Neither dose of AZD6280 affected the individual CogState variables, except for the statistically significant effect of AZD6280 40 mg associated with worse performance on one-card learning accuracy compared to placebo (AZD6280 40 mg vs. placebo: -0.09 vs. -0.02, [acr(%)], p=0.0018). However, the effect size of AZD6280 was significantly less than that of lorazepam 2 mg (lorazepam vs. placebo: -0.14 vs. -0.02, [acr(%)], p<0.0001). Moreover, lorazepam was also associated with extensive impairments on the other CogState cognitive parameters compared to placebo (lorazepam *vs.* placebo, [unit], *p*-value): reaction time of correct responses in the detection task (0.12 *vs.* 0.03, [log(msec)], p<0.0001), reaction time of correct responses in the identification task (0.08 *vs.* 0.02, [log(msec)], p<0.0001), moves per second (mps) in the chase test (-0.20 vs. 0.03, [mps], p<0.0001), and the sum of errors in GMLT (22.2 vs. 4.5, p=0.0003). Lorazepam also reduced the number of words recalled in both the ISLT immediate-recall (lorazepam *vs.* placebo: 25.2 vs. 35.3, p<0.001) and the ISLT delayed-recall (lorazepam *vs.* placebo: 4.8 vs. 10.1, p<0.001).

Several CNS effects of the different compounds are plotted against their impact on SPV. The regression analyses for ΔLog(Sway)-ΔSPV and ΔTracking-ΔSPV are plotted in Figure 5, in combination with the calculated population regression lines. The absolute slopes of the regression lines were significantly smaller for the relations of ΔLog(Sway)-ΔSPV (AZD6280 10 mg vs. lorazepam 2 mg: -0.00056 vs. -0.00157, p=0.0099; AZD6280 40 mg vs. lorazepam 2 mg: -0.00080 vs. -0.00157, p=0.0135), ΔTracking-ΔSPV (AZD6280 10 mg vs. lorazepam 2 mg: 0.03474 vs. 0.06453, p=0.0168; AZD6280 40 mg vs. lorazepam 2 mg: 0.03080 vs. 0.06453, p=0.0006), and ΔSmooth-ΔSPV (AZD6280 10 mg vs. lorazepam 2 mg: 0.06146 vs. 0.01083, p=0.0232; AZD6280 40 mg vs. lorazepam 2 mg: 0.06106 vs. 0.01083, p<0.0001) for either dose of AZD6280 than for lorazepam 2 mg, but the relations of $\Delta VAS_{alertness}$-ΔSPV are comparable between AZD6280 and lorazepam.

As is shown in Figure 6, the effect size on SPV generally grows with the increase of concentration with either AZD6280 or lorazepam. The range of effect size is generally comparable between lorazepam and AZD6280 on SPV. However, when the effect size is higher than 30 deg/sec (i.e. SPV reduction > 30 deg/sec), the corresponding range of normalized drug concentration is much larger with AZD6280 than that with lorazepam. Moreover, lorazepam produces a relatively sharp decline of SPV with concentration elevation, while the concentration-effect profile of AZD6280 is less steep. The same concentration-effect profile also applies to the profiles of body sway and VAS alertness, except that AZD6280 had much lower maximal effect on the two PD parameters within the observed range of plasma concentrations (Figure 6). These findings indicate that AZD6280 is pharmacologically less potent than lorazepam, and that higher concentrations of the new compound are needed to reach the same effect as the benzodiazepine.

SAFETY

The administration of single dose AZD6280 10 mg or 40 mg were safe and well tolerated in the 17 healthy male subjects. In general, Treatment-emergent AEs (frequency [incidence]) occurred more frequently with AZD6280 40 mg (25 [75.0%])

than with AZD6280 10 mg (7 [43.8%]), but were milder and less frequent than with lorazepam 2 mg (39 [93.8%]). The frequency and severity of AEs with low dose (10 mg) AZD6280 were comparable to those with placebo (8 [47.1%]). Compared to lorazepam 2 mg, AZD6280 40 mg (lorazepam vs. AZD6280 40mg) caused fewer gastrointestinal (2 [12.5%] vs. 1 [6.3%]) and less frequent and less intensive neurological events (particularly indicative of sedation) (14 [87.5%] vs. 11 [68.8%]). No clinically significant abnormalities in vital signs, laboratory or ECG results were identified during the study.

DISCUSSION

The current study compared the CNS effects of AZD6280, a novel $\alpha_{2,3}$ subtype-specific GABA-A receptor modulator intended for anxiolytic use, with those of lorazepam. A comprehensive selection of neurophysiological and neuropsychological tests was employed to address the pharmacodynamic effects of the two GABA-ergic compounds on various brain domains. As a positive control, therapeutic dose of lorazepam was designed to benchmark the effect of clinically relevant GABA-A agonism on each pharmacodynamic measure.

Single doses of AZD6280 10 mg, AZD6280 40 mg, and lorazepam presented distinct pharmacodynamic effect profiles on the central nervous system. These effect profiles are likely to result from differences in selectivity and potency of the compounds to modulate the subtypes of the GABA-A receptor complex (AZD6280 vs. lorazepam) as well as from differences in exposure levels of the same drug (AZD6280 10 mg vs. 40 mg). The highest dose of AZD6280 in this study caused SPV reductions that on average were somewhat smaller than those of lorazepam. This indicates that the treatments might not be fully equipotent. SPV has been suggested as a pharmacodynamic biomarker for clinical anxiolysis of benzodiazepines and novel $\alpha_{2,3}$-subtype selective GABA-A receptor agonists [4,13,22]. The concentration-effect profiles of lorazepam and AZD6280 on SPV provide a better approach for the evaluation of dose equivalence, because they show the entire range of drug plasma levels and effects. Noteworthy, the range of effect size is comparable between lorazepam 2 mg and AZD6280 40 mg on SPV, although fewer individual SPV measurements reach the level that is regularly attained after lorazepam. Also, a larger range of AZD6280 concentrations was linked to significant effect on SPV (i.e. >30 deg/sec SPV reduction) compared with lorazepam (Figure 6). These findings and the known relevance of SPV effect suggest that the anxiolytic effect of peak concentrations of AZD6280 40 mg may be similar to the effects of lorazepam 2 mg. Moreover, the SPV effects of AZD6280 diminish at relatively high plasma levels, which are compatible with partial agonism, with a maximum effect that seems to be smaller than with high doses of the full agonist lorazepam.

The CNS-PD parameters other than SPV are linked to various clinical side-effects of benzodiazepines, including postural instability, visuo-motor coordination, hypnotic effects and cognitive impairments. In Figure 6, the ranges of AZD6280 and lorazepam concentrations are identical to the concentration-effect plots delineated for SPV; however, larger relative differences are seen in the maximally attainable effect on sway between lorazepam and AZD6280, versus the difference seen in peak SPV effects. The smaller maximum effect of AZD6280 on body sway indicates less impairment of postural stability despite of equal or even higher normalized concentrations of the new compound compared to lorazepam. These findings can be interpreted by the superior pharmacological selectivity of AZD6280 over the non-selective GABA-A agonism of lorazepam.

Moreover, the relations between drug-induced SPV reduction and the drug effects on these side-effect-related CNS-PD parameters were proposed to quantitatively compare the pharmacodynamic selectivity of a GABA-A-ergic drug with benzodiazepine, regardless of dose or dose equivalence. In this study, AZD6280 demonstrated relatively flat regression lines in the scatter graphs for body sway, tracking or smooth pursuit against SPV. The slopes for these regression lines were significantly different from those observed after lorazepam. Such a SPV-dominant effect profile is distinguishable from that of benzodiazepines and is consistent with our previous reports regarding other GABA $\alpha_{2,3}$-selective partial agonists [13]. On the other hand, the similar slopes of the regression lines between the two doses of AZD6280 support that such SPV-relative indices are independent from dose or dose equivalence.

In line with their distinct pharmacodynamic profile, lorazepam and AZD6280 differed considerably in their profiles of CNS depression. Fewer treatment-emergent adverse events, mostly 'neurological disorders' as defined in MedDRA-ergic, occurred with the highest dose of AZD6280 than with lorazepam. Lorazepam caused remarkable concentration-dependent response profiles on adaptive tracking, body sway, smooth pursuit, visual analogue scales for alertness, 'feeling high', external and internal perceptions, and various cognitive functions. The effects of AZD6280 40 mg on these parameters were smaller and often not statistically significant. All these findings suggest that lorazepam induces substantial impairments in a wide range of CNS domains, but the effects of AZD6280 40 mg were less extensive. These results are consistent with those reported in previous single-dose studies with lorazepam and other $\alpha_{2,3}$-selective GABA-A partial agonists [14,15,16]. Thus the abovementioned pharmacodynamic effect profiles probably reflect the characteristics of the entire family of $\alpha_{2,3}$-selective GABA-A partial agonists, which may translate into a reduced propensity to cause sedative side effects.

AZD6280 and lorazepam also showed different effects on the EEG power spectrum. The effect of AZD6280 40 mg on EEG Delta band was opposite to that of lorazepam and may relate to differences in the sedative effects of the compounds. In

the EEG bands between 4 Hz and 35 Hz, the EEG responses to AZD6280 were generally consistent with but smaller than those to lorazepam. Lorazepam was associated with an increase in EEG power in the Gamma band, while the effect of AZD6280 was negligible with either dose. The similarities of EEG signatures between AZD6280 and lorazepam in the medium frequency bands, as well as those between the two doses of AZD6280, may reflect common pharmacological properties underlying an anxiolytic action. Furthermore, the relatively low effects of AZD6280 on the EEG parameters may be explained by the partial GABA-A modulation of this new compound, compared with the full agonism of lorazepam.

In conclusion, the SPV responses to AZD6280 suggest potential anxiolytic effect of the compound, while the absent or smaller effects of AZD6280 on subjective alertness, visuo-motor coordination, postural balance, psychomotor and cognitive functions indicates a more favorable and concentration-related side-effect profile compared to that of lorazepam. Overall, the pharmacodynamic profile of AZD6280 is consistent with the specificity and selectivity of this compound at the $\alpha_{2,3}$ GABA-A receptor subtypes. This pharmacodynamic profile, combined with a good tolerability profile, support further clinical development of AZD6280 as a potential fast-onset anxiolytic at doses around 10 to 40 mg.

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Table 1 · Mean (SD) of the dose-normalized pharmacokinetic parameters after administration of AZD6280 10 mg and AZD6280 40 mg (n=16)

	Unit	AZD6280 10mg	AZD6280 40mg	Lorazepam 2mg
C_{max},norm*	ng/mL/mg	9.9 (2.4)	8.2 (2.4)	NC
AUC_{0-t},norm	h·ng/mL/mg	41.0(8.6)	37.9 (10.0)	NC
$AUC_{0-\infty}$,norm	h·ng/mL/mg	46.0 (10.8)	42.2 (11.9)	NC
C_{max}	ng/mL/mg	98.5(24.3)	328(97.5)	21.9(4.67)
AUC_{0-t}	h·ng/mL/mg	397(100)	1515(400)	235(47.8)
$AUC_{0-\infty}$	h·ng/mL/mg	444(125)	1686(477)	360(103)
$AUC_{1/2}$	h	7.0(1.2)	6.7(0.9)	13.0(2.6)
CL/F	L/hr	24.9(9.55)	25.8(8.09)	5.96(1.56)

*NC=not calculated; *'norm' indicates dose-normalized pharmacokinetic parameter*

Table 2 · Treatment contrasts as Estimated difference for the PD parameters

Parameters	Lorazepam 2mg	AZD6280 10mg	AZD6280 40mg
	vs. Placebo		
SPV (deg/sec)	-62.9 (-75.2, -50.6)	-22.6 (-34.6, -10.6)	-50.0 (-62.1, -37.8)
	p<0.0001	p=0.0005	p<0.0001
SacInacc (%)	0.7 (0.1, 1.3)	-0.4 (-0.9,0.2)	-0.4 (-1.0, 0.2)
	p=0.0206	p=0.2191	p=0.1590
SacRT (sec)	0.029 (0.023, 0.035)	0.003 (-0.003, 0.009)	0.004 (-0.002, 0.010)
	p<0.0001	p=0.2964	p=0.1548
Sway (%)	89.0%(62.8%, 119.6%)	-1.0%(-14.8%, 15.1%)	21.1%(4.3%, 40.7%)
	p<0.0001	p=0.8965	p=0.0134
Smooth (%)	-10.8 (-14.2, -7.3)	-1.1 (-4.6, 2.3)	-4.0 (-7.5, -0.6)
	p<0.0001	p=0.5103	p=0.0238
Tracker (%)	-9.53 (-11.9, -7.21)	-0.63 (-2.95, 1.69)	-2.65 (-4.97, -0.33)
	p<0.0001	p=0.5806	p=0.0266
$VAS_{alertness}$ (mm)	-5.6 (-9.1, -2.1)	-0.5 (-3.9, 2.9)	-2.7(-6.1, 0.7)
	p1=0.0024	p=0.7691	p=0.1178
$VAS_{calmness}$ (mm)	2.2(-0.8, 5.3)	1.4 (-1.6, 4.5)	1.2 (-1.8, 4.3)
	p=0.1429	p=0.3402	p=0.4114
VAS_{mood} (mm)	-0.7 (-2.5, 1.2)	0.5 (-1.4, 2.4)	0.0 (-1.8, 1.9)
	p=0.4733	p=0.5847	p=0.9723
$VAS_{external}$ log(mm)	0.10 (0.05, 0.16)	0.01 (-0.04,0.07)	0.05 (-0.00, 0.10)
	p=0.0004	p=0.5931	p=0.0719
$VAS_{internal}$ log(mm)	0.07 (0.03, 0.11)	0.01 (-0.02,0.05)	0.01 (-0.03, 0.05)
	p=0.0007	p=0.4781	p=0.5087
VAS_{high} log(mm)	0.12 (0.02, 0.22)	-0.01 (-0.11,0.08)	0.10 (0.00, 0.19)
	p=0.0168	p=0.7881	p=0.0474

1. *The p-value represent the result of statistical comparison between the corresponding active treatment (i.e. AZD6280 40 mg, AZD6280 10 mg, or lorazepam 2 mg) and placebo for each pharmacodynamic parameter*

Figure 1 · Mean (standard error as error bars) concentration-time profiles of AZD6280 10 mg, AZD6280 40 mg and lorazepam

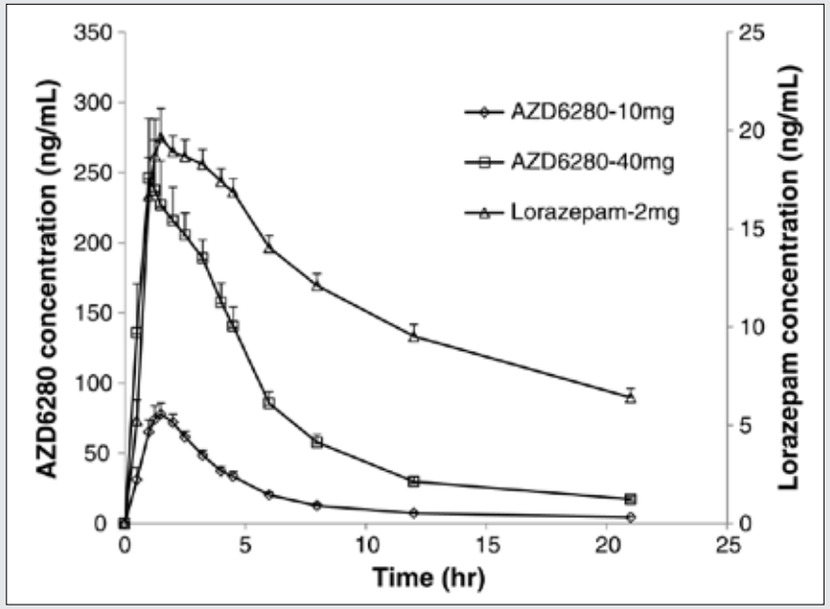


Figure 2 · Mean (standard error) profiles of objective pharmacodynamic parameters after the treatments of placebo, lorazepam 2 mg, AZD6280 10 mg, and AZD6280 40 mg

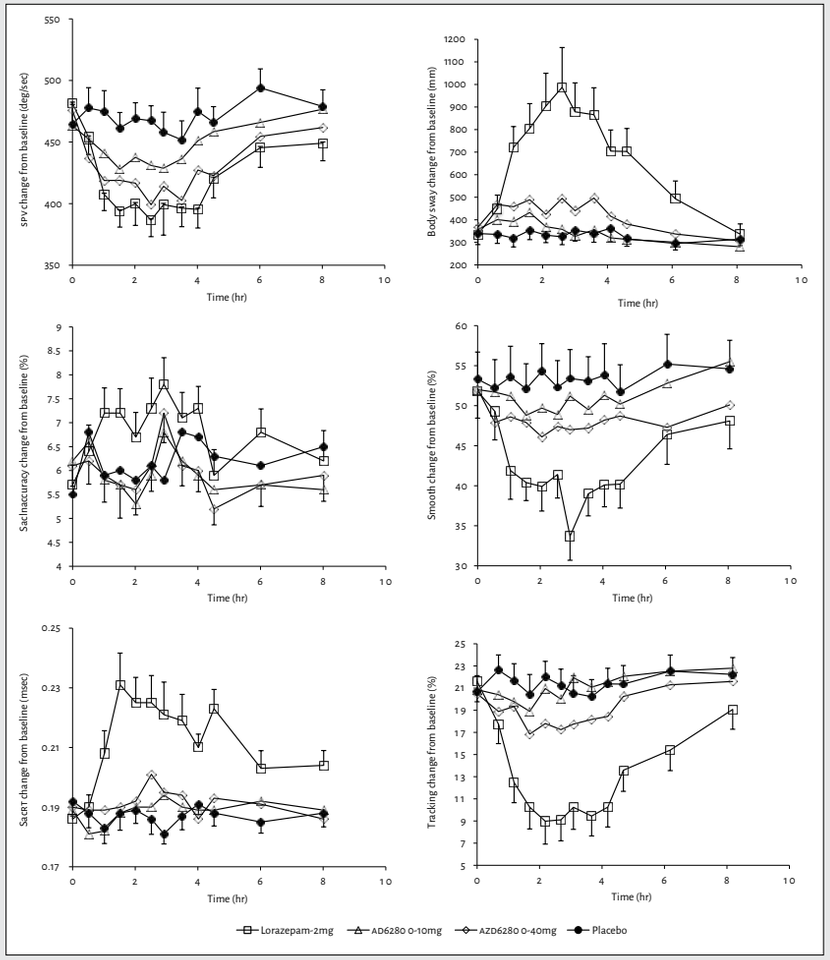


Figure 3 · Arithmetic mean (standard error) profiles of subjective pharmacodynamic paramters (i.e. visual analogue sub-scales) after the treatments of placebo, lorazepam 2 mg, AZD6280 10 mg, and AZD6280 40 mg

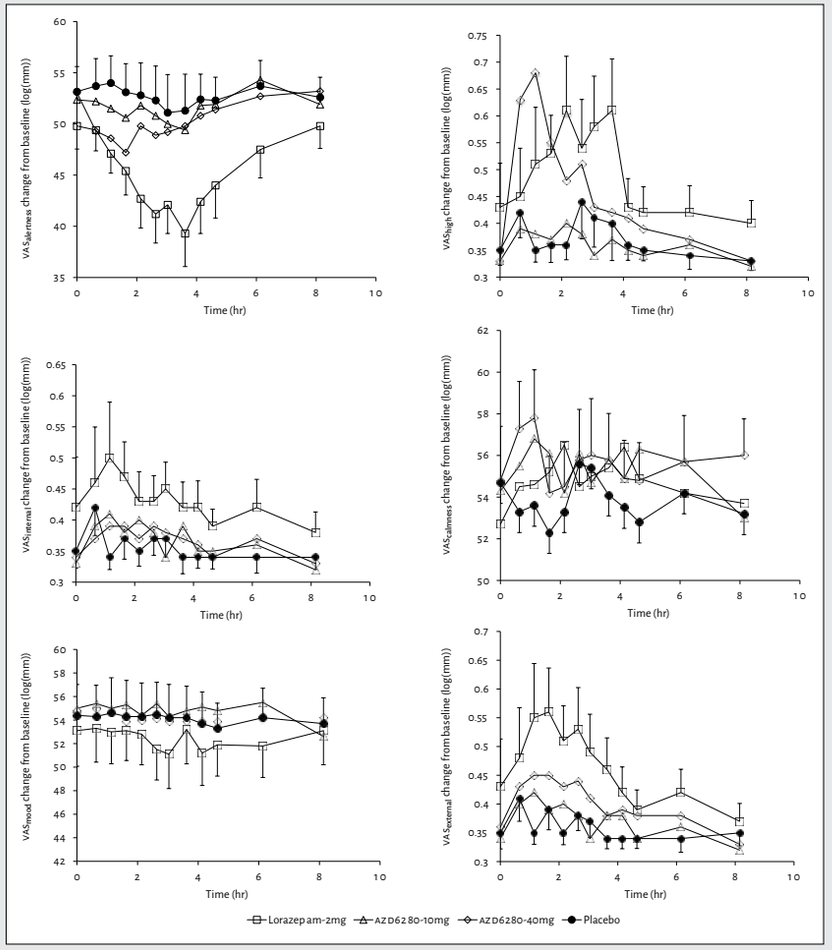
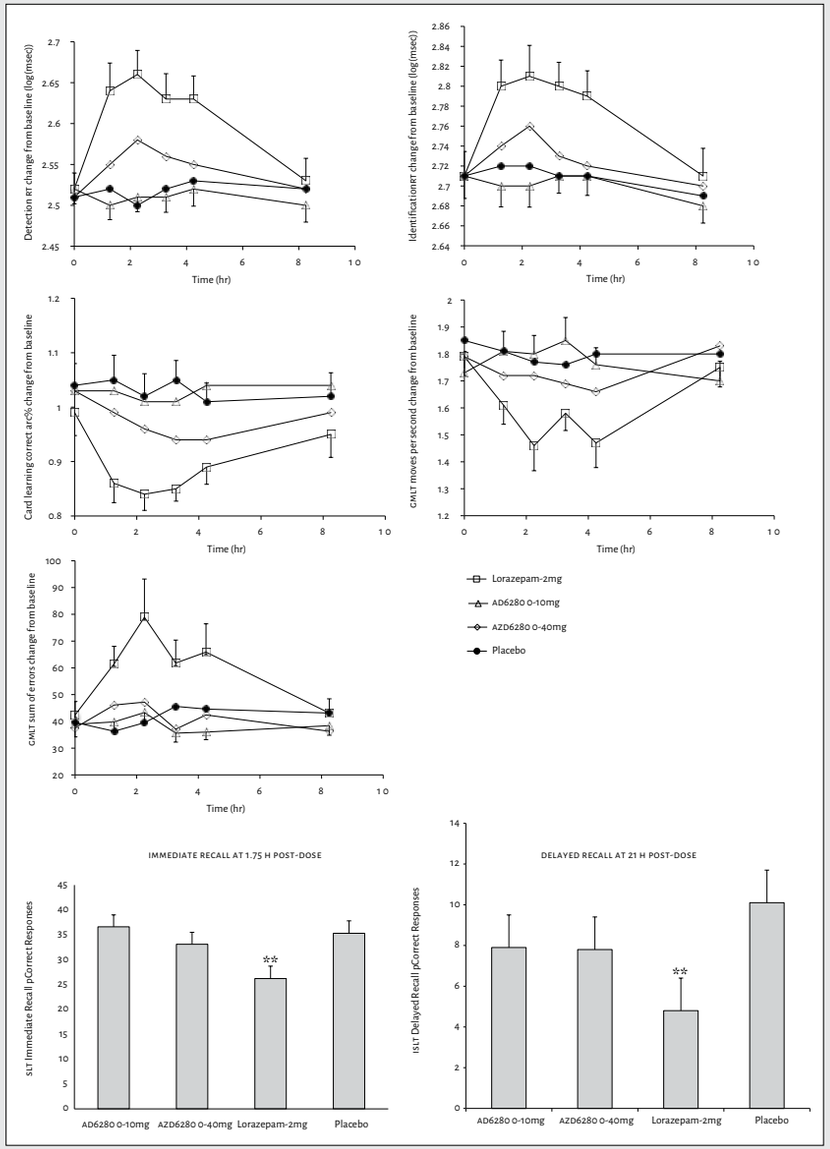


Figure 4 · Means (standard error) profiles of CogState parameters after the treatments of placebo, lorazepam 2 mg, AZD6280 10 mg, and AZD6280 40 mg



*ISLT=International Shopping List Task; ** represent 'p<0.001' compared to the placebo arm.*

Figure 5 · The ΔLOG(Sway)-ΔSPV, $\Delta VAS_{alertness}$-ΔSPV, ΔTRACK-ΔSPV, ΔSMOOTH-ΔSPV relations of AZD6280 10 mg and 40 mg vs. lorazepam 2 mg.

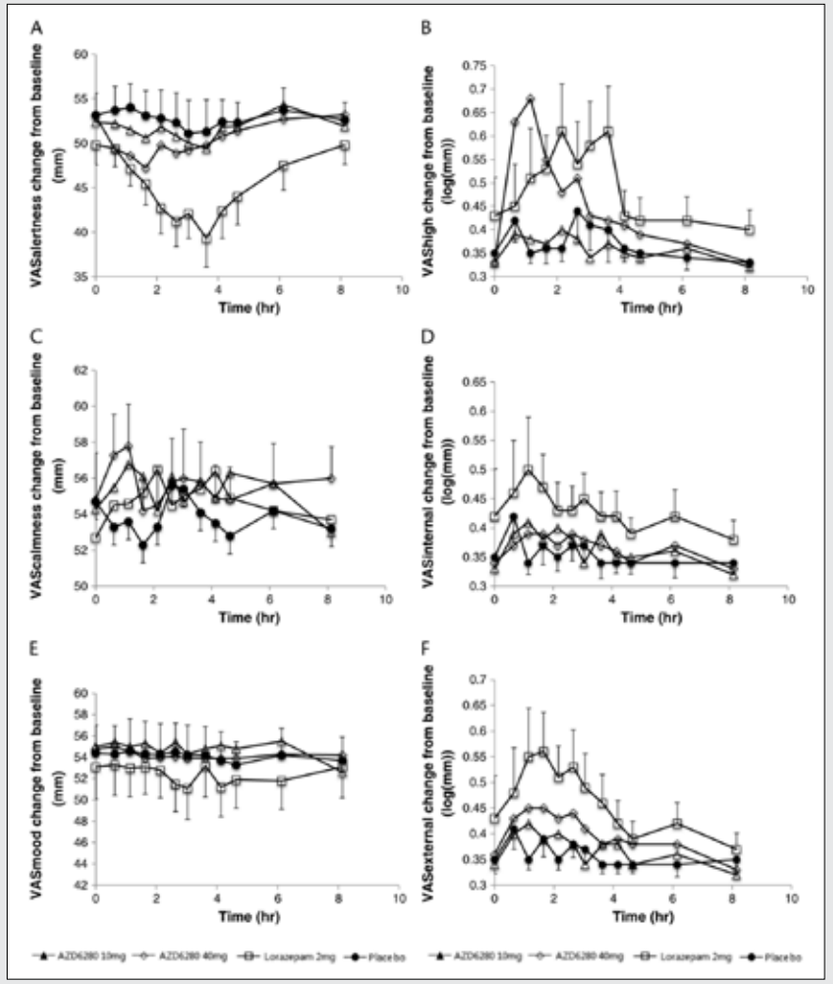
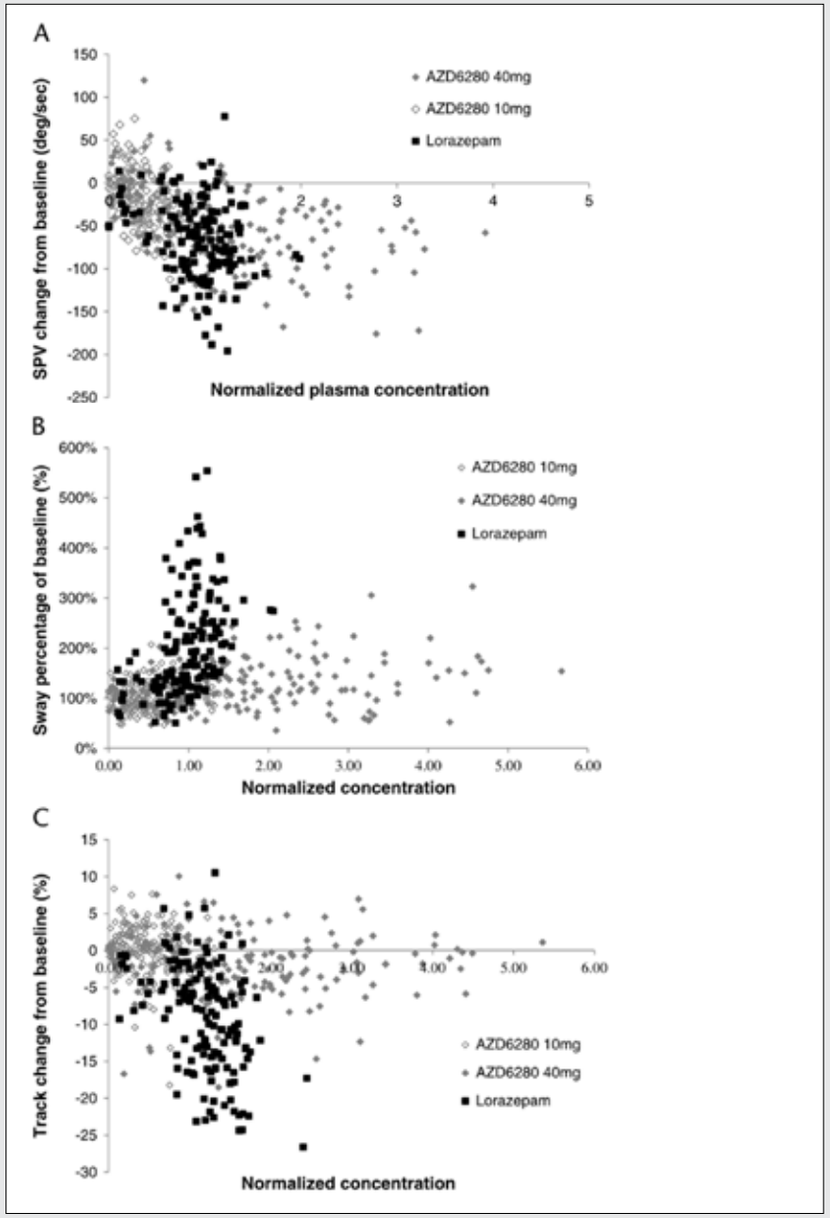


Figure 6 · Normalized concentration-effect profiles of lorazepam and AZD6280 on saccadic peak velocity (SPV), body sway (sway), and adaptive tracking (track)



IV

NS11821, A PARTIAL SUBTYPE-SELECTIVE GABA-A AGONIST, ELICITS SELECTIVE EFFECTS ON THE CENTRAL NERVOUS SYSTEM IN RANDOMIZED CONTROLLED TRIAL WITH HEALTHY SUBJECTS

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Xia Chen[1,2], Rob GJA Zuiker[1], Ole Østerberg[3], Naheed R Mirza[3], Pierandrea Muglia[3], Marieke de Kam[1], Erica S Klaassen[1] and Joop MA van Gerven[1]

1. Centre for Human Drug Research (CHDR), Leiden, The Netherlands | 2. Clinical Pharmacological Research Center (CPRC), Peking Union Medical College Hospital, Beijing, China | 3 NeuroSearch A/S, Hellerup, Denmark

ABSTRACT

NS11821 is a partial GABA-A agonist with relatively dominant $\alpha_{2,3}$ and α_5 subtype efficacy but negligible α_1 agonism. This first-in-human study was performed in healthy male subjects using a single-dose, parallel, double blind, placebo-controlled, randomized, dose-escalation study design. In total six cohorts (n=48) were enrolled. The eight subjects of each cohort received NS11821 (10 mg, 30 mg, 75 mg, 150 mg, 300 mg or 600 mg) or placebo in a 6:2 ratio. At low dose levels, NS11821 had a relatively low exposure and a more-than-proportional increase of AUC and C_{max}, probably due to poor solubility. Saccadic peak velocity (SPV) decreased in a dose-related manner while limited impairments were seen on body sway and the visual analogue scale (VAS) for alertness. The most common adverse events were somnolence and dizziness, which were more prominent with the higher doses. Although no positive control was used in this study, the results were compared *post hoc* to a CHDR dataset for lorazepam 2 mg. The maximum SPV effects seemed comparable to the typical effects lorazepam, whereas the other CNS effects were smaller. These results support the pharmacological selectivity of NS11821 and show that pharmacodynamic effective doses of NS11821 were safe and well tolerated in healthy subjects.

INTRODUCTION

Benzodiazepines (BZDs) are one of the most commonly prescribed anxiolytic drugs, although therapeutic guidelines generally limit their use to several weeks. The use of BZDs is restricted by tolerance and dependence, as well as concomitant psychomotor impairments. In general, the effect profile of BZDs is attributed to their non-selective agonism at the α_1, α_2, α_3, and α_5 subunit-containing GABA-A receptors. Preclinical studies have linked these subtypes to different pharmacological aspects of BZDs: 1) α_1-containing receptors are associated with sedative and motor effects (McKernan et al., 2000; Rowlett et al., 2005); 2) α_2- and α_3-containing receptors are related to anxiolysis and analgesia (Knabl et al., 2008; Knabl et al., 2009); and 3) α_5-containing receptors are involved in amnesic effects (Atack et al., 2006; Ballard et al., 2009). In order to minimize the untoward depressive effects on the central nervous system (CNS), several novel $\alpha_{2,3}$-subtype selective GABAergic compounds are being developed in preclinical and clinical phases and they are expected to deliver anxiolytic effects with less adverse effects.

According to the *in vitro* two-electrode voltage-clamp electrophysiological assessments performed on receptors expressed in oocytes, although the *in vitro* binding affinity is generally comparable (Ki [nM] = 1.6, 9.7, 3.8, 2.5 for α_1, α_2, α_3 and α_5 subunits, respectively, for different human GABA-A receptor subtypes expressed in HEK cells), NS11821 has relatively higher maximum efficacy for GABA-A α_2, α_3 and α_5 over GABA-A α_1 receptors: compared to diazepam, NS11821 showed 17%, 40% and 41% relative efficacy at the α_2, α_3 and α_5 subunits, but 4% relative efficacy for the α_1 subunit, respectively (Neurosearch data on file). The EC50 of NS11821 was 59, 73 and 44 nM for the *in vitro* pharmacological effects on human GABA-A α_2ß2γ2s, α_3ß2γ2s and α_5ß2γ2s receptors, respectively. (Neurosearch data on file). Such a profile is translated to low propensity for sedative effects with retained anxiolytic activity: in both the rat conditioned emotional response (CER) test and the plus-maze task (Davis, 1990; Rodgers and Dalvi, 1997), NS11821 dose dependently reduced anxiety-like behavior with a minimum effective dose (MED) of 3 mg/kg, corresponding to a human equivalent dose (HED) of 29 mg. On the other hand, NS11821 was found to increase exploratory motility at doses between 3-30 mg/kg in mice, but significantly reduce motor activity in rats at doses higher than 30 mg/kg. In the rotarod test, NS11821 had no significant effect on rotarod performance in rats up to 100 mg/kg. In contrast, diazepam significantly reduced exploratory motility and rotarod performance at doses≤ 3 mg/kg. In addition, the passive avoidance memory test in mice has been shown sensitive to BZD-induced anterograde amnesia when the drug was administered prior to the learning session. Compared with a single dose of chlordiazepoxide, NS11821 demonstrated little effect on memory in mice with doses from 10 to 100 mg/kg, while chlodiazepoxide did impaire memory as measured by the test. With regard to safety, the no-observed adverse-effect level

(NOAEL) in rats was 10 mg/kg after repeat doses, corresponding to a HED of 1.61 mg/kg (97 mg) (Neurosearch data on file). Taken together, the above findings inferred potential anxiolytic effect of NS11821, with reduced sedative and memory-impairing effects at its pharmacolocally active doses.

Using the Food and Drug Administration guidelines for first-in-man studies, a starting dose of NS11821 10 mg was selected. In this study, NS11821 was orally administrated to healthy male volunteers in six single ascending doses (10 mg, 30 mg, 75 mg, 150 mg, 300 mg and 600 mg) and compared to placebo. The objective was to evaluate the safety, tolerability and pharmacokinetic (PK) profile of the compound as well as to estimate the maximum tolerable dose. The study included a validated battery of CNS measurements, including saccadic eye movement, smooth pursuit, body sway, adaptive tracking, tapping, visual analogue scales (VAS) and memory tests, to evaluate the pharmacodynamic (PD) profile of NS11821. In previous studies conducted in healthy volunteers, BZDs have demonstrated robust effects on VAS-alertness, postural stability, memory and neurophysiological functions. These diverse effects across a wide range of different CNS-regions are thought to account for the widespread distribution of GABA-A receptors throughout the brain, and the non-selective, full agonism of BZDs on these receptors.

Out of the many tests used to evaluate the CNS effects of BZDs, saccadic peak velocity (SPV) and the visual analogue scale (VAS) for alertness were identified as the most sensitive parameters for BZDs (de Visser et al., 2003). Both tests showed consistent effects to various BZDs at different doses. Studies with different $\alpha_{2,3}$ subtype selective GABA-A agonists suggest that impairments of subjective alertness and body sway have been primarily attributed to α_1 stimulation, reduction of SPV seems related to mainly reflect α_{2-3} stimulation (Chen et al., 2012), and memory effects could be related to α_5 stimulation (Collinson et al., 2002). As a result, we employed this battery to measure the pharmacodynamic effects of NS11821 in healthy volunteers. In addition, a *post hoc* comparison was performed with a historic data set based on a considerable number of similar studies conducted at CHDR with a therapeutic dose of the full benzodiazepine lorazepam.

METHODS

DESIGN

This was a single-dose, parallel, double blind, placebo-controlled, randomized, dose escalation study in healthy male subjects. There were 8 subjects per dose cohort. Decisions for dose escalation were based on investigator blinded interim assessments of PK, PD and safety results.

SUBJECTS

Forty eight healthy male volunteers were recruited from the Centre for Human Drug Research (CHDR) database. All volunteers gave written informed consent and were medically screened before entry into the study. Subjects were not allowed to smoke more than five cigarettes per day and had to refrain from smoking during the study days. In the 48 hours prior to the study days they were asked not to drink alcohol and to avoid xanthine- containing drinks until the end of the study days. The use of medication was not allowed during the study period (except occasional use of paracetamol, up to 1.5 g per day). The study was approved by the Medical Ethics Review Board of Leiden University medical Centre, the Netherlands.

TREATMENTS

A total of six study cohorts (n=48) received capsules containing 10 mg, 30 mg, 75 mg, 150 mg, 300 mg and 600 mg NS11821 or placebo with 250 mL of water. In each cohort, six subjects were randomized to receive a single dose of NS11821 and two subjects received placebo. A standard lunch was served 2 hr post-dose together with approximately 200 mL water.

SAFETY

Adverse events, electrocardiogram (ECG), blood pressure and heart rate measurements were collected throughout the study. Twelve lead ECG recordings were made using Electrocardiograph Marquette 5000/5500 (USA). Continuous real time telemetry (1 lead ECG) and pulse oxymetry were performed with GE Marquette (USA) and DASH 4000 (USA) respectively. Blood pressure and heart rate were assessed using a Nihon Kohden BSM 1101K monitor (Japan) or a Dash 4000 (USA). All ECG, blood pressure and heart rate measurements were made after the subject had been resting in a supine position for at least 5 minutes. In addition, for the evaluation of orthostatic blood pressure and heart rate, subjects were required to stand for 3 minutes prior to a second assessment.

PHARMACOKINETICS

Whole blood samples and urine samples were taken for assay of the parent drug NS11821 and its metabolite NS14606. Blood samples were taken 1 hour pre-dose and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 15, 22, 34 and 48 hours post-dose. The blood was drawn in 10 mL K2 EDTA tubes and then centrifuged (2000 G, 10 minutes, at 2-8°

C), transferred to 3.5 mL Sarsted tubes and stored at -80° C within 30 minutes after sampling. All urine voids were collected throughout the study days and combined in intervals: last 2 hours pre-dose, first 10 hours post-dose, 10-12 hours post-dose and 12-22 hours post-dose. Two 6 ml samples (duplicate) from each time interval were stored at -40° C. Analysis of the blood and urine samples was performed at PRA International (Assen, the Netherlands), using LC MS/MS and validated bio analytical methods. The lower limit of quantification (LLOQ) for plasma NS11821 and NS14606 was set to 0.1 ng/mL.

PHARMACODYNAMICS

All subjects were trained to be familiarized with the psychometric tests during the screening sessions to minimize learning effects preceding the study. During the treatment period, pharmacodynamic measurements were performed twice pre-dose and 0.5, 1, 1.5, 2, 3, 4, 6 and 10 hours post-dose. The CNS tests were performed in a quiet room with subdued illumination with only one subject in the same room per session. Each session consisted of the following sequence of tests: saccadic eye movements; smooth pursuit; pharmaco-EEG, body sway with eyes closed; VAS Bond and Lader; VAS Bowdle; adaptive tracking and tapping. Cognitive function tests were performed at 2 and 6 h post-dose.

SACCADIC EYE MOVEMENT

Measurements of saccadic eye movements were recorded as previously described (de Haas et al., 2008; de Haas et al., 2009). Average values of saccadic peak velocity (SPV), latency (= reaction time) and inaccuracy were calculated for all artefact free saccades. SPV is closely related to their anxiolytic properties (de Visser et al., 2003) and its measurement has been validated as the most sensitive biomarker for the effects of BZDs (de Visser et al., 2003; van Steveninck et al., 1991; van Steveninck et al., 1992; van Steveninck et al., 1999).

SMOOTH PURSUIT

Smooth pursuit was measured as previously described (de Haas et al., 2009). The time in which the eyes were in smooth pursuit of the target was calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies was the target parameter.

BODY SWAY

Two minute body sway measurements were performed as previously described (de Haas et al., 2009). Body sway is a measure of postural stability that has previously been shown to be sensitive to BZDs (van Steveninck et al., 1996).

VISUAL ANALOGUE SCALE

The visual analogue scales (VAS) in this study were used as previously described by Norris (Norris, 1971). The Bond and Lader VAS was performed to measure subjective alertness, mood and calmness (de Haas et al., 2009). The Bowdle VAS evaluates psychedelic effects clustered into three distinct total sum scores: internal perception (reflects inner feelings that do not correspond with reality, including mistrustful feelings), external perception (reflects a misperception of an external stimulus or a change in the awareness of the subject's surroundings) and feeling high (Zuurman et al., 2008).

ADAPTIVE TRACKING

The adaptive tracking test will be performed as originally described by Borland and Nicholson (Borland and Nicholson, 1975; Van Steveninck AL, 1993), using customised equipment and software (based on TrackerUSB hard-/software (Hobbs, 2004, Hertfordshire, UK)). The average performance and the standard deviation of scores over a 3.5-minute period will be used for analysis. This 3.5-minute period is including a run in time of 0.5 minute, in this run in time the data is not recorded. Adaptive tracking is a pursuit-tracking task. A circle moves randomly about a screen. The subject must try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. Conversely, the velocity is reduced if the test subject cannot maintain the dot inside the circle.

TAPPING

The test has been adapted from the Halstead Reitan Test Battery (Andrew, 1977), and evaluates motor activation and fluency. The volunteer is instructed to tap as quickly as possible with the index finger and to rest the wrist on the table. The space bar is used as tapping device and each session contains five performances of 10 seconds. The mean tapping rate and the standard deviations for the dominant hand are used for statistical analysis.

PHARMACO-EEG

Pharmacoelectroencephalography (pharmaco-EEG) recordings were performed as previously described (de Haas et al., 2010). EEG recordings were made at Fz, Cz, Pz and Oz. For each lead, fast Fourier transform analysis was performed to obtain the sum of amplitudes in the delta, theta, alpha and beta frequency ranges. The duration of EEG measurements was 64 s per session. Change in amplitudes in the beta frequency band of the EEG is found to be a relevant measure of the pharmacological effect intensity of BZDs (Mandema et al., 1992).

VISUAL VERBAL LEARNING TESTS (VVLT)

Short term and long term memory was similarly tested as recently described in another publication (de Haas et al., 2009). The 30 word memory learning test was performed at 2 h post-dose with 'Immediate Recall' immediately hereafter. Approximately 4 h after start of the Immediate Recall test, 'Delayed Recall' was performed and followed by the 'Delayed Recognition'.

STATISTICAL ANALYSIS

PHARMACOKINETICS

The plasma PK parameter estimates were calculated in WinNonlin Version 5.2 (Pharsight Corporation, USA) using non compartmental analysis of the individual plasma concentrations of NS11821 and NS14606. The area under the curve (AUC) was calculated using the linear trapezoidal method. Terminal rate constants were estimated by fitting a linear regression of log concentration against time. Other parameters determined were: maximum plasma concentrations (C_{max}), time to maximum plasma concentration (T_{max}) and elimination half-life ($T_{1/2}$).

PHARMACODYNAMICS

The PD parameters were analyzed by mixed model analyses of covariance (ANCOVA) with treatment, time and treatment by time as fixed effects, with subject as random effect, and with the baseline value as covariate, where baseline was defined as the average of the available values obtained prior to dosing. The six NS11821 treatments (10 mg, 30 mg, 75 mg, 150 mg, 300 mg and 600 mg) were compared to placebo and used as contrasts within the ANCOVA model. The cognitive function test resulted in single measured pharmacodynamic data for which the fixed factor treatment ANOVA model was used. All variables were analyzed untransformed except for body sway and EEG results, which were log transformed prior to analysis to correct for the expected log normal distribution. Treatment effects were reported as contrasts where the average of the measurements up to last time point, were calculated within the statistical model. Contrasts were reported along with 95% confidence intervals (CIs) and analyses were two sided with a significance level of 0.05. All calculations were performed using SAS for windows V9.1.2 (SAS Institute, Inc., Cary, NC, USA).

Pre-study power calculation based on previous studies conducted at CHDR (de Haas et al., 2007; de Haas et al., 2008; de Haas et al., 2009) with lorazepam 2 mg, revealed that 6 subjects in each group, should provide 80% power, to detect a reduction in SPV of at least 50.3 degrees per second after active treatment compared to placebo, assuming a common standard deviation of 15.0 degrees per second using a two group t-test with a 5% two sided significance level. Similarly, seven subjects in each group should provide an 80% power to detect a difference of -11.2 mm in VAS-alertness assuming that the common standard deviation is 6.5 mm using a two group t-test with a two-sided significance level of 5%.

POST HOC COMPARISON WITH HISTORIC LORAZEPAM DATA SET

No active control, e.g. benzodiazepine, was used in this first in man study for NS11821. Such a control might have complicated the blinded effect assessments and decisions on dose escalations. Recently, we reported a comparative study of several different subtype selective GABA-A partial agonists with lorazepam, which had been used as a positive control in a number of trials that basically employed the same methods that were also used in this first-in-man study of NS11821 (Chen et al., 2012). The combined lorazepam data from this historic data set were used for a *post hoc* comparison of the pharmacodynamic effects of NS11821 with those of lorazepam.

In the *post hoc* analysis the relationship of individual changes from baseline against the change from baseline of SPV (ΔSPV) was done for: body sway (Δsway), tracking (Δtracking), VAS-alertness (ΔVAS-alertness), and smooth pursuit (Δsmooth). These data were then compared to the profile from a full, non-selective GABA-A agonist (Lorazepam 2 mg). The slopes of these regression lines are thought to reflect the relation between the effect profile of anxiolysis and sedation (Chen et al., 2012). A mixed effect model was used, where the fixed factors were treatment and treatment by SPV, whereas the random factors were subject slope and intercept. The estimate of the slopes of the regression lines of these ΔSPV-relative effect profiles were compared between three high doses of NS11821 (150 mg, 300 mg, 600 mg) and lorazepam.

RESULTS

SUBJECTS

Sixty seven male volunteers were medically screened after given written informed consent and forty eight were randomized. None of these subjects dropped out of the study; all randomized subjects completed all study days and had a follow up visit. On average (min-max) subjects were 23 (18-41) years old with a weight of 75 (57-101) kg, and a body mass index of 22.9 (18.1-29.7) kg/m^2. The mean body measures were generally comparable among the seven treatment groups, Table 1.

CLINICAL OBSERVATIONS / SAFETY

All forty-eight subjects were included in the safety analysis. No serious adverse events occurred during the study. Neither did subjects withdraw from the study due to adverse events. No clinically significant changes in vital signs or ECG

characteristics were noted for any of the NS11821-treated groups. The highest dose of NS11821 (600 mg) caused the most gastrointestinal and neurological events, with ≥50% subjects experiencing nausea, vomiting, fatigue, dizziness, and/or somnolence. The incidences of these AEs were higher than those reported in the placebo group and were all judged 'probably' treatment-related by the investigator. None of the AEs required medical intervention. Except for two AEs occurring in one subject dosed with NS11821 600 mg, all AEs were classified mild in intensity. This subject suffered from moderate dysphagia, nausea and subsequent vomiting, while trying to swallow the study medication (twelve capsules).

PHARMACOKINETICS

One subject in the 600 mg NS11821 dose group vomited shortly after taking the study medication and the pharmacokinetic data from this subject were excluded from the PK analysis. Figure 1 depicts the average plasma concentration-time curves for NS11821 and its metabolite NS14606 for all 6 doses. Table 2 summarizes the pharmacokinetic parameters of NS11821 and NS14606 by dose. The absorption time was dose dependent; T_{max} increase from 0.5 to 4.0 hr following doses of 10 to 600 mg. A more than proportional increase in exposure was seen; AUC_{inf}/dose increase with a factor 10 from 0.9 at 10 mg to 9.7 at 600 mg. There was considerable variability between subjects was seen for all the pharmacokinetic parameters.

PHARMACODYNAMICS

A total of three subjects, who were administered with NS11821 600 mg, vomited post-dose during performance of the PD tests. Their pharmacodynamics data were excluded from the PD datasets before the statistical analysis.

Compared to placebo, NS11821 600 mg elicited statistically significant effects on a variety of pharmacodynamic parameters, Table 3. At a dose level of 150 mg NS11821 affected EEG ß-band power of the frontal-central leads statistically significantly, a trend was seen at lower doses. NS11821 300 mg was associated with significant effects on SPV, tracking, and EEG ß-band power of the frontal-central leads and the parietal-occipital leads. NS11821 300 mg also reduced the frequency of tapping, with an effect size similar to NS11821 600 mg. No significant effects were observed on body sway or smooth pursuit with any dose of NS11821.

The effects of lorazepam 2 mg are also presented in Figure 2. These results were based on historic data for a number of studies with GABA-A subtype selective partial agonists, in which lorazepam 2 mg was used as a positive control (Xia-Chen et al. 2012). Effects of 2 mg lorazepam on SPV appear to be in line with the effects of higher doses of NS11821 (300-600 mg). Apart from the effect of 600 mg NS11821 on VAS-alertness, lorazepam effects on body sway and VAS-alertness clearly distinct from the effects observed from NS11821.

In addition to the above mentioned psychomotor effects, NS11821 reduced the response accuracy of immediate recall in a dose-dependent manner (600 mg: -7.2 words [-10.7; -3.7], p=0.0001; 300 mg: -3.0 [-5.7, -0.3], p=0.0305). Doses of 75-600 mg NS11821 decreased the number of delayed recalled words in a dose dependent manner without reaching statistical significance, while 75 and 300 mg of NS11821 impaired delayed recognition statistically significantly; 75mg: -5.6 [-9.7,-1.4], p=0.0095 and 300mg -5.2 [-9.4,-1.1], p=0.0143; respectively (Figure 1, Panel D). A historic comparison between the memory effects of lorazepam 2 mg and NS11821 300 mg and 600 mg was made with a previous study that used the same word memory test (De Haas et al. 2009). This analysis suggests that the average percentage decrease relative to placebo is in the same order of magnitude among lorazepam 2 mg, NS11821 300 mg and 600 mg, for immediate recall (-61%, -78% and -48%, resp.), delayed recall (-46%,-67% and -48%) and delayed recognition (-76%,-79% and -82%).

DISCUSSION

This study investigated the pharmacokinetic, pharmacodynamics as well as safety and tolerability profiles of NS11821 after single oral doses. As a novel subtype selective GABA-A agonist, NS11821 was administered from a lower-than-MED dose to a dose 6 times higher than its human equivalent dose of the NOAEL with a dose escalation factor between 2 and 3.

The systemic exposure of NS11821 (AUC and C_{max}) increased approximately by a factor 10 following administration of 10 to 600 mg, a considerable variability between subjects was seen, Coefficient of Variation (CV) for the AUC and C_{max} was more than 30%. The half-life increased with increasing dose levels. In addition, the time to maximum plasma concentration was dose dependent and increased with increasing dose levels. This complex absorption profile may be due to low solubility properties of the compound in gastric fluid.

NS14606, the hydroxyl metabolite of NS11821, accounts for 30-50% of the parent compound. The ratios of active metabolite to parent compound are generally similar among the six doses. As is shown in Figure 1, the plasma concentration of NS11821 and NS14606 underwent multi-phase declines after T_{max}. Taken together, all these factors contribute to the complex non-linear pharmacokinetic profile of NS11821/NS14606 pharmacokinetics. Future studies of NS11821 should explore additional formulations, as for example acidic solutions of NS11821 to investigate how solubility affects the bioavailability of NS11821.

The study did not show clear pharmacodynamic effects with the lower doses of NS11821 (i.e. 10 mg, 30 mg, and 75 mg). However, single doses of NS11821 150 mg and higher caused statistically significant effects on several pharmacodynamic parameters in a dose dependent manner. NS11821 600 mg demonstrated the most extensive and robust effects, with impairments on SPV, VAS-alertness, and adaptive

tracking, as well as increase on VAS internal, VAS feeling high and the spectrum power of EEG beta bands. However, this high dose required the administration of a large number of tablets, which was associated with nausea and vomiting in a substantial proportion of subjects. Consequently, the results were affected by low numbers of observations, and by adverse events. NS11821 300 mg showed smaller effects on SPV and adaptive tracking and did not differ from placebo on VAS internal perception or VAS-alertness. Studies with other GABA-A subtype selective partial agonists have suggested that effects on VAS-alertness and adaptive tracking are closely linked to GABA-A α_1 subunit modulation (de Haas et al., 2010, Chen et al., 2014, Chen et al., 2015), whereas SPV is primarily sensitive to drug effects on the GABA-A $\alpha_{2,3}$ subunits (Chen et al., 2012). NS11821 300 mg and NS11821 600 mg are both effective on SPV, indicating potential anxiolysis of the two doses.

GABA-A α_5-agonism is considered to be associated with the memory-impairing effects of lorazepam (Chen et al., 2014, Chen et al., 2015). NS11821 has comparable effect potency at the GABA-A α_5-subunit and the GABA-A $\alpha_{2,3}$ subunits. This could cause memory deficits with clinically anxiolytic doses of NS11821. This seems to be corroborated by a historic comparison with lorazepam 2 mg, which suggests that the two highest doses cause roughly similar memory deficits. However, the memory effects need to be interpreted with caution. In particular, dose-relatedness wasn't always very consistent. For instance, two doses (i.e. 75 mg and 300 mg) of NS11821 were linked to considerable reduction in delay word recall compared to placebo. The study also showed similar average responses between NS11821 10 mg and NS11821 300 mg in immediate recall, and demonstrated comparable responses among NS11821 10 mg, 150 mg and placebo in delay recall (Figure 2, Panel D). These findings support the contribution of inter-subject variation to the observation of inter-treatment differences in this parallel-group study. Consequently, quantitative conclusions about memory effects of NS11821 are currently not warranted, and more dedicated studies with larger sample size are needed to further investigate these effects.

One limitation of this study is the lack of positive control to confirm the sensitivity of the pharmacodynamic measurements and benchmark the effect size of NS11821 at different doses. To compensate this deficiency, we calculated the sample size on a power level of 80% based on previous studies with benzodiazepines (Chen et al., 2012, Chen et al., 2014, Chen et al., 2015) and compared the results of this study with those studies in the statistical analysis. Figure 3 provide further information regarding the ΔSPV-relative pharmacodynamic profiles. The three high doses of NS11821 (150 mg, 300 mg, 600 mg) showed similar flatness in the ΔSPV-Δlog(Sway) relation, the ΔSPV-ΔVAS-alertness relation, and the ΔSPV-Δsmooth relation. A certain reduction of SPV is accompanied with smaller change of body sway, VAS-alertness or smooth pursuit, compared to the ΔSPV-relative responses of these PD parameters after lorazepam 2 mg. For the relation between ΔSPV and ΔTracking, the slope of the regression line is marginally smaller with NS11821 300 mg versus lorazepam, but comparable between either 150 or 600 mg and lorazepam (Table 4). Normalized by the effect size on SPV, the ΔPD-ΔSPV relations compare the anxiolytic potency of a treatment versus its effect potency on one of other CNS regions. Based on the ΔPD-ΔSPV profiles of NS11821 at different doses, NS11821 300 mg showed the most prominent SPV effect against the other CNS-PD effects. As such, NS11821 300 mg is thought to be an effective anxiolytic dose with minimal off-target CNS-effects. With NS11821 150 mg, the compound only had marginal effect on saccadic peak velocity, and adaptive tracking performance was even better than during placebo at some time points. These observations may provide some explanation for the lack of significant difference between NS11821 150 mg and lorazepam 2 mg. When subjects are dosed with NS11821 300 mg, both α_1-containing receptors and $\alpha_{2,3,5}$-containing receptors are modulated, but pharmacological selectivity is preserved, causing minimal effects on body sway and VAS-alertness. Such SPV-relative pharmacodynamic effect profiles are distinct from those of a non-selective partial GABA-A agonist, where the slopes of the ΔPD-ΔSPV regression lines are theoretically comparable between the partial agonist and the full agonist. The effects of NS11821 600 mg seemed non-selective. These observations should be interpreted carefully since the occurrence of nausea and vomiting may have interfered with the pharmacodynamic measurements.

In conclusion, the effects of NS11821 on the pharmacodynamic biomarkers indicate entry of the compound into the central nervous system and a concentration dependent effect profile. The dose related effect of NS11821 on SPV suggests potential anxiolytic effect; while the minimal effects of NS11821 150 mg, 300 mg and 600 mg on subjective alertness, postural balance, psychomotor and cognitive functions imply reduced side effects of this compound. Pharmacological subtype selectivity is further confirmed by the response relation of various CNS pharmacodynamic biomarkers versus SPV as compared to lorazepam 2 mg. The absence of pharmacodynamic effect and the placebo-like AE profile seen with NS11821 10 mg, 30 mg, and 75 mg may be caused by low exposure of this compound. Single oral doses of NS11821 10-600 mg were safe and well tolerated in the healthy male participants of this study. Modifying the formulations of NS11821 may result better bioavailability and dose-proportionality.

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Table 1 · Summary of subject characteristics

Cohort	n	Age (yrs)*	BMI (kg/m^2)*	Height (m)*	Weight (kg)*
Placebo	12	21.8 (18-29)	22.1 (18.1-28.1)	1.81 (1.72-1.93)	72.7 (59.2-88.2)
1: 10 mg NS11821	6	25.2 (20-41)	23.8 (21.5-28.7)	1.83 (1.78-1.88)	79.7 (68.6-101.4)
2: 30 mg NS11821	6	26.2 (19-39)	24.3 (21.1-29.7)	1.78 (1.71-1.85)	77.6 (61.7-98.1)
3: 75 mg NS11821	6	22.5 (18-31)	22.9 (20.6-25.2)	1.80 (1.67-1.89)	74.5 (57.5-89.0)
4:150 mg NS11821	6	20.0 (19-22)	23.3 (20.8-25.5)	1.81 (1.76-1.87)	76.2 (68.4-89.2)
5:300 mg NS11821	6	20.3 (18-24)	23.0 (19.6-25.6)	1.83 (1.73-1.90)	76.5 (65.0-87.5)
6:600 mg NS11821	6	22.7 (18-26)	22.1 (19.2-24.9)	1.84 (1.77-1.92)	74.5 (64.5-83.0)

**mean (min-max)*

Table 2 · Summary of pharmacokinetic parameters of NS11821

Cohort	T_{max} (h)	C_{max} (ng/ml)	CV% for C_{max}	$C_{max/D}$	$T_{1/2}$* (h)	AUC_{inf} (h*ng/ml)	CV% for AUC_{inf}	AUC_{inf}/D	M/P Ratio	CL_R (l/h)
1: 10 mg	0.50	9.37	53	0.94	0.62	8.98	23	0.90	0.39	-
2: 30 mg	1.0	22.8	59	0.76	0.95	38.8	44	1.3	0.32	0.021
3: 75 mg	0.99	77.2	147	1.0	1.9	110	77	1.5	0.42	0.021
4:150 mg	0.75	236	111	1.6	3.2	419	90	2.8	0.31	0.0067
5:300 mg	2.5	477	49	1.6	5.0	1440	50	4.8	0.44	0.0061
6:600 mg	4.0	1430	83	2.4	4.4	5790	93	9.7	0.49	0.0046

*For C_{max}, AUC, CL_R the geometric mean values are presented, for T_{max} the median and for all other parameters the mean value is presented; *The half-life of the elimination phases;* M/P *ratio* = $AUC_{NS14606}/AUC_{NS11821}$

Table 3 · Pharmacodynamic effect on saccadic peak velocity, smooth pursuit, body sway, visual analogue scales and tapping

Variable	10 mg NS11821 -Placebo	30 mg NS11821 -Placebo	75 mg NS11821 -Placebo	150 mg NS11821 -Placebo	300 mg NS11821 -Placebo	600 mg NS11821 -Placebo
Saccadic peak velocity (deg/s)	-5.4 (-25.7; 14.9) p=0.5940	6.3 (-14.1; 26.7) p=0.5364	-7.8 (-28.1; 12.6) p=0.4439	-16.8 (-37.2; 3.5) p=0.1023	**-23.1 (-43.5; -2.7) p=0.0279**	**-41.4 (-68.7; -14.1) p=0.0040**
Smooth pursuit (%)	-0.6 (-4.1; 3.0) =0.7468	-2.3 (-5.9; 1.2) p=0.1916	-3.2 (-6.8; 0.4) p=0.0785	-0.5 (-4.0; 3.1) p=0.7853	-3.4 (-7.1; 0.2) p=0.0643	-1.1 (-5.7; 3.6) p=0.6458
Body Sway (mm)	11.05% (-9.78; 6.70) p=0.3133	7.44% (-12.5; 31.98) p=0.4842	19.14% (-2.99; 46.34) p=0.0926	8.11% (-12.5; 33.57) p=0.4599	15.84% (-5.84, 42.53) p=0.1590	24.68% (-5.24, 64.04) p=0.1119
VAS-Alertness (mm)	-0.2 (-1.9; 1.5) p=0.8047	-0.6 (-2.3; 1.1) p=0.4738	-1.2 (-2.9; 0.6) p=0.1830	-1.5 (-3.2; 0.2) p=0.0868	-0.5 (-2.3; 1.2) p=0.5422	**-3.6 (-5.8; -1.4) p=0.0023**
VAS Internal (log(mm))	0.001 (-.008; 0.009) p=0.9057	-.000 (-.009; 0.008) p=0.9283	0.005 (-.003; 0.013) p=0.2324	0.004 (-.004; 0.012) p=0.3510	0.002 (-.007; 0.010) p=0.6910	**0.012 (0.001; 0.023) p=0.0281**
VAS Feeling high (log(mm))	0.006 (-.067; 0.080) p=0.8636	0.000 (-.073; 0.073) p=1.0000	0.016 (-.057, 0.090) p=0.6573	0.047 (-.026; 0.121) p=0.1986	0.048 (-.025; 0.121) p=0.1941	**0.095 (0.000; 0.190) p=0.0491**
Adaptive tracking (%)	0.38 (-1.66; 2.43) p=0.7054	-0.49 (-2.58; 1.60) p=0.6363	-1.67 (-3.71; 0.37) p=0.1052	-1.47 (-3.53; 0.59) p=0.1565	**-2.97 (-5.00; -0.94) p=0.0054**	**-8.55 (-11.2; -5.93) p=<.0001**
Mean of 5 tapping trials (taps/10 sec)	-2.24 (-5.85; 1.38) p=0.2174	-1.57 (-5.02; 1.89) p=0.3641	2.16 (-1.27; 5.59) p=0.2097	1.16 (-2.32; 4.64) p=0.5039	**-3.60 (-7.05; -0.15) p=0.0413**	-3.07 (-7.55; 1.41) p=0.1734
Pharmaco-EEG EEG Beta Fz-Cz (uV)	3.59% (-6.09; 14.27) p=0.4711	8.36% (-1.51; 19.21) p=0.0968	6.78% (-2.91; 17.44) p=0.1708	**16.20% (5.58; 27.89) p=0.0030**	**16.79% (5.92; 28.78) p=0.0027**	**58.31% (38.41; 81.06) p=<.0001**
EEG Beta Pz-Oz (uV)	3.72% (-8.87; 18.05) p=0.5712	13.59% (-0.19; 29.27) p=0.0532	5.25% (-7.52; 19.79) p=0.4279	8.98% (-4.32; 24.13) p=0.1889	**15.20% (1.09; 31.29) p=0.0346**	**36.64% (14.52; 63.02) p=0.0010**

Differences in LSM values are shown for each contrasts with (95% CI) and P-value; LSM=Least square mean; CI=Confidence interval; NS=NS11821; PBO=placebo; SPV=Saccadic Peak Velocity; VAS=Visual Analogue Scale

Table 4 · Results of the linear model for Saccadic Peak Velocity change from baseline and other pharmacodynamic parameter change from baseline by treatment

Relation	Slope of NS11821	NS11821 vs. Lorazepam	P-value
ΔSmooth/ΔSPV			
NS11821 150 mg	0.01118	-0.09867	0.0449
NS11821 300 mg	0.02222	-0.08764	0.0680
NS11821 600 mg	0.03822	-0.07164	0.1321
ΔSway/ΔSPV			
NS11821 150 mg	-0.00084	0.00221	0.0276
NS11821 300 mg	0.000347	0.0034	0.0004
NS11821 600 mg	-0.00053	0.002525	0.0077
ΔVAS-alertness/ΔSPV			
NS11821 150 mg	0.03906	-0.08692	0.1285
NS11821 300 mg	-0.01465	-0.14062	0.0108
NS11821 600 mg	0.00655	-0.11942	0.0279
ΔTracking/ΔSPV			
NS11821 150 mg	0.04592	-0.01128	0.6112
NS11821 300 mg	0.01835	-0.03885	0.0681
NS11821 600 mg	0.04672	-0.01049	0.6112

Figure 1 · Mean (SD) drug concentrations profile of NS11821 (panel A) and NS14606 (panel B) following oral administration of 10-600 mg NS11821 (logarithmic scale).

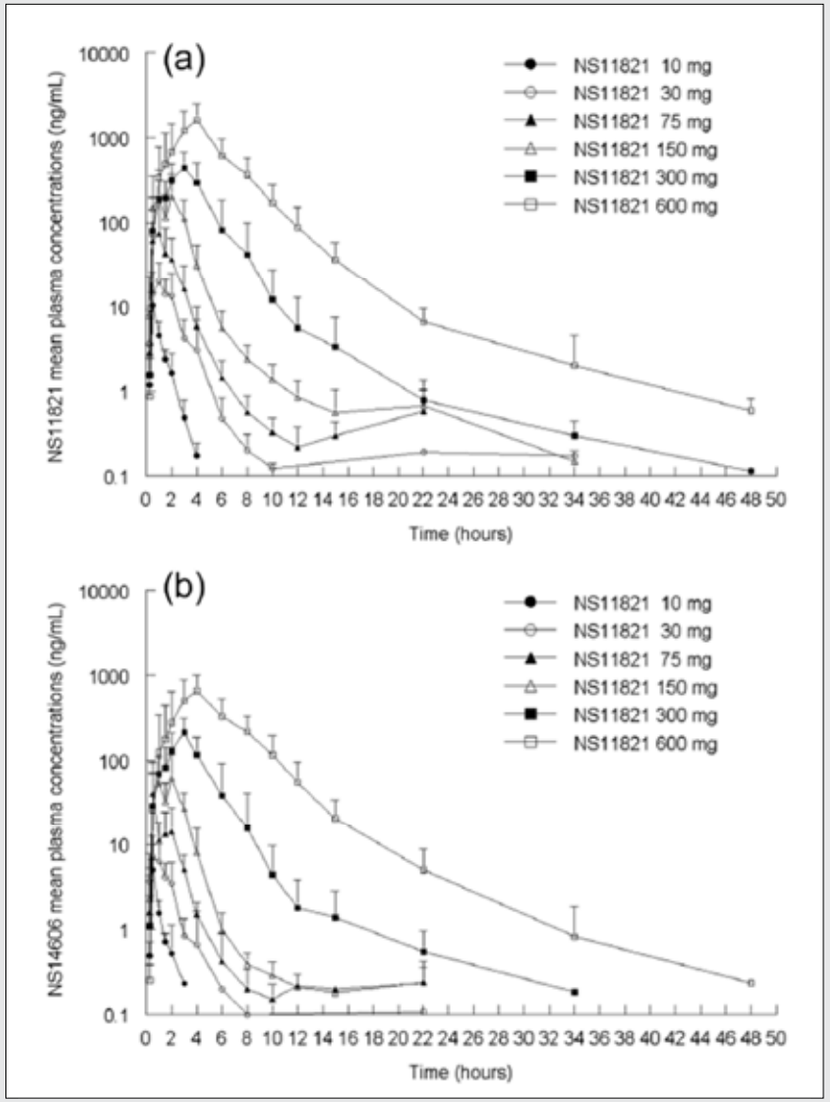
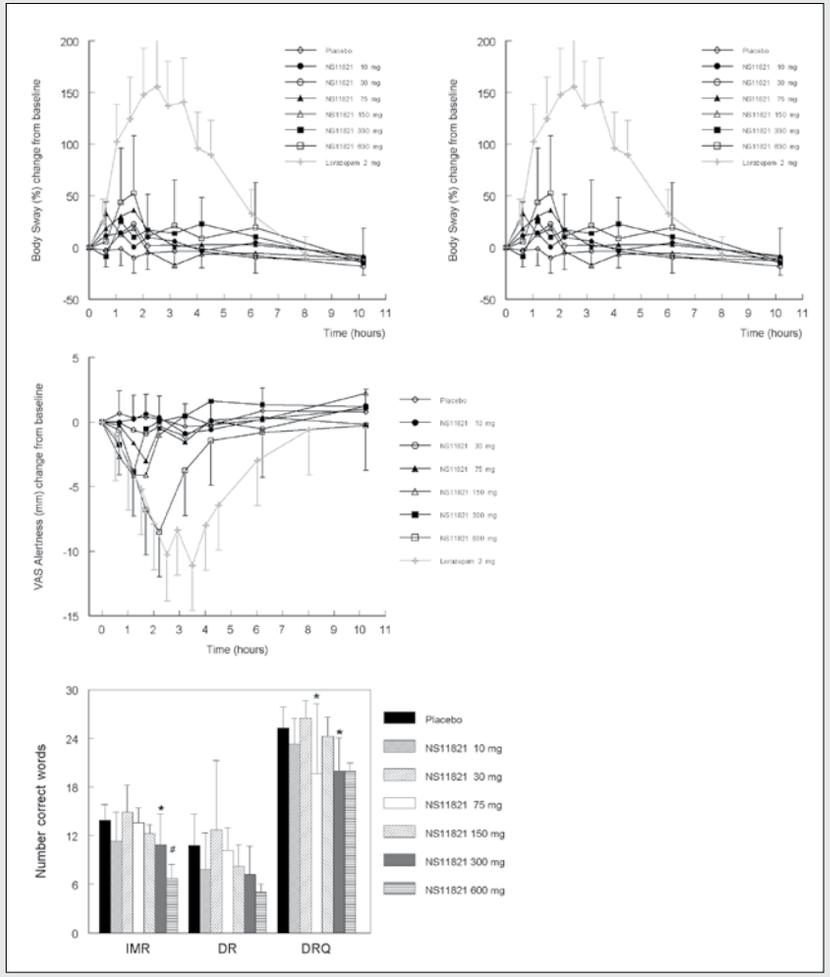
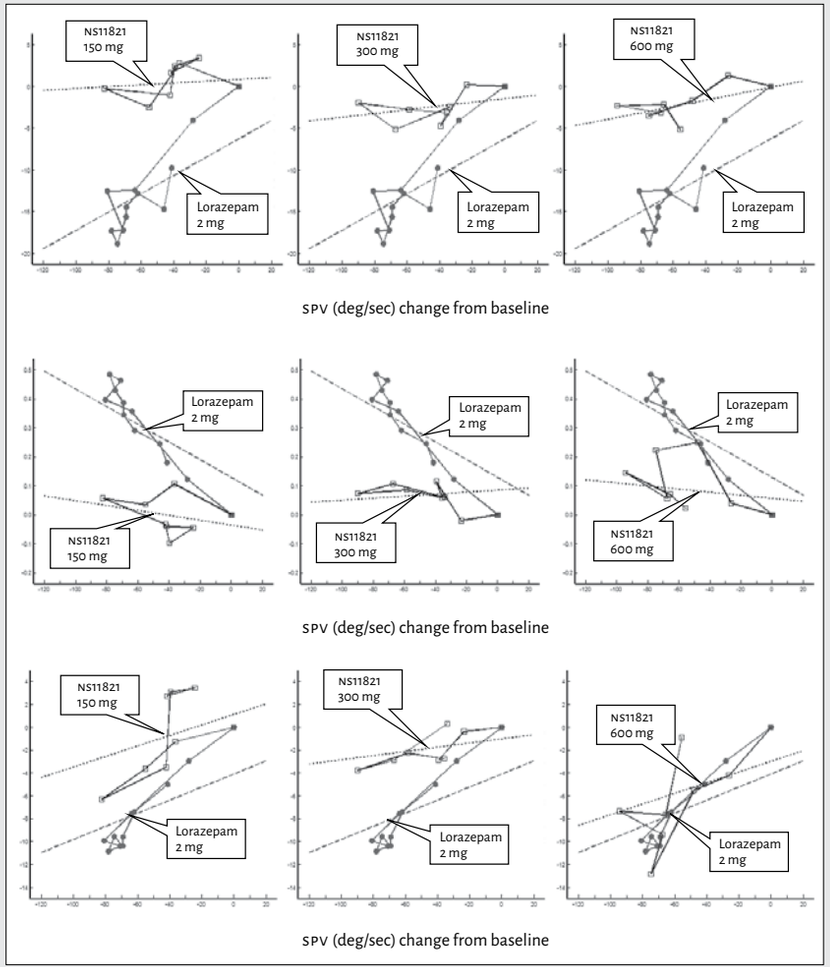


Figure 2 · Pharmacodynamic profile for NS11821 with additional historic lorazepam 2 mg data for: saccadic peak velocity (panel A), body sway (panel B), $VAS_{alertness}$ (panel C), and visual verbal learning test (panel D) (the y-axis presents the LSMs change from baseline profile with 95% CI error bars)



$p < 0.05$ and # $p = 0.001$: comparing NS11821 to placebo. IMR=Immediate Recall, DR= Delayed Recall and DRQ= Delayed Recognition for number of correct words

Figure 3. ΔPD-ΔSPV relative effect profile of NS11821 150 mg, NS11821 300 mg, and NS11821 600 mg vs. lorazepam 2 mg, respectively.



V

AN OVERVIEW OF THE CNS-PHARMACODYNAMIC PROFILES OF NON-SELECTIVE AND SELECTIVE GABA AGONISTS

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Chen Xia[1,2], Sanne de Haas[2], Marieke de Kam[2], Joop van Gerven[2]

1. Clinical Pharmacological Research Center (CPRC), Peking Union Medical College Hospital, Beijing, China | 2. Centre for Human Drug Research (CHDR), Leiden, The Netherlands

ABSTRACT

Various $\alpha_{2,3}$ subtype selective partial GABA-A agonists are in development to treat anxiety disorders. These compounds are expected to be anxiolytic with fewer undesirable side effects, compared to nonselective GABA-A agonists like benzodiazepines. Several $\alpha_{2,3}$ subtype selective and nonselective GABA-A agonists have been examined in healthy volunteers, using a battery addressing different brain domains. Data from five placebo-controlled double-blind studies were pooled. Lorazepam 2mg was the comparator in three studies. Three $\alpha_{2,3}$-selective GABAA agonists (i.e., TPA023, TPACMP2, SL65.1498), one α1-selective $GABA_A$ agonists (zolpidem), and another full agonist (alprazolam) were examined. Pharmacological selectivity was assessed by determination of regressionlines for the change from baseline of saccadic-peak-velocity-(ΔSPV-)relative effect,relative to changes in different pharmacodynamic endpoints (ΔPD). SPV was chosen for its sensitivity to the anxiolysis of benzodiazepines. Slopes of the ΔSPV-ΔPD relations were consistently lower with the $\alpha_{2,3}$ selective GABA-A agonists than with lorazepam, indicating that their PD effects are less than their SPV-effects. The ΔSPV-ΔPD relations of lorazepam were comparable to alprazolam. Zolpidem showed relatively higher impairments in ΔPD relative to ΔSPV, but did not significantly differ from lorazepam. These PD results support the pharmacological selectivity of the $\alpha_{2,3}$-selective GABA-A agonists, implying an improved therapeutic window.

INTRODUCTION

Anxiety is a *psychological* and *physiological* state with *somatic*, *emotional*, *cognitive*, and *behavioral* components [1], which dominates thinking and leads to disturbance of daily functioning. Serotonergic antidepressants, either selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs), are currently prescribed as the 1st-line treatment for several anxiety disorders. However, the slow onset of therapeutic effect and the presence of sexual side-effects prevent these drugs from more extensive use and lead to lack of treatment compliance [2]. Moreover, SSRIs/SNRIs cause transient increase of anxiety during the first few weeks of administration. All these clinical experiences provide space for the use of benzodiazepines (BZDs) in acute anxiety episodes.

Benzodiazepines are the most commonly prescribed anxiolytic drugs, although treatment guidelines generally limit their use to several weeks to prevent the occurrence of tolerance and dependence. Benzodiazepines are allosteric modulators of the GABA-A receptors that affect the central nervous system (CNS) as full GABAergic agonists [3]. As a consequence, these drugs have detrimental effects on alertness, memory, postural stability and muscle tone. In loss-of-function studies conducted in point-mutated mice [4], different subtypes of GABA-A receptors have been found responsible for the specific aspects of benzodiazepine pharmacology: 1) α_1-containing receptors are associated with sedative effects of benzodiazepines [5,6]; 2) α_2/α_3-containing receptors are related to anxiolysis and analgesia [7,8]; and 3) α_5-receptors are associated with cognition [9,10]. BZDs exert their CNS actions in a concentration-related manner [11]. The anxiolytic, hypnotic, muscle relaxant, and *amnesic* effects of BZDs generally appear concomitantly, and the onset and duration of action of the compounds correlates closely with their pharmacokinetic properties. The effect profile of BZDs has been attributed to their non-selective agonism at the α_1, α_2, α_3, and α_5 subunit-containing GABA-A receptors. To improve the pharmacological and functional selectivity, novel GABAergic anxioselective compounds are evaluated using recombinant human GABA-A receptors during preclinical development. The GABAergic effect profile of a compound is characterized by the affinity of the ligand for the receptor, and by the *in vitro* efficacy of the compound at each GABA-A receptor subtype. In the past years, several partial GABA-A-agonists have been developed, which have a relatively high *in vitro* efficacy at α_2/α_3 subtypes compared with α_1 or α_5 subtypes. Such α_2/α_3 subtype-selective partial GABA agonists are anticipated to have favorable therapeutic effect and to be less sedating or cognition-impairing (Table 1).

Based on non-clinical investigations with *in vitro* assays and animal models of anxiety, the human pharmacology of novel GABAergic agents is approached through sequential clinical studies regarding pharmacokinetics, receptor occupancy, and pharmacodynamics (PD) in healthy volunteers. Direct links have been

proposed between plasma drug concentration and receptor occupancy [4], as well as between plasma drug concentration and pharmacodynamic parameters [12,13,14,16]. Such pharmacokinetic/pharmacodynamic (PK/PD) relationships warrant the assessment of surrogate biomarkers in healthy volunteers treated with single doses of selective novel GABAergic compound(s).

More than 170 pharmacodynamic tests or test variants have been developed to assess the CNS effects of benzodiazepines [11]. De Visser et al analyzed the inter-study consistence, sensitivity, and pharmacological specificity of the frequently used biomarkers. Saccadic peak velocity (SPV) and visual analogue scale of alertness ($VAS_{alertness}$) were identified as the most sensitive parameters for benzodiazepines. Both tests showed consistent effects to a variety of benzodiazepines at different doses.

During the past fifteen years, the Centre for Human Drug Research (CHDR) has established a selection of computerized neuropsycho-pharmacodynamic tests called the Neurocart battery. The components of this battery target a variety of neurophysiological and/or neuropsychological domains (Table 2). Of this battery, adaptive tracking, saccadic eye movements and body sway was proved sensitive to the sedating effects of sleep deprivation [15], as well as benzodiazepines and other GABAergic drugs. In the recent years, the Neurocart battery was used in a series of phase I studies to assess CNS pharmacodynamics of partial $\alpha_{2,3}$ subtype selective GABA-A agonists. Both non-selective and/or selective GABA-A agonists were administered as single oral dose to healthy volunteers. Clear distinctions of effect profile were observed in these trials [12,13,14]. The objective of this manuscript was to characterize the pharmacodynamic effect profiles of novel anxioselective GABA-A agonists and identify suitable biomarkers to distinguish $\alpha_{2,3}$ subtype-specific GABA-A agonists from full GABA-A -agonists like benzodiazepines.

METHODS

Five clinical studies, all of which are published [12,13,14,16,31], were conducted at the CHDR in healthy volunteers after approval from the Ethics Review Board of Leiden University Medical Centre. All subjects provided written inform consent for study participation. Each trial was designed as single-dose, cross-over or parallel-armed, randomized, double-blind, placebo- and/or positive-controlled study. The subjects took single oral doses of a selective GABAergic compound, placebo, and/or a non-selective benzodiazepine. Three studies used lorazepam 2 mg as a positive control, whereas in the studies with zolpidem 10 mg and alprazolam 1 mg, these drugs were the only GABAergic study medications. Data of all studies came from the same research center and were pooled from the studies-specific electronic databases kept by the center. *In vitro* pharmacological parameters of novel compounds were extracted from the Investigator's Brochures and published articles. These parameters provide reliable information about the subtype selectivity of each compound, but it is more difficult to compare the pharmacological properties between the drugs. Due to the diversity of cell types and GABA-A receptor homologies used in the whole-cell patch clamping assays, the links between *in vitro* pharmacology and human *in vivo* effects are considered less quantitative and semi-quantitative comparisons are preferred.

TREATMENTS

Three novel drugs designed to be $\alpha_{2,3}$ subtype-selective were dosed in three of the abovementioned studies (for each dose group, the number of study participants is provided in parentheses): TPA023 0.5 mg, 1.5 mg (n=12)[12]; TPACPM2 (MK0343) 0.25 mg, 0.75 mg (n=12)[13]; SL65.1498 2.5 mg, 7.5 mg, and 25 mg (n=20)[14]. Zolpidem is a hypnotic with a high affinity for α_1-subtypes, and alprazolam is a nonselective GABAergic anxiolytic. Zolpidem 10 mg (N=14)[16] and alprazolam 1 mg (N=20) were administered in another two studies, respectively.

PHARMACODYNAMIC ASSESSMENTS

SACCADIC EYE MOVEMENT

Saccadic eye movements are very sensitive to a variety of mostly CNS-depressant drugs [17;18]. Saccadic peak velocity has been shown to be closely related to the anxiolytic properties of benzodiazepines [4]. Since partial $\alpha_{2,3}$ subtype selective GABA-A-agonists are developed to be anxiolytic, it was expected that these compounds would reduce saccadic peak velocity, similar to what is typically observed with benzodiazepines. Therefore, saccadic peak velocity was used as a biomarker for the anxiolytic properties of the GABA-A agonists, to which all other pharmacodynamics effects were compared in this meta-analysis. Recording and analysis of saccadic eye movements was conducted with a microcomputer-based system for sampling and analysis of eye movements. The program for signal collection and the AD-converter was from Cambridge Electronic Design (CED Ltd., Cambridge, UK), the amplifiers were supplied by either Nihon Kohden (Nihon Kohden, Life Scope EC, Tokyo, Japan) or Grass (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA) and the sampling and analysis scripts were developed at CHDR (Leiden, the Netherlands).

SMOOTH PURSUIT

The same systems as used for saccadic eye movements were also used for measuring smooth pursuit. For smooth pursuit eye movements, the target moves sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, in steps of 0.1 Hz. The amplitude of target

displacement corresponds to 22.5 degrees eyeball rotation to both sides. Four cycles were recorded for each stimulus frequency. The method has been validated at CHDR by van Steveninck based on the work of Bittencourt [19] and the original description of Baloh [20].

VISUAL ANALOGUE SCALES (VAS)

Visual analogue scales as originally described by Norris [21] were used previously to quantify subjective effects of benzodiazepines [18]. From the set of sixteen scales three composite factors were derived as described by Bond and Lader [22], corresponding to alertness, mood and calmness. These factors were used to quantify subjective drug effects [23].

BODY SWAY

The body sway meter measures of body movements in a single plane, providing a measure of postural stability. Body sway was measured with an apparatus similar to the Wright ataxiameter, which integrates the amplitude of unidirectional body movement transferred through a string attached to the subject's waist. Two minute measurements were made in the antero-posterior direction with eyes open and closed, with the subject standing comfortably on a firm surface with their feet slightly apart. The method has been used before to demonstrate postural instability due to benzodiazepines [24,25].

ADAPTIVE TRACKING

The adaptive tracking test as developed by Hobbs & Strutt was used, according to specifications of Borland and Nicholson [27]. The adaptive tracking test is a pursuit-tracking task. A circle of known dimensions moves randomly across a screen. The test subject must try to keep a dot inside the moving circle by operating a joystick. If this effort was successful, the speed of the moving circle increases. Conversely, the velocity was reduced if the test subject cannot maintain the dot inside the circle. The adaptive tracking test is a measure of visuomotor coordination that has proved to be very sensitive of various psychoactive drugs [26]. Table 3 summarizes the pharmacodynamic tests used in the different studies.

STATISTICAL ANALYSIS

Individual graphs are generated for each pharmacodynamic variable (y-axis) versus SPV change from baseline (x-axis). Summary graphs are generated with Lorazepam and one other treatment per graph, for all GABAergic treatments.

A regression analysis of change from baseline of body sway (Δsway), tracking (ΔTrack), VAS alertness ($\Delta VAS_{alertness}$), or VAS calmness ($\Delta VAS_{calmness}$) against the change from baseline of SPV (ΔSPV) was performed with a mixed effect model on the available individual data. The fixed factor was the GABAergic treatment and treatment by saccadic peak velocity, while the random factors were subject slope and intercept. The values of body sway were analyzed after log-transformation, while the other parameters were taken without transformation. The estimate of the slopes of the linear relations of these ΔSPV-relative effect profiles were compared between each dose of subtype-selective GABA-A agonists and lorazepam. The estimates of slopes, their estimated difference and the p-values were tabulated. Thereafter, summary plots were generated, combined with the population regression line as calculated in the regression.

All statistical analyses were carried out with SAS for Windows v9.1.3 (SAS institute, inc., Cary, NC, USA).

RESULTS

ΔSPV-ΔSWAY RELATION (Δ=CHANGE FROM BASELINE)

Average changes from baseline of body sway against SPV within the investigational time course (i.e. 6 hours post-dose) were plotted by study. Figure 1 demonstrates clear distinctions between the ΔSPV-relative effect profile of lorazepam 2 mg and most doses of the $\alpha_{2,3}$-subtype selective compounds (i.e. TPA023 1.5 mg, TPACMP2 0.75 mg). The full GABA-A agonist alprazolam is similar to lorazepam. The slope of the ΔSPV-Δsway plots for zolpidem is slightly steeper than for lorazepam.

As was revealed by the statistical analysis using the mixed linear model (Table 4), the estimated differences of the slope of regression lines are statistically significant between lorazepam and the $\alpha_{2,3}$ subtype selective partial GABAergic treatment of TPA023 1.5 mg, TPACMP2 0.75 mg, and SL65.1498 25 mg. There is no statistically significant difference between the slopes for lorazepam and alprazolam, and the difference with zolpidem suggested by the average plots (Figure 1) is not confirmed by the model (Table 4).

ΔSPV-$\Delta VAS_{ALERTNESS}$ RELATION

Figure 2 plots the average values of $\Delta VAS_{alertness}$ vs. ΔSPV obtained from individual subjects per study. As was found for the ΔSPV-Δsway relations, a similar difference to lorazepam was observed with novel subtype selective GABAergic compounds. The slopes of the regression line of the ΔSPV-Δsway relation for TPA023 1.5 mg, and SL65.1498 25 mg are statistically shallower than the slope for lorazepam, respectively. No statistical differences can be demonstrated for TPACMP2 0.75 mg, alprazolam 1 mg, or zolpidem 10 mg.

ΔSPV-ΔSMOOTH RELATION

Figure 3 and Table 4 provide the ΔSPV-relative effect profiles and the slopes and intercept for smooth pursuit after alprazolam, zolpidem, and SL65.1498. Smooth

pursuit was not determined with the other partial agonists. Statistically significant differences is found in the slope of regression lines with SL65.2498 25 mg. Zolpidem and alprazolam show comparable slopes to lorazepam.

ΔSPV-ΔPD relations vs in vitro pharmacological properties

This analysis surmises that comparisons of ΔSPV-ΔPD profiles represent the underlying pharmacological characteristics of subtype selective and non-selective GABA-A agonists. A further corroboration of this approach could be provided by a comparison of ΔSPV-ΔPD profiles with the underlying pharmacological properties. This should be possible in principle, but the quantitative preclinical information provided in Table 1 was derived from different sources which in themselves were incomparable, despite the fact that all programs used oocyte-clamp assays to characterize the different GABAergic compounds. Some of these differences could be diminished by calculation of the ratio of relative efficacy on the α_1 GABA-A subunit to that on the α_2 subunit, as a benchmark of α_2-specificity of the GABAergic compounds. This calculated ratio is provided in Table 1. Although the number of compounds in this overview is too small for any meaningful statistical evaluation, it is interesting that the four compounds for which this could be calculated, showed a close relationship between α_1/α_2-efficacy ratio's and ΔSPV-ΔVAS alertness ratio's with borderline statistical significance (r^2=0.86, two-sided p=0.0727). Due to the absence of *in vitro* pharmacological data and the difference of experimental settings of the trail with alprazolam, alprazolam was not included into the present analysis.

DISCUSSION

This analysis was performed to explore the Central Nervous System (CNS) effects of various GABAergic agents and characterize the pharmacodynamic effect profiles of these compounds in healthy volunteers and correlate such profiles to their pharmacological properties.

A battery of CNS pharmacodynamic tests was administered to healthy volunteers who were dosed with GABAergic compound(s). The composition of the CNS battery was based on the sensitivity of the measurements to non-selective GABAergic treatments, and on the coverage of a wide range of different CNS domains (Table 2). This approach enabled us to identify unique effect profiles for pharmacologically distinct GABAergic treatments, including 1) traditional, pharmacologically non-selective, full GABAergic compounds at their clinical dose(s) (i.e. lorazepam 2 mg and alprazolam 1 mg), 2) a marketed GABAergic compound with high α_1-subtype affinity (i.e. zolpidem 10 mg), and 3) several novel, $\alpha_{2,3}$-subtype selective GABAergic compounds at different investigational doses.

The new class of partial subtype selective GABA agonists were expected to be anxiolytic but less sedating and cognition-impairing, as indicated by the preclinical *in vitro* and *in vivo* data. The anxiolytic effects of non-selective GABAergic agonists are accompanied by somnolence, impaired locomotion, and cognitive disturbance. These clinical side-effects are reflected by the pharmacodynamics effects of lorazepam or alprazolam on $VAS_{alertness}$ (measure of subjective sedation), body sway (measure of postural instability) and adaptive tracking (measure of visuo-motor coordination). Memory testing was not performed frequently and consistently enough to allow a comparative analysis among the different compounds. However, the original publication of the TPA023-study provides indications that the partial subtype selective GABA agonist has fewer cognitive effects than the partial subtype selective GABA agonist. In this study, lorazepam 2 mg showed clear memory reductions, which did not occur with a dose of TPA023 1.5 mg that caused comparable SPV reductions [12].

Saccadic peak velocity (SPV) has previously been shown to be closely related to the anxiolytic doses of benzodiazepines [11], and SPV was therefore used as a reference parameter. As expected, SPV showed significant responses to almost every GABAergic compound investigated in these six studies [12,13,14]. In contrast to lorazepam or alprazolam, which influenced each output parameter of the saccadic eye movement test (i.e. SPV, Saccadic reaction time, and inaccuracy), the α_1-(zopidem) or $\alpha_{2,3}$-subtype selective GABAergic compounds (TPA023, TPACMP2, SL65.1498) only affected SPV.

At their highest investigational dose, the effect size of TPA023 and TPACMP2 on SPV was comparable to the effects observed with lorazepam or alprazolam; whereas the effect of SL65.1498 was only marginally significant on SPV. In almost all these cases, the impact on other CNS effects was lower. This by itself is an indication of pharmacological selectivity, but a comparison based merely on overall or maximum effects could obscure some of the more subtle pharmacological differences (like the findings of SL65.1498 study) when the pharmacodynamic biomarker is less sensitive to the drug or if the dose of a drug is subtherapeutic. The relationships between the ΔSPV effects and other pharmacodynamic (ΔPD) effects provide a complete profile of the differential effects, at each time point after drug administration. These outputs reflect the degree of $\alpha_{2,3}$ selectivity, and may therefore also be indicators for anxioselectivity. Based on these perceptions, a GABAergic compound with 'flat' regression lines in the ΔSPV-relative plotting graphs would show anxiolysis with reduced off-target effects in clinical settings. For most of the novel compounds described in this overview, there are no clinical reports of anxiolytic effects or improved tolerability. However, a recent article on TPA023, the oldest compound in this meta-analysis, reported reduced anxiety in a preliminary clinical trial at doses that were also used in our pharmacodynamic studies [4]. No detailed comparative information is available on the therapeutic window in these clinical trials. We found that the ΔSPV-relative effect profiles of $\alpha_{2,3}$ subtype-specific GABAergic compounds are similar among each other, but different from lorazepam 2 mg. The

absolute slopes of the regression lines for the ΔSPV-ΔPD relations are generally lower with the selective GABA-A agonists than with the benzodiazepines. The results of alprazolam were comparable to lorazepam, which provides additional confidence that the analyses reflect pharmacological differences as well as similarities. Zolpidem seemed to be the only major exception, since this α_1 subtype-selective GABAergic compound produced considerably steeper average slopes for certain ΔSPV-relative profiles than lorazepam or alprazolam; whereas the statistical population model did not reveal statistically significant differences between zolpidem and the benzodiazepines. This could reflect a limitation of the population model for ΔSPV-ΔPD relationships, which was chosen to be simple and unbiased, but necessarily had to ignore some rather complex individual response relationships. The analyses were based on linear slope estimates without a fixed intercept. In reality however, all individual data points started at a fixed intercept (at T=0, when ΔSPV and ΔPD were both zero), and in many cases the ΔSPV-ΔPD relationships were not linear, and zolpidem even formed loops when the SPV effect displayed a different time-course than the PD effect. In almost all other cases however, the statistical analyses and the graphical representations of the average relationships provide accurate representations of the individual plots.

This meta-analysis indicates that comparisons of ΔSPV-ΔPD profiles are able to identify pharmacological differences between subtype selective and non-selective GABA-A agonists. A comparison of ΔSPV-ΔPD profiles with the underlying pharmacological properties was refuted by the very small number of compounds for which this could be compared. Nonetheless, strong relationships (with an R-value of 0.93) between the α_1/α_2-ratio's of the four compounds for which this could be determined, and their ΔSPV-$\Delta VAS_{alertness}$ ratios. Clearly this remains to be confirmed with larger numbers of compounds. Still, the consistent ΔSPV-relative profiles of the selective GABAergic compounds suggest potential links between the preclinical profiles and the ΔSPV-relative pharmacodynamics profiles of these compounds. Moreover, TPACMP2 showed a distinct ΔSPV-$\Delta VAS_{alertness}$ relation but shared a similar ΔSPV-Δsway relation with the other $\alpha_{2,3}$ subtype-selective GABAergic agonists. The relatively large amount of sedation with TPACMP2 could reflect the relatively high ratio of α_1/α_2-efficacy of TPMCMP2 compared to the other compounds. Similarly, the large efficacy of zolpidem is compatible with its steep ΔSPV-$\Delta VAS_{alertness}$ ratio and the strong hypnosedative effect of this z-hypnotic in the clinic.

CONCLUSION

TPA023, TPACMP2, and SL65.1498 are members of the novel experimental drug family of $\alpha_{2,3}$-subtype selective receptor agonists. *In vitro* pharmacological properties of these compounds indicate higher binding affinity and relative efficacy at the $\alpha_{2,3}$-subunits. *In vivo* preclinical studies with animal models translated such pharmacological properties into potential of anxiolysis and relatively reduced off-target effects in comparison with non-selective full GABAergic agonists like benzodiazepines.

The Neurocart battery is a collection of validated tests amenable to the effects of various CNS-acting drugs. Components of this battery were shown to be sensitive to different rapid-onset CNS-effects of the benzodiazepines, in which reduction of saccadic peak velocity displays features of a GABAergic anxiolytic biomarker, whereas impairments of body sway, adaptive tracking, and memory are translated to effects that are less desirable for an anxiolytic drug. Most novel GABAergic compounds showed dose-dependent responses to saccadic peak velocity, but did not affect the other CNS-effects to the same extent, indicative of the pharmaco-selectivity of these new compounds. Moreover, the ΔSPV-relative effect profiles provide information about dose potency and effect specificity. This battery is suitable to not only present the general depressive effects of benzodiazepines but demonstrate the pharmacological selectivity and specificity of the novel GABAergic compounds. Comparative effect profiling as used in these studies can provide clear indications for the pharmacological selectivity and specificity of novel GABAergic compounds in healthy volunteers. This is a valuable approach for the early drug development of this new drug class, which will hopefully contribute novel anxiolytics with an improved therapeutic window to patients with anxiety disorders.

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Table 1 · In vitro pharmacological properties of the GABAergic compounds

	α_1		α_2		α_3		α_5		$\alpha_1/\alpha_{2\text{-ratio}}$
Compound	**K_i (nM)**	**Efficacy° (%)**	**K_i (nM)**	**Efficacy° (%)**	**K_i (nM)**	**Efficacy° (%)**	**K_i (nM)**	**Efficacy° (%)**	
TPA023*[27]	0.27	0#	0.31	11	0.19	21	0.41	5	0
TPACMP2*[13]	0.22	18	0.40	23	0.21	45	0.23	18	0.78
SL65.1498#[28]	17	45	73	115	80	83	215	48	0.39
Zolpidem	20[30]	75§[29]	400[30]	78§[29]	400[30]	80§	5000[30]	9§[29]	0.96

*° Relative efficacy is defined as the extent of the potentiation of GABA-A EC_{20}-equivalent current produced by the compound compared to that produced by a non-selective full-agonist (chlordiazepoxide/diazepam); * Mean values of 3 experiments in Xenopus oocytes with human recombinant $\alpha\beta_3\alpha_2$ receptors; efficacy relative to chlordiazepoxide; # Mean values of 3 experiments in HEK293 cells with recombinant rat receptors $\alpha\beta_2\alpha_2$; efficacy relative to chlordiazepoxide; § Mean values of 3 experiments in Xenopus oocytes with human recombinant $\alpha\beta_2\alpha_2$ receptor; efficacy relative to diazepam*

Table 2 · Component tests of the Neurocart battery and the related CNS domains

Neurocart test	Targeted function	Related CNS areas
Saccadic eye movement	Neurophysiologic function	Superior colliculus, Substantia Nigra, amygdala
Smooth pursuit	Neurophysiologic function	Midbrain
Adaptive tracking	Visuo-motor coordination	Neocortex, basal nuclei, brain stem, cerebellum
Body sway	Balance	Cerebellum, brain stem
Visual verbal learning test (VVLT)	Memory	Hippocampus
VAS Bond and Lader	Alertness, mood, calmness	Cortex, prefrontal cortex
VAS Bowdle	Feeling high, internal and external perception	Cortex, prefrontal cortex, amygdala

Table 3 · Use of pharmacodynamic tests in each study

Study	CHDR99112	CHDR0102	CHDR0105	CHDR0614	CHDR0407
Compound	**TPA023**	**TPACMP2**	**SL65.1498**	**Alprazolam**	**Zolpidem**
Comparator	**Lorazepam**	**Lorazepam**	**Lorazepam**	**NA**	**NA**
SEM	Done	Done	Done	Done	Done
Sway	Done	Done	Done	Done	Done
VASBL	Done	Done	Done	Done	Done
Smooth	ND	ND	Done	Done	Done
Track	ND	ND	ND	Done	Done

ND=Not Done; NA=Not Applicable; SEM= Saccadic eye movement; Smooth=Smooth pursuit; Sway=Body sway; VAS BL=VAS Bond and Lader; Track= Adaptive tracking;

Table 4 · Results of the linear model for Saccadic Peak Velocity change from baseline and Log Body sway change from baseline by treatment with treatment by spv change from baseline as interaction

Treatment	ΔSPV-relative relation	Item	Estimate of treatment	Estimate of Lorazepam	P-value
TPA023 1.5 mg	ΔSway-ΔSPV	Slope	-0.00048	-0.00305	<0.0001
		Intercept	-0.01316	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.03312	0.126	0.0001
		Intercept	0.4551	-4.4739	0.0021
TPACMP2 0.75 mg	ΔSway-ΔSPV	Slope	-0.00027	-0.00305	<0.0001
		Intercept	0.03784	0.1292	0.0009
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.09884	0.126	0.2525
		Intercept	-1.4465	-4.4739	0.0397
SL65.1498 25 mg	ΔSway-ΔSPV	Slope	-0.00128	-0.00305	0.0003
		Intercept	0.0222	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.04193	0.126	0.0009
		Intercept	0.2453	-4.4739	<0.0001
	ΔSmooth-ΔSPV	Slope	0.01554	0.1099	<0.0001
		Intercept	-1.4483	-6.2553	<0.0001
Alprazolam 1 mg	ΔSway-ΔSPV	Slope	-0.00204	-0.00305	0.0667
		Intercept	0.001788	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.0734	0.126	0.0763
		Intercept	-0.628	-4.4739	0.0254
	ΔTrack-ΔSPV	Slope	0.0747	0.0572	0.1545
		Intercept	0.3023	-4.0742	<0.0001
	ΔSmooth-ΔSPV	Slope	0.08077	0.1099	0.2808
		Intercept	-1.4025	-6.2553	0.0002
Zolpidem 10 mg	ΔSway-ΔSPV	Slope	-0.0033	-0.00305	0.7336
		Intercept	0.06014	0.1292	0.0127
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.1526	0.126	0.5231
		Intercept	-3.2697	-4.4739	0.5219
	ΔTrack-ΔSPV	Slope	0.0489	0.0572	0.6240
		Intercept	-0.9123	-4.0742	<0.0001
	ΔSmooth-ΔSPV	Slope	0.09771	0.1099	0.7412
		Intercept	-3.8439	-6.2553	0.0815

Figure 1 · ΔLogSway (log mm)-ΔSPV (deg/sec) relative effect profile of TPA0231.5 mg, TPACMP2 0.75 mg, SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg vs. lorazepam 2 mg, respectively.

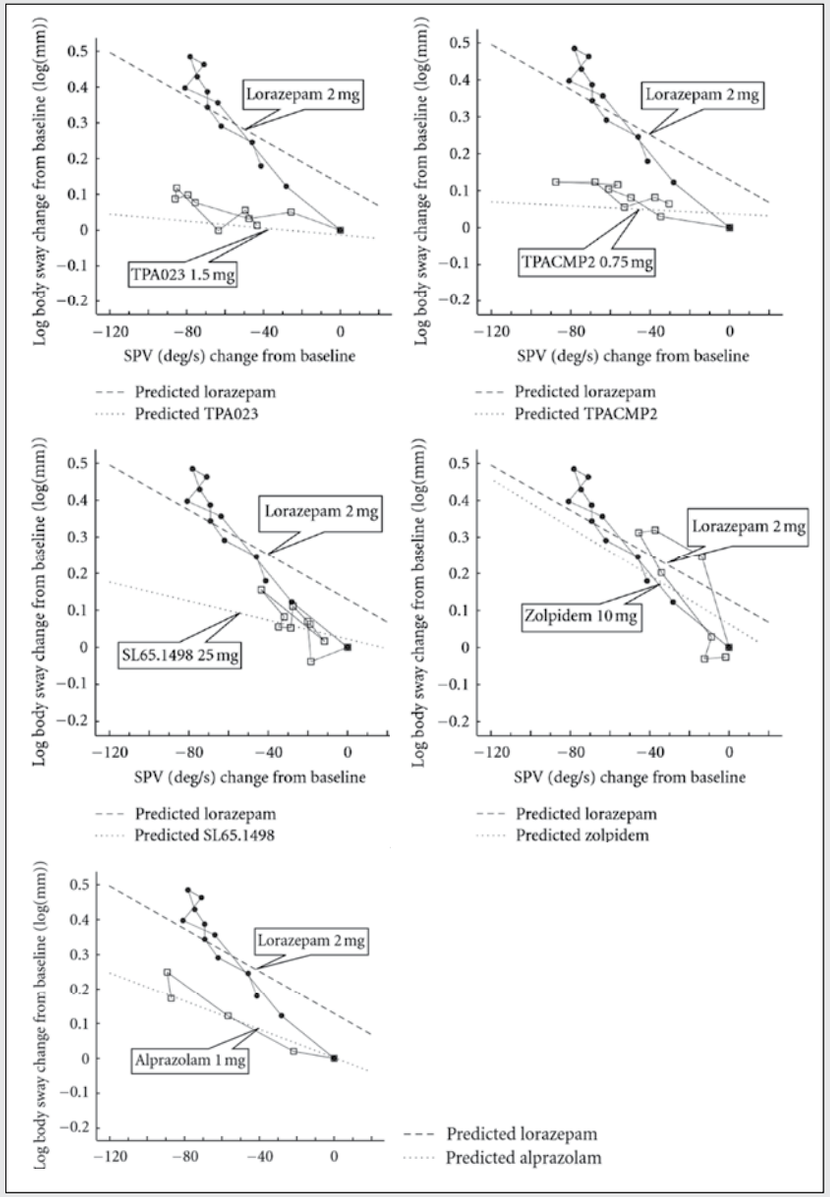


Figure 2 · $\Delta VAS_{alertness}$-ΔSPV relative effect profile of TPA0231.5 mg, TPACMP2 0.75 mg, SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg vs. lorazepam 2 mg, respectively.

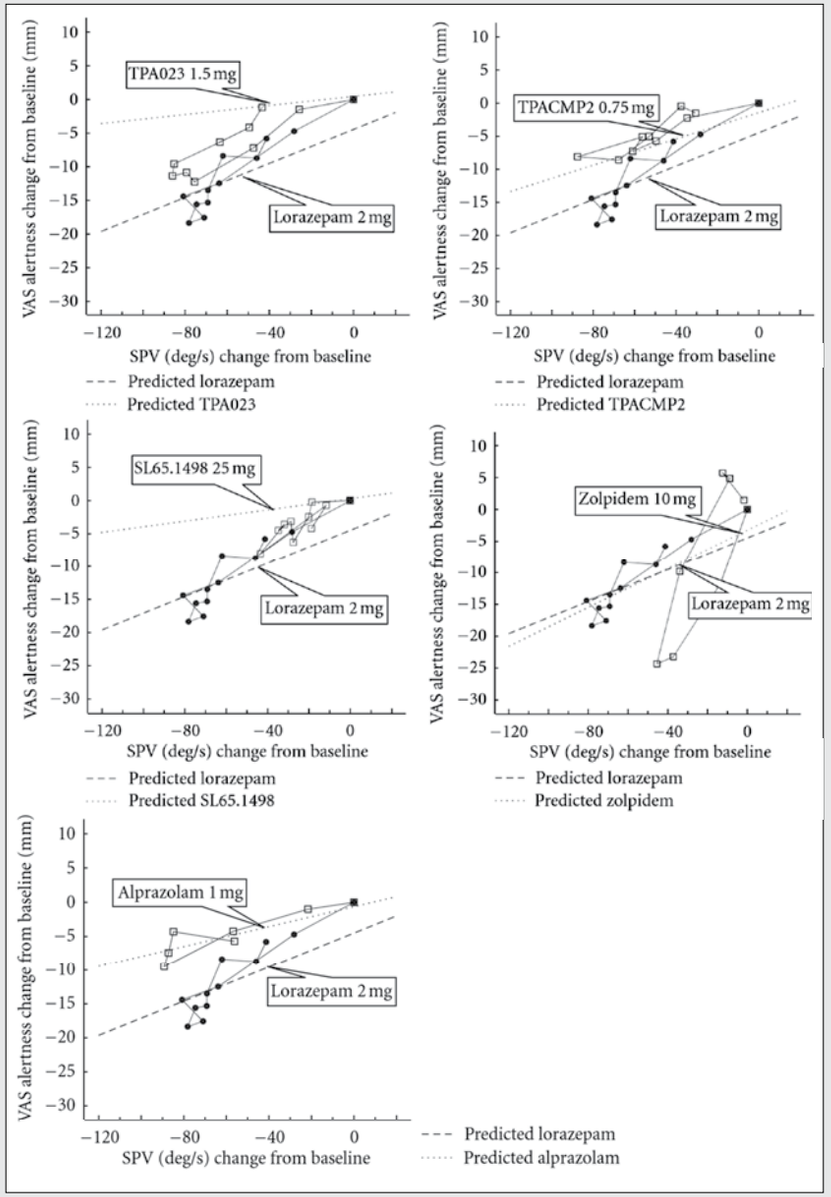
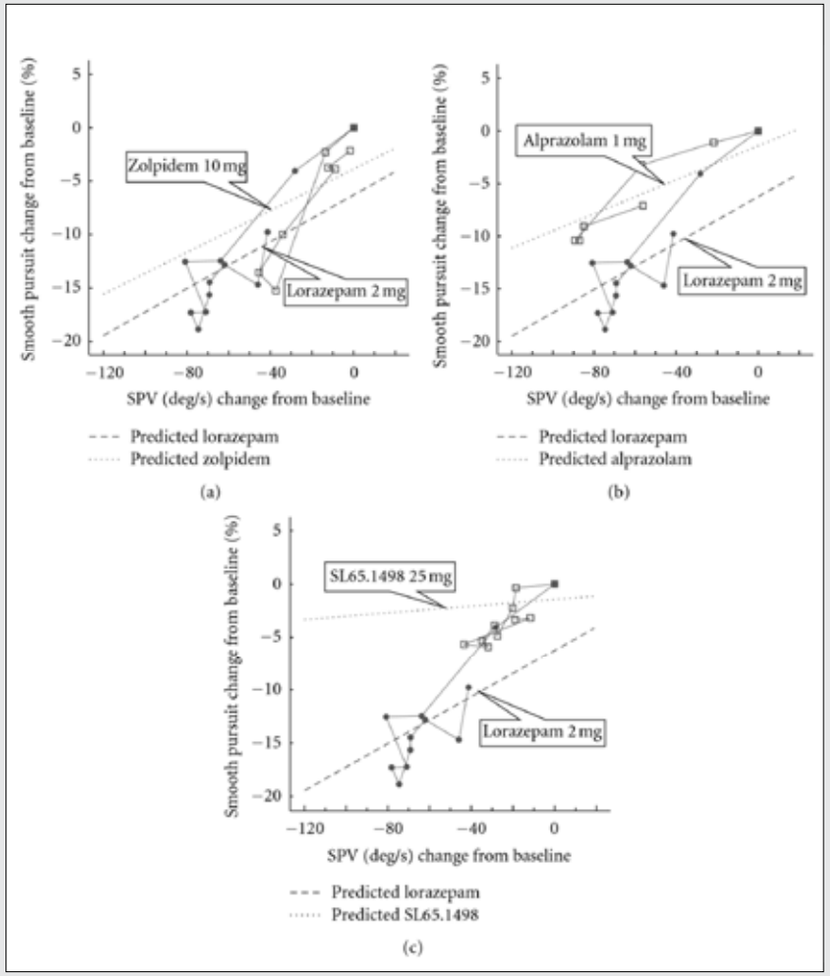


Figure 3 • ΔSMOOTH-ΔSPV relative effect profile of SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg vs. lorazepam 2 mg, respectively.



VI

THE EFFECTS OF THE NONSELECTIVE BENZODIAZEPINE LORAZEPAM AND THE α2/α3 SUBUNIT-SELECTIVE GABA-A RECEPTOR MODULATORS AZD7325 AND AZD6280 ON PLASMA PROLACTIN LEVELS

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Erik T. te Beek[1], Xia Chen[1,2], Gabriël E. Jacobs[1,3], Kimberly J. Nahon[1], Marieke L. de Kam[1], Jaakko Lappalainen[4], Alan J. Cross[4], Joop M.A. van Gerven[1] & Justin L. Hay[1]

1. Centre for Human Drug Research, Leiden, the Netherlands | 2. Clinical Pharmacological Research Center (CPRC), Peking Union Medical College Hospital, Beijing, China | 3. Department of General Hospital Psychiatry, Free University Medical Centre, Amsterdam, the Netherlands | 4. AstraZeneca Pharmaceuticals, Wilmington de, USA

ABSTRACT

Compounds with selectivity for GABA-A receptor subtypes may differ significantly from nonselective benzodiazepines in their dopaminergic effects in vivo. To explore the exact role of the GABA-A receptor subtypes in the regulation of prolactin secretion and the differential effects of selective and nonselective GABA receptor modulators, the effects of the nonselective benzodiazepine lorazepam, as well as two novel α_2/α_3 subunit-selective GABA-A receptor modulators AZD7325 and AZD6280, on prolactin levels were measured in healthy male volunteers. Following administration of lorazepam at 2 mg doses and AZD6280 at 10 mg and 40 mg doses, prolactin levels increased significantly compared with placebo (difference 42.0%, 19.8% and 32.8% respectively), suggesting that the α_2 and/or α_3 receptor subtypes are involved in GABAergic modulation of prolactin secretion, although possible roles of the α_1 and α_5 receptor subtypes are not excluded. The increases in prolactin levels after administration of AZD7325 at 2 mg and 10 mg doses (difference 7.6% and 10.5% respectively) did not reach statistical significance, suggesting that doses of AZD7325 or intrinsic efficacy at the α_2 and α_3 receptor subtypes may have been too low.

INTRODUCTION

A series of studies in animal models has indicated that certain effects of benzodiazepines may be attributable to efficacy at specific GABA-A receptor subtypes, such as sedation (α_1 receptor subtype) [1, 2], anxiolysis (α_2 and α_3 receptor subtypes) [3-6] and learning and memory (α_5 receptor subtype) [7, 8]. Accordingly, it has been suggested that compounds with high efficacy at α_2 and/or α_3 receptor subtypes and low efficacy at α_1 and α_5 receptor subypes may exhibit significant anxiolysis, with less sedation than nonselective benzodiazepines [9]. In addition, because γ-aminobutyric acid (GABA) is one of the major inhibitors of dopamine neurotransmission, it has also been suggested that compounds with selectivity for GABA-A receptor subtypes may differ significantly from nonselective benzodiazepines in their effects on dopamine neurotransmission [9]. In the ventral tegmental area, dopaminergic neurons express α_3 receptor subtypes, whereas GABAergic interneurons express α_1 receptor subtypes [10]. Benzodiazepines inhibit the dopaminergic neurons through efficacy at α_3 receptor subtypes, but simultaneous efficacy at α_1 receptor subtypes on the GABAergic interneurons results in disinhibition of the dopaminergic neurons [9, 10]. Thus, compounds with high efficacy at α_3 receptor subtypes and low efficacy at α_1 receptor subtypes may attenuate dopamine neurotransmission without counteractive disinhibition. Such compounds may have therapautic potential in disorders such as schizophrenia [9].

To evaluate if compounds with selectivity for GABA-A receptor subtypes differ from nonselective benzodiazepines in their dopaminergic effects in vivo, we evaluated the effects of lorazepam and two novel selective modulators of α_2 and α_3 receptor subtypes on the activity of the tuberoinfundibular pathway, which represents the most readily available dopaminergic pathway for evaluation in vivo, by measuring circulating prolactin levels. The tuberoinfundibular dopaminergic pathway is the primary physiological inhibitory control mechanism of prolactin secretion [11, 12]. GABA may directly inhibit the activity of the hypothalamic tuberoinfundibular dopaminergic pathway, with a resulting increase in prolactin secretion [12-16]. It has also been reported that GABA exerts a dual control [17] and can also have an inhibitory effect on prolactin release by acting at GABA receptors in the anterior pituitary gland [12, 16], but this effect is less clear *in vivo* [18] than *in vitro*. Only a few studies have evaluated the direct effects of GABAergic drugs on circulating basal prolactin levels in healthy subjects. Diazepam [19, 20] and bromazepam [21] did not significantly affect prolactin levels, while temazepam was found to increase prolactin levels only to a small extent [22]. Alprazolam at high doses increased prolactin levels [23], while lower doses had no significant effect [24]. Zolpidem and bretazenil stimulated nocturnal secretion of prolactin [25, 26], while sodium valproate decreased prolactin levels [27]. The effect sizes in these studies, if any, were very small, especially compared with the potent prolactin-elevating effects of dopamine D_2 receptor antagonists.

To explore the exact role of the various GABA-A receptor subtypes in the regulation of prolactin secretion and the differential effects of selective and nonselective GABA receptor modulators, we report the effects of two novel α_2/α_3 subunit-selective GABA-A receptor modulators, AZD7325 [28, 29] and AZD6280 [28, 30], and a therapeutic dose of lorazepam on prolactin secretion. These studies were part of larger phase I pharmacokinetic and pharmacodynamic studies of these compounds, which will be reported elsewhere [31, 32].

METHODS

STUDY DESIGN

In total, 32 healthy male volunteers were planned to participate in two parallel double-blind, placebo-controlled, randomized, cross-over studies. To be eligible for inclusion in both studies, subjects were required to be aged between 18 and 55 years, with a body mass index (BMI) of 18 to 30 kg/m² and refrain from alcoholic beverages, smoking and caffeine-containing products during study days. Both studies were approved by the medical ethics review board of the Leiden University Medical Center. Prior to medical screening, all subjects gave written informed consent. Both studies had an identical design, except the administered drugs. In the first study, 16 subjects were administered single oral doses of 2 mg lorazepam, 2 mg AZD7325, 10 mg AZD7325 or placebo, during four separate study periods. In the second study, 16 subjects were administered single oral doses of 2 mg lorazepam, 10 mg AZD6280, 40 mg AZD6280 or placebo, during four separate study periods. Study periods were scheduled in randomized order using a Williams Latin square design and were separated by a washout time of at least 7 days. On study days, subjects fasted for minimally 2.5 hours after a light standard breakfast until dose administration (which generally occured between 11h00m and 12h00m AM) and continued fasting until 4 hours after dose administration.

POWER CALCULATION

A power calculation using data from a previous study [33] indicated that the present study (n = 32 subjects receiving lorazepam, power 80% and alpha 0.05) was powered to detect an increase of 12.5% or a decrease of 11% in prolactin concentration after administration of lorazepam, compared with placebo.

PLASMA CONCENTRATION OF PROLACTIN

Venous blood samples for analysis of prolactin concentration were collected prior to study drug administration and at ½, 1, 1¼, 1½, 2, 2½, 3¼, 4, 4½, 6, 8, 12 and 21 hours after study drug administration. Plasma concentrations of prolactin were determined using an electrochemiluminescence immunoassay (ECLIA) with a lower detection limit of 0.047 ng/mL (Elecsys Prolactin II assay, Roche Diagnostics GmbH, Mannheim, Germany).

STATISTICAL ANALYSIS

Prolactin measurements up to 8 hours after administration of lorazepam or placebo were compared with a mixed model analysis of variance with treatment, period, time and treatment by time as fixed factors, and subject, subject by treatment and subject by time as random factors, and the pre-value (measurement prior to study drug administration) as covariate. Prolactin measurements were log-transformed prior to analysis to correct for the log-normal distribution of the data. Estimates of treatment differences and back-transformed estimates of the difference in percentage with corresponding 95% confidence intervals (95% CI) and p-values were calculated. All calculations were performed using SAS for Windows version 9.1.3 (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS

SUBJECTS

Subjects had a mean age of 28.1 years (range 18-54), weight of 76.1 kg (range 62.0-89.5) and body mass index (BMI) of 23.0 kg/m² (range 19.1-26.7). Two subjects withdrew informed consent after completion of study period 1 for reasons unrelated to study drug administration. Another subject tested positive for THC in study period 2 and was excluded from participation. Pharmacodynamic data from these subjects were not used for further analysis. All three subjects were replaced. Therefore, in total, 32 subjects completed the study.

PLASMA CONCENTRATION OF PROLACTIN

Plasma concentrations of prolactin after administration of lorazepam, AZD7325 and AZD6280 are shown in Figure 1 and Table 1. Following administration of 2 mg lorazepam, prolactin levels increased with 42.0% compared with placebo (95% CI 31.4/53.5%, $p < 0.001$), which remained elevated until at least 8 hours after dose administration. Following administration of 2 mg and 10 mg AZD7325, prolactin levels increased with 7.6% and 10.5%, respectively, compared with placebo. Both increases did not reach statistical significance, although the 10.5% increase after the 10 mg dose has a p-value of 0.0536. Following administration of 10 mg AZD6280, prolactin levels increased significantly compared with placebo (difference 19.8%

versus placebo, 95% CI 8.2/32.6%, $p = 0.0007$). A larger increase was observed after administration of 40 mg of AZD6280 (difference 32.8% versus placebo, 95% CI 20.0/47.0%, $p < 0.0001$). Prolactin levels after administration of lorazepam were significantly higher than those after AZD7325 at 2 and 10 mg doses and AZD6280 at 10 mg doses, but were not significantly different from those after AZD6280 at 40 mg doses.

DISCUSSION

Compounds with high efficacy at α_2 subunit-containing GABA-A receptor subtypes and low efficacy at α_1 receptor subtypes may differ significantly from nonselective benzodiazepines in their effects on dopaminergic circuits [9]. Such compounds may thus have therapeutic potential in disorders such as (certain aspects of) schizophrenia [9]. The present study was performed to evaluate the effects of two novel α_2/α_3 subunit selective GABAergic drugs on the activity of the tuberoinfundibular dopaminergic pathway, by measuring circulating prolactin levels in healthy male volunteers, compared with lorazepam and placebo.

After administration of placebo, prolactin levels showed an initial decrease with a return to baseline values at the end of the study day, which is consistent with a normal circadian rhythm [12, 34]. Also, a peak in prolactin levels was observed 6 hours after dose administration, which probably reflects normal prolactin release following food consumption [35, 36.]

After administration of a single oral dose of 2 mg lorazepam, an increase of 42.0% in prolactin levels was observed. The magnitude of the effects of lorazepam on prolactin levels was rather small, especially in comparison to the much more potent prolactin-elevating effects of dopamine D_2 receptor antagonists. Haloperidol at 3 mg doses increases prolactin levels with 130.9% [37]. Thus, the effects of lorazepam administration on prolactin secretion are not likely to produce clinically relevant hyperprolactinaemia in men. However, our studies showed clear results in comparison with other studies that evaluated the effects of GABAergic drugs on basal prolactin levels in healthy subjects. The benzodiazepines diazepam and bromazepam showed no significant effects on prolactin levels under resting conditions [19-21], whereas temazepam caused a small increase in prolactin levels of roughly 21.4 mU/L (which would correspond to roughly 1 ng/mL), but only at a single time point 1 hour after dose administration [22]. In contrast, our study demonstrates that lorazepam increases prolactin levels with roughly 5-6 ng/ml, which remain elevated until at least 8 hours after dose administration. The dose of lorazepam (2 mg) used in our study is roughly equipotent with the doses of diazepam (10 mg), bromazepam (3 mg) and temazepam (20 mg) used in these earlies studies, although estimates of benzodiazepine dose equivalencies differ somewhat between various authors [38-40]. Dose dependency of the effects on prolactin secretion has been demonstrated with the benzodiazepine alprazolam, which causes an increase of prolactin levels with roughly 9-10 ng/mL at relatively high doses (3 mg) [23], while doses in the lower therapeutic range (0.5 mg) demonstrated no effects [24]. The different findings might be explained by the small sample sizes used in the earlier studies (6-10 subjects in most studies) and statistical power may thus have been too small.

The increase in prolactin levels following administration of the GABA agonist lorazepam in our study suggests that the postulated stimulatory effect of GABA transmission (by suppressing the tuberoinfundibular dopaminergic neurons in the hypothalamus) exceeds the postulated inhibitory effect of GABA transmission (directly at the anterior pituitary gland). The preferential effect of lorazepam on the tuberoinfundibular dopaminergic neurons might result from differences in affinity for the pituitary and hypothalamic GABA binding sites, as has been shown for the GABA agonist muscimol and antagonist bicuculline [41], both of which have higher affinity for the binding sites in the mediobasal hypothalamus than for the binding sites in the anterior pituitary. However, effects of benzodiazepines on the activity of the tuberoinfundibular dopaminergic neurons have not been confirmed *in vivo* in man. A recent positron emission tomography (PET) study using the dopamine D_2 receptor ligand [^{11}C]FLB457 in healthy subjects has demonstrated that single oral doses of 2.5 mg lorazepam induce a statistically significant decrease in dopamine D_2 receptor binding potential (BP_{ND}) in the medial temporal and dorsolateral prefrontal cortex [42], but effects on the hypothalamus were not reported. Although a decrease in BP_{ND} (i.e. suggesting dopamine release) in the cerebral cortex does not imply that lorazepam inhibits the tuberoinfundibular dopaminergic pathway in the hypothalamus, it does confirm that lorazepam can alter dopamine levels in extrastriatal areas in humans *in vivo*.

The present study evaluated the effects of two novel α_2/α_3 subunit-selective GABA-A receptor modulators, AZD7325 and AZD6280, on prolactin levels. Administration of 2 mg and 10 mg AZD7325 produced small increases in prolactin levels, which did not reach statistical significance, although the 10.5% increase after the 10 mg dose is in line with the other effects and has a *p*-value of 0.0536. Administration of 10 mg and 40 mg AZD6280 produced statistically significant increases in prolactin levels of 19.8% and 32.8%, respectively. These findings suggest that the α_2 and/or α_3 receptor subtypes are involved in GABAergic modulation of the tuberoinfundibular dopaminergic pathway. Indeed, α_2 and α_3 subunit-containing GABA-A recepors have been shown to be expressed in the arcuate nucleus and hypothalamus 43. However, it is not excluded that α_1 or α_5 receptor subtypes, which are also expressed in the arcuate nucleus and hypothalamus [43], are also involved in the control of prolactin secretion. The nonbenzodiazepine GABA agonist zolpidem (10 mg), which has modest selectivity for α_1 receptor subtypes [44],

increased nocturnal prolactin levels by two-fold 26. The effects of AZD7325 on prolactin secretion were less clear than those of AZD6280. Similarly, in other studies [31, 32], AZD7325 also caused fewer effects than AZD6280 on peak velocity of saccadic eye movements, which is one of the most consistent and sensitive biomarkers for the effects of nonselective benzodiazepines [45] and α_2/α_3 subtype-selective compounds [46] in healthy volunteers. These differences may be related to the lower dosages of AZD7325 used.

Comparison of the effects of 2 mg lorazepam with 40 mg AZD6280 indicated no statistically significant difference. The lower prolactin levels after AZD7325 at 2 and 10 mg doses and AZD6280 at 10 mg doses are likely related to dose. However, dose equivalencies of lorazepam, AZD7325 and AZD6280 are not known. Thus, these results do not fully exclude potential differences between nonselective benzodiazepines and selective α_2/α_3 subunit-containing GABA-A receptor modulators on prolactin secretion. In addition, our study measured prolactin levels only in healthy male volunteers. These results cannot readily be extrapolated to females, because the regulation of prolactin secretion in females is different, with the notable influence of estrogens.

In conclusion, the nonselective benzodiazepine lorazepam and the novel α_2/α_3 subunit-selective GABA-A receptor modulator AZD6280 at 40 mg doses both increase plasma prolactin levels in healthy male subjects. The increases in prolactin levels after administration of the novel α_2/α_3 subunit-selective GABA-A receptor modulator AZD7325 did not reach statistical significance, which may be related to the lower dosages used. These results indicate that the α_2 and/or α_3 receptor subtypes are involved in GABAergic modulation of prolactin secretion.

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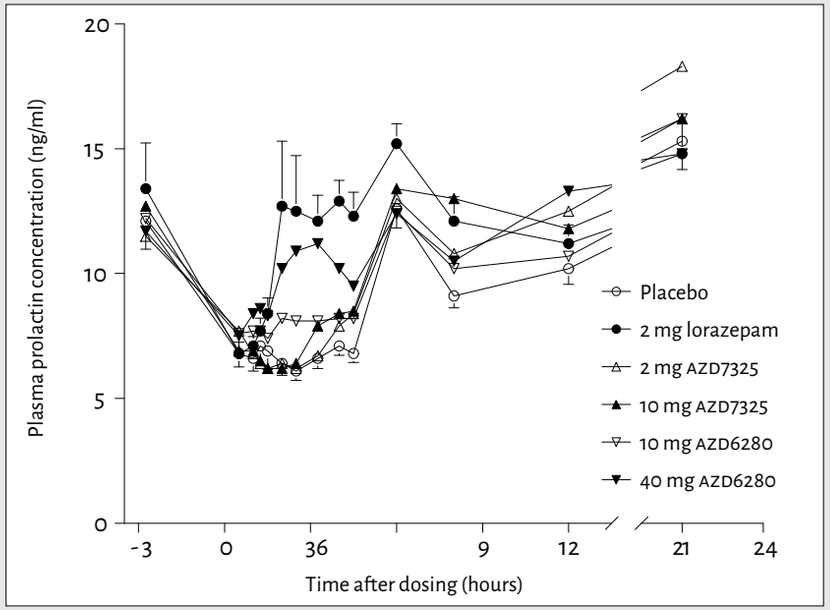
Table 1 · Comparison of prolactin levels after administration of lorazepam, AZD7325 and AZD6280 compared with placebo. Treatment differences are expressed as percentages with 95% confidence intervals and p-values.

Treatment comparisons	Percentage difference (95% CI)	p-value
Lorazepam versus placebo	42.0 (31.4/53.5)	<0.0001
AZD7325 2 mg versus placebo	7.6 (-2.8/19.1)	0.1566
AZD7325 10 mg versus placebo	10.5 (-0.2/22.3)	0.0536
AZD6280 10 mg versus placebo	19.8 (8.2/32.6)	0.0007
AZD6280 40 mg versus placebo	32.8 (20.0/47.0)	<0.0001
AZD7325 2 mg versus lorazepam	-24.2 (-31.6/-16.1)	<0.0001
AZD7325 10 mg versus lorazepam	-22.2 (-29.7/-13.9)	<0.0001
AZD6280 10 mg versus lorazepam	-15.7 (-23.8/-6.7)	0.0012
AZD6280 40 mg versus lorazepam	-6.4 (-15.5/3.6)	0.1957

Figure 1 · Time course of plasma concentration of prolactin after administration of single oral doses of 2 mg lorazepam, 2 mg AZD7325, 10 mg AZD7325, 10 mg AZD6280 and 40 mg AZD6280 (AT T = 0 hours). Means are presented with standard errors of the mean (SEM) as error bars.



VII

PHARMACODYNAMIC RESPONSE PROFILES OF ANXIOLYTIC AND SEDATIVE DRUGS

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Xia chen[1,2], Freerk Broeyer[2], Marieke de Kam[2], Joke Baas[3], Adam Cohen[2], Joop van Gerven[2]

1. Phase I Unit, Clinical Pharmacology Research Center, Peking Union Medical College Hospital, Beijing, China | 2. Centre for Human Drug Research, Leiden, The Netherlands | 3. Department of Experimental Psychology, Faculty of Social Sciences, Utrecht University, The Netherlands

ABSTRACT

Introduction: Centrally-acting acutely anxiolytic drugs, like benzodiazepines, barbiturates and gabapentinoids, affect various central nervous system (CNS) functions, which reflect not only their anxiolytic effects but also neuropsychological side-effects. To validate the pharmacodynamic biomarkers for GABAergic anxiolytics, this study determined the pharmacodynamics of two anxiolytics and a non-anxiolytic control and linked them to their anxiolytic and sedative effects, during an anxiety-challenge study day. **Methods:** Twenty healthy volunteers were randomized in this placebo-controlled, double-blind, four-way cross-over study with single-dose alprazolam (1 mg), diphenhydramine (50 mg), pregabalin (200 mg) or placebo. The Neurocart® was used in-between repeated fear-potentiated-startle assessments. Thus the potential influence of anxiety on CNS pharmacodynamic markers could be examined. **Results:** Compared to placebo, $VAS_{calmness}$ increased with alprazolam (2.0 mm) and pregabalin (2.5 mm) but not with diphenhydramine. Saccadic-peak-velocity (SPV) declined after alprazolam (-57 deg/sec) and pregabalin (-28 deg/sec), more than with diphenhydramine (-14 deg/sec); so did smooth-pursuit. The average responses of SPV and smooth-pursuit were significantly correlated with the drug-induced increases in $VAS_{calmness}$. The SPV-relative responses of $VAS_{alertness}$, body-sway and adaptive-tracking also differed among alprazolam, pregabalin, and diphenhydramine. **Conclusions:** Compared with the antihistaminergic sedative diphenhydramine, alprazolam and pregabalin caused larger SPV reduction, which was correlated with simultaneous improvement of subjective calmness, during a study day in which anxiety was stimulated repeatedly. The different effect profiles of the three drugs are in line with their pharmacological distinctions. These findings corroborate the profiling of CNS effects to demonstrate pharmacological selectivity, and further support SPV as biomarker for anxiolysis involving GABAergic neurons. The study also supports the use of prolonged mild threat to demonstrate anxiolytic effects in healthy volunteers.

INTRODUCTION

Centrally-acting acute anxiolytic drugs, like benzodiazepines, barbiturates and gabapentinoids, have an impact on a range of central nervous system (CNS) functions, which reflect not only their anxiolytic effects but also side effects like sedation, postural instability and visuomotor and memory impairment [1]. It would be useful to identify the CNS activities for those compounds that are more closely linked to reduction of anxiety than to general CNS depression.

Pharmacodynamic (PD) approaches have been increasingly employed in early human pharmacology studies to obtain in vivo pharmacological information of different drugs acting on the central nervous system and of the systems with which the drugs interact. The general aim of these methodology is to obtain information about the pharmacological characteristics of a drug (such as blood-brain barrier penetration, target engagement and mechanistically meaningful activity), which underlie its therapeutic effects [2-4]. The use of appropriate biomarkers may be especially useful for anxiety disorders, where therapeutic exploratory studies in patients can be difficult to achieve a clinically meaningful end-point due to the nature of subjective assessments, the relatively large size and probability of placebo effect, and other ethical or practical issues [5,6]. Of no doubt, a validated biomarker in early human pharmacology studies would serve as a useful tool for the development of new therapeutic anxiolytics.

It has been well established that benzodiazepines (BZDs) exert their pharmacological effects through positive allosteric modulation of the GABA-A receptors. Recent years, the experiments on GABA-A receptor subtype-gene knock-out mouse lines has greatly facilitated the identification of GABA-A receptor subtypes that mediates BZDs-induced sedation (α_1 GABA-A receptors), anxiolysis (α_2 and α_3 GABA-A receptors), or memory impairment (α_5 GABA-A receptors) [7-9]. To address the effects of BZDs in human pharmacological studies, a collection of pharmacodynamic measurements were employed and evaluated for their pharmacokinetic/pharmacodynamic relationship with BZDs, which include objective measures such as electroencephalography, semi-subjective measures such as psychomotor performance, and subjective measures such as mood/sedation scales [10-13]. Despite of the acceptable sensitivity and the observed exposure-response relationship of these PD measurements for the effects of BZDs, as well as the potential involvement of eye movement in anxiety disorder and related neuropsychiatric disturbance, increasing attention has been paid to evaluate the relevance of these PD parameters to the pharmacological effects of established or novel anxiolytic drugs. The exact clinical relevance of quantitative electroencephalogram (EEG), for example, to the anxiolytic, anticonvulsant, sedative and hypnotic actions of benzodiazepines, have not yet clearly been elucidated [14].

The Centre for Human Drug Research (CHDR) (Leiden, The Netherlands) has developed a Neurocart battery of validated computerized tests for the assessments of various CNS functions. These tests have been shown to be sensitive to various aspects of sedation [15] and have been used in early studies of psychoactive drugs as pharmacodynamic biomarkers for postural (in)stability (body sway test), eye-hand cooperation (adaptive tracking test), subjective feelings of alertness, mood and calmness (visual analogue scale [VAS] Bond & Lader), and for neurophysiologic functions (saccadic eye movement and smooth pursuit eye movement tests) [6]. Our previous studies showed that the Neurocart battery presents distinct pharmacodynamic response-patterns to different subtype-selective partial GABA-A agonists and non-selective benzodiazepine anxiolytics [16-19], which may imply potential GABA-A subtype specificity of these PD markers. Normally, this test battery does not provide any clear information about the specific anxiolytic properties of drugs, as measured by $VAS_{calmness}$. Benzodiazepines or selective serotonin reuptake inhibitor (SSRIs), for instance, don't cause consistently significant increases of subjective calmness in healthy volunteers, when the measurement was performed in stress-free experimental settings [5,6]. Such findings can be true for SSRIs that have a slow onset of action and can even worsen anxiety symptoms during initial treatment [20], but is not expected for fast-acting anxiolytic drugs like benzodiazepines [21]. We therefore combined the Neurocart test battery with a modified fear-potentiated-startle (FPS) paradigm [22]. In this way, we could compare our more general CNS test battery with a specific anxiety test, which in some studies [23-24], but not all [25], has been shown to be sensitive to anxiolytic drugs. To this end, we administered two sedating anxiolytic drugs (alprazolam and pregabalin) and a sedating non-anxiolytic (diphenhydramine) at therapeutic doses to healthy volunteers.

METHODS

ETHICS

The study was approved by the Medical Ethics Review Board of Leiden University Medical Centre (LUMC), and was conducted according to the principles of the Helsinki Declaration and the International Conference on Harmonization/Good Clinical Practice (ICH/GCP).

DESIGN

This was a single-center, randomized, placebo-controlled, four-way crossover, double-blind study conducted in twenty healthy subjects. The scheme of this study included a screening period of maximally 14 days, four treatment periods separated by three washout periods of at least 3 days, and a telephone follow-up.

SUBJECTS

Ten men and ten women, aged between 18 and 40 years, with a BMI between 18 and 30 kg/m², without any clinically significant abnormalities, were recruited. All volunteers provided written inform consent. Their eligibilities were evaluated before being randomized into the study. Subjects were instructed not to use alcoholic beverages from 24 hours before admission until the next morning of each study day. No xanthine or tobacco containing products were allowed from 22:00 in the evening before each study day and during stay in the research unit. They were asked to keep a normal day/night pattern from two weeks before the first study day until the last study day.

SAMPLE SIZE DETERMINATION

As was shown in Grillon et al [23], the mean effect of the THREAT-SAFE difference between unpredictable threat and a neutral context seen under placebo was about 15 μV ± 8.5 μV whereas the effect under 1 mg alprazolam was around 5 μV ± 8.5 μV (mean ± standard deviation). This leads to an alprazolam effect of 10 μV over placebo. Given that the within patient variability is normally not substantially greater than the between patient variability a residual standard deviation of 10 μV was assumed. Based on these assumptions, a sample size of 16 subjects was obtained to ensure a power of at least 80% with a two-sided alpha level of 5%. For the Neurocart end points, using data from previous studies [17-19], the same sample size of 16 was determined to have equal to or greater than 80% power to detect the mean differences of 1.244 in VAS alertness and 20.577 in saccadic peak velocity (SPV), respectively assuming standard deviations of 1.663 (VAS alertness) and 27.429 (SPV) between placebo and lorazepam 2 mg using a paired t-test with a 0.050 two-sided significance level. Considering the possibility of drop-out and the sample should be a multiple of four (to keep the study design balanced the sample size), a total sample size of 20 subjects was finally decided for the study.

TREATMENTS

The study treatments were assigned according to a randomization schedule, which consisted of five blocks of the fully balanced 4*4 William Latin Squares. Each subject received single oral dose of over-capsulated pregabalin 200 mg, alprazolam 1 mg, diphenhydramine 50 mg or matching placebo in a fasted state at about 8 to 9 AM on each treatment period.

SAFETY

Adverse events, electrocardiograms (ECGs) and vital signs, as well as safety laboratory assays were frequently evaluated during the study. Twelve-Lead ECG recording was made using Nihon Kohden Cardiofax with Ecaps 12 software devices (Nihon Kohden, Tokyo, Japan). Vital signs (pulse rate and blood pressure) were taken using a Nihon-Kohden BSM-1101K monitor or a Colin Pressmate BP 8800. All blood pressure, pulse rate, and ECG recordings were done after subject was resting in a supine position for at least 5 minutes. Safety laboratory tests on blood or urine samples were performed in the Central Clinical Laboratories of LUMC.

PHARMACOKINETIC MEASUREMENTS

For the determination of drug concentrations, two venous blood samples of 5 and 2 ml were collected into ice-bathed Li-Hep tubes (Becton and Dickinson 367684 & 368200, respectively) within 0.5 hour pre-dose and at 0.5, 1.25, 1.75, 2.25, 3, 4, 6, and 8 hours post-dose. The samples were centrifuged (2000G, 15 min, 4°C). The obtained plasma was transferred into two polypropylene Sarstedt 2 ml tubes and stored at -20°C until analysis.

Plasma pregabalin concentrations were determined at AAI Pharma GmbH & Co KG, Neu-Ulm, Germany, using LC-MS/MS on a Finnigan LCQ system. A Phenomenex Gemini (50 x 3.0 mm i.d., 5µm) was used as the HPLC column. The quantification range was from 1.00 to 1000 µg/L. The intra- and inter-assay variability was 2.1-10.5% and 0.9-6.6%, respectively. Plasma alprazolam and diphenhydramine concentrations were determined at the pharmacy of the Groningen University Medical Centre, Groningen, the Netherlands, using LC-MS/MS. All experiments were performed on a ThermoFisher (San Jose, USA) triple quadrupole LC-MS/MS with a Finnigan™ Surveyor® LC pump and a Finnigan™ Surveyor® autosampler which was set at 20 °C. Lower limit of Quantification (LLOQ) was 1.00 µg/L for alprazolam and 5.00 µg/L for diphenhydramine, respectively. Intra- and inter- assay variability were 2.1-7.2% and 0.0-3.3%, respectively, for alprazolam and 2.0-3.3% and 0.0-2.0%, respectively for diphenhydramine.

PHARMACODYNAMIC MEASUREMENTS

A training session of the pharmacodynamic tests (i.e. the Neurocart battery and the FPS paradigm) was performed during the screening. The purpose was to familiarize the subjects with the tests and prevent potential learning effect. In each study period, the FPS paradigm was carried out around 1 hour after dosing; while the Neurocart battery was assessed at pre-dose and 0.5, 1.25, 1.75, 2.25, 3, 4, 6, and 8 hours post-dose in the following sequence of tests: body sway, VAS Bond & Lader, saccadic eye movements, smooth pursuit eye movements, and adaptive tracking. At each assessment, one subject was assigned to a quiet room with ambient illumination.

Pharmaco- electroencephalograph (EEG) approach is currently widely used, and the empirical relation between this measure and other agonist effects of benzodiazepines has been reported. However, the main purpose of this study was to compare the sensitivity and specificity of the Neurocart PD measurements versus those of the FPS measurements to the effects of sedating, hypnotic, and anxiolytic drugs. As the flowcharts of the study days were already quite busy with the combination of the non-EEG PD tests and the FPS paradigm, and the device used for generation of electronic shocks in the FPS paradigm may interfere with the pharmaco-EEG measurements, the EEG measures were omitted from the study design for the sake of smooth operation.

BODY SWAY

Body sway was measured with an apparatus similar to the Wright ataxiameter [26], which integrates the amplitude of unidirectional body sway. The measurements were made in the antero-posterior direction with eyes closed for 2 minutes. The subject was asked to stand comfortably on a floor with his/her feet slightly apart. Body sway measures postural (in)stability. It has demonstrated considerable sensitivity to the effect of benzodiazepines [27].

VISUAL ANALOGUE SCALES OF BOND & LADER (VAS B&L)

Visual analogue scales, as originally described by Norris [28], were presented on a computer screen. Three composite factors were derived from the sixteen items, corresponding to alertness, mood and calmness, respectively. These factors quantify subjective feelings and have been extensively used to delineate subjective effects of a variety of sedative agents [6].

SACCADIC EYE MOVEMENTS

Saccadic eye movements were evaluated using a computer-based system composed of 1) stimulus display and signal collection (Nihon Kohden Corporation, Tokyo, Japan), 2) signal amplification (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA), 3) data recording (Cambridge Electronics Design, Cambridge, UK), 4) disposable silver-silver chloride electrodes (Medicotest N-00-S, Olstykke, Denmark), as well as 5) the sampling and analysis scripts developed by CHDR (Leiden, the Netherlands). The parameters of this test were the average values of saccadic peak velocity (SPV, degree/msec), reaction time (msec) and inaccuracy (%) of all artefact-free saccades that were calculated on each session. Saccadic peak velocity appears to be the most sensitive measure for the sedative effect of benzodiazepines [6] and has been found to be a promising biomarker for the anxiolytic component of benzodiazepines and some newly developed compounds with potential anxiolytic effect [16-19].

SMOOTH PURSUIT EYE MOVEMENTS

The same system as used for saccadic eye movements was also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moved sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, by step of 0.1 Hz. The amplitude of target displacement corresponded to 22.5 degrees eyeball rotation to both sides. Four cycles were recorded for each stimulus frequency. The method has been validated at the CHDR by van Steveninck *et al.* [29] based on the work of Bittencourt *et al.* [30] and the original description of Baloh *et al.* [31]. The time in which the eyes were in smooth pursuit of the target were calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies were used as the parameter.

ADAPTIVE TRACKING

The adaptive tracking test was performed as originally described by Borland and Nicholson [32], using customised equipment and software (Hobbs, 2004, Hertfordshire, UK). After a 0.5-minute run-in time without data-recording, the average performance over the rest 3.0 minutes was scored and was used as the test parameter. Adaptive tracking is a pursuit-tracking task. The subject was required to operate a joystick and try to keep a dot inside a circle moving randomly on the computer screen. If he/she succeeded, the speed of the moving circle increases, and vice versa.

FEAR POTENTIATED STARTLE (FPS) PARADIGM

The FPS paradigm is extensively described elsewhere [22]. In brief, the test contained three contexts, which differed in the possibility of electronic shocks signaled by a computer displayed verbal instruction: 'No shock' for the Neutral (N) context, 'Shock only during cue' for the Predictable (P) context, and 'Shock at any time' for the Unpredictable (U) context. Duration of each context was 90-100 sec, during which six startle probes were administered together with the assessment of startle response. Intervals between startle probes varied between 12 and 18 sec (16 sec on average). The FPS session consisted of two blocks with the following orders of contexts: (1) P-N-U-N-U-N-P and (2) U-N-P-N-P-N-U. The order of these two blocks was counterbalanced across the subjects. A total of 12 shocks were administered during each FPS test session.

The shocks were delivered through two medal electrodes located on the inner side of one of the subjects' forearms. Shock stimuli were delivered using a Digitimer DS7A constant current stimulator (Digitimer Ltd, Hertfordshire, England). Stimulation consists of short trains (total duration maximally 750 ms) of brief (2 ms) pulses. The maximum current intensity delivered during the study was 7 mA.

STATISTICAL ANALYSIS

PHARMACOKINETICS

The plasma concentrations of pregabalin, diphenhydramine and alprazolam were summarized by time points, and graphically presented as mean concentration-time profiles. The error bars represent the standard deviation (SD) at each time point.

PHARMACODYNAMICS

Body sway values were log-transformed prior to analysis to correct for the expected log-normal distribution of the data [17-19]. The effects of the four treatments on the pharmacodynamic measurements were compared with a mixed model analysis of variance. In this statistic model, treatment, period, time and treatment by time were set as fixed factors; and the random factors were subject, subject by treatment and subject by time; the baseline value was included as covariate, where baseline is defined as the average of the available measures obtained prior to dosing. The following contrasts were requested to demonstrate the effects of the active treatments: placebo-pregabalin, placebo-alprazolam, and placebo-diphenhydramine.

A summary table of the analysis results was generated with estimates of the difference between each active treatment and placebo and a back-transformed estimate of the difference in percentage for Body Sway, 95% confidence intervals (in percentage for Body Sway) and Least Square Means (geometric means for Body Sway), and the p-value of the contrasts. Least Square Means graphs were generated, with the Least Square Means of the analysis of the data as change from baseline.

Previous studies suggested good sensitivity of SPV to the effect of BZDs [6] and $\alpha_{2,3}$ subtype-selective GABA-A receptor modulators [17-19,33-35]. There is a close association between the effect size of benzodiazepines for SPV-reduction and their administered doses [6]. Based on the putative link between GABA-A $\alpha_{2,3}$ receptors and anxiety [36,37], this supports the consideration of SPV as a biomarker of clinical anxiolysis associated with GABA $\alpha_{2,3}$ activation [16], and the predictivity of SPV was supported by the selective SPV-reduction caused by TPA023 [17], combined with early clinical findings of this partial GABA $\alpha_{2,3}$ agonist [36]. BZDs also affected body sway, $VAS_{alertness}$, adaptive tracking, and $VAS_{calmness}$, suggesting impairment of postural balance, subjective alertness, eye-hand coordination, and subjective calmness, respectively [17-19,33,34]. Given the clinical relevance of these pharmacodynamic parameters, scatter plots of each pharmacodynamic measurement against simultaneously obtained SPV values were depicted to demonstrate SPV-normalized effect profiles with the study treatments. Moreover, a regression analysis was performed using the mixed model with treatment as the fixed factor

and SPV change from baseline and intercept as the random factors. Comparisons were made between each two active treatments with regards to the estimates of the slopes of the regression line obtained from each relative effect profile. The estimates of the slopes and their estimated difference were tabulated with the p-values. The slopes of these regression lines can be regarded as a measure of pharmacological selectivity of the drugs in respective of their anxiolytic effect [16].

RESULTS

SUBJECTS

Twelve men and ten women participated in the study. Ten subjects of each gender completed the study. The two drop-outs withdrew for personal reasons unrelated to the study, and were replaced by male subjects who received the same order of study treatments. Subjects had an average age of 22 years (range 18-36), and BMI of 23.3 kg/m^2 (range 18.1-29.6). Data from all treated subjects were used in the analyses of safety and pharmacokinetics. Subjects who completed the study per protocol were included in the pharmacodynamic analysis.

SAFETY

No serious adverse events were observed during the study. Neither were subjects discontinued their study due to AEs. The most frequently reported adverse events were 'somnolence', 'dizziness', 'fatigue' and 'headache'. Alprazolam was associated with the largest number of CNS-related AEs (n=21 in 14 out of 21 [66.7%] subjects), followed by diphenhydramine (n=19 in 16 out of 21 [76.2%] subjects), pregabalin (n=15 in 9 out of 20 [45.0%] subjects) and placebo (n=14 in 11 out of 20 [55.0%] subjects). Most AEs were attributed to the CNS-depressant effects of the study treatments. No ECG or laboratory abnormalities were judged clinically significant.

PHARMACOKINETICS

Sixty-two concentration-time profiles were obtained (20 for pregabalin, 21 for diphenhydramine and 21 for alprazolam). Following single-dose oral administration, peak plasma concentrations of all three active treatments were reached at 2-3 hours post-dose. Mean (standard deviation, SD) C_{max} was 4.87 (0.94), 91.47 (29.85) and 15.17 (2.10) mg/L for pregabalin, diphenhydramine and alprazolam, respectively. Figure 1 showed the average concentration-time profiles of pregabalin, diphenhydramine and alprazolam.

PHARMACODYNAMICS

The profiles of the CNS pharmacodynamic parameters (Figure 2 and Figure 3) showed that peak effects of the study treatments were usually observed around the point of T_{max}. Table 1 summarized the results of statistical comparisons between each active drug and placebo. Compared to placebo, $VAS_{calmness}$ increased statistically significantly with alprazolam (2.0 mm) and pregabalin (2.5 mm), but not with diphenhydramine (1.1 mm). In the meantime, saccadic peak velocity (SPV) declined after alprazolam (-57 deg/sec) and pregabalin (-28 deg/sec), more than by diphenhydramine (-14 deg/sec); so did smooth pursuit. The average responses of SPV were significantly correlated with the drug-induced increases in $VAS_{calmness}$.

To further characterize the pharmacodynamic profiles of these compounds, various CNS pharmacodynamic effects were compared with the corresponding drug-induced SPV reductions. According to the analyses about SPV-relative effect profiles (Table 2), the SPV-normalized impairment of adaptive tracking was higher after diphenhydramine and alprazolam, compared to that of pregabalin. The estimated slope for the regression line Δsway/ΔSPV was rather flat with pregabalin and significantly smaller than alprazolam and diphenhydramine. The slope for the $\Delta VAS_{alertness}/\Delta SPV$ relation was larger with pregabalin and alprazolam than with diphenhydramine. No significant difference was found among alprazolam, diphenhydramine, and pregabalin in the relative effect profiles of $\Delta VAS_{calmness}$ versus ΔSPV. The results of the FPS paradigm were reported in a separate article [22].

DISCUSSION

In this study, a set of neuropsycho-pharmacodynamic tests (i.e., the Neurocart battery) was performed to characterize the CNS profiles of three clinically anxiolytic and/or hypnotic drugs. Therapeutically relevant doses were administered as a single dose, because all drugs had a rapid onset of effects. The aim was to identify response patterns that are shared by fast-acting anxiolytics (alprazolam and pregabalin) but differ from sedative effects (diphenhydramine).

For the assessment of fear-potentiated startle, none of the treatments reliably reduced either fear- or anxiety-potentiated startle. Alprazolam and diphenhydramine reduced overall baseline startle. Pregabalin did not significantly affect any of the physiological measures [22]. On the other hand, as a full GABA-A agonist, alprazolam induced robust effects on most CNS parameters. Such generalized CNS depressive pharmacodynamics is similar to that of other benzodiazepines [29,33,34] and can be explained by the non-selective modulation of alprazolam on

different GABA-A receptor subtypes, which constitute the most widely distributed inhibitory receptors in the CNS. Pregabalin and its congener gabapentin are more selective and affect the α2 subunit of the voltage-dependent calcium channel. Contrary to benzodiazepines, 'gabapentinoids' don't bind to GABA receptors, but both drug classes lead to a decrease of the stimulatory neurotransmitters that are involved in anxiety, such as glutamate and the monoamines [38]. In this study, pregabalin was associated with moderate reduction of SPV and smooth pursuit, as well as statistically significant increase of $VAS_{calmness}$. Diphenhydramine, acting as an antagonist at the histamine H_1 receptors, slightly reduced SPV, but it did not influence $VAS_{calmness}$. As an indication that the 50 mg dose was functionally relevant, diphenhydramine showed a prominent effect on adaptive tracking.

An important finding of this study was the improvement of subjective calmness after a single dose of pregabalin and alprazolam. Moreover, the increase of $VAS_{calmness}$ was significantly correlated with SPV reductions. The literature is less clear about the subjective effects of anxiolytic drugs in healthy volunteers. In general, inconsistent changes of $VAS_{calmness}$ have been reported for single doses of lorazepam (2 mg) and some $\alpha_{2,3}$-subtype selective GABA-A agonists [17-19,33,34], even at dosages that are clinically more anxiolytic than the relatively low doses of alprazolam 1 mg or pregabalin 200 mg employed in the current study. These inconsistencies suggest that $VAS_{calmness}$ is a less reliable biomarker in studies where anxiety is not specifically stimulated. In such 'normal' drug studies, healthy subjects can experience different levels of anxiety, for instance depending on how familiar they are with these experiments, which may affect their sensitivity to anxiolytic drug effects. In the current study, subjects were repeatedly exposed to fear potentiated startle tests, which include unpleasant electrical shocks. We assume that this has induced a mild anticipatory anxiety in the study subjects [39], which was suppressed by the anxiolytic drugs but not by the sedative antihistamine.

On the other hand, the partial effect profiles of diphenhydramine and pregabalin and the more general CNS depression caused by alprazolam seems to match their pharmacological characteristics. Strictly speaking, a reliable comparison of pharmacological effect profiles is only justified across a wider dose range or at least at roughly equipotent dosages. Although it is difficult to establish dose equivalence across different drugs classes, all doses were in their therapeutic range. We tried to solve this further by looking at relative effect profiles across the entire profile of the plasma concentrations of the investigated drugs [16]. With this approach, the concern regarding dose equivalence in PD comparisons is overcome by transforming from dose-based PD-effect relationship to exposure-based PD effect relationship. SPV is one of the most sensitive pharmacodynamic biomarkers for anxiolytic doses of benzodiazepines [6]. Therefore, SPV was used to benchmark anxiolytic effects and was compared by linear regression with a second CNS biomarker to depict a drug effect on another CNS domain.

As can be seen in Table 1 and 2, alprazolam and diphenhydramine lead to comparable impairments on body sway (measure of postural stability) relative to their effects on saccadic peak velocity. In contrast, the effect of pregabalin on body sway was less remarkable than SPV. The differential effects of pregabalin on these two pharmacodynamic parameters seem to be consistent with the clinical behavior of this compound, which, compared to benzodiazepines, shows a larger therapeutic window between anxiolysis and ataxia [40]. The slopes of the $\Delta VAS_{alertness}/\Delta SPV$ regression lines are comparable among the study treatments. This is different from our previous findings between selective and non-selective GABA-A receptor agonists [16]. As subjects were physically and mentally stressed by electronic shocks of the fear-potentiated-startle paradigm [22], this challenge probably increased the baseline level of $VAS_{alertness}$ and hence reduced the responses to the investigated anxiolytic/hypnotic drugs. In addition, a distinct relationship was seen in the ΔSPV-relative effect profiles of ΔTracking among the three compounds. The steeper slope of the ΔTracking/ΔSPV regression line after diphenhydramine reflects its minimal effect on SPV but substantial effect on tracking. Such a profile is linked to the clinical properties of diphenhydramine: it shows considerable hypnotic effects at the dose of 50 mg, but does not lead to anxiety relief. Known side-effects of this compound, including drowsiness and motor impairment, are attributed to its inverse agonism at the histamine H_1-receptors distributed in the brain.

Taken together, the results of present study supports the combination a physically stressful procedure to the subjective assessment of anxiolysis. Consistently, the simultaneous reduction of SPV and the correlation between these two PD measurements provide further confirmation for the use of these biomarkers for clinically relevant anxiolytic effects. The sensitivity of the experiment appears to have been increased by the constant mild anticipation of shock during repeated FPS testing. The different effect profiles of the three drugs are in line with their pharmacological distinctions. These findings corroborate the profiling of CNS effects to demonstrate pharmacological selectivity, optimize the previous use of EEG/psychomotor/subjective pharmacological assessments [41] to a more pharmacological mechanism-based PD marker selection, and warrant the extension from a single, less reliable, subjective assessment to the combination of a stress-challenged subjective measurement and a neurophysiological test for the evaluation and extrapolation of clinical anxiolysis.

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Table 1 · Summary of the analysis results for CNS-pharmacodynamic parameters. (The results are presented as the estimated differences between each active treatment and placebo in the least square mean [LSM] change from baseline and the 95% confidence intervals [CIs] of the differences. The results of body sway are presented as the differences of LSM proportional change from baseline and their 95% CIs.)

Parameter (unit)	Pregabalin vs. Placebo	Alprazolam vs. Placebo	Diphenhydramine vs. Placebo
Body sway (mm)	12.27% (-2.37%, 29.11%) p=0.1026	34.43% (16.90%, 54.59%) p<0.0001	12.25% (-2.35%, 29.03%) p=0.1021
Saccadic Inaccuracy (%)	0.4 (-0.2, 0.9) p=0.1670	0.8 (0.3, 1.4) p=0.0021	0.3 (-0.2, 0.8) p=0.1827
Saccadic Peak Velocity (deg/sec)	-27.7 (-35.9, -19.5) p<0.0001	-56.9 (-65.0, -48.8) p<0.0001	-13.8 (-21.7, -5.9) p=0.0010
Saccadic Reaction Time (sec)	0.001 (-0.006, 0.009) p=0.7032	0.010 (0.003, 0.017) p=0.0082	0.002 (-.005, 0.009) p=0.6109
Smooth pursuit (%)	-5.1 (-7.8, -2.5) p=0.0003	-6.8 (-9.5, -4.2) p<0.0001	-0.5 (-3.1,2.1) p=0.7149
Adaptive tracking (%)	-1.04 (-2.30, 0.22) p=0.1039	-5.04 (-6.30, -3.78) p<0.0001	-2.64 (-3.92, -1.36) p=0.0001
VAS Alertness (mm)	-2.3 (-5.7, 1.0) p=0.1676	-4.5 (-7.8, -1.1) p=0.0096	-1.0 (-4.4,2.3) p=0.5377
VAS Calmness (mm)	2.5 (0.4, 4.7) p=0.0201	2.0 (-0.1, 4.1) p=0.0606	1.1 (-1.0, 3.2) p=0.3066
VAS Mood (mm)	0.7 (-0.5,2.0) p=0.2483	-0.1 (-1.4, 1.1) p=0.8633	0.4 (-0.8, 1.7) p=0.5059

CNS=central nervous system

Table 2 · Summary of Relative Effect Profile Among The Three Active Treatments. (The results are presented as least square mean [LSM] estimates of the slope of regression line. The p-values are presented for the comparisons of each two active treatments.)

				P-value		
	ALP	DPH	PRG	ALP-DPH	ALP-PRG	DPH-PRG
ΔSway/ΔSPV	-0.00208	-0.00186	-0.00106	0.5733	0.0055	0.0716
ΔTracking/ΔSPV	0.07785	0.06189	0.03056	0.1526	<0.0001	0.0133
ΔVAS alertness/ΔSPV	0.07227	0.01491	0.06061	0.0008	0.4540	0.0156
ΔVAS calmness/ΔSPV	-0.03626	-0.02776	-0.05070	0.6564	0.4123	0.2834

Figure 1 · Average plasma concentration-time profiles with standard deviation (SD) error bars of each compound after single oral administration (population estimates superimposed)

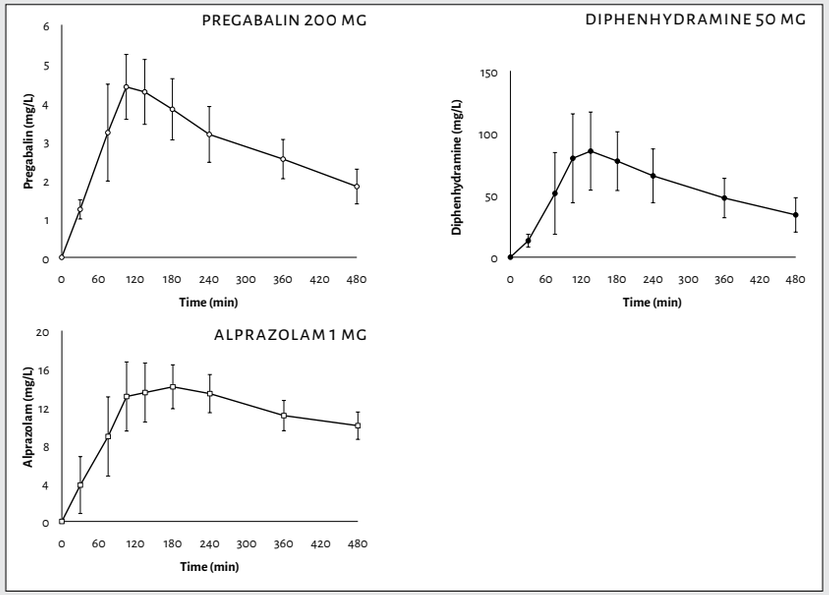
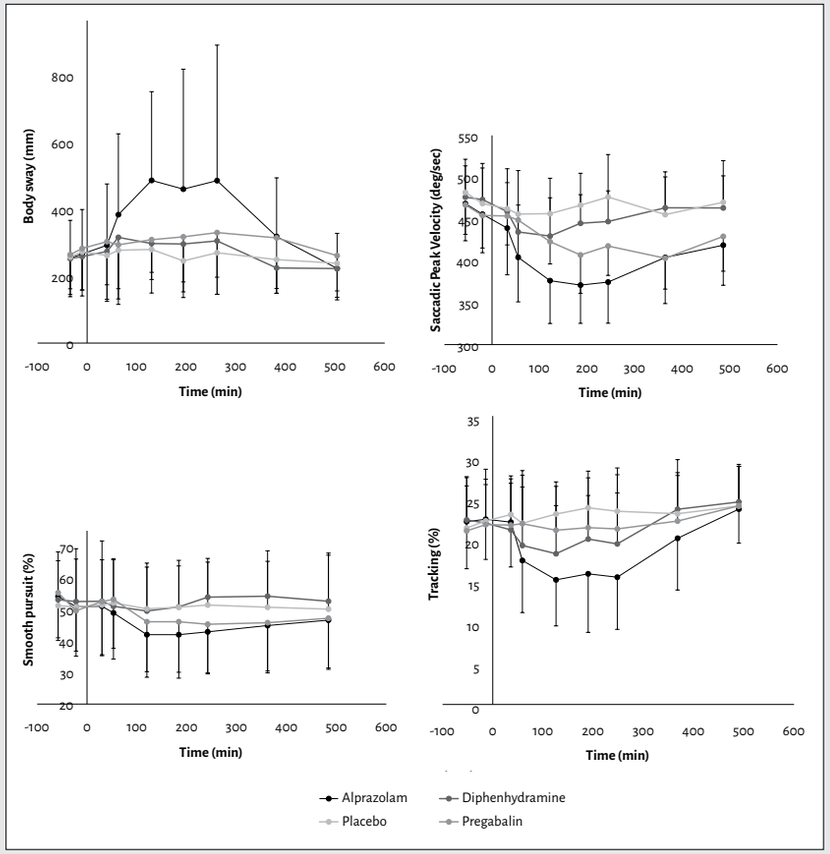
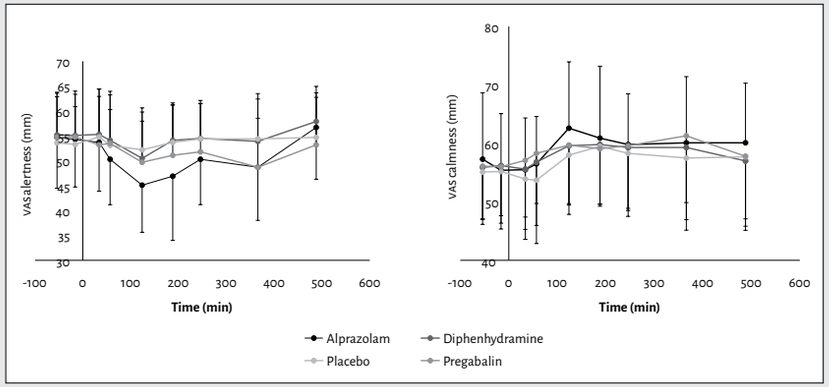


Figure 2 · Graph of means of objective CNS-pharmacodynamic parameters with standard deviation as error bars



2a. Body Sway; 2b. Saccadic Peak Velocity; 2c. Smooth Pursuit; 2d. Adaptive Tracking

Figure 3 · Graph of means of subjective CNS-pharmacodynamic parameters with standard deviation as error bars



3a. Visual Analogue Scale of Alertness; 3b. Visual Analogue Scale of Calmness

VIII
DISCUSSION AND CONCLUSION

For more than two decades, no mechanistically novel anxiolytic agents have been approved and launched into the market for the treatment of anxiety disorders. Such situation may be attributed to the lack of a solid understanding on the underlying pathophysiology of anxiety disorders, as well as the insufficiency in the development and application of valid animal models and their inability to reliably predict clinical anxiolytic effects in humans [1]. In addition, the term 'anxiety disorders' actually represents a heterogeneous group of illnesses that share a core phenomenology of both excessive fear and anxiety in terms of apprehension and worry about future events. Psychiatrists are still struggling to define the appropriate nosological classification of these disorders and current diagnostic classifications lack a robust neurobiological basis for clinical anxiety-related phenomena. The changing diagnostic landscape and uncertain boundaries between both the various anxiety disorders and mood disorders introduce further challenges for drug development [1]. Meanwhile, the search of novel pharmacotherapies for various anxiety disorders is driven by the growing medical need derived from clinically available drugs for the improvement of their effectiveness and/or for the reduction of their side-effect profiles [2].

The pharmacotherapeutic pipeline of anxiolytic treatments in development can be outlined into three major trends: 1) exploration of compounds acting on novel targets that address the underlying neural circuits of anxiety disorders, in which the glutamate, various neuropeptides and the endocannabinoid systems show particular promise as the targets of future drug development [4-6]; 2) design of compounds with established mechanism of action for anxiety but have modified or additional pharmacological properties than the traditional drugs: the development of subtype-selective GABA(A)-ergic partial agonists is an example of this approach; likewise, the recently marketed multi-target serotoninergic compounds, such as vortioxetine, vilazodone, and agomelatine [1], have been proved effective as antidepressant agents, and their efficacy on anxiety disorders has been shown in small population of patients; 3) repositioning of registered drugs for other indications in the treatment of anxiety disorders, such as clinical trials investigating the effects of antipsychotic drugs on anxiety disorders, and the approval of pregabalin by the European Medicines Agency for treatment of GAD in 2006 is a successful example of this approach [1,3].

Benzodiazepines were discovered by serendipity in the 1950s. Thereafter, due to the widespread therapeutic use of GABAergic agents on anxiolysis, sedation, seizure suppression, muscle relaxation, etc., as well as the cumulating understanding about GABA(A) receptor subunit neurophysiology and subtype-specific pharmacology, GABA(A) receptors have become a highly appreciated target in preclinical-to-clinical translational strategies. In the area of anxiety disorders, increasing evidence

from neuroscience indicates that anxiety disorders result from a functional imbalance in the modulation of brain circuits that regulate the emotional response to potentially threatening stimuli. In this context, the inhibitory network of GABAergic neurotransmission system is proposed to contribute to the pathogenesis of anxiety and hence serves as a promising therapeutic target for the treatment of human anxiety disorders [7].

In addition to anxiolytic effects, benzodiazepines also display potent sedative-hypnotic properties. For anxiety-related symptomatology like insomnia, these properties are useful. However, for the management of daytime anxiety, such effects are undesirable. The sedative effects and their ensuing cognition impairment and the potential for tolerance development and abuse liability are the major obstacles against wide and long-term use of benzodiazepines in the treatment of anxiety disorders. Previous research suggested these untoward effects are associated with the off-target pharmacological activities of benzodiazepines on the GABA(A) receptors containing α_1 and α_5 subunits [8-11]. As a result, novel GABA(A)-ergic α_1- and α_5-subtype sparing partial agonists, with either disproportional binding affinity or disproportional *in vitro* efficacy at the benzodiazepine-targeted GABA(A)-ergic receptor subtypes, are expected to separate anxiolytic effects from the BZDs-induced sedative and cognition-impairing effects.

Across the industry, the most common reason for developmental failure in Phase 2 in was lack of efficacy [12]. There are many areas of uncertainty regarding the translation of preclinical pharmacology data to human. These questions cannot be readily answered unless we know whether the drug actually expressed the intended pharmacology by modulating its target(s). In the entire process of clinical drug development, the demonstration of pharmacological effects with clinically tolerable doses is termed as proof-of-mechanism (POM) study. Generally speaking, these types of studies should comprise three goals: 1) observing drug exposure at the target site of action; 2) detecting drug interaction with the intended drug target; and 3) exploring effect of the drug on human biology using biomarker(s). Such investigational approach may be especially useful for anxiety disorders, in which therapeutic exploratory studies in patients can be difficult to achieve a clinically meaningful end-point due to the nature of subjective assessments, the relatively large sample size, the high probability of placebo effect, and other ethical or practical issues [13,14].

This thesis presents the early-phase proof-of-mechanism studies evaluating the pharmacokinetics and pharmacodynamics of three $\alpha_{2,3}$-subunit-selective GABA(A) agonists (i.e., AZD7325, AZD6280 and NS11821), in at least two dose levels, compared with active control (lorazepam), in its therapeutic dose, and placebo control. Most

of the studies were single-dose, double-blind, randomized, cross-over trials in healthy volunteers. A number of validated pharmacodynamic measurements were taken to address the effects of these novel drugs on psychomotor, neurophysiological, and neuroendocrine functions.

The results of each study provided a comprehensive picture about the pharmacological 'fingerprint' of the investigated compounds on a variety of CNS domains [15-17]. The concept of pharmacological selectivity was demonstrated by the relatively dominant effects of these novel compounds on saccadic eye movements, which measure the $\alpha_{2,3}$-subtype GABA(A) receptor related pharmacodynamic responses, in comparison with their minimal or none effects on postural stability, subjective alertness (i.e., measurements reflecting GABA(A) receptor α_1-subtype modulation) and cognition (i.e., GABA(A) α_5-subtype specific effects) [18]. In contrast, lorazepam-induced SPV reduction was generally consistent with its effect size on the other non-SPV neurophysiologic biomarkers. Considering the potential relation between SPV decline and clinical anxiolysis [19], the similarity in the effect size of these GABA(A) subtype-selective agonists on SPV implied the possibility of comparable anxiolytic effect between the novel compounds at certain dose levels and the active control, and was therefore taken as supportive evidence for future dose selection and the decision of further clinical development. Meanwhile, the flat concentration-effect curves of the novel GABA(A)-ergic compounds on subjective alertness, visuo-motor coordination, postural balance, and cognition indicate relatively favorable clinical side-effect profiles of these drugs versus the traditional non-subtype-selective full GABA(A) agonists, such as lorazepam. However, since the dose potency of the novel GABA(A)-ergic drugs might not be equivalent to that of lorazepam 2 mg, the lack of effects on the abovementioned CNS domains cannot be directly interpreted as improvement of adverse effects. In order to resolve this problem, we incorporate these pharmacodynamic (PD) measurements into an SPV-normalized regression model.

As is indicated in the previous chapters, the abovementioned repeat pharmacodynamic measurements all presented a clear dose/exposure-response relationship in healthy volunteers administered with benzodiazepines and subtype-selective GABAergic compounds. The PD-SPV regression models established on simultaneously measured pharmacodynamic endpoints actually reflect the relative effect profiles of the investigated drug across a wide range of plasma drug concentrations. The effect size on SPV was used as the normalizer because SPV has been shown associated with $\alpha_{2,3}$ GABA(A) receptor subtype modulation [20]. Interestingly, recent studies [21] reported quantitative correlation between disturbed performance in saccadic eye movement paradigm and the severity of various anxiety disorders. These results suggested measurements of saccadic eye movements might also

serve as neuropathophysiological biomarkers for the status or severity of anxiety. Moreover, two additional findings suggested performance of saccadic eye movement may be a predictive biomarker for clinical anxiolytic effect: 1) TPA023, a previously developed GABA(A) receptor $\alpha_{2,3}$ subtype-selective agonist, induced significant SPV reduction and minimal sway impairment and no memory change in single-dose study performed in healthy volunteers [22], has demonstrated a better-than-placebo anxiolytic effect in its phase 2 proof-of-efficacy studies [19]; 2) our study with both GABA(A)-ergic and non-GABA anxiolytic compounds showed similar SPV-depressive effects by alprazolam and pregabalin with single doses of these drugs at their clinically anxiolytic doses [23].

The pooled data analysis on the studies of this GABA(A) modulator family was performed to not only summarize the common pharmacological characteristics of these compounds but also evaluate the sensitivity and specificity of the selected CNS-pharmacodynamic measures. Three $\alpha_{2,3}$-selective GABA(A) agonists (i.e., TPA023, TPACMP2, SL65.1498), one α_1-selective GABA(A) agonists (zolpidem), and another full GABA(A) agonist (alprazolam) were examined through this approach. Pharmacological selectivity was assessed by determination of regression lines for the change of a pharmacodynamic endpoint (ΔPD) versus the change from baseline of SPV (ΔSPV). The absolute slopes of the ΔPD-ΔSPV relations were consistently lower with the $\alpha_{2,3}$ selective GABA(A) agonists than with lorazepam, indicating that their effects on non-SPV pharmacodynamic measurements are less than their effects on SPV. The ΔSPV-ΔPD relations of lorazepam were comparable to those of alprazolam. In contrast, zolpidem, an α_1 selective GABA(A) agonist, showed relatively higher impairments in the α_1-relevant PD parameters relative to the effect on SPV, although its ΔPD-ΔSPV profiles did not statistically significantly differ from those of lorazepam. These ΔPD-ΔSPV findings support the pharmacological selectivity of the $\alpha_{2,3}$-selective GABA(A) agonists, implying that the clinical anxiolytic effect of these drugs might be accompanied with fewer untoward side effects on psychomotor and cognitive function.

In summary, the development of novel GABAergic compounds can be structured, step-by-step, as the preclinical-to-clinical translation process depicted in Figure 1. First of all, the neurobiological investigation about anxiety and the clinical experience with benzodiazepines both cast light on the GABAergic neurotransmission system as a potential pathway for new drug development. Further knock-in animal studies suggest that the pharmacological selectivity of a ligand for a certain GABA(A) receptor subtype can be achieved either by affinity differentiation (i.e., forming or not forming a receptor-ligand complex) or by efficacy differentiation (i.e., eliciting or not eliciting a biological response after binding to the receptor) [24]. Using 18^F-flumazenil as the tracer, a positron emission computed tomography

(PET) study provides information on the dose-dependency or exposure-dependency of the drug's *in vivo* GABA(A) receptor occupancy, and thereby helps to determine the dose range to be administered in future clinical development. In a clinical pharmacology study, the compound is assessed for its pharmacological effects and pharmacokinetic exposures within the tolerated dose range, at which considerable receptor occupancy can be reached based on the findings of previous neuroimaging study. The observed effects indicate biological interactions between the ligand and the targeted receptors. More specifically, in the case of GABA(A)-ergic novel compounds, the subtype-specific pharmacodynamic biomarkers, in conjunction with the simultaneously measured plasma drug concentrations, allows addressing the effect amplitude and effect potency of GABA(A) receptor subtype modulation elicited by the investigated drug and the active control, and demonstrates the PK/PD profile distinctions that one would expect between full agonist and partial agonist [25]. Also, the relationship of these effects builds up a bridge that connects the *in vitro* pharmacological activity to the *in vivo* physiological responses and supports the concept of pharmacological selectivity for $\alpha_{2,3}$-subtype selective GABA-A agonists in general and, in the case of this thesis, AZD7325, AZD6280 and NS11821.

The results of our research were informative and affected the decision of further clinical development of each specific novel compound: 1) since 10 mg AZD7235 was associated with 80-90% receptor occupancy, the small effect size of 2 mg and 10 mg AZD7325 indicated insufficient receptor modulation of the compound at the investigated dose [26]; 2) for NS11821, the pharmacodynamic effects observed at the moderate-to-high dose levels in the first-in-human study helped to identify and select the pharmacologically active doses for future clinical trials [27]; 3) for AZD6280, the pharmacodynamic effect size on SPV was similar to that of lorazepam, suggesting potentially comparative clinical anxiolytic effect, while the ignorable effects of this compound on body sway and $VAS_{alertness}$ were thought to predict a reduced profile of CNS side-effects [25]. Such clinical pharmacological profile was considered promising for further development and future clinical doses were probably limited to the range of 10 to 40 mg.

In order to link the neurophysiological and neuropsychological biomarkers to the pathophysiological alteration of anxiety patients in fear extinction, we integrated a fear-potentiated-startle (FPS) paradigm with the Neurocart pharmacodynamic set, and, in particular, with the repeated assessments of subjective calmness ($VAS_{calmness}$). The FPS paradigm is a procedure that mimics the fear extinction experiment in rodents [28] and aims to induce stressfulness in human. To evaluate the feasibility and utility of this approach, a validation study was conducted in healthy vopunteers. The PD effects of three marketed comparator drugs (i.e. two anxiolytic drugs and one hypnotic drug) were characterized by applying them as

pharmacological probes in the FPS study. The findings of this study corroborated the sensitivity and specificity of the CNS-PD measures to single therapeutic dose of GABAergic (alprazolam) and non-GABAergic (pregabalin) anxiolytic compounds, and reinforced the clinical relevance of saccadic eye movement measurements to clinical anxiolysis. In conjunction with the FPS paradigm, significant increase of subjective calmness was observed with the two anxiolytic drugs, which warrants the use of stress-challenged subjective measurements and neurophysiological tests for the prediction of clinical anxiolytic drug effect [24].

Last but not least, the exploration of potential endocrine biomarkers regarding the differential effects of selective and nonselective GABA receptor modulators suggested a compensatory approach for the pharmacodynamic evaluation of novel anxiolytic agents. The overall effects of the nonselective benzodiazepine lorazepam, as well as two novel $\alpha_{2,3}$ subunit-selective GABA(A) receptor modulators AZD7325 and AZD6280, on prolactin levels were measured within 8 hours post-dose in healthy male volunteers. Following administration of lorazepam at 2 mg and AZD6280 at 10 mg and 40 mg, prolactin levels increased significantly compared with placebo (difference 42.0%, 19.8%, and 32.8%, respectively), suggesting that the α_2 and/or α_3 receptor subtypes are involved in GABAergic modulation of prolactin secretion, although possible roles of the α_1 and α_5 receptor subtypes cannot be excluded. The increases in prolactin levels after administration of AZD7325 at 2 mg and 10 mg doses (difference 7.6% and 10.5%, respectively) did not reach statistical significance. Such results were consistent with the non-significant responses observed on the other neurophysiological and neuropsychological measurements with AZD7325 [15], reinforcing the conclusion that the investigated doses of AZD7325 or the intrinsic efficacy of AZD7325 at the α_2 and α_3 receptor subtypes may have been too low [29].

CONCLUSION

The GABAergic system has been implicated in the pathogenesis of various anxiety disorders. Pharmacological treatments, like benzodiazepines, have been proven to target the GABA(A) receptors and exert quick-onset anxiolytic effect in anxiety patients. However, the side effects of these non-selective GABA(A)ergic compounds, such as sedation, postural imbalance, or potential abuse, limit their use for clinical anxiolysis. Based on the understanding of benzodiazepines' mechanism of action, the emergence of $\alpha_{2,3}$ subtype-selective GABA(A) modulator is expected to provide a novel pharmacological approach that alleviates anxiety symptoms but spares the common undesired side effects. Most of these compounds are still in early clinical development, in which stage proof-of-mechanism studies are usually performed

in healthy volunteers. The findings from our studies consistently present a similar pattern in the pharmacodynamic effect profiles of the $\alpha_{2,3}$ subtype-selective GABA(A) modulators versus those of the non-selective full GABA(A) agonist, lorazepam. Future application of anxiogenic symptom provocation models that combine subjective measurements and/or neuroendocrine biomarker assays may provide further construct validity for clinical anxiolytic effects of $\alpha_{2,3}$ subtype-selective GABA(A) modulators. Also, such findings are expected to provide insights into the translation of preclinical pharmacological properties of $\alpha_{2,3}$ subtype-selective GABA(A)-ergic compounds to clinical effects in patients with anxiety disorders through human pharmacology studies.

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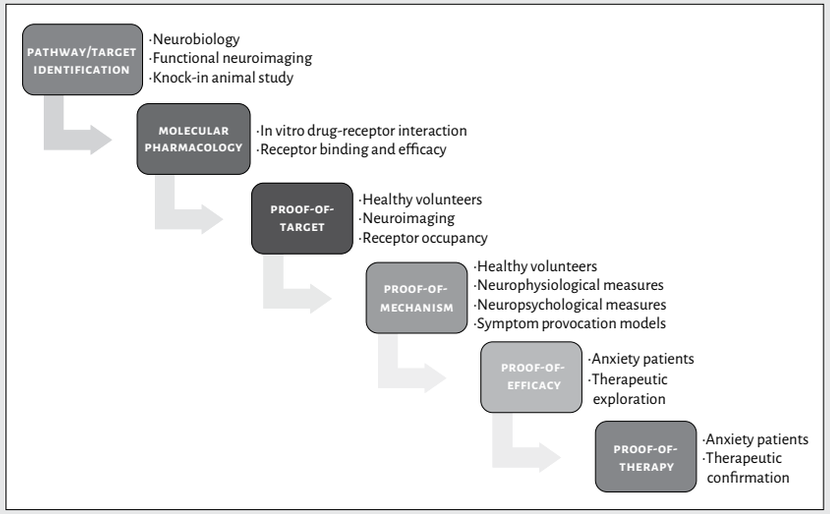
Figure 1 · Schematic graph about the developmental steps of GABAergic novel compounds from pathway/target identification to clinical research



ENGLISH SUMMARY

Anxiety disorders are highly prevalent psychiatric disorders that are associated with significant personal and societal costs. The transition from adaptive negative affect such as fear and anxiety to an anxiety disorder in humans is mediated by an interplay between psychosocial factors and a wide array of neurobiological alterations.

The introduction of this thesis (**chapter 1**) provides a detailed overview of the definition, classification, neurobiology and current psychopharmacological treatment of anxiety disorders. On a conceptual level, anxiety disorders result from disruptions of highly interconnected neuronal circuits that normally serve to process the stream of potentially threatening stimuli detected by the human brain from the outside world. Perturbations in any of these circuits cause imbalance in the entire system, resulting in a fundamental misinterpretation of sensory information as threatening and leading to inappropriate emotional hyperarousal, physical symptoms and behavioral responses that are characteristic of anxiety disorders. Although monoamine modulating drugs such as the selective serotonin reuptake inhibitors (SSRI's) and gamma-aminobutyric acid (GABA) agonists are widely applied to modulate central emotional processing centers in patients with anxiety disorders, their effectiveness is limited in a large proportion of patients due to either inefficacy or untoward effects. This obviously unmet clinical need in the treatment of anxiety disorders opens an opportunity for novel pharmacological approaches.

As the predominant inhibitory neurotransmitter system in the human brain, the GABAergic system has been implicated in the pathophysiology of anxiety disorders. Evidence from preclinical studies suggests distinct physiological effects of the benzodiazepines-targeted α1, α2, α3, and α5 GABA(A) receptor subunits: α2/α3-subunits predominantly mediate analgesia and anxiolysis, while α1- and α5-subunits are associated with sedation and cognition, respectively. The relatively high affinity or *in vitro* efficacy of novel α2,3 subtype-selective GABAergic receptor modulators therefore represents a potentially useful innovative pharmacological approach for the treatment of anxiety disorders. This thesis is largely devoted to the early development of these innovative compounds, and to methods to show their effects in humans.

In the subsequent chapters, we report three first-in-human (FIH) clinical pharmacology studies which evaluated the pharmacokinetics and pharmacodynamics of the α2,3-subunit-selective GABA(A) agonists AZD7325 (**chapter 2**), AZD6280 (**chapter 3**) and NS11821 (**chapter 4**), respectively. Because of their pharmacological selectivity at the α2,3 GABA(A) receptor subtypes, these compounds are expected to elicit clinical anxiolysis without inducing unwanted sedative effects in humans. Therefore, these studies aimed to characterize the pharmacodynamic effects and evaluate the pharmacologically active doses/exposure levels of these compounds by applying Neurocart, a battery of previously validated pharmacodynamic measurements

that assess different functional central nervous system (CNS) domains. In all studies, at least two dose levels were explored and were compared with placebo and the non-selective GABA-A receptor agonist lorazepam as active control. The results of these studies demonstrate compound-specific effect profiles on the neurophysiological functions postural balance, visuo-motor coordination, cognition and subjective feelings for most compounds. Moreover, the concept of pharmacological selectivity is demonstrated by the relatively dominant effects of these novel compounds on saccadic eye movements, which reflects the GABA(A) α2,3-subtype receptor related pharmacodynamic responses, in comparison with their minimal or absent effects on postural stability and subjective alertness (i.e., α1-subtype receptor modulation) and cognition (i.e., α5-subtype-specific effects). In contrast, lorazepam-induced SPV reduction is generally similar to its effect size on the other non-SPV neurophysiologic biomarkers, indicating a comparable interaction with different GABA(A) receptor subtypes. These findings are corroborated by the α2,3-subtype-selective GABA(A) partial agonist TPA023, which previously demonstrated SPV reduction in healthy volunteers that translated to a clinical anxiolytic effect in patients with generalized anxiety disorder. Therefore, similar effect sizes of the evaluated α2,3 GABA(A) subtype-selective agonists on SPV suggest potentially efficacious anxiolytic effects comparable to the clinically effective dose of non-subtype-selective GABA(A) modulator, lorazepam. On the other hand, the flat concentration-effect curves of the α2,3-selective GABA(A)-ergic compounds on subjective alertness, visuo-motor coordination, postural balance and cognition, indicate a relatively favorable clinical side-effect profile of these drugs versus the traditional non-subtype-selective full GABA(A) agonists, such as lorazepam. Taken together, the demonstration of an equipotent α2,3 GABA(A) effect in the absence of either α1 or α5 effects provides support to further pursue clinical development and can potentially guide future dose selection for studies in both healthy volunteers and patients with anxiety disorders.

In **chapter 5**, we present a pooled data analysis based on studies with the α2,3 subtype-selective GABA(A) modulator family that were previously published by our group. The pharmacological selectivity of three α2,3-selective GABA(A) agonists (i.e., TPA023, TPACMP2, SL65.1498), one α1-selective GABA(A) agonist (zolpidem), and another non-selective GABA(A) agonist (alprazolam) were examined by modeling their regression lines for the effect on one of the (unwanted) pharmacodynamic endpoints (ΔPD) versus the simultaneous (desired) effect on SPV (ΔSPV). The absolute slope of the relation between the unwanted and desired pharmacodynamics effect (ΔPD-ΔSPV) was consistently lower with the α2,3 selective GABA(A) agonists than with lorazepam. Moreover, the ΔSPV-ΔPD relations of lorazepam were comparable to those of alprazolam, but slightly lower than zolpidem. Together, these ΔPD-ΔSPV findings further support the pharmacological selectivity of the α2,3-selective GABA(A) agonists, and as a consequence, imply

that the clinical anxiolytic effect of these drugs might be accompanied with fewer untoward side effects on psychomotor and cognitive function compared to the non-selective benzodiazepines.

Next to the neurophysiological, emotional and cognitive effects that were investigated in previous chapters, anxiety responses are also characterized by neuroendocrine reactions. This was explored further in **chapter 6**, which focused on potential potential peripheral neuroendocrine biomarkers for the effects of selective and non-selective GABA receptor modulators. The effects of two novel α2,3 subunit-selective GABA(A) receptor modulators, AZD7325 and AZD6280, on serum prolactin levels were evaluated in healthy male volunteers, compared with the non-selective GABA(A) modulator lorazepam. Prolactin levels increased significantly after administration of AZD6280 and lorazepam, whereas increases in prolactin levels after administration of AZD7325 did not reach statistical significance, probably because the dosages were too low. These findings suggest that the α2 and/or α3 receptor subtypes are involved in GABAergic modulation of prolactin secretion, although possible roles of the α1 and α5 receptor subtypes cannot be excluded. The observed drug effects on serum prolactin levels support the use of serum prolactin level as a neuroendocrine biomarker complementary to the validated pharmacodynamic measurements in clinical pharmacology study of novel anxiolytic agents.

The previous chapters mainly describe the pharmacological effects of GABA-ergic compounds on the CNS. To get an impression of their potential anxiolytic effects, anxiety and fear can be examined in healthy volunteers. Finally in **chapter 7**, the fear-potentiated-startle (FPS) paradigm is used to experimentally simulate conditioned and unconditioned threat in healthy volunteers. Conceptually the former scenario represents fear whereas the latter relates to anxiety. In this study, FPS is combined with saccadic and smooth pursuit eye-movement tests, visual analogue scales measuring subjective alertness, visuo-motor coordination and postural balance to evaluate anxiolytic drug effect on the FPS-stimulated neurophysiological and neuropsychological responses. The PD effects of two anxiolytic drugs (alprazolam and pregabalin) and one hypnotic drug (diphenhydramine) were characterized in the presented study. None of the treatments reliably reduced either fear or anxiety-potentiated startle responses, probably due to methodological complexity and the variability of startle responses between and within study participants. However, decrease of subjective calmness from baseline was evident after the stressful FPS procedure during the placebo treatment, while alprazolam and pregabalin maintained subjective calmness to its baseline level following FPS. Such findings corroborate the sensitivity and specificity of the CNS-PD measures to a single therapeutic dose of GABAergic (alprazolam) and non-GABAergic (pregabalin) anxiolytic compounds. In fact, clinically available anxiolytic drugs, such as benzodiazepines or SSRIs also do not consistently induce

significant increases of subjective calmness in healthy volunteers under stress-free experimental conditions. Therefore, the measurable effects on subjective calmness, as well as the test procedure modification with FPS integration, may warrant the use of stress-challenged subjective measurements and neurophysiological tests for the simulation of clinical anxiolytic drug effect.

CONCLUSION

The GABAergic system has been implicated in the pathogenesis of various anxiety disorders. Clinically effective pharmacological treatments like benzodiazepines have been demonstrated to target the GABA(A) receptors, by which they exert acute anxiolytic effects in patients with anxiety disorders. However, the side effects of these non-selective GABA(A)-ergic compounds, such as sedation, postural imbalance, cognitive effects and potential abuse limit their use in clinical practice. Based on the understanding of benzodiazepines' mechanism of action, the emergence of α2,3 subtype-selective GABA(A) modulators is expected to provide a novel pharmacological approach that alleviates anxiety symptoms but spares the common undesired side-effects. Most of these compounds are still in early clinical development, in which stage proof-of-mechanism studies are usually performed in healthy volunteers. The findings from our studies consistently present a similar pattern in the pharmacodynamic effect profiles of the α2,3 subtype-selective GABA(A) modulators versus those of the non-selective full GABA(A) agonist, lorazepam. Future application of anxiogenic symptom provocation models that combine subjective measurements and/or neuroendocrine biomarker assays may provide further construct validity for clinical anxiolytic effects of α2,3 subtype-selective GABA(A) modulators. Also, such findings are expected to provide insights into the translation of preclinical pharmacological properties of α2,3 subtype-selective GABA(A)-ergic compounds to clinical effects in patients with anxiety disorders through human pharmacology studies.

Overall, the work in this thesis illustrates an important step in a structured translational process of novel subtype-selective GABAergic compounds from preclinical development to early phase clinical trials : 1) **pathway identification** of the gabaergic neurotransmission system informed by the neurobiology of anxiety and clinical efficacy of benzodiazepines; 2) **novel drug design and discovery** based on knock-in animal studies that suggest the distinct pharmacological activities of various GABA(A) receptor subtypes; 3) **proof-of-target study** using neuroimaging tools to demonstrate the drug's *in vivo* GABA(A) receptor occupancy; 4) **proof-of-mechanism study** assessing the drug's pharmacodynamic effects and pharmacokinetic exposures within the tolerated dose range; 5) **proof-of-efficacy study** exploring the drug's clinical efficacy in the target patient population; 6) **proof-of-therapy study** confirming the drug's clinical utility and effectiveness in in clinical practice.

NEDERLANDSE SAMENVATTING

Angststoornissen zijn veel voorkomende psychiatrische aandoeningen die persoonlijk lijden en hoge maatschappelijke kosten met zich meebrengen. De overgang van adaptieve negatieve emoties zoals vrees en angst naar een angststoornis wordt bij mensen medebepaald door psychosociale factoren en een breed scala aan neurobiologische veranderingen.

Het inleidende hoofdstuk van dit proefschrift (**hoofdstuk 1**) geeft een gedetailleerd overzicht van de definitie, classificatie, neurobiologie en de actuele farmacotherapie van angststoornissen. Op conceptueel niveau zijn angststoornissen het gevolg van een verstoring van onderling sterk verbonden hersencircuits die onder normale omstandigheden potentieel bedreigende stimuli uit de buitenwereld verwerken. Verstoringen in één of meer van deze circuits kunnen ertoe leiden dat het gehele systeem uit balans raakt. Sensorische informatie wordt dan ten onrechte als bedreigend geïnterpreteerd, wat leidt tot de extreme emotionele en lichamelijke reacties en de bijbehorende gedragingsveranderingen die kenmerkend zijn voor angststoornissen. Gamma-aminoboterzuur (GABA)-agonisten en monoaminerge geneesmiddelen zoals de selectieve serotonine heropnameremmers (SSRI's), worden frequent toegepast om de centrale emotieverwerkende centra in patiënten met angststoornissen te beïnvloeden. De toepasbaarheid van dergelijke behandelingen is echter bij veel patiënten beperkt als gevolg van onvoldoende werkzaamheid en/of ongewenste bijwerkingen. Er bestaat dus duidelijk een behoefte aan betere geneesmiddelen voor de behandeling van angststoornissen en geeft aanleiding tot innovatieve farmacologische benaderingen in de ontwikkeling van nieuwe anxiolytica.

Het GABA-erge systeem is het belangrijkste inhibitoire systeem in de menselijke hersenen. GABA is in verband gebracht met de pathofysiologie van angststoornissen en vormt daarmee een belangrijk aangrijpingspunt voor farmacologische behandelingen. Preklinisch onderzoek suggereert duidelijk te onderscheiden fysiologische functies voor de verschillende GABA(A) receptor subtypes, namelijk α1, α2, α3, en α5, die aangrijpingspunten zijn voor de benzodiazepines. Hierbij lijken de α2/α3-subtypes vooral betrokken te zijn bij pijnstilling en anxiolyse, terwijl de α1- en α5-subtypes samenhangen met respectievelijk de sederende en cognitieve effecten van benzodiazepines. Een groep nieuwe α2,3 subtype-selectieve GABA-agonisten hebben een relatief hoge receptoraffiniteit dan wel *in vitro* werkzaamheid voor GABA(A) α2,3, waarmee zij een innovatieve en potentieel nuttige aanvulling kunnen zijn in de farmacotherapie van angststoornissen. Dit proefschrift is grotendeels gewijd aan de vroege ontwikkeling van dergelijke innovatieve middelen, en aan manieren om hun effecten bij mensen aan te tonen.

In de volgende drie hoofdstukken worden klinisch farmacologische studies beschreven waarin drie verschillende α2,3-subtype selectieve GABA(A) agonisten voor het eerst worden toegediend aan menselijke vrijwilligers. In deze *first in human* (FIH) studies werden de farmacokinetiek en de farmacodynamiek van de α2,3-subtype selectieve GABA(A) agonisten AZD7325 (**hoofdstuk 2**), AZD6280 (**hoofdstuk 3**) and NS11821 (**hoofdstuk 4**) onderzocht. Vanwege hun farmacologische selectiviteit voor de α2,3 GABA(A) receptorsubtypes, verwacht men een klinisch anxiolytische werking zonder de ongewenste sederende effecten van de benzodiazepines. In het bijzonder waren deze studies gericht op het karakteriseren van de farmacodynamische effecten en het vaststellen van de farmacologisch actieve doses en plasmaconcentraties van deze middelen. Voor dit doel werd de NeuroCart toegepast, een testbatterij bestaande uit eerder gevalideerde farmacodynamische metingen die verschillende functionele domeinen van het centraal zenuwstelsel (CZS) kwantificeren. In deze studies werden tenminste twee verschillende doseringen van de nieuwe geneesmiddelen onderzocht en werden deze doorgaans vergeleken met placebo enerzijds en met de niet-selectieve GABA-A receptoragonist lorazepam als 'actieve controle' anderzijds. De resultaten van deze studies tonen voor de meeste stoffen een duidelijke farmacologische selectiviteit. Vergeleken met benzodiazepines, zijn er relatief sterke effecten op de saccadische oogbewegingen (saccadic peak velocity, SPV), die vooral een maat zijn voor GABA(A) α2,3-subtype activiteit. Daarentegen worden nauwelijks tot geen effecten gevonden op houdingsstabiliteit en subjectieve alertheid (α1-subtype effect) en cognitie (α5-subtype effect). Bij lorazepam is de afname van de SPV in het algemeen van dezelfde orde van grootte als het effect op de andere genoemde neurofysiologische biomarkers, wat erop wijst dat lorazepam de verschillende GABA(A) receptorsubtypes in ongeveer dezelfde mate beïnvloedt. Deze bevindingen zijn in lijn met de eerder onderzochte effecten van de α2,3-subtype-selectieve GABA(A) partiële agonist TPA023, waarbij een vermindering van de SPV bij gezonde proefpersonen werd gevonden en bij patiënten met een gegeneraliseerde angststoornis klinisch relevante anxiolyse werd aangetoond. Vergelijkbare effectgroottes van de drie onderzochte α2,3 GABA(A) subtype-selectieve agonisten op SPV suggereren dat deze stoffen potentieel een even krachtige anxiolytische werking zouden kunnen hebben als een klinisch effectieve dosis van de niet-subtype selectieve GABA(A) agonist lorazepam. Met betrekking tot de bijwerkingen, wijzen de relatief vlakke concentratie-effect curves van de α2,3 GABA(A) -erge middelen voor subjectieve alertheid, visuo-motorische coördinatie, houdingsbalans en cognitie op een gunstig klinisch bijwerkingenprofiel in vergelijking met traditionele niet-subtypeselectieve GABA(A) agonisten zoals lorazepam. Al met al vormen de gerapporteerde studies een goede basis voor de verdere klinische ontwikkeling van deze middelen, waarbij de α2,3 GABA(A) effecten even krachtig zijn als de niet-selectieve GABA(A) agonisten terwijl er geen sprake is α1 of α5 effecten. Overigens kunnen deze resultaten ook worden toegepast bij het selecteren van de optimale dosering in toekomstige studies met gezonde vrijwilligers en patiënten met angststoornissen.

In **hoofdstuk** 5 wordt een gecombineerde analyse gepresenteerd, gebaseerd op data afkomstig uit studies met α2,3 subtype-selectieve GABA(A) modulatoren die eerder door onze onderzoeksgroep zijn gepubliceerd. De farmacologische selectiviteit van drie α2,3 subtype-selectieve GABA(A) agonisten (TPA023, TPACMP2 en SL65.1498), een α1-selectieve GABA(A) agonist (zolpidem) en een niet-selectieve GABA(A) agonist (alprazolam), werd onderzocht door de regressielijn te modelleren voor een van de (ongewenste) farmacodynamische eindpunten (ΔPD) versus het gelijktijdige (gewenste) effect op SPV (ΔSPV). De absolute helling van de verhouding tussen de ongewenste en ongewenste farmacodynamische effecten (ΔPD/ΔSPV) was lager voor α2,3 subtype-selectieve GABA(A) agonisten dan voor lorazepam. Verder waren de ΔPD/ΔSPV verhoudingen van lorazepam vergelijkbaar met die van alprazolam, maar iets lager dan voor zolpidem. Deze bevindingen bieden verdere steun aan de hypothese dat α2,3 subtype-selectieve GABA(A) agonisten inderdaad farmacologisch selectief zijn en dat het klinische anxiolytische effect van deze middelen gepaard zou kunnen gaan met minder ongewenste bijwerkingen op de psychomotorische en cognitieve functies dan de niet-selectieve benzodiazepines.

Naast de neurofysiologische, emotionele en cognitieve reacties die in voorgaande hoofdstukken werden bestudeerd, vormen neuro-endocriene responsies een belangrijk onderdeel van angstreacties. Derhalve werden potentiële perifere neuro-endocriene biomarkers voor de effecten van selectieve en niet-selectieve GABA receptormodulatoren in **hoofdstuk 6** verkend. Het effect van twee α2,3 subtype-selectieve GABA(A) receptormodulatoren, AZD7325 en AZD6280, op de serum prolactineconcentraties werd bij gezonde proefpersonen vergeleken met de niet-selectieve GABA(A) receptormodulator lorazepam. AZD6280 en lorazepam leidden tot een significante stijging van de prolactineconcentratie terwijl de stijging van de prolactineconcentratie na toediening van AZD7325 geen statistische significantie bereikte mogelijk vanwege een te lage dosering. Deze resultaten suggereren dat het GABA(A) α2 en/of α3 receptorsubtype betrokken is bij prolactinesecretie, al kan eventuele betrokkenheid van de receptorsubtypes α1 en α5 niet met zekerheid worden uitgesloten. Deze effecten op serum prolactine ondersteunen het integreren van prolactine als neuro-endocriene biomarker met gevalideerde farmacodynamische metingen bij de klinisch farmacologische evaluatie van nieuwe anxiolytica.

In de voorafgaande hoofdstukken zijn vooral de farmacologische effecten van GABA-erge stoffen op het CZS bestudeerd. Om een indruk te krijgen van hun potentiële anxiolytische effecten, kunnen tevens de effecten op angstreacties bij gezonde vrijwilligers worden onderzocht. In **hoofdstuk 7** wordt het *fear-potentiated-startle* (FPS) paradigma toegepast om geconditioneerde en ongeconditioneerde bedreigende stimuli experimenteel bij gezonde vrijwilligers te simuleren. Geconditioneerde bedreiging komt conceptueel overeen met vrees (Engels: *fear*), terwijl ongeconditioneerde bedreiging meer overeenkomsten heeft met angst (Engels: *anxiety*). In deze studie worden de farmacodynamische effecten van twee klinisch effectieve anxiolytica (alprazolam en pregabaline) en een slaapmiddel (difenhydramine) onderzocht door FPS te combineren met neurofysiologische en neuropsychologische metingen, zoals saccadische oogbewegingen (SPV), subjectieve alertheid, houdingstabiliteit en oog-handcoördinatie. Geen van deze middelen verminderden de vrees of angst-gerelateerde schrikreactie (Engels: *startle response*), vermoedelijk als gevolg van de methodologische complexiteit en de grote intra-individuele en interindividuele variatie in schrikreacties. Wel ervoeren deelnemers die placebo hadden gekregen een duidelijke afname van de subjectief ervaren kalmte na de stressvolle FPS procedure, terwijl na toediening van alprazolam of pregabaline de subjectieve kalmte vergelijkbaar bleef met de uitgangswaarde. Deze bevindingen bevestigen de sensitiviteit en specificiteit van farmacodynamische CZS-metingen na toediening van een therapeutische dosis van een GABA-erg anxiolyticum (alprazolam) en een anxiolyticum met een ander werkingsmechanisme (pregabaline). Daarnaast is bekend dat klinisch effectieve anxiolytica zoals de benzodiazepines of SSRI's bij gezonde vrijwilligers onder stressvrije experimentele condities evenmin de subjectieve kalmte consistent vergroten. Bij het evalueren van anxiolytische geneesmiddeleffecten onder stressvolle omstandigheden valt daarom te overwegen om subjectieve metingen en neurofysiologische tests te combineren met FPS.

CONCLUSIE

Het GABA-erge systeem speelt een rol in de pathogenese van angststoornissen. Klinisch effectieve geneesmiddelen zoals de benzodiazepines hebben hun anxiolytische werking te danken aan een interactie met de GABA(A) receptoren. De toepassing van deze niet-selectieve GABA-erge middelen in de klinische praktijk wordt echter beperkt door bijwerkingen zoals sedatie, verstoorde lichaamsbalans en cognitieve effecten, en potentieel misbruik en verslaving. Huidige inzichten in het werkingsmechanisme van de benzodiazepines maken een nieuwe farmacologische benadering mogelijk, waarbij de α2,3 subtype-selectieve GABA(A) receptormodulatoren angst zouden kunnen reduceren zonder de ongewenste bijwerkingen van de benzodiazepines te hebben. De meeste van deze nieuwe middelen bevinden zich in de vroege klinische geneesmiddelontwikkeling, waarin overwegend mechanistische studies worden uitgevoerd bij gezonde vrijwilligers. De resultaten van onze studies laten consequent eenzelfde patroon zien in de farmacodynamische effecten van α2,3 subtype-selectieve GABA(A) receptormodulatoren vergeleken met de niet-selectieve GABA(A) agonist lorazepam. In de toekomst kunnen provocatiemodellen voor het induceren van angstsymptomen

bijdragen aan de constructvaliditeit en klinische toepasbaarheid van deze vroege bevindingen bij gezonde vrijwilligers. Bovendien kunnen dergelijke bevindingen inzichten opleveren voor de vertaalslag van de preklinische farmacologie van α2,3 subtype-selectieve GABA-erge middelen naar klinische effecten in patiënten met angststoornissen.

De beschreven studies illustreren een belangrijke stap in een gestructureerd translationeel proces van preklinische geneesmiddelontwikkeling naar vroege klinische studies voor nieuwe subtype selectieve GABA-erge middelen: 1) **identificatie van de betrokken signaalroutes** (Engels: *pathway identification*) van het GABA-erge neurotransmittersysteem vanuit de neurobiologie van angst en de klinische effecten van benzodiazepines; 2) **geneesmiddelontdekking en ontwerp** op basis van *knock-in* dierstudies die laten zien dat de verschillende GABA(A) receptor subtypes een te onderscheiden farmacologische activiteit vertonen; 3) **proof-of-target studies** waarin met behulp van beeldvormende technieken (Engels: *neuro-imaging*) wordt aangetoond met welke GABA(A) receptorsubtypen het middel *in vivo* daadwerkelijk bindt; 4) **proof-of-mechanism studies** waarin de farmacodynamische effecten en de farmacokinetische blootstelling binnen de bandbreedte van verschillende doseringen wordt vastgesteld; 5) **proof-of-efficacy studies** waarin de klinische werkzaamheid van het middel wordt verkend binnen de groep patiënten voor wie het middel uiteindelijk bedoeld is; **6) proof-of-therapy studies** die bevestigen dat het middel inderdaad bruikbaar en effectief is in de klinische praktijk.

CURRICULUM VITAE

Xia Chen was born on 15 February 1976 in Beijing. Following high school, she studied Clinical Medicine and graduated from Peking Union Medical College as a medical doctor in the year of 2002. Thereafter, she started her professional career in the department of Neurology in Peking Union Medical College Hospital. In 2007, she came to the Netherlands and began her PhD study in Centre for Human Drug Research (CHDR, Leiden) under the supervision of Prof. Joop van Gerven and Prof. Adam Cohen. Dr. Gabriel Jacobs was her co-promoter. Her research focused on the clinical pharmacology of novel anxiolytic drugs, especially the alpha2,3-subtype selective GABA(A) receptor agonists. She is currently working in the Phase I unit of Peking Union Medical College Hospital as a study physician.

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