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EFFECTS OF BENZODIAZEPINES AND VALPROIC ACID ON BRAIN ALDEHYDE REDUCTASE AND A PROPOSED MECHANISM OF ANTICONVULSANT ACTION*

MARTIN JAVORS and V. GENE ERWIN
School of Pharmacy, University of Colorado, Boulder, CO 80309, U.S.A.

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Abstract—Benzodiazepines (clonazepam, diazepam, flurazepam, fosazepam, lorazepam, nitrazepam, oxazepam and RO7-5205) were shown to inhibit the activity of brain aldehyde reductase obtained from DBA/2J mice with the IC_{50} values (concentration of inhibitor at 50 per cent of control activity) ranging from 0.24 to 7.0 mM. ED_{50} Values of these benzodiazepines for protection against maximal electroshock-induced convulsions were determined for DBA/2J mice which were pretreated with either saline or β-diethylaminoethyl diphenylpropylacetate (SKF-525A), an inhibitor of microsomal drug-metabolizing systems. Spearman rank order and Pearson correlation coefficients between the IC_{50} values for inhibition of aldehyde reductase activity and the ED_{50} values for protection against maximal electroshock-induced convulsions were calculated to be 0.62 and 0.82, respectively, for a group of eight benzodiazepines. When the animals were pretreated with SKF-525A, the correlation coefficients were 0.83 and 0.71, respectively. R_m values, indicators of relative lipid solubility, were measured for these benzodiazepines. Correlations between R_m values and IC_{50} values or ED_{50} values were not significant at the 95 per cent confidence level.

Valproic acid inhibited DBA/2J mouse brain aldehyde reductase activity with an IC_{50} value of 7×10^{-5} M. Data presented in this study are consistent with the hypothesis that highly reactive aldehyde intermediates of biogenic amine metabolism may be implicated in anticonvulsant drug action.

Compounds from all the major classes of anticonvulsant drugs have been shown to inhibit NADPH-dependent aldehyde reductase (EC 1.1.1.2) from brain. Erwin *et al.* [1] reported that bovine brain aldehyde reductase activity *in vitro* was markedly inhibited by several barbiturates, and they suggested that the ionized form of the barbiturates was responsible for the inhibition. Erwin and Deitrich [2] extended these studies to include the hydantoins, succinimides and oxazolidinediones when they observed that these compounds also inhibited bovine brain aldehyde reductase activity *in vitro*. Similarly, Turner and Tipton [3] reported that sodium barbitone inhibited the activities of the low K_m and high K_m forms of porcine brain NADPH-dependent aldehyde reductase *in vitro*. Bronaugh and Erwin [4] demonstrated the inhibition of monkey brain aldehyde reductase activity by barbiturates and phenytoin.

More recently, several biogenic acids have been shown to be potent inhibitors of brain aldehyde reductase activity [5–7]. While it has not been reported whether these biogenic acids have anticonvulsant activity, studies have shown antiseizure effects of other carboxylic acids [8–11]. One of these carboxylic acids is dipropylacetic acid (valproic acid), a compound useful in the prophylactic treatment of absence epileptic seizures. Of all the anticonvulsant drugs thus far tested, valproic acid has proved to be the most potent inhibitor of brain aldehyde reductase activity *in vitro* [12, 13].

This study was undertaken in order to examine the effects of the benzodiazepines on brain aldehyde reductase activity and on maximal electroshock (MES)-induced convulsions in DBA/2J mice, and to evaluate any correlation that may exist between these variables.

MATERIALS AND METHODS

Sepharose 4B-200, blue dextran, NAD^+, $NADP^+$, NADPH, DEAE-cellulose and 4-carboxybenzaldehyde were purchased from the Sigma Chemical Co., St. Louis, MO. All chemicals and reagents were of the highest quality obtainable from commercial sources. Thin-layer chromatography (t.l.c.) plates, silica gel 60 F_{254}-silanized, were obtained from Merck & Co., Inc., Rahway, NJ, and DBA/2J mice were purchased from The Jackson Laboratories, Bar Harbor, ME. β-Diethylaminoethyl diphenylpropylacetate (SKF-525A) was the gift of Smith Kline & French Laboratories, Philadelphia, PA; valproic acid was the gift of Abbott Laboratories, North Chicago, IL; fosazepam was the gift of Hoechst–Roussel Pharmaceuticals, Somerville, NJ; and lorazepam and oxazepam were the gifts of Wyeth Laboratories, Philadelphia, PA. "Placebo for diazepam," nitrazepam, clonazepam, diazepam, flurazepam and RO7-5205 were the gifts of Hoffman–La Roche Inc., Nutley, NJ.

Aldehyde reductase activity was assayed spectrophotometrically using a Beckman model 25 spectrophotometer. The standard reaction mixture consisted of partially purified enzyme protein, 0.1 mM NADPH cofactor, 0.1 mM 4-carboxybenzaldehyde

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(4-CBA) and 50 mM monobasic sodium phosphate. The sodium phosphate buffer was adjusted to pH 7.0 with sodium hydroxide at room temperature. The final volume of the reaction mixture was 2 ml, and the assay was performed at 37°. The reaction was initiated by the addition of NADPH, and the initial rates of NADPH oxidation were followed at 340 nm.

Purification of brain aldehyde reductase from DBA/2J *mice.* Equal numbers of adult male and female DBA/2J mice, weighing between 20 and 30 g, were decapitated; the brains were rapidly removed and placed in a buffer solution containing 10 mM monobasic sodium phosphate and 0.5 mM mercaptoethanol. This buffer was adjusted to pH 7.0 with sodium hydroxide at room temperature. All enzyme isolation procedures were conducted at 0–4°.

A 25% (w/v) homogenate was prepared by homogenizing 40–60 g of brain tissue, in the above buffer solution, in a Waring blendor at medium speed for 1 min. The homogenate was centrifuged at 53,700 *g* for 1 hr in a Beckman model L3-50 ultracentrifuge using a type 19 rotor. The resultant supernatant fluid was added with stirring to an aqueous suspension of 100 g of Sepharose 4B-200, to which blue dextran had been chemically attached according to the methods of Ryan and Vestling [14] and Porath *et al.* [15]. This mixture was slowly stirred for 30 min and the blue dextran Sepharose 4B-200, which bound the aldehyde reductase, was washed in a Buchner funnel with 2 liters of 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM mercaptoethanol, and with 500 ml of the same buffer containing 1.0 mM NAD^+ and 1.0 mM lactate in order to remove NAD^+-dependent dehydrogenase enzymes. Residual NAD^+ and lactate were removed from the blue dextran Sepharose by washing with the above buffer until the filtered wash solution measured less than 0.05 absorbance at 280 nm.

Aldehyde reductase activity was eluted from the blue dextran Sepharose with 400 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM mercaptoethanol and 0.6 mM NADPH. The resultant enzyme solution was concentrated to approximately 10 ml using an Amicon filter apparatus with a UM-10 membrane, which blocks the passage of compounds with a molecular weight of 10,000 daltons or greater.

The concentrated enzyme solution was applied to a 25 × 250 mm DEAE–cellulose column, and aldehyde reductase activity passed through the column. The DEAE–cellulose was prepared by washing with 0.1 N sodium hydroxide, 0.1 N hydrochloric acid and water, followed by equilibration with 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM mercaptoethanol. The flow rate was 3.5 ml/min, and the eluent was continuously monitored spectrophotometrically at 280 nm, using an ISCO model UA-5 absorbance monitor to observe the elution of proteins.

Fractions containing aldehyde reductase activity were combined and stored at 0–4°. Under these conditions, the enzyme activity is stable for at least 6 months. The enzyme was obtained with approximately 21 per cent yield and a specific activity of 2.5 μmoles NADPH/min per mg protein. This yield and final specific activity exceed that obtained previously in our laboratory using other procedures [1, 2, 4]. The enzyme was specific for NADPH and readily utilized long-chain aliphatic and "aromatic" aldehydes as substrates.

Determination of IC_{50} *values of benzodiazepines and valproic acid for the inhibition of partially purified brain aldehyde reductase activity.* The concentrations of inhibitors were chosen such that the inhibition was between 16 and 84 per cent of control velocity. The IC_{50} values represent benzodiazepine concentrations which produce 50 per cent inhibition of partially purified brain aldehyde reductase activity. The IC_{50} values were estimated utilizing at least four concentrations of inhibitor, and each value is the average of two or more separate estimations using different enzyme preparations.

Benzodiazepines were dissolved in Tween 20 and brought to a final volume with a solution containing 50 mM monobasic sodium phosphate (the pH had been adjusted to 7.0 with sodium hydroxide at room temperature). The final concentration of Tween 20 in the reaction cuvette was maintained constant at 10% with the use of a buffered Tween 20 solution without benzodiazepine.

Probit values for per cent of control velocity in the presence of benzodiazepine were plotted against the concentrations of inhibitors, and a best fit line was drawn using the method of least squares. The IC_{50} values of the benzodiazepines and valproic acid were calculated from the least squares line.

Determination of ED_{50} *values for protection against maximal electroshock-induced convulsions.* Determinations of ED_{50} values [16] of benzodiazepines for protection from maximal electroshock (MES)-induced seizures were made using the method of Swinyard [17]. Male and female DBA/2J mice in equal numbers were given intraperitoneal injections of a benzodiazepine 1 hr before receiving an electric shock administered via a corneal electrode. The benzodiazepines were dissolved in Roche "Placebo for diazepam,"* a solvent used commercially to dissolve diazepam. This solvent will protect mice against MES-induced seizures at doses greater than 0.7 ml; however, no protection was afforded at the doses (0.1–0.2 ml) used in this study. The electroshock was delivered with a Wahlquist Instrument Co. power supply which provided a current intensity of 50 mA with a duration of 0.2 sec, at a frequency of 60 Hz. A positive tonic convulsion was characterized by the classical hindlimb extension. At least eight mice were used per dosage, and at least three dosages of each drug were used per ED_{50} determination.

All experiments were conducted between the hours of 12:00 noon and 6:00 p.m. in order to minimize the effects of diurnal rhythms, and mice were caged in groups of four of the same sex, at a constant temperature of 72 ± 2° with a 12 hr light–dark cycle. Mice were given water and food (Wayne Lab Blox) *ad lib.*, and all animals weighed between 20 and 30 g.

* Roche "Placebo for diazepam" contains: propylene glycol USP, 40%; ethyl alcohol USP, 10.55%; benzyl alcohol NF, 1.5%; sodium benzoate USP, 4.88%; benzoic acid USP, 0.12%; and water for injection USP, q.s.

The age of the mice ranged from 55 to 85 days.

The calculation of the ED_{50} values and 95 per cent confidence limits was done according to the method of Litchfield and Wilcoxon [18]. The percentages of animals protected for each dosage group were changed to probits, and the doses, in μmoles drug per kg body wt, were changed to $\log_{10}$. Probits were plotted against $\log_{10}$ doses and a best fit line was drawn to the data points using the least squares method. Using least square lines, ED_{50} values and 95 per cent confidence limits were then calculated.

In experiments utilizing SKF-525A, water solutions of this compound were administered at a dose of 20 mg/kg, intraperitoneally, 30 min prior to benzodiazepine injection.

Determination of R_m *values.* R_m values, indicators of lipid solubility of compounds, were determined using the chromatographic methods of Biagi *et al.* [19] and Hung and Raynaud [20]. R_m values are calculated according to the following formula:

$$R_m = \log\left(\frac{1}{R_f} - 1\right),$$

where R_f is the distance traveled from the baseline by the drug divided by the distance traveled by the solvent front.

R_m values were determined using silanized silica gel t.l.c. plates (Merck, precoated T.L.C. plates, Silica gel 60 F_{254}-silanized, layer thickness 0.25 mm, size 20 × 20 cm). Drugs were dissolved in methanol solutions and spotted on the baseline of the t.l.c. plates. The plates were then placed in a sealed chromatographic tank containing an acetone–water solution, and the sides of the tank were lined with filter paper that was saturated with the solvent solution. After the chromatographic run, at room temperature, the plates were removed from the tank and dried at ambient temperatures. Compounds were visualized as dark spots under a u.v. lamp, against a fluorescing background.

Each drug was chromatographed three times at several acetone concentrations, and the average R_m value for each concentration of acetone was plotted against the percentage of acetone in the solvent mixture. A best fit line was drawn using the method of least squares, and the R_m value for zero per cent acetone was calculated.

Table 1. IC_{50} Values of benzodiazepines and valproic acid for inhibition of brain aldehyde reductase activity*

Drug	IC_{50} Values ($M \times 10^4$)
Nitrazepam	2.46
Clonazepam	2.63
RO7-5205	17.7
Lorazepam	19.5
Oxazepam	29.7
Diazepam	46.8
Fosazepam	54.9
Flurazepam	71.1
Valproic acid	0.70

* The IC_{50} estimations for inhibition of partially purified DBA/2J mouse brain aldehyde reductase activity were determined as described in the text and in the legend to Fig. 1. The IC_{50} values reported are averages of at least two IC_{50} estimations per inhibitor using different enzyme preparations.

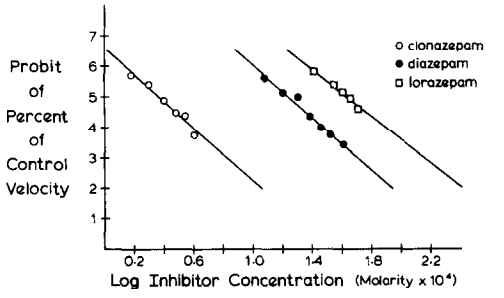

Fig. 1. Estimation of IC_{50} for inhibition of brain aldehyde reductase from DBA/2J mice. Aldehyde reductase assays were conducted as described in the text with each final reaction mixture containing Tween 20, 10% (w/v). The benzodiazepines were dissolved in a 20% (w/v) Tween 20 solution containing 50 mM monobasic sodium phosphate adjusted to pH 7.0 with sodium hydroxide. The concentration of the inhibitor was varied, and the rate of NADPH disappearance was measured at 340 nm for each inhibitor concentration. The probit of per cent of control velocity was plotted against the log of the inhibitor concentration. The inhibitor concentration which corresponded to a probit value of 5.0 was taken as the IC_{50} estimation.

RESULTS

As shown in Table 1 and Fig. 1, valproic acid inhibited aldehyde reductase with an IC_{50} value of 7×10^{-5}M, and the K_i value was determined to be 2.5×10^{-5}M. The IC_{50} values of the benzodiazepines ranged from 2.46×10^{-4}M, for nitrazepam, to 7.11×10^{-3}M, for flurazepam. Inhibition of aldehyde reductase activity by clonazepam was reversible by dilution (Table 2).

The ED_{50} values of the benzodiazepines for protection of DBA/2J mice against MES-induced convulsions are presented in Table 3. The method described by Swinyard [17] for the determination of anticonvulsant ED_{50} values for protection of animals against MES-induced seizures involves administration of the drug 1 hr prior to MES. Garattini *et al.* [21] have demonstrated, 1 hr after the intravenous injection of diazepam, the presence of measurable brain levels of diazepam, *N*-demethyl-diazepam and oxazepam. These compounds, as well as another derivative of diazepam, *N*-methyloxazepam, are known to possess anticonvulsant activity in the mouse [22]. Therefore, the anticonvulsant ED_{50} values in Table 3 are undoubtedly the result of a combination of compounds, i.e. the originally administered drug and its *in vivo* metabolites. In order to minimize the *in vivo* formation of benzodiazepine metabolites, SKF-525A (20 mg/kg) was administered intraperitoneally 0.5 hr prior to the administration of benzodiazepine, and a new set of ED_{50} values (SKF-ED_{50} values) was determined (Fig. 2). The SKF-ED_{50} values for the benzodiazepines are shown in Table 4. These values probably reflect more accurately the direct result of the originally administered drug [23–25].

Table 2. Reversibility of benzodiazepine inhibition of brain aldehyde reductase activity*

	Clonazepam	Per cent of control activity	NADPH (moles oxidised/min)
Control		100.0	1.318
Assay I	8×10^{-4} M	0.0	0.0
Assay II	4×10^{-4} M	22.8	0.306
Assay III	2.7×10^{-4} M	48.8	0.643

* Determination of aldehyde reductase activity was performed as described in the text with clonazepam as inhibitor. The concentration of clonazepam, prior to dilution, was sufficient to inhibit the reaction completely (Assay I). The Assay I mixture was diluted by a factor of 2 by adding an equal volume of reaction medium such that the 4-carboxybenzaldehyde, NADPH and Tween 20 concentrations remained constant at 10^{-4} M and 12 per cent, respectively (Assay II). Assay III was a further dilution of Assay II by a factor of 1.5.

Table 3. ED_{50} Values of benzodiazepines for protection of DBA/2J mice against maximal electroshock-induced convulsions*

Drug	ED_{50} Value (moles/kg) (confidence limits)	
Clonazepam	8.03	(7.60–8.49)
Nitrazepam	11.3	(8.88–14.4)
Oxazepam	16.4	(15.7–17.2)
Lorazepam	22.6	(20.9–24.5)
Diazepam	29.1	(24.5–34.5)
R07-5205	57.2	(51.8–63.4)
Fosazepam	104.0	(92.0–118)
Flurazepam	227.0	(213–242)

* Anticonvulsant ED_{50} values were determined according to the method of Swinyard [17]. The calculation of the ED_{50} values and of the 95 per cent confidence limits was done according to the method of Litchfield and Wilcoxon [18].

Table 4. ED_{50} Values of benzodiazepines for protection against maximal electroshock-induced convulsions of DBA/2J mice pretreated with SKF-525A*

Drug	ED_{50} Value (moles/kg) (confidence limits)	
Nitrazepam	5.41	(4.83–6.06)
Clonazepam	8.06	(7.10–9.16)
Lorazepam	10.3	(9.19–11.5)
Oxazepam	17.8	(16.0–19.8)
Diazepam	27.2	(25.2–29.3)
RO7-5205	45.9	(39.3–53.6)
Flurazepam	56.7	(44.2–72.8)
Fosazepam	101.8	(90.4–115)

* Determination of anticonvulsant SKF-ED_{50} values is described in the legends of Fig. 2 and Table 3, as well as in Materials and Methods.

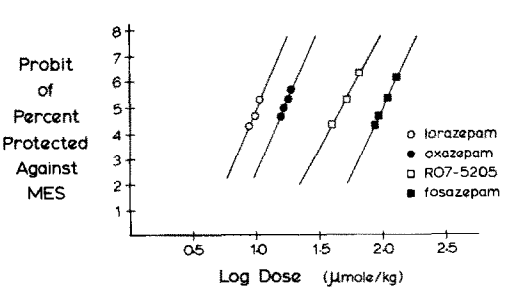

Fig. 2. Determination of ED_{50} values for protection of DBA/2J mice pretreated with SKF-525A against maximal electroshock (MES)-induced convulsions. At least eight mice were used at each drug dosage level, and a minimum of three dosage levels was utilized per ED_{50} determination. Determinations presented here are for animals pretreated with a 20 mg/kg intraperitoneal dose of SKF-525A 30 min prior to the benzodiazepine. The probit of the per cent of mice protected against MES-induced, hindlimb-extension convulsions was plotted against the log of the dose of benzodiazepine. The dose that corresponded to a probit value of 5.0 was taken as the SKF-ED_{50} value. The ED_{50} values for protection of DBA/2J mice against MES-induced convulsions were similarly obtained except that the mice were not pretreated with SKF-525A.

Pretreatment of animals with SKF-525A lowered the anticonvulsant ED_{50} values of nitrazepam, lorazepam and flurazepam, while it did not alter the ED_{50} values of clonazepam, oxazepam, diazepam, fosazepam and RO7-5205.

In the course of these studies it was of interest to determine whether binding of the benzodiazepines might be related to lipophilic interactions with the enzyme. Consequently, R_m values which indicate the relative lipid solubilities of several benzodiazepines were estimated; the values are presented in Table 5. Values ranged from 0.712 for fosazepam, a water-soluble benzodiazepine, to 2.193 for flurazepam.

Two types of correlation analysis were used to evaluate the date [26]. The Spearman rank order correlation coefficients (r_s) and the Pearson correlation coefficients (r_p) are presented in Table 6.

The R_m values for the benzodiazepines (Table 5) did not correlate significantly with either the IC_{50} values for the benzodiazepines for inhibition of brain aldehyde reductase activity, the ED_{50} values, or the SKF-ED_{50} values of the benzodiazepines for protection of DBA/2J mice against MES-induced seizures. This implies that the order of potency of the benzodiazepines in these parameters is not related to their lipid solubility, but is a more specific binding phenomenon.

Table 5. R_m values of benzodiazepines*

Drug	R_m value
Fosazepam	0.712
Oxazepam	1.283
Lorazepam	1.369
Nitrazepam	1.438
Clonazepam	1.579
Diazepam	1.652
Flurazepam	2.193

* R_m values were measured by thin-layer chromatography on silanized silica gel plates run in a mixture of acetone–water solutions in varying proportions, as described in Materials and Methods. R_m values (average of three determinations) were plotted against the percentage of acetone in the mixture and extrapolated to 0% (v/v) acetone to give R_m (H_2O). $R_m = \log(1/R_f - 1)$.

The correlation analysis between ED_{50} and SKF-ED_{50} values and the IC_{50} values for the benzodiazepines produced r_s and r_p values that were statistically significant; for example, the r_s values were 0.62 and 0.83, respectively.

DISCUSSION

These studies were conducted in order to examine further the hypothesis that inhibition of brain aldehyde reductase activity might be responsible for the anticonvulsant action of the benzodiazepines [2, 13]. As reported in the results, the benzodiazepines were shown to be moderately potent reversible inhibitors of DBA/2J mouse brain aldehyde reductase activity *in vitro* (Tables 1 and 2), and to protect DBA/2J mice against MES-induced seizures (Tables 3 and 4). A modest correlation was observed between these parameters (Table 6). The r_p value for the correlation between the IC_{50} values and the SKF-ED_{50} values of the benzodiazepines is 0.71, and r_p^2 equals 0.50. Of course, this significant covariance does not indicate any causal relationship between brain aldehyde reductase inhibition and anticonvulsant activity. It is consistent, however, with the working hypotheses that drug-induced alterations in the brain levels of β-hydroxylated biogenic aldehydes [27, 28] may be involved in the mechanism of action of anticonvulsant compounds [2]. The concentration of these β-hydroxylated biogenic aldehydes might be increased *in vivo* by the inhibition of brain aldehyde reductase activity. These biogenic aldehydes have been shown to form tetrahydroisoquinoline alkaloids *in vivo* [29, 30], to inhibit Na^+-K^+-ATPase activity from mouse brain and to inhibit norepinephrine uptake into rat brain synaptosomes [31].

To date, no one has been able to demonstrate *in vivo* that an anticonvulsant drug will increase the concentration of dihydroxyphenylglycolaldehyde (DHPGA), the biogenic aldehyde of norepinephrine. Several investigators have used brain homogenates to show this effect [29, 32–34]. A concern in the present study is that the concentration of benzodiazepine required to block convulsions is considerably lower than those needed to inhibit aldehyde reductase *in vitro*. Studies are currently in progress to determine whether any of the anticonvulsants make aldehyde reductase rate-limiting in the intact brain.

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Table 6. Spearman rank order and Pearson correlation coefficients for *in vitro* and *in vivo* parameters*

Parameters correlated	r_p	No. of drugs tested	P	r_s	No. of drugs tested	P
ED_{50} vs IC_{50}	0.82	8	<0.05	0.62	8	<0.10
SKF-ED_{50} vs IC_{50}	0.71	8	<0.05	0.83	8	<0.01
R_m vs IC_{50}	0.19	7	NS	0.14	7	NS
R_m vs ED_{50}	0.42	7	NS	0.14	7	NS
R_m vs SKF-ED_{50}	−0.35	7	NS	−0.07	7	NS

* Correlation coefficients (r_s and r_p) were determined using two methods, the Spearman rank order and the Pearson methods, according to the procedures of Brown and Hollander [26]. P values were taken from statistical tables. NS = not significant.

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