Mechanism-Based Modeling of Functional Adaptation Upon Chronic Treatment with Midazolam

Adriaan Cleton,[1] Jonas Ödman,[1] Piet Hein Van der Graaf,[1] Wim Ghijsen,[4] Rob Voskuyl,[2,3] and Meindert Danhof[1,5]

Received September 22, 1999; accepted December 10, 1999

Purpose. A mechanism-based model is applied to analyse adaptive changes in the pharmacodynamics of benzodiazepines upon chronic treatment in rats.

Methods. The pharmacodynamics of midazolam was studied in rats which received a constant rate infusion of the drug for 14 days, resulting in a steady-state concentration of 102 ± 8 ng·ml^{-1}. Vehicle treated rats were used as controls. Concentration-EEG effect data were analysed on basis of the operational model of agonism. The results were compared to data obtained *in vitro* in a brain synaptoneurosomal preparation.

Results. The relationship between midazolam concentration and EEG effect was non-linear. In midazolam pre-treated rats the maximum EEG effect was reduced by 51 ± 23 μV from the original value of 109 ± 15 μV in vehicle treated group. Analysis of this change on basis of the operational model of agonism showed that it can be explained by a change in the parameter tissue maximum (E_m) rather than efficacy (τ). In the *in vitro* studies no changes in density, affinity or functionality of the benzodiazepine receptor were observed.

Conclusions. It is concluded that the observed changes in the concentration-EEG effect relationship of midazolam upon chronic treatment are unrelated to changes in benzodiazepine receptor function.

KEY WORDS: benzodiazepines; pharmacokinetics; EEG; operational model of agonism; receptor binding; muscimol-induced Cl^- uptake.

INTRODUCTION

Benzodiazepines are widely prescribed for their anxiolytic, muscle relaxant, sedative-hypnotic and anticonvulsant effects. Their clinical usefulness however is limited by functional tolerance development upon chronic treatment (1). Much research has been conducted on the mechanisms involved in tolerance development, focussing specifically on the functionality of the GABA-benzodiazepine receptor complex (2). The extrapolation from *in vitro* test-systems to the *in vivo* situation remains a complex issue, however. It is therefore not fully understood which mechanisms explain, in a quantitative manner, functional tolerance development *in vivo*.

Recently, we have successfully developed a new mechanism-based model (based on the operational model of agonism (3)) to characterise functional adaptation of midazolam (4). As pharmacodynamic endpoint, the EEG was used, which reflects benzodiazepine-induced enhancement of GABA-ergic inhibition in a quantitative manner (5,6). The operational model of agonism is based on receptor theory and contains two important parameters. The first is the parameter tissue maximum (E_m), that represents the maximal primary response that can be achieved in the system by receptor activation. The second is an efficacy parameter (τ) that describes the efficiency of the transduction of receptor occupation into the pharmacological response and that is closely related to the intrinsic efficacy of a given ligand (3). Briefly in our previous investigation it was shown by application of a stepwise approach, that the observed reduction in the maximum EEG effect (α, i.e., intrinsic activity) of midazolam in the kindling model of epilepsy can be explained by a change in the parameter tissue maximum (E_m) rather than the efficacy parameter (τ). These findings were confirmed by the results from *in vitro* investigations, showing no changes in the density, affinity and functionality of the benzodiazepine receptor in a synaptoneurosomal preparation. Furthermore, it was shown by simulation that the observed reduction in maximum EEG effect should also occur for other benzodiazepines with a different intrinsic efficacy.

In the present investigation we have applied the same approach to analyse changes in the concentration-EEG effect relationship of midazolam following continuous exposure to the drug for 14 days by chronic infusion. On the basis of this model predictions are made regarding changes in the pharmacodynamics of other benzodiazepines with different intrinsic efficacy. These predictions show that the absolute change in intrinsic activity upon chronic treatment will be smaller for partial agonists than for full agonists.

METHODS

Animals

Adult male Wistar rats (Harlan, C.P.B., Zeist, The Netherlands), weighing 200–225 g, were used. The animals were housed individually in plastic cages, at 21°C and a 12-h light–dark cycle (lights on: 8:00 am to 8:00 pm). Food (Standard Laboratory Rat, Mouse and Hamster Diets, RMH-TM, Hope Farms, Woerden, The Netherlands) and tap water were available *ad libitum*. The Committee on Animal Experimentation of Leiden University approved the protocol of this study.

EEG-Effect Measurement

The pharmacodynamics of midazolam was characterised in two groups of 8 rats on the basis of quantitative EEG monitoring as described previously (7). Briefly, one week before the

[1] Division of Pharmacology, Leiden Amsterdam Center for Drug Research, Leiden University, P.O. Box 9503, 2300 RA Leiden, The Netherlands.
[2] Stichting Epilepsie Instellingen Nederland, Achterweg 5, 2103 SW Heemstede, The Netherlands.
[3] Department of Physiology, Leiden University, The Netherlands.
[4] Institute of Neurobiology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands.
[5] To whom correspondence should be addressed. (e-mail: m.danhof@lacdr.leidenuniv.nl)

ABBREVIATIONS: α, upper asymptote of concentration-effect relationship; E_0, no drug response; n_H, midpoint slope of concentration-effect relationship; EC_{50}, midpoint of concentration-effect relationship; E_m, maximal achievable effect in the system; K_A, agonist equilibrium/dissociation constant; n, slope index; τ, efficacy parameter; K_E, concentration of agonist-receptor complex required to produce half-maximal effect; C.I., confidence interval; MVOF, minimum value of objective function.

0724-8741/00/0300-0321$18.00/0

experiment cortical electrodes for EEG recording were implanted under anaesthesia with 0.8 ml·kg^{-1} Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and 0.25 ml·kg^{-1} Nembutal (Sanofi, Maassluis, The Netherlands). The EEG signal from the fronto-central lead on the left hemisphere was continuously recorded during the time course of the experiment and subjected to on-line Fast Fourier Transformation. Amplitudes in the β frequency band (11.5–30 Hz) was used a measure of the drug effect intensity.

Chronic Treatment

In the rats in which the pharmacodynamics of midazolam were to be determined *in vivo*, indwelling canulas were implanted one day before experimentation. The right jugular vein was used for drug administration and the right femoral artery for serial collection of blood samples. On the first day the animals were placed in a home-made cage and connected to a single channel fluid swivel (22 ga. no. 050-0022, Plato, Diemen, The Netherlands) for continuous administration of midazolam or vehicle (8.1 μl/min, 0.25 mg/ml). In order to monitor blood concentrations during drug treatment, blood samples were taken at 1, 2, 3, 4, 5, 6 hours and 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 14 days after starting the continuous infusion. Shortly after blood sampling, EEG activity was determined over a period of 15 minutes.

In rats that were to be used for preparation of synaptoneurosomes, midazolam was administered by way of chronic implants, rather than by continuous infusion (see below).

Pharmacokinetic-Pharmacodynamic Experiment

Immediately after chronic treatment with midazolam or vehicle, a bolus dose of midazolam was administered. Midazolam was dissolved in equimolar hydrochloric acid and administered in a volume of 250 μl over 10 minutes, using a syringe infusion pump (Razel, A99, Stamford, Connecticut, U.S.A.)

All experiments started between 8.30 and 9.30 a.m. to standardise a possible influence of diurnal rhythms. EEG recording was started at least 45 minutes before the administration of the bolus dose of midazolam and lasted approximately six hours. During the experiments the animals were conscious and freely moving.

Arterial samples for the determination of midazolam blood concentrations were drawn at predefined time-points during and after the infusion. The samples were hemolyzed immediately in glass tubes containing 0.5 ml Millipore water and stored at −20°C. HPLC analysis was performed according to Mandema *et al.* (8).

Twenty-four hours after drug administration, a 3 ml blood sample was drawn and spiked with midazolam to contain 0.4 mg·ml^{-1}, for determination of the plasma-to-blood ratio (P/B) and the extent of plasma protein binding (f_u) of midazolam (5). Since it has been shown that the protein binding of benzodiazepines is concentration independent (9), the P/B ratio and protein binding were determined at only one concentration.

Experiments in Synaptoneurosomes

In order to determine the *in vitro* radioligand binding characteristics and the muscimol-stimulated $^{36}Cl^-$ flux, a separate group of 9 animals was continuously exposed to midazolam. In this group a sustained release preparation, which consisted of sealed silastic tubing (0.062 inch i.d. × 0.095 inch o.d., silastic, medical grade tubing, Dow Corning Corporation, Midland, U.S.A.) and which contained midazolam, was implanted under the skin at the back (10). The control animals were shared with a previous study (4). In order to monitor the blood concentrations during drug treatment, blood samples were taken at the same time points as with the continuous infusion.

Synaptoneurosomes for radioligand binding and measurement of $^{36}Cl^-$ uptake were prepared according to the method of Schwartz *et al.* (11) with some modifications, as described in detail in the previous study (4). Muscimol-induced $^{36}Cl^-$ uptake was determined in the concentration range of 0–150 μM. Modulation of muscimol-stimulated $^{36}Cl^-$ uptake was determined in the absence and presence of 1 μM midazolam.

For radioligand binding studies, saturation studies were performed in 75 μl synaptoneurosome preparation (89–216 μg protein), containing 100 μM GABA, 2-150 nM [^{3}H]flunitrazepam (specific activity 84.0 Ci·mmol^{-1}).

DATA ANALYSIS

In Vivo Data

The pharmacokinetics of midazolam after the bolus infusion was quantified in individual rats. The blood-concentration-time profiles of midazolam were described by a poly-exponential equation for intravenous infusion (12):

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T}(1 - e^{-\lambda_i \cdot t}) \qquad (t < T) \tag{1}$$

$$C(t) = \sum_{i=1}^{n} \frac{C_i}{\lambda_i \cdot T}(e^{-\lambda_i \cdot (t-T)} - e^{-\lambda i \cdot t}) \qquad (t \geq T) \tag{2}$$

where C(t) is the concentration at time t, T the infusion duration and C_i and λ_i are the coefficients and exponents of the equation, respectively. Total blood clearance (Cl), the elimination half-life ($t_{1/2}$) and the volume of distribution at steady-state (V_{dss}) were calculated by standard methods from the coefficients and the exponents of the fitted functions (12). The functions were fitted to the data with weight factor y^{-2}, using the non-linear least squares regression program Siphar (Simed SA, Creteil, France). In each individual rat the fitted function of the concentration-time profile was used to calculate the concentrations at the measured effect-time points.

During the continuous treatment the clearance was calculated on basis of the following equation:

$$Cl = \frac{R_0}{Css} \tag{3}$$

in which Cl is the clearance at steady-state, R_0 the infusion rate of midazolam and C_{ss} the blood concentration at steady-state.

The Hill equation was fitted to all individual concentration-effect data points:

$$E_C = E_0 + \frac{\alpha \cdot C^{nH}}{EC_{50}^{nH} + C^{nH}} \tag{4}$$

in which E_C is the EEG effect at midazolam concentration C, α the upper asymptote (intrinsic activity), EC_{50} the midpoint location, n_H the midpoint slope and E_0 the no-drug response.

Subsequently, the concentration-effect data were fitted to the following form of the operational model of agonism (3), as described previously (4):

$$E_C = E_0 + \frac{E_m \cdot \tau^n \cdot C^n}{(K_A + C)^n + \tau^n \cdot C^n} \quad (5)$$

where E_m is the maximum effect achievable in the system, K_A the agonist equilibrium/dissociation constant, n the slope index for the occupancy-effect relationship and τ the efficacy parameter, which is defined by the ratio of total receptor concentration ($[R_0]$) and the concentration of agonist-receptor complex required to produce half-maximal effect (K_E):

$$\tau = \frac{[R_0]}{K_E} \quad (6)$$

The intrinsic activity of a drug (α) relates to system maximum (E_m), efficacy (τ) and slope factor (n) according to equation 7 (Ref. 13):

$$\alpha = E_M \cdot \frac{\tau^n}{1 + \tau^n} \quad (7)$$

All fitting procedures, except the pharmacokinetic part and the receptor-binding characteristics, were performed by use of the non-linear mixed effect modelling software package NONMEM (NONMEM project group, University of California, San Francisco, CA).

The statistical models used in this analysis were described in full detail in a previous study (4). In short, the models take into account both intra- and interindividual variation. For convenience, interindividual variability was always expressed as coefficient of variation (c.v.) in this study. An effect of chronic treatment on any of the parameters in equation 4 or 5, is expressed as a difference δ from control.

In the fitting procedure the value of K_A was fixed to 82.12 ng·ml^{-1}, as determined previously in brain homogenates (14). Also, since there was no difference between the control groups from the previous study and the present study, the system maximum and its interindividual variability were fixed to the previously determined values of 110 μV and 13% respectively (4). In a stepwise procedure, the operational model of agonism was first fitted to the combined data from the midazolam-pretreated and control rats, assuming that the chronic treatment had no effect on any of the three parameters E_m, τ and n. Subsequently, changes in the values of both τ and E_m were allowed, and finally only in E_m.

In Vitro Data

The receptor binding characteristics of the radioligand [^{3}H]-flunitrazepam were determined by fitting the following equation to the data from the saturation experiment:

$$B = \frac{B_{max} \cdot [A]^n}{K_d^n + [A]^n} \quad (10)$$

in which B is the number of receptors occupied, B_{max} is the total number of specific binding sites, K_d is the ligand concentration at which 50% of the receptors is occupied, n is the slope factor and [A] is the ligand concentration.

The muscimol-stimulated $^{36}Cl^-$ uptake was fitted with the following equation using a population approach.

$$v = \frac{v_{max} \cdot C}{EC_{50} + C} \quad (11)$$

in which v is the $^{36}Cl^-$ uptake, v_{max} is the maximal $^{36}Cl^-$ uptake, EC_{50} is the muscimol concentration at which 50% of the uptake is obtained and C is the muscimol concentration. The effect of the chronic treatment was characterised by the term δ, reflecting the midazolam-induced change in either v_{max} or EC_{50}.

STATISTICAL ANALYSIS

The pharmacokinetic parameter estimates of the different treatments as well as receptor binding characteristics were statistically compared using the parametric one-way analysis of variance (ANOVA) or a non-parametric Kruskall-Wallis test, if more appropriate. A significance level of 5% was selected. All data are reported as mean ± S.E. unless indicated otherwise.

RESULTS

Chronic Treatment

In Fig. 1 the averaged concentration and effect data during continuous treatment are presented. The constant i.v. infusion of midazolam during 14 days resulted in a steady-state concentration of 102 ± 8 ng·ml^{-1}. Therefore the clearance of midazolam, as determined by the ratio of infusion rate and steady-state concentration (Eq. 3), was found to be 88 ml·min^{-1}·kg^{-1}. The β activity in the EEG increased to 59 ± 5 μV above baseline EEG and remained constant during the 14 days midazolam treatment. For the animals treated with midazolam from subcutaneous implants, a comparable steady-state concentration (93 ± 9 ng·ml^{-1}) was observed (Fig. 1). Based on the weight difference of the tubes prior to implantation and after removal, the average release of midazolam was estimated to be 5.4 ± 0.2 mg·day^{-1} (n = 9).

Pharmacokinetics

In all individual animals, the concentration–time profiles after the bolus injection were described most adequately by a bi-exponential equation. The averaged pharmacokinetic parameters are summarised in Table 1. No statistical differences were observed between the treatment groups. The free fraction in plasma (f_u) and the plasma-to-blood ratio (P/B) of midazolam were determined *in vitro*. The f_u values for the control and midazolam treated groups did not differ significantly (8.2 ± 2.1% *versus* 7.0 ± 1.3%). The P/B ratios were also equal (1.4 ± 0.1 in both groups).

Pharmacodynamics

Chronic treatment with midazolam caused a statistically significant reduction δ of 51 ± 23 μV of the value of α, compared to the value of 109 ± 15 μV in control animals ($\delta_\alpha \neq 0$, $p < 0.05$) (Table 2). The other parameters were not affected.

Mechanism-Based Modelling

The data were fitted simultaneously to the operational model of agonism in a stepwise procedure. In the first run,

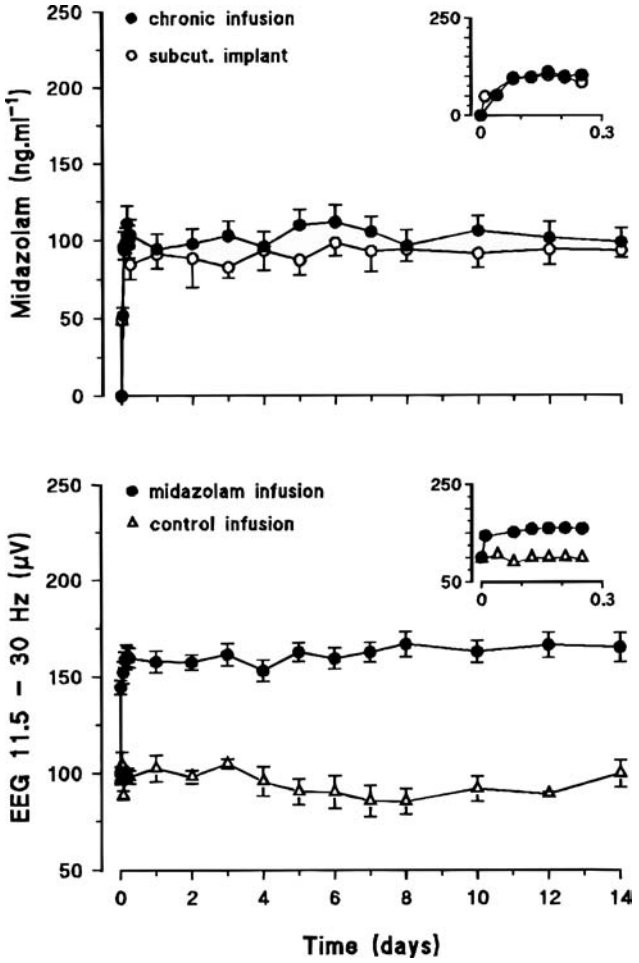

Fig. 1. Upper panel: time *versus* concentration profile (mean ± S.E.) during the 14 days treatment with midazolam by chronic infusion (○) and by release from subcutaneous implants (○). Lower panel: time *versus* EEG profile (mean ± S.E.) during the 14 days infusion with midazolam (○) or vehicle (△). The inserts show the time course on the first day on an extended time scale.

assuming identical values of the parameters reflecting system maximum (E_m), efficacy (τ) and slope (n) in midazolam pre-treated and control rats, the model converged, yielding estimates for the different parameters (Table 3). In the second run, now allowing differences in both E_m and τ, a considerable improvement of the goodness-of-fit was observed, as reflected in the reduction of the MVOF from 4608.0 to 4555.3 ($p < 0.05$).

Table 1. Pharmacokinetic Parameter Estimates After the Administration of 10 mg·kg^{-1} Midazolam over 10 Minutes in Control Animals and Animals Chronically Treated with Midazolam for 14 Days (Mean ± S.E., n = 8)

	Control	Midazolam (chronic infusion)
Cl(ml^{-1}·min^{-1}·kg^{-1})	83 ± 4	93 ± 6
Vd_{ss}(l·kg^{-1})	1.7 ± 1	2.0 ± 1
$t_{1/2}$ (min)	28 ± 5	33 ± 4

Table 2. Hill Equation Parameter Estimates for *In Vivo* Effect of Midazolam After Chronic Infusion (Mean ± S.E. of estimate#, n = 8)

	Population mean	δ (chronic infusion)
Intrinsic activity (α)	109 ± 15	−51 ± 23*
(μV)	(29%)	($-97 < \delta_\alpha < -5$)
EC_{50}	82.5 ± 11	9 ± 29
(ng.ml^{-1})	(94%)	($-49 < \delta_{EC50} < 67$)
Hill factor	1.2 ± 0.1	−0.1 ± 0.1
	(41%)	($-0.3 < \delta_{nH} < 0.1$)

Note: The effect of the chronic treatment is reflected in the estimate of the difference (δ). In parenthesis are either shown the c.v. (population mean), describing the interindividual variation, or the 95% confidence intervals of δ.
* $p < 0.05$ $\delta = 0$, determined by NONMEM.

Chronic midazolam treatment produced a significant reduction δ of 41.5 μV in E_m (95% C.I. $-74.3 < \delta_{Em} < -8.7$, $p < 0.05$), yielding values of 110 ± 10 μV *versus* 74 ± 7 μV for the control and midazolam treated rats, respectively. In contrast, the reduction in τ of −0.19 ± 0.42 in midazolam pre-treated animals was not significant. Therefore, in the final run only a difference in E_m was allowed. There was no further reduction of the goodness-of-fit criterium (MVOF = 4556.1) and a similar reduction in E_m (δ_{Em} −43.0 ± 12.9 μV) was obtained. Also, the obtained values of τ and n were very similar to the first run. The results of the final run are illustrated in Fig. 2.

Receptor Binding

Binding of [^{3}H]flunitrazepam to the GABA-benzodiazepine receptor complex was measured in rat synaptoneurosomes. There was no influence of chronic midazolam treatment on the [^{3}H]flunitrazepam binding characteristics, as for control and midazolam-treated animals a K_d of 37.5 ± 5 *versus* 29.5 ± 3 nM, a B_{max} of 1355 ± 150 *versus* 1426 ± 188 fmol·mg protein^{-1} and a slope factor of 1.3 ± 0.1 *versus* 1.3 ± 0.1 was observed. Non-specific binding was low, about 24% of the total binding at 70 nM [^{3}H]flunitrazepam.

Muscimol-Stimulated $^{36}Cl^-$ Influx

Muscimol stimulated the $^{36}Cl^-$ flux in a concentration-dependent manner as shown in Fig. 3. Table 4 gives the characteristic parameters of $^{36}Cl^-$ uptake. A significant decrease δ in the maximal $^{36}Cl^-$ influx was observed after chronic midazolam administration (δ_{vmax} −8.7 ± 4.1 nmol·mg protein^{-1}·5 sec^{-1}), resulting in the maximal uptakes of 75.9 *versus* 67.1 nmol·mg protein^{-1}·5 sec^{-1} in control and midazolam-treated animals, respectively. A slightly lower potency of muscimol in saline-treated animals was observed. EC_{50} values were 4.08 ± 0.12 μM *versus* 4.85 ± 0.12 μM (mean ± S.E. mean), respectively. In Fig. 3 the enhancement of muscimol-stimulated $^{36}Cl^-$ uptake by 1 μM midazolam is illustrated. Midazolam lowered the EC_{50} value from 4.10 ± 0.43 to 1.78 ± 0.21 μM. However, the degree of enhancement was not affected by chronic midazolam administration, as δ_{EC50} was −0.15 ± 0.35 (95% C.I. $-0.85 < \delta_{EC50} < 0.55$, Table 4).

Table 3. Operational Model of Agonism Pharmacodynamic Parameter Estimates, Reflecting the Effect of Chronic Midazolam Treatment on the Different Parameters (Mean ± S.E. of Estimate, n = 8)

	τ	δ_τ	n	E_m (μV)	δ_{Em} (μV)	MVOF
I	2.18 ± 0.23 (36%)		3.19 ± 0.59 (85%)	110 (13%)		4608.0
II	2.41 ± 0.24 (40%)	−0.19 ± 0.42	3.37 ± 0.54 (91%)	110 (13%)	−41.5 ± 16.4**	4555.3*
III	2.37 ± 0.23 (40%)		3.38 ± 0.55 (94%)	110 (13%)	−43.0 ± 12.9**	4556.1*

Note: In the first run (I) it was assumed that chronic treatment does not affect any of the pharmacodynamic parameters. Subsequently a change in both τ and E_m (II) or E_m alone (III) was assumed. MVOF (i.e. minimum value of objective function) reflects the goodness-of-fit. No statistical differences between the MVOF values between II and III were observed.
* $p < 0.05$ *versus* I.
** $p < 0.05$ $\delta \neq 0$, determined by NONMEM.

DISCUSSION

In the present investigation functional tolerance development for benzodiazepines was investigated by the application of mechanism-based modelling *in vivo*, in combination with *in vitro* studies. The main result emerging from this study was that chronic treatment is associated with a decrease in the system maximum rather than the efficacy parameter τ, without affecting the pharmacokinetics of midazolam. Midazolam was continuously administered via

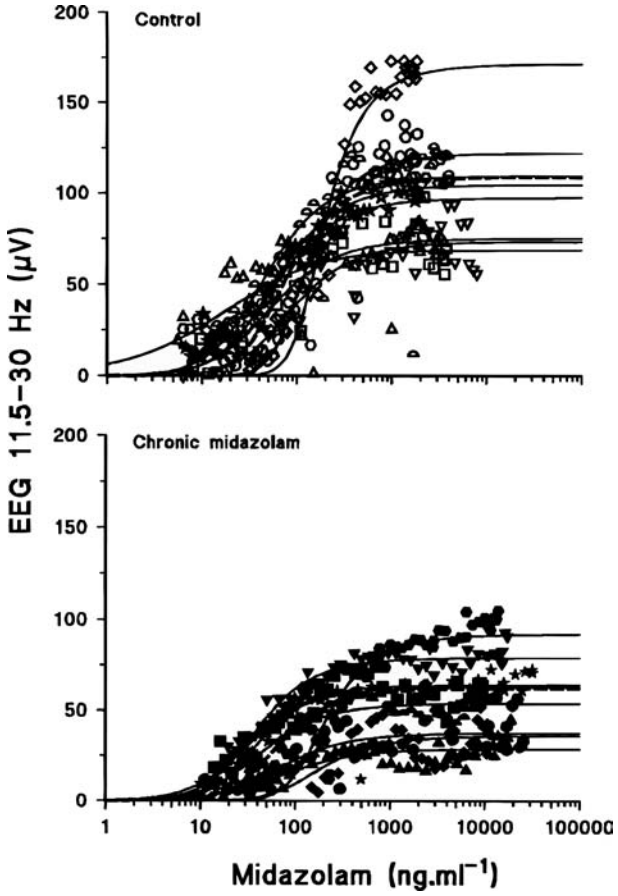

Fig. 2. Individual blood concentration-effect relationships for the EEG effect (β activity) following intravenous infusion of 10 mg·kg^{-1} midazolam for 2 minutes. The solid lines are individual post-hoc Bayesian estimates, obtained by fitting the operational model of agonism to the data, with a change in the parameter system maximum (E_m) between midazolam treated and control rats. The dashed lines represent the population mean. Different symbols indicate different animals.

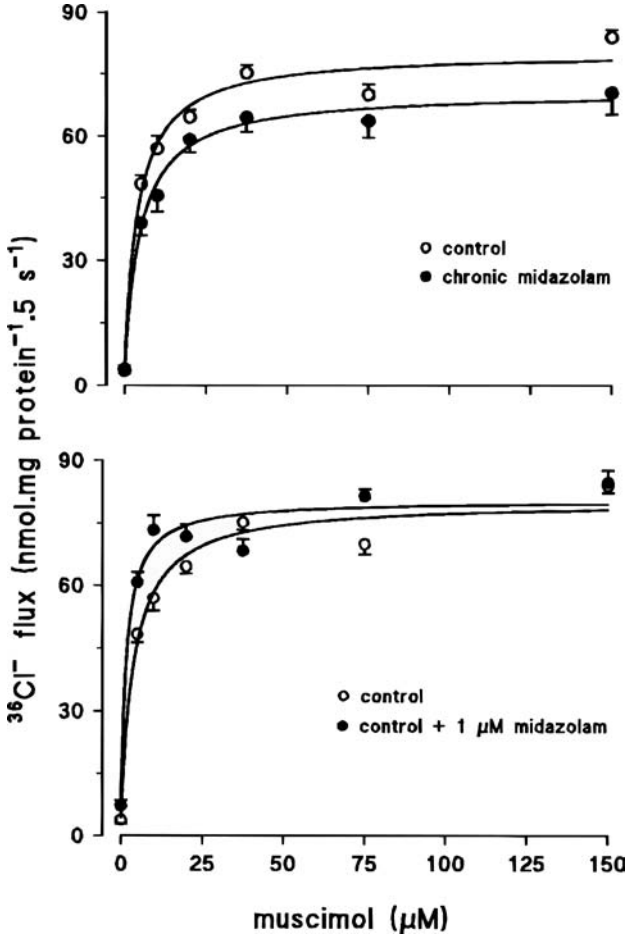

Fig. 3. Concentration–response curves for muscimol-stimulated $^{36}Cl^-$ uptake. The upper panel shows the influence of chronic midazolam treatment for 14 days, the lower graph reflects the modulation of the uptake by 1 μM midazolam in control animals. The lines represent the population fits (mean ± S.E., n = 9).

Table 4. Parameter Estimates for the Effect of Chronic Treatment on the Muscimol-Stimulated $^{36}Cl^-$ Uptake in Synaptoneurosomes (Mean ± S.E.# of Estimate, n = 9)

	Population mean	δ (subcutaneous implants)
basal uptake	4.2 ± 0.9	−0.6 ± 1.2
(nmol·mg protein^{-1}·5 s^{-1})	(0%)	
V_{max}	75.9 ± 1.2	−8.7 ± 4.1*
(nmol·mg protein^{-1}·5 s^{-1})	(11%)	($-16.9<\delta_{vmax}<-0.5$)
EC_{50}	4.10 ± 0.43	0.76 ± 0.68
(μM)	(17%)	
$EC_{50}^{\#}$	1.78 ± 0.21	−0.15 ± 0.35
(μM)	(0%)	

Note: δ reflects the effect of chronic treatment on the population parameter estimates. $EC_{50}^{\#}$ is the potency of muscimol obtained in the presence of 1 μM midazolam.
* $p < 0.05$ $\delta \neq 0$,# determined by NONMEM.

an intravenous infusion or a subcutaneous implant (10), which allowed for the maintenance of constant steady-state midazolam concentrations during the 14 days of treatment. Following the subsequent intravenous infusion of a bolus dose of midazolam, no difference in the pharmacokinetic parameters was observed, indicating that no dispositional tolerance had developed.

Although the sigmoidal relationship between the blood concentrations and the change in β activity in the EEG could be described successfully by the Hill equation, a disadvantage of analysis with this empirical equation is, that it provides limited insight in the relation between receptor pharmacology and the *in vivo* pharmacodynamics. This complicates the comparison of the changes observed *in vivo* with the results obtained *in vitro* in the brain synaptoneurosomal preparation. Therefore, we applied the operational model of agonism (3), that proved to be successful in a previous study on the reduction in efficacy of midazolam induced by epileptic activity (4), to obtain more insight in the factors determining the drug-induced change in drug concentration-effect relationship.

A stepwise approach was used to fit the operational model of agonism to the data, to identify the parameters that account for the observed change in the maximum effect α and to determine the changes quantitatively. In theory the decrease in maximum EEG effect can be explained by a change in one of the four parameters E_m, τ, K_A and n or in combination (Ref. 3; Eq. 7). Since K_A is not related to the intrinsic activity (α), no changes in this value were considered. Instead the value of K_A was fixed to 82.12 ng·ml^{-1}, as determined previously in radioligand binding studies (14). The validity of this assumption is confirmed by the lack of changes observed in the [^{3}H]flunitrazepam binding experiments. Furthermore, since no changes in the value of n_H were observed in the tolerant animals a change in n can be ruled out as well (13). Therefore, in the modelling procedure only changes in the parameters E_m and τ were considered. This analysis revealed clearly that the decrease in intrinsic activity could be exclusively explained by a decrease in the tissue maximum E_m, without a change in the efficacy parameter τ.

The absence of a change in the efficacy parameter (τ) is in agreement with the observations *in vitro*. Chronic treatment did not alter receptor affinity and density. Also, there were no changes in the coupling between the GABA and benzodiazepine receptor, as reflected by the enhancement of muscimol-stimulated $^{36}Cl^-$ uptake in the presence of 1 μM midazolam.

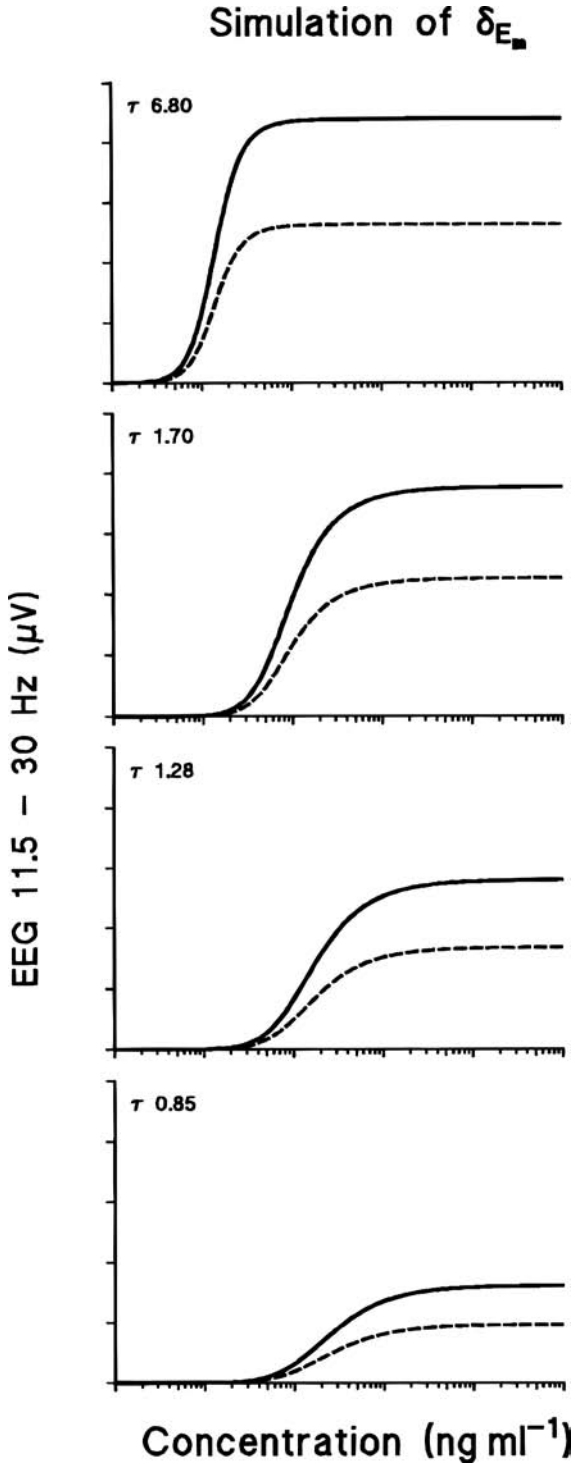

Fig. 4. Simulation of the concentration-EEG relationships of 4 benzodiazepine agonists based on the operational model of agonism (Eq. 5). The solid lines were simulated with the values for E_m 110 μV, K_A 82.12 ng·ml^{-1} and slope factor 3.38. The values of the intrinsic efficacy were 6.80, 1.70, 1.18 and 0.85 respectively. The dashed lines represent the simulated concentration-effect relationships in the situation of a 40% decrease in the system maximum (E_m) for the different agonists.

An important question is at which rate functional adaptation to midazolam develops. In the present investigation no systematic change in the EEG effect was observed during the chronic treatment (Fig. 1). This may be explained, however, by the fact that the observed functional adaptation is reflected in a change in the pharmacodynamic parameter 'maximum EEG effect' (α) rather than the EC_{50} (Table 2). Consequently, the predicted change in EEG effect at the constant plasma concentration of 100 ng $\cdot$ ml^{-1} is small and within the range of the random variation. It is therefore impossible to estimate, on the basis of the present data, the rate at which functional tolerance develops. In a previous investigation it has been shown however that functional adaptation to the EEG effect of midazolam may develop rapidly (i.e., within hours) (15).

An intriguing question is how functional adaptation would affect the concentration-EEG effect relationships of benzodiazepines with an intrinsic efficacy different from midazolam. Several studies indicate that partial benzodiazepine agonists may be less liable to tolerance development (16–18) and it has been suggested that this is directly related to the degree of $GABA_A$ receptor activation (19). Our studies on basis of the operational model of agonism indicate that the pharmacodynamics of partial benzodiazepine agonists are indeed less sensitive to the influence functional adaptation. Simulations of a certain decrease in tissue maximum (E_m) for 4 agonists with different intrinsic efficacies (i.e., different τ values) show the same relative decrease in maximal effect for all agonists (Fig. 4). In absolute terms, however, the decrease is large for a full agonist, but small for a partial agonist. Thus, it is predicted that in the experimental situation tolerance development will be seen particularly for full agonists, whereas for less efficacious agonists it may become undetectable.

In conclusion, application of principles from receptor theory in pharmacokinetic-pharmacodynamic modelling *in vivo* in combination with receptor studies *in vitro*, revealed that impaired benzodiazepine-modulated GABAergic inhibition after chronic treatment, can be explained by a change in $GABA_A$ receptor functioning. There were no concomitant changes in benzodiazepine receptor density or efficiency of the coupling between $GABA_A$ and benzodiazepine receptors. This approach also appears to offer a theoretical basis for the observations that partial agonist are less liable to tolerance development.

ACKNOWLEDGMENTS

The authors are grateful for the technical assistance of Erica Tukker and Elly Besselsen. We also thank Hofmann-La Roche AG (Basel, Schweiz and Mijdrecht, The Netherlands) for their generous gift of midazolam. This study was supported by Netherlands Organization for Scientific Research (NWO) grant 903-52-201.

REFERENCES

1. D. W. Gallager, R. J. Marley, and T. D. Hernandez. Biochemical and electrophysical mechanisms underlying benzodiazepine tolerance and dependence In: J. Pratt, (eds), *The biological bases of drug tolerance and dependence* Academic Press, London, 1991, pp. 49–70.
2. M. A. Hutchinson, P. F. Smith, and C. L. Darlington. The behavioural and neuronal effects of chronic administration of benzodiazepine anxiolytic and hypnotic drugs. *Prog. Neurobiol.* **49**:73–97 (1996).
3. J. W. Black and P. Leff. Operational models of pharmacological agonism. *Proc. R. Soc. Lond. B.* **220**:141–162 (1983).
4. A. Cleton, P. H. Van der Graaf, R. Voskuyl, and M. Danhof. Mechanism-based modeling of the adaptive changes in the pharmacodynamics of midazolam in the kindling model of epilepsy. *Pharm Res.* **16**:1702–1709 (1999).
5. J. W. Mandema, L. N. Samsom, M. C. Dios-Vièitez, M. Hollander-Jansen, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the EEG effect of benzodiazepines. Correlation with receptor binding and anticonvulsant activity. *J. Pharmacol. Exp. Ther.* **257**:472–478 (1991).
6. J. W. Mandema, M. T. Kuck, and M. Danhof. Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration-EEG effect relationship. *Br. J. Pharmacol.* **105**:164–170 (1992).
7. J. W. Mandema and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the central nervous system effect of heptabarbital using aperiodic EEG analysis. *J. Pharmacokinet. Biopharm.* **5**:459–481 (1990).
8. J. W. Mandema, E. Tukker, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the EEG effects of midazolam in individual rats: influence of rate and route of administration. *Br. J. Pharmacol.* **102**:663–668 (1991).
9. L. J. Moschitto and D. J. Greenblatt. Concentration-independent plasma protein binding of benzodiazepines. *J. Pharm. Pharmacol.* **35**:179–180 (1983).
10. D. W. Gallager, A. B. Malcolm, S. A. Anderson, and S. F. Gonsalves. Continuous release of diazepam: electrophysiological, biochemical and behavioral consequences. *Brain Res.* **342**: 26–36 (1985).
11. R. D. Schwartz, P. D. Suzdak, and S.M. Paul. γ-Aminobutyric acid (GABA)- and barbiturate-mediated $^{36}Cl^-$ uptake in rat brain synaptoneurosomes: evidence for rapid desensitization of the GABA receptor-coupled chloride ion channel. *Mol. Pharmacol.* **30**:419–426 (1986).
12. M. Gibaldi and D. Perrier. Non-compartmental analysis based on statistical moment theory. In: *Pharmacokinetics* (2nd edition), Marcel Dekker, New York, 1982, pp. 409–424.
13. J. W. Black, P. Leff, N. P. Shankley, and J. Wood. An operational model of pharmacological agonism: the effect of E/[A] curve shape on agonist dissociation constant estimation. *Br. J. Pharmacol.* **84**:561–571 (1985).
14. A. Hoogerkamp, R. H. G. P. Arends, A. M. Bomers, J. W. Mandema, R. A. Voskuyl, and M. Danhof. Pharmacokinetic/pharmacodynamic relationship of benzodiazepines in the direct cortical stimulation model of anticonvulsant effect. *J. Pharmacol. Exp. Ther.* **279**:803–812 (1996).
15. A. Cleton, D. Mazee, R.A. Voskuyl, and M. Danhof. Rate of change of blood concentration is a major determinant of the pharmacodynamics of midazolam in rats. *Br. J. Pharmacol.* **127**:227–235 (1999).
16. J. Auta, P. Giusti, A. Guidotti, and E. Costa. Imidazenil, a partial positive allosteric modulator of $GABA_A$ receptors, exhibits low tolerance and dependence liabilities in the rat. *J. Pharmacol. Exp. Ther.* **270**:1262–1269 (1994).
17. W. Löscher, C. Rundfeldt, D. Hönack, and U. Ebert. Long-term studies on anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. II. The novel imidazoquinazolines NNC 14-0185 and NNC 14-0189. *J. Pharmacol. Exp. Ther.* **279**:573–581 (1996).
18. C. Rundfeldt, P. Wlaf, D. Hönack, and W. Löscher. Anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. Comparison of diazepam, bretazenil and abecarnil. *J. Pharmacol. Exp. Ther.* **275**:693–702 (1995).
19. E. Costa. From $GABA_A$ receptor diversity emerges a unified vision of GABAergic inhibition. *Ann. Rev. Pharmacol. Toxicol.* **38**:321–350 (1998).