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General Principles

Experimental Selection, Quantification, and Evaluation of Antiepileptic Drugs

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The Epilepsy Branch of the National Institute of Neurological Disorders and Stroke established an Anticonvulsant Drug Development Program in October 1974. The Anticonvulsant Screening Project (ASP) for this program was awarded to the University of Utah. Over the past 20 years, a variety of *in vivo* animal models have been utilized in order to identify and evaluate chemicals with anticonvulsant efficacy and minimal toxicity. Over 16,000 chemicals supplied by medicinal chemists and pharmaceutical companies around the world have been subjected to anticonvulsant identification tests. Approximately 2,700 of these exhibited sufficient anticonvulsant activity to warrant subjecting them to anticonvulsant quantification studies, and 130 of the latter were also evaluated for tolerance, metabolism, and mechanisms of action. The Epilepsy Branch subjected 11 of the latter group to clinical trial; as of July 1993, six Investigational New Drug Applications have been filed with the United States Food and Drug Administration (FDA). Three (felbamate, gabapentin, and lamotrigine) of the six have also received FDA Advisory Panel approval and one, felbamate (Felbatol; Carter Wallace, Inc.), received marketing approval July 30, 1993. It is of particular interest that the anticonvulsant activity of these and other novel anticonvulsants was originally identified in the well-established animal models of epilepsy described herein.

The maximal electroshock and subcutaneous pentylenetetrazol (Metrazol) tests have been used extensively for identifying new anticonvulsant drugs (17). This is due in part to the predictive nature of these two tests. For example, it is generally agreed that substances which obtund only the tonic extension of maximal seizures (e.g., phenytoin) may be clinically useful in generalized tonic–clonic seizures, whereas substances which only elevate minimal seizure threshold (e.g., ethosuximide) may be useful in generalized absence seizures. Similarly, substances which obtund seizures in kindled rats (e.g., valproate) may be useful in complex partial seizures. However, because valproate is effective in all three of these *in vivo* animal models, it may be expected to have some utility in generalized tonic–clonic, generalized absence, and complex partial seizures. It is clear from this brief discussion that a battery of tests must be used in order to reveal the overall anticonvulsant potential of a test substance. The tests used in the ASP to identify and evaluate the antiepileptic potential of candidate chemicals are described in this chapter. Upon receipt at the laboratories of the ASP, the test substance is subjected to a large number of testing procedures according to the paradigm summarized in Fig. 1. The details of many of these procedures are outlined in Table 1 and discussed below.

MATERIALS AND METHODS

Experimental Animals

Adult male CF No. 1 albino mice (18–25 g) and adult male Sprague–Dawley albino rats (100–150 g) are used

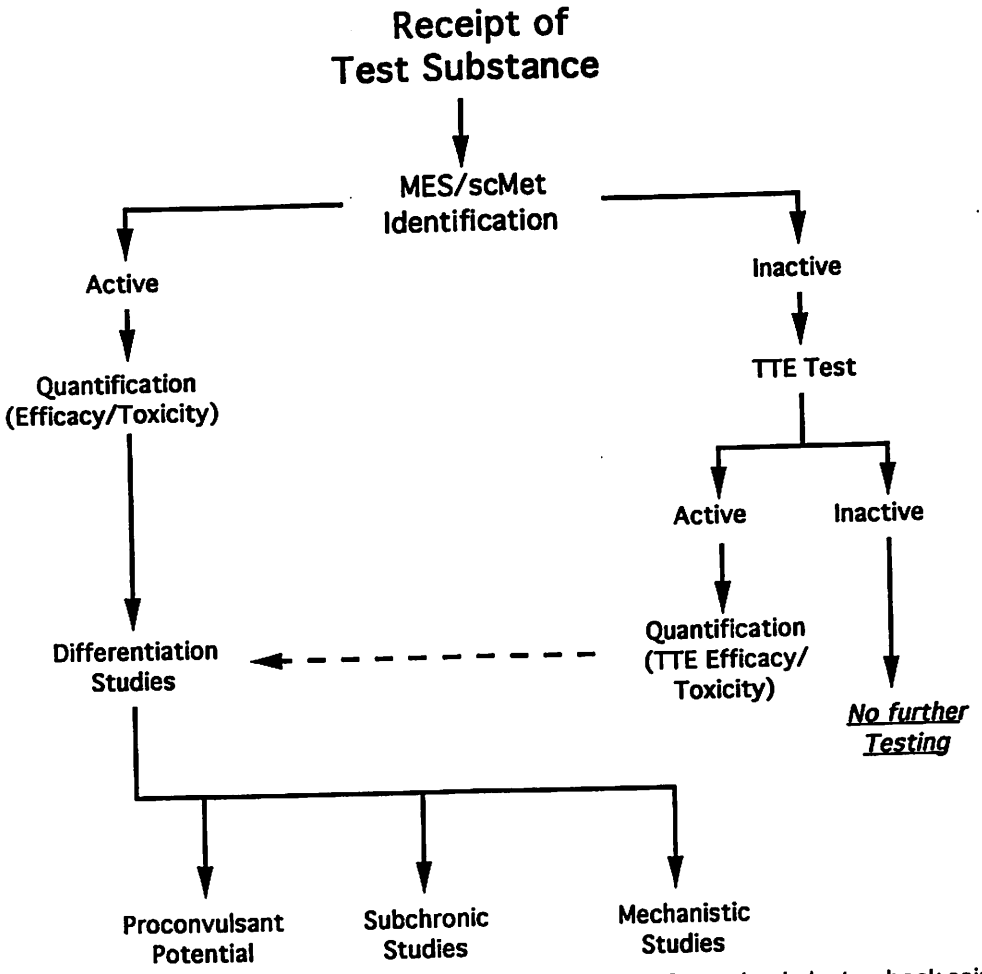

FIG. 1. Anticonvulsant screening project testing paradigm. MES, maximal electroshock seizure; scMet, subcutaneous pentylenetetrazol (Metrazol); TTE, threshold tonic extension.

as experimental animals. These particular strains are preferred for anticonvulsant studies because they are docile and easy to handle. Moreover, CF No. 1 mice rarely succumb to induced seizures (19). Animals of the same sex, age, and weight are employed to minimize biological variability (22). The animals are maintained on a 12-hr light/dark cycle and allowed free access to food and water, except during the short time they are removed from their cages for testing. Animals newly received in the laboratory are allowed 24 hr to compensate for the food and water restriction incurred during transit. This is necessary because such restriction increases the severity of maximal electroshock seizures (3). All animals are maintained and handled in a manner consistent with the recommendations in HEW publication (NIH) No. 8623, "Guide for the Care and Use of Laboratory Animals." Animals are generally used only once and then disposed of in a humane manner. In those instances where they are used a second time, at least a 1-week interval is allowed for the animal to eliminate the test drug.

Convulsant Chemicals

For tests based on chemically induced seizures, the convulsant chemical is prepared in a concentration that will induce convulsions in more than 97% of animals when injected in mice in a volume of 0.01 ml/g body weight or in rats in a volume of 0.02 ml/10 g body weight. For mice, pentylenetetrazol and picrotoxin are dissolved in 0.9% saline sufficient to make a 0.85% and 0.032% solution, respectively. For rats, pentylenetetrazol is given in a concentration of 3.5%. Bicuculline is dissolved in 1.0 ml of warmed 0.1 N hydrochloric acid with the aid of a micro-mixer and sufficient 0.9% saline added to make a 0.027% solution. The solution is used within 30 min. All chemical convulsants are administered subcutaneously (s.c.) into a loose fold of skin in the midline of the neck. No other drugs or chemicals are injected in the same subcutaneous site. The judicious selection of injection sites avoids false-positives induced by vasoconstrictor substances retarding the absorption of the convulsant agents. Because the doses employed in the above tests induce convulsions

TABLE 1. *Overview of testing procedures*[a]

I. Anticonvulsant identification
  a. Mice i.p.
    Dose range: 30, 100, and 300 mg/kg
    Tests: MES, s.c. Met, rotorod
    Time of test: 0.5 and 4 hr
  b. Rats p.o. (compounds active in a.)
    Dose: 30 mg/kg
    Tests: MES or s.c. Met and minimal neurotoxicity
    Time of test: 0.25, 0.5, 1, 2, and 4 hr
  c. Mice i.p. (compounds inactive in a.)
    Dose: 100 mg/kg
    Test: TTE
    Time of test: 0.25, 0.5, 1, 2, and 4 hr
II. Anticonvulsant quantification
  a. Mice i.p.
    TPE: MES, s.c. Met, TTE, rotorod
    ED_{50}: MES, s.c. Met, TTE
    TD_{50}: Rotorod
  b. Rats p.o.
    TPE: MES, s.c. Met, minimal neurotoxicity
    ED_{50}: MES, s.c. Met
    TD_{50}: minimal neurotoxicity
III. Anticonvulsant differentiation
  a. Mice i.p.
    ED_{50}: s.c. Bic, s.c. Pic
    ED_{50}: Frings AGS
  b. Rats p.o.
    GHB-altered electroencephalogram
    Expression of corneal kindling
    Acquisition of corneal kindling
IV. Proconvulsant potential
  a. Mice i.p.
    Timed i.v. infusion of pentylenetetrazol
V. Subchronic studies
  a. Rats p.o., *in vivo* tolerance
    Effect of subchronic dosing on MES or s.c. Met efficacy
    Effect of subchronic dosing on hexobarbital sleep time
  b. Rats p.o., liver studies
    Effect of subchronic dosing on sALT activity
    Effect of subchronic dosing on various drug metabolizing enzymes
VI. Mechanism studies (*in vitro*)
  a. Imaging studies
    Cl^-, K^+, Ca^{2+}, and Na^+ flux
  b. Patch-clamp electrophysiology studies
    Cl^-, K^+, Ca^{2+}, and Na^+ currents

[a] MES, maximal electroshock seizure test; s.c. Met, subcutaneous pentylenetetrazol (Metrazol) seizure threshold; TTE, threshold tonic extension test; TPE, time of peak effect; ED_{50}, median effective dose; TD_{50}, median toxic dose; s.c. Bic, subcutaneous bicuculline test; s.c. Pic, subcutaneous picrotoxin test; AGS, audiogenic seizure susceptible; GHB, gamma-hydroxybutyrate; sALT, serum alanine aminotransferase.

in over 97% of animals, it is unnecessary to run control groups simultaneously with the test groups.

Preparation of Test Drugs

Test drugs soluble in water are administered in 0.9% saline solution; those insoluble in water are administered as a suspension in 0.5% methylcellulose. The test substance is given in a concentration that permits optimal accuracy of dosage without the volume contributing excessively to total body fluid. Thus, the volume employed in mice is 0.01 ml/g body weight, and the volume in rats is 0.04 ml/10 g body weight. Test drugs are administered either intraperitoneally (i.p.) or orally (p.o.), as indicated in Table 1.

Determination of Acute Toxicity

Abnormal neurological status disclosed by the rotorod test (4) is commonly taken as the endpoint for minimal neurotoxicity in mice. Abnormal neurological status disclosed by the positional sense test, muscle tone test, or the gait and stance test is taken as the endpoint for minimal neurotoxicity in rats. Inability of a rat to perform normally in at least two of these tests indicates that the animal has some neurological deficit. The names assigned to the above tests are those employed in the authors' laboratories and do not necessarily refer to the specific neurological reflexes involved (17).

Rotorod Test

The rotorod test is used exclusively in mice to assess minimal neurotoxicity. When a normal mouse is placed on a rod 1 inch in diameter that rotates at a speed of 6 rpm, the mouse can maintain its equilibrium for long periods of time. Neurological deficit is indicated by inability of the animal to maintain its equilibrium for 1 min in each of three trials.

Positional Sense Test

If the hind leg of a normal mouse or rat is gently lowered over the edge of a table, the animal will quickly lift its leg back to a normal position. Neurological deficit is indicated by inability of the animal to correct rapidly such an abnormal position of the limb.

Gait and Stance Test

Neurological deficit is indicated by a circular or zigzag gait, ataxia, abnormal spread of the legs, abnormal body posture, tremor, hyperactivity, lack of exploratory behavior, somnolence, stupor, catalepsy, and so on.

Muscle Tone Test

Normal animals have a certain amount of skeletal muscle tone which on handling is apparent to the expe-

rienced technician. Neurological deficit is indicated by a loss of skeletal muscle tone characterized by hypotonia or flaccidity.

Anticonvulsant Identification

At the present time, no single laboratory test will, in itself, establish the presence or absence of anticonvulsant activity or fully predict the clinical potential of a test substance. In the ASP, three tests are used for the routine identification of anticonvulsant activity: the maximal electroshock seizure test, the s.c. pentylenetetrazol seizure threshold test, and the threshold tonic extension test.

Maximal Electroshock Seizure (MES) Test and Subcutaneous Pentylenetetrazol Seizure Threshold Test

In the MES test, a 60-Hz alternating current (mice 50 mA, rats 150 mA) is delivered for 0.2 sec through corneal electrodes by means of an apparatus similar to that originally designed by Woodbury and Davenport (21). At the time of administration of the test substance, a drop of 0.5% tetracaine in saline is applied to the eyes of all animals assigned to any electroshock test. Immediately before the placement of corneal electrodes, a drop of electrolyte (saline) is placed on each eye. The animals are restrained by hand and released immediately following stimulation to permit observation of the seizure throughout its entire course. Abolition of the hind-limb tonic extensor component is taken as the endpoint for this test. Absence of this component suggests that the test substance has the ability to prevent the spread of seizure discharge through neural tissue.

In the s.c. pentylenetetrazol test, a convulsive dose (CD97) of pentylenetetrazol (85 mg/kg in mice, 70 mg/kg in rats) is injected subcutaneously. The animals are placed in isolation cages and observed for the next 30 min for the presence or absence of an episode of clonic spasms persisting for at least 5 sec. Absence of a clonic seizure suggests that the test substance has the ability to raise the seizure threshold.

In identification studies involving mice, the ASP routinely employs the MES and s.c. pentylenetetrazol tests. Sixteen mice are randomly divided into three groups of four, eight, and four mice each; each group is then given 30, 100, or 300 mg/kg, respectively, of the test substance i.p. Thirty minutes after administration of the test substance, all animals are subjected to the rotorod test; one animal in the 30 and 300 mg/kg group and three animals in the 100 mg/kg group are then subjected to the MES test, and one animal in each group is subjected to the s.c. pentylenetetrazol test. Four hours after drug administration, all remaining animals in each group are subjected to the rotorod test; these animals are then subjected to the MES and pentylenetetrazol test as indicated above. Thus, it requires only 16 mice to cover the dose range of 30, 100, and 300 mg/kg and the time periods of 0.5 and 4 hr.

TABLE 2. *Anticonvulsant identification in mice after intraperitoneal administration*

Test[a]	Dose (mg/kg)	Results[b] 0.5 hr	Results[b] 4 hr
Toxicity	30	0/4	0/2
	100	0/8	0/4
	300	4/4	0/2
MES	30	1/1	0/1
	100	3/3	0/3
	300	1/1	1/1
s.c. Met	30	0/1	0/1
	100	0/1	0/1
	300	0/1	0/1

[a] MES, maximal electroshock seizure test; s.c. Met, subcutaneous pentylenetetrazol (Metrazol) seizure threshold test.
[b] Number protected or toxic/number tested.

An example of results obtained with the MES and s.c. pentylenetetrazol screening procedures are shown in Table 2. As can be seen, the test substance is effective in nontoxic doses against the MES test but ineffective by the s.c. pentylenetetrazol test; minimal neurotoxicity is greater than 100 mg/kg but less than 300 mg/kg. The test substance also appears to have a relatively rapid onset and short duration of action, because both the anticonvulsant and neurotoxic effects are obviously greater at 30 min than at 4 hr.

In identification studies employing rats, a dose of 30 mg/kg of the test substance is administered orally to five groups of four rats per group. At various times after administration (0.25, 0.5, 1, 2, and 4 hr), animals are evaluated for neurological deficit and then subjected to the MES test. The ratios of animals protected or toxic to animals tested are determined.

The initial identification studies in rats provide information relative to whether or not the test substance is active and/or toxic in a dose of 30 mg/kg after oral administration. It also discloses the time of onset, the approximate time of peak effect (TPE), and the duration of anticonvulsant activity and/or neurotoxicity. Anticonvulsant identification results obtained in rats (MES test) with the same test substance described in Table 2 are shown in Table 3. As can be seen from these data, the test substance is active in rats by the MES test; some anticonvulsant activity is present within 15 min, the TPE is in the range of 1 to 2 hr, and the duration of action is about 3.5 hr. Moreover, no evidence of neurological deficit (toxicity) is observed at this dose. For the test substance profiled in Tables

TABLE 3. *Anticonvulsant identification in rats after oral administration*

Test[a]	Dose (mg/kg)	Number protected or toxic/ number tested 0.25 hr	0.5 hr	1 hr	2 hr	4 hr
MES	30	1/4	2/4	4/4	4/4	2/4
s.c. Met	—	—	—	—	—	—
Toxicity	30	0/4	0/4	0/4	0/4	0/4

[a] MES, maximal electroshock seizure test; s.c. Met, subcutaneous pentylenetetrazol (Metrazol) seizure threshold test.

2 and 3, the results suggest that further experimental work is justified because the favorable anticonvulsant profile indicates possible clinical usefulness in generalized tonic–clonic and perhaps complex partial seizures.

Threshold Tonic Extension (TTE) Test

As shown in Fig. 1, only those compounds which are inactive in the traditional identification tests (MES and s.c. pentylenetetrazol) are subjected to the TTE test. This test is a nonselective, electroconvulsive seizure model that, for the most part, identifies substances that block seizures induced by maximal electroshock and/or s.c. pentylenetetrazol stimulation. However, this test will also identify a small number of compounds that are inactive in the MES and s.c. pentylenetetrazol tests. Ongoing investigations are attempting to determine whether these "novel" compounds will afford any significant benefit over existing anticonvulsants. The primary technical difference between the TTE test and the MES test is the current employed. The TTE test uses sufficient current to elicit threshold hind-limb tonic extension in 97% of the animals challenged, whereas the MES test uses a supramaximal current (four to five times threshold).

Twenty mice are pretreated i.p. with 100 mg/kg of the test substance. At various times (0.25, 0.5, 1, 2, and 4 hr) after pretreatment, individual mice (four at each time point) are challenged with sufficient current to elicit a tonic extension seizure (12.5 mA; 0.2 sec via corneal electrodes). Animals not displaying hind-limb tonic extension are considered protected.

As shown in Table 4, this test has the potential for identifying anticonvulsant activity in compounds which are inactive in the MES and s.c. Met tests. After i.p. administration of 100 mg/kg of the test substance profiled in this table, anti-TTE activity was present within 15 min, maximum after 1 hr, and gone after 2 hr. As can also be seen, the compound was inactive by the MES and s.c. pentylenetetrazol tests. Furthermore, when the test substance was rescreened at the same time points as used in the TTE test, it was still inactive by the MES test. Whether this substance is active in other anticonvulsant models has yet to be established.

The results from these identification tests [MES and s.c. pentylenetetrazol (mice and rats), TTE (mice), neurological deficit (mice and rats)] provide important preliminary information pertaining to oral bioavailability, species variation, duration of action, toxicity, efficacy, and overall potential of novel anticonvulsant substances.

Quantification of Experimental Results

Anticonvulsant quantification in mice reveals more about the TPE and details the median effective dose (ED_{50}) by the MES, s.c. pentylenetetrazol, or TTE tests; the median toxic dose (TD_{50}) by the rotorod test; the 95% confidence intervals; the slopes of the regression lines; and protective indices (PIs) which define the ratio between the TD_{50} and ED_{50}. The TPE data provide further insight into the time of onset and the duration of anticonvulsant and toxic activity. Anticonvulsant quantification is also conducted in rats after p.o. administration to delineate anticonvulsant activity and neurotoxicity via a different route of administration in another rodent species and to develop dose information prerequisite to subsequent chronic toxicity studies.

Time of Peak Effect (TPE)

All quantitative studies are performed at the TPE. To determine the TPE for anticonvulsant activity, five

TABLE 4. *Threshold tonic extension, MES, and s.c. Met identification and rotorod toxicity in mice after intraperitoneal administration*

Test[a]	Dose (mg/kg)	Number protected or toxic/ number tested 0.25 hr	0.5 hr	1 hr	2 hr	4 hr
TTE	100	2/4	3/4	4/4	0/4	0/4
Toxicity	30	—	0/4	—	—	0/2
	100	—	0/8	—	—	0/4
	300	—	0/4	—	—	0/2
MES	30	—	0/1	—	—	0/1
	100	—	0/3	—	—	0/3
	300	—	0/1	—	—	0/1
(rescreen)	100	0/4	0/4	0/4	0/4	0/4
s.c. Met	30	—	0/1	—	—	0/1
	100	—	0/1	—	—	0/1
	300	—	0/1	—	—	0/1

[a] MES, maximal electroshock seizure test; s.c. Met, subcutaneous pentylenetetrazol (Metrazol) seizure threshold test; TTE, threshold tonic extension test.

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