Pharmacodynamics of the anticonvulsant effect of oxazepam in aging BN/BiRij rats

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1 The purpose of this investigation was to examine the influence of increasing age on the pharmacokinetics and the time course of the anticonvulsant response of oxazepam in BN/BiRij rats as an animal model of aging.

2 Oxazepam was administered intravenously in a dose of 12 mg kg⁻¹ body weight and the anticonvulsant effect intensity was measured as elevation above baseline of a threshold for induction of localized seizure activity (TLS). Direct cortical stimulation with ramp shaped electrical pulse trains of increasing intensity was used to determine this threshold.

3 The pharmacological effect vs. time profile showed in young rats an anticonvulsant component followed by proconvulsant component which is suggestive for the occurrence of acute tolerance and/or withdrawal syndrome. With increasing age the proconvulsant component disappeared, resulting in a monophasic effect profile (anticonvulsant effect only) at the age of 35 months with significantly higher anticonvulsant effect intensity immediately following drug administration. No age-related changes in the pharmacokinetic parameters of oxazepam were observed.

4 In five animals of each age group, benzodiazepine receptor binding characteristics were determined *in vitro* with [³H]-flunitrazepam as a ligand. Both receptor density and affinity did not show age-related changes. Available literature data on post-receptor events do not indicate conclusive age-related changes.

5 It is concluded, that the observed change in the pharmacodynamics of anticonvulsant effect of oxazepam can be explained by the disappearance of the tolerance/withdrawal phenomenon. This is compatible with a decreased efficiency of homeostatic control mechanisms in the elderly.

Keywords: Pharmacokinetics; pharmacodynamics; oxazepam; homeostatic reserve capacity; aging; anticonvulsant effect

Introduction

It has been frequently reported, that with increasing age there is an increase of the sensitivity of the brain to the pharmacological actions of benzodiazepines, especially for sedative effects and the effects on psychomotor function (Castleden *et al.*, 1977; Reidenberg *et al.*, 1978; Kanto *et al.*, 1981; Swift *et al.*, 1981; 1985a,b; Cook, 1986). Thus far, the influence of increasing age on the anticonvulsant effect of benzodiazepines has not been studied extensively. This may be relevant, because with increasing age there is an increase in the incidence of epilepsy and in the use of antiepileptic drugs (Hauser & Kurland, 1975; Troupin & Johannessen, 1990). Moreover, the mechanism of the anticonvulsant effect of benzodiazepines may be in part different from that of most of the other effects of these drugs, because of the involvement of micromolar affinity benzodiazepine receptors (Bowling & De Lorenzo, 1982).

The purpose of this investigation was to study the influence of increasing age on the pharmacodynamics of the anticonvulsant effect of oxazepam *in vivo*. Since changes in pharmacodynamics may occur simultaneously at different organizational levels (i.e. receptor binding characteristics, post-receptor events or homeostatic mechanisms), it was decided to study the pharmacodynamics initially *in vivo*, i.e. in the complete living system. Anticonvulsant effect intensity was determined on the basis of the threshold for induction of a localized seizure reaction upon direct cortical stimulation with ramp shaped electrical pulse trains of increasing inten-

sity (Voskuyl *et al.*, 1989). In combination with the determination of blood concentrations and the application of simultaneous pharmacokinetic-pharmacodynamic modelling concepts, this allows the determination of concentration vs. anticonvulsant effect relationships in individual rats (Dingemans *et al.*, 1990). In addition, benzodiazepine receptor binding characteristics were determined *in vitro* with [³H]-flunitrazepam as radioligand.

Methods

Animals

Five groups of male BN/BiRij rats (TNO Institute of Ageing and Vascular Research, Leiden, The Netherlands) of different ages (4, 12, 24, 31 and 35 months; *n* = 8, 8, 7, 6, 10 respectively) were used. The 10, 50 and 90% survival ages of the male BN/BiRij rats are 38.1, 31.7 and 22.8 months, respectively. During the period in which the experiments were performed, the rats were kept solitary in Makrolon cages and in a normal 12 h light-dark cycle (light between 07 h 00 min and 19 h 00 min). The temperature was maintained at 22–23°C. They were allowed free access to water (acidified, pH 3–4) and food (Standard diet for Rat, Mouse and Hamster, AM 1410, Hope Farms, Woerden, The Netherlands).

Animals experiments

Clinical biochemical/pathological evaluation In order to be able to determine the health status of the animals, several

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clinical biochemistry parameters were measured. Blood concentrations of urea nitrogen, aspartate aminotransferase, alanine aminotransferase, glucose, total plasma protein and albumin concentration, 24 h urine production, osmolality, and creatinine clearance were determined as described before (Stijnen *et al.*, 1992).

After the pharmacokinetic/pharmacodynamic evaluation, the animals were killed, the liver was weighed and the heart, the lungs, the kidneys, the liver, the brain and macroscopically visible abnormalities were evaluated by a pathologist, who was unaware of the outcome of the pharmacokinetic/pharmacodynamic evaluation.

Pharmacokinetic/pharmacodynamic evaluation Three weeks before the experiment, two electrodes were implanted under halothane anaesthesia in the skull over the motor area of the frontoparietal cortex as described by Voskuyl *et al.* (1989). A bipolar pulse train (duration of one pulse 2 ms, frequency 50 pulses per s) of linearly increasing amplitude (0–2400 μ A in 20 s), applied to the electrodes, was used as the convulsive stimulus and the threshold to induce localized seizure activity was determined, by measuring the stimulus current corresponding to the (visually determined) point at which clonic forelimb activity started. Since the convulsive threshold, which would be measured repeatedly in the experiment, decreases in the initial test sessions (Voskuyl *et al.*, 1989), this threshold was determined twice daily during the two weeks before the actual experiment until a stable baseline threshold was reached.

One day before the experiment, a polyethylene cannula was implanted in the right jugular vein under light halothane anaesthesia. On the day of the experiment the threshold to induce localized seizure activity was determined five times before the administration of oxazepam; the mean threshold value was used as the baseline level. Anticonvulsant effect intensities after administration of oxazepam were expressed as the elevation in threshold over this baseline level.

In vivo experiment Oxazepam, dissolved in 100 μ l dimethylacetamide, was administered via the cannula in the jugular vein as an intravenous bolus dose of 12 mg kg⁻¹ in about 1 min to the rats of the five different age groups. The animals of a 4-month-old control group received only the solvent. The seizure threshold was determined repeatedly (38 times) and 16 blood samples of 100 μ l were collected from an incision in the tail during a period of 7 h after administration (in 5 of the 35-month-old rats the experiment was extended to a total of 11 h). Blood samples were immediately haemolyzed by addition of 500 μ l of distilled water and stored at –20°C until analysis. In order to determine *in vivo* plasma protein binding, a second oxazepam bolus dose (6 mg kg⁻¹) was administered to the rats one day after the experiment and 1 min thereafter a blood sample of about 2 ml was withdrawn from an incision in the tail. Plasma was separated from the blood samples and stored at –20°C until analysis. A few days later the brain was removed in order to determine the *in vitro* benzodiazepine receptor binding characteristics. The brains were stored at –70°C until analysis.

Receptor binding The *in vitro* benzodiazepine receptor binding characteristics were determined at 0°C with [³H]-flunitrazepam as a ligand applying the method described by Hollander-Jansen *et al.* (1989).

Drug analysis

Whole blood oxazepam concentrations were measured by a high performance liquid chromatography (h.p.l.c.) method described by Dingemans *et al.* (1988) with slight modifications (oxazepam concentrations were measured in 100 μ l whole blood instead of in 100 μ l plasma and the 100 μ l samples were diluted with distilled water instead of borate buffer). The h.p.l.c. system (Kratos Analytical Instru-

ments, Ramsay, U.S.A.) consisted of a Spectroflow 400 solvent delivery system, a Promis automatic sample injector and a Spectroflow 757 u.v. detector, set at 240 nm. A Z-module containing a Radial-Pak C-18 cartridge, particle size 10 μ m, was used (Waters Ass., Milford, U.S.A.). For data processing a Shimadzu C-R3A reporting integrator was applied. Protein binding of oxazepam was determined by means of ultrafiltration with the Amicon MPS-1 system (Grace B.V., Rotterdam, The Netherlands). The concentrations of oxazepam in the ultrafiltrate were measured in the same way as the blood samples.

Data analysis

Pharmacokinetics The area under the oxazepam concentration-time curve (AUC) was calculated by the linear trapezoidal rule with extrapolation to infinity, using the elimination rate constant *k*. This elimination rate constant was determined from the slope of the terminal phase of the log concentration vs. time profile. The elimination half life was calculated as 0.693/*k*. Total body clearance was calculated as dose/AUC and the apparent volume of distribution (*V*_{area}) as dose/AUC**k*.

In vitro benzodiazepine receptor binding characteristics The receptor binding data were subjected to a Langmuir-type equation (Hollander-Jansen *et al.*, 1989):

$$\text{number of receptors occupied} = \frac{B_{\max} \times C}{K_D + C}$$

in which *B*_{max} represents the total number of specific binding sites, *K*_D the apparent dissociation constant and *C* the free flunitrazepam concentration, applying a least squares non-linear regression algorithm (Siphar, Simed SA, Creteil, France).

Statistics

Differences between age groups were statically evaluated by one-way analysis of variance followed by the Student *t* test with multiple comparison. Student's *t* test was applied to test whether the anticonvulsant and the proconvulsant effect in the mean effect vs. time profiles were significantly different from zero effect. *P* values lower than 0.05 were judged to be significant.

Chemicals

[³H]-flunitrazepam (specific activity 87 Ci mmol⁻¹) was obtained from New England Nuclear ('s-Hertogenbosch, The Netherlands) and N,N-dimethylacetamide (DMA) from Merck Schuchardt (München, Germany).

Results

Clinical biochemical/pathological evaluation

The results of the clinical biochemical indices are shown in Table 1. The creatinine clearance and the osmolality decreased during aging. In the other parameters very few changes were found in both the mean and the individual values, except for the values for blood aspartate and blood alanine aminotransferase in the 35-month-old animals, where the high s.e.mean is caused by the extremely high individual values in one animal.

Three 35-month-old animals appeared to show pathological abnormalities. Two animals suffering from large meningeal granular cell tumours, with invasion into brain tissue in one of them and the other animals displayed a small granular cell tumour, leukaemia and liver abnormalities. These three animals were excluded from the calculations in

Table 1 Effect of age on selected clinical biochemical indices

Age (months)	4	12	24	31	35
Number of animals	8	8	7	6	10
Body weight (g)	243 ± 10	397 ± 21 ^a	377 ± 8 ^a	429 ± 14 ^a	376 ± 12 ^a
Liver weight (g)	8.6 ± 0.7	10.5 ± 0.6	10.9 ± 0.6 (n = 6)	14.1 ± 0.7 ^{a,b}	13.0 ± 1.0 ^a
Liver wt/body wt (%)	3.49 ± 0.18	2.63 ± 0.06 ^a	2.90 ± 0.12 (n = 6)	3.29 ± 0.16	3.46 ± 0.25
Blood aspartate aminotransferase (iu l ⁻¹)	101.5 ± 9.7	89.9 ± 5.5	81.5 ± 4.7	81.5 ± 4.1	163 ± 79
Blood alanine aminotransferase (iu l ⁻¹)	38.4 ± 1.0	39.5 ± 1.0	36.7 ± 2.1	38.2 ± 4.9	68 ± 38
Plasma albumin (mg ml ⁻¹)	33.4 ± 0.6	32.8 ± 0.8	33.2 ± 0.9	35.2 ± 1.5	28.6 ± 2.8
Plasma total protein (mg ml ⁻¹)	82.8 ± 1.1	87.7 ± 1.5	88.4 ± 3.4	90.3 ± 4.7	80.9 ± 2.0
Blood glucose (mmol l ⁻¹)	5.0 ± 0.4	5.5 ± 0.2	5.4 ± 0.3	4.9 ± 0.4	4.8 ± 0.3
Blood urea nitrogen (mmol l ⁻¹)	9.5 ± 0.2	9.9 ± 0.6	8.4 ± 0.3 ^a	8.5 ± 0.6	8.2 ± 0.2 ^a
Creatinine clearance (ml h ⁻¹ kg ⁻¹)	297 ± 18	215 ± 11 ^a	234 ± 22	158 ± 23 ^a	152 ± 9 ^{a,b,c}
24 h urine production (ml)	8.6 ± 0.5	11.4 ± 1.0	12.0 ± 0.6 ^a	14.0 ± 2.7	12.7 ± 1.5
Osmolality (mOsm l ⁻¹)	2210 ± 110	1727 ± 53 ^a	1530 ± 120 ^a	1340 ± 100 ^{a,b}	1241 ± 71 ^{a,b}

Results are presented as mean ± s.e.mean. ^aSignificantly different from 4-month value, $P < 0.05$; ^bSignificantly different from 12-month value, $P < 0.05$; ^cSignificantly different from 24-month, $P < 0.05$.

Figure 1 Blood concentration (●) vs. time and anticonvulsant effect (continuous line) vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg⁻¹, in a typical 4-month-old male BN/BiRij rat. Anticonvulsant effect intensities (μA) were expressed as the elevation in seizure threshold over baseline level. At $t = 0$ min oxazepam was administered.

Figure 2 Total blood concentration vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg⁻¹, in male BN/BiRij rats of five different ages. The mean values of all animals in each age group are given: (+) 4-months-old, (Δ) 12-months-old, (○) 24-months-old, (◇) 31-months-old, (▽) 35-months-old.

the pharmacokinetic/pharmacodynamic evaluations on the basis of these findings.

Pharmacokinetic/pharmacodynamic evaluation

In vivo experiment In Figure 1 the blood concentration and anticonvulsant effect vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg in a typical 4-month-old male BN/BiRij rat are shown.

The mean oxazepam blood concentration vs. time profiles of the five age groups were identical (Figure 2), which is

reflected in comparable pharmacokinetic parameters in the age groups (Table 2); the protein binding also showed no age-related changes (Table 2).

The anticonvulsant effect vs. time profile (Figure 1) showed a biphasic effect, first an increase in seizure threshold, later followed by a transient drop below the baseline value. The drop below the baseline was not found in the 4-month-old male control animals which received only the solvent.

Figure 3 shows the mean anticonvulsant effect vs. time profiles in the five age groups. The curves for the ages of 4, 12, 24 and 31 months are very similar: the shape is com-

Table 2 The influence of aging on pharmacokinetic parameters of oxazepam following an intravenous dose of 12 mg kg⁻¹

Age (months)	4	12	24	31	35
Number of animals	8	8	7	6	7
Total body clearance (ml min ⁻¹ kg ⁻¹)	20.4 ± 0.9	15.2 ± 1.7	19.4 ± 1.1	18.7 ± 1.9	20.4 ± 2.5
Volume of distribution (l kg ⁻¹)	2.52 ± 0.19	2.40 ± 0.22	2.27 ± 0.20	2.42 ± 0.29	2.56 ± 0.15
Elimination half-life (min)	95 ± 11	105 ± 6	81 ± 5	90 ± 7	92 ± 8
Plasma protein binding (%)	88.4 ± 1.5	91.3 ± 1.2	93.6 ± 0.5	93.1 ± 1.0	92.4 ± 0.5

The results are presented as mean ± s.e.mean.

Figure 3 Anticonvulsant effect vs. time profiles after administration of oxazepam as an intravenous bolus dose of 12 mg kg^{-1} , in male BN/BiRij rats of five different ages. Anticonvulsant effect intensities (μA) were expressed as the elevation in seizure threshold over baseline level. The mean values of all animals in each age group are given: (+) 4-months-old, (Δ) 12-months-old, (O) 24-months-old, ($\diamond$) 31-months-old, (∇) 35-months-old. The last eight values for the 35-month-old rats were measured in 5 out of the total of 7 animals.

parable, but the curves tend to move to higher anticonvulsant effect values with increasing age. The curve for the 35-month-old animals shows relatively high anticonvulsant effect values and a slower decline in anticonvulsant effect with time. Moreover, the effect does not drop below the baseline as in the other age groups. The anticonvulsant effect was significantly different from zero effect in all age groups, whereas the proconvulsant part was only significant in the 4-, 12-, and 24-month-old rats.

The mean baseline seizure threshold value for all animals was $837 \pm 40 \mu\text{A}$ (mean $\pm$ s.e.mean; $n = 36$). No systematic trend during aging was found for the threshold values. For all age groups the means of the highest anticonvulsant effect values in the individual animals were calculated (Table 3). These values showed an age-related tendency to increase, with the value in the 35-month-old rats significantly higher than that in the 4-month-old animals.

In all individual animals the oxazepam blood concentration at which the anticonvulsant effect crossed the baseline value was determined graphically (Table 3). In one animal in both the 4- and the 12-month-old group and three animals in both the 24- and the 31-month-old group, the effect did not drop below the baseline level but only returned to that value. In those animals the concentration at which the effect returned to the baseline was determined. No age-related change was found in the concentration at zero effect.

In vitro benzodiazepine receptor binding characteristics No age-related changes were found in the total number of specific binding sites and the apparent dissociation constant (Table 4).

Discussion

In this investigation we determined simultaneously the time course of the plasma concentration and the anticonvulsant effect intensity following a single dose of oxazepam. Of considerable interest is the observation, that at least in young rats, the time course of the pharmacological response shows a biphasic character: anticonvulsant effect followed by proconvulsant effect. This is in contrast to the situation in Wistar rats, where only an anticonvulsant effect was observed (Dingemans *et al.*, 1990). The biphasic character of the anticonvulsant effect is highly suggestive of an acute tolerance/withdrawal syndrome. This is also confirmed by the results of some pilot experiments, in which no proconvulsant effect was observed at low concentrations of oxazepam during slow intravenous infusion. Thus it appears that initially a decrease from high concentrations to lower values is required for the proconvulsant effect to occur (Hoogerkamp *et al.*, unpublished observations). The finding of a tolerance/withdrawal phenomenon is compatible with observations by Lister & Nutt (1986).

The pharmacological effect vs. time profile in the 35-month-old animals was quite different from that in the other age groups. Immediately following administration of oxazepam the anticonvulsant effect intensity was significantly higher and also the proconvulsant component was no longer present. Finally the anticonvulsant effect in the 35-month-old animals showed a slower decrease over time compared to the other ages. The lack of the biphasic effect profile in the older rats is compatible with the disappearance of the tolerance/withdrawal phenomenon with increasing age. The question is whether this also contributes to the initial increased effect intensity that is observed.

In theory the underlying mechanism of the observed change in anticonvulsant effect intensity can be a change in pharmacokinetics (resulting in higher concentrations at the site of action) or an increased brain sensitivity. Between the different age groups no important differences in pharmacokinetics were observed (Figure 2, Table 2). As the plasma protein binding is also unaffected by age, it is unlikely that a change in distribution is the cause of the increased effect intensity. Thus it appears that in the different age groups the concentrations of oxazepam immediately following administration are quite similar. Therefore the increase in anticonvul-

Table 3 The influence of aging on the highest anticonvulsant effect values and the oxazepam blood concentrations at which the effect crossed the baseline value

Age (months)	4	12	24	31	35
Number of animals	8	8	7	6	7
Highest anticonvulsant effect (μA)	262 ± 37	350 ± 37	365 ± 53	400 ± 15	549 ± 65^a
Concentration at baseline crossing (mg l^{-1})	1.44 ± 0.23	1.52 ± 0.31	1.44 ± 0.30	1.39 ± 0.29	

The results are presented as mean $\pm$ s.e.mean. ^aSignificantly different from 4-month value, $P < 0.05$.

Table 4 The influence of aging on the *in vitro* benzodiazepine receptor binding characteristics

Age (months)	4	12	24	31	35
Number of animals	5	5	5	5	5
Total number of specific binding sites (ng mg^{-1})	0.55 ± 0.05	0.53 ± 0.22	0.54 ± 0.08	0.44 ± 0.05	0.52 ± 0.03
Apparent dissociation constant (ng ml^{-1})	4.0 ± 0.8	4.7 ± 0.9	3.3 ± 0.8	3.5 ± 0.8	5.8 ± 1.3

The results are presented as mean $\pm$ s.e.mean.

sant effect intensity in the 35-month-old rats appears to be due to a change in the pharmacodynamics of oxazepam rather than a change in its disposition, which is in agreement with observations by Kitani *et al.* (1989).

The next important question relates to the mechanism of the increase in brain sensitivity. In theory, the higher brain sensitivity we found can be caused by changes in drug-receptor interaction, post-receptor events or changes in homeostatic control mechanisms (Danhof, 1989). In the present study we did not observe age-related changes in the *in vitro* receptor binding characteristics as has been reported in other studies (Heusner & Bosmann, 1981; Barnhill *et al.*, 1990). Other data on post-receptor events (GABA receptor binding and chloride channel functioning) have not provided evidence for clear age-related changes (De Blasi *et al.*, 1982; Concas *et al.*, 1988; Ito *et al.*, 1988; Barnhill *et al.*, 1990). This leaves therefore the possibility of changes at the level of homeostatic control mechanisms as a realistic explanation for the higher brain sensitivity. Alterations in the production and/or release of an endogenous ligand for the benzodiazepine receptor with inverse agonist properties have been postulated as a mechanism for the occurrence of the tolerance/withdrawal phenomena (Baldwin & File, 1988). Interestingly, the presence of endogenous *n*-butyl β carboline-3-carboxylate, which possesses inverse agonist properties, has indeed been demonstrated (Novas *et al.*, 1988; Medina *et al.*, 1989). In man also, the occurrence of tolerance/withdrawal phenomena with regard to the pharmacological effects of benzodiazepines appears to be ubiquitous (Bliding, 1974; Greenblatt *et al.*, 1977, 1981; Macleod *et al.*, 1977; Greenblatt & Shader, 1978; Ellinwood *et al.*, 1983, 1985, 1987; Salzman *et al.*, 1983; Ashton *et al.*, 1988). Thus a decrease in the efficiency of homeostatic control mechanisms may be a realistic explanation for the increase in sensitivity to the pharmacological actions of benzodiazepines in the elderly. This would be in line with the idea of an impaired maintenance of homeostasis in the elderly in general (Shock, 1961, 1983; Bender, 1969; Crooks, 1983). In agreement with our data in rats, data from

a spontaneous reporting system maintained by the Food and Drug Administration in the U.S.A. showed a much lower frequency of withdrawal after cessation of benzodiazepine therapy in the older age group (Tanner *et al.*, 1989).

In the present investigation also detailed information on the influence of aging on the pharmacokinetics of oxazepam was obtained. The estimates of the pharmacokinetic parameters in young male BN/BiRij rats are similar to those observed in female Wistar rats (Dingemanse *et al.*, 1988, 1990). Interestingly, the pharmacokinetics of oxazepam were found not to change with increasing age, as is also the case in man (Greenblatt *et al.*, 1980; Murray *et al.*, 1981; Ochs *et al.*, 1981). In man oxazepam is eliminated primarily by phase II metabolic reactions, which are known to be relatively insensitive to changes that occur with increasing age. In rats however, the metabolism is quite different with phase I metabolic reactions being more prominent (Sisenwine *et al.*, 1972; Greenblatt, 1981; Sisenwine & Tio, 1986). Thus it appears that the similarities in the effect of aging on the pharmacokinetics of oxazepam are merely a coincidence.

Summarising, the biphasic pharmacological effect vs. time profiles (anticonvulsant effect followed by 'proconvulsant' effect) observed in the 4- to 31-month-old BN/BiRij rats after administration of an intravenous oxazepam bolus dose of 12 mg kg⁻¹ are suggestive for acute tolerance development and withdrawal. The disappearance of this biphasic profile in the 35-month-old animals and the increase in brain sensitivity are compatible with a decreased efficiency of homeostatic control mechanisms in the elderly.

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