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Blockade of sustained repetitive action potentials in cultured spinal cord neurons by zonisamide (AD 810, CI 912), a novel anticonvulsant

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Zonisamide is a novel anticonvulsant that prevents seizures in laboratory animals and in man. Zonisamide (3 $\mu\text{g/ml}$ and above) blocked the sustained firing of action potentials induced by depolarizing steps of current injected across the membrane of intracellularly recorded spinal cord neurons. Responses to GABA and glutamate were not altered by zonisamide, and spontaneous synaptically evoked activity was not reduced until higher concentrations of zonisamide (10 $\mu\text{g/ml}$) were applied.

INTRODUCTION

Zonisamide (AD 810, CI 912) (1,2-benzisoxazole-3-methanesulfonamide, Fig. 1) is a novel anticonvulsant compound that has recently been tested in controlled clinical trials^{11,13}. Clinical studies demonstrate significant therapeutic effects against partial seizures. The clinical and animal pharmacological profile of zonisamide is very similar to that of phenytoin or carbamazepine¹¹.

Phenytoin, carbamazepine, and valproate are effective therapy against generalized tonic-clonic seizures and block sustained repetitive firing of sodium action potentials in vitro at doses that do not affect postsynaptic inhibitory or excitatory neuro-

transmitter responses^{3,5-7}. The blockade of repetitive firing appears to be a use-dependent block of Na^+ channels, shifting these channels into a more inactivated state^{3,4,14,15}. Phenytoin and carbamazepine displace the specific binding of batrachotoxinin to sodium channels of mammalian neurons¹⁶ and may have their effects on sodium action potentials by acting at this molecular site. Such actions may be important correlates of the anticonvulsant activity of these compounds^{1,3}. The current electrophysiological experiments were performed to see if zonisamide affects sodium-dependent action potentials of mammalian neurons at relevant concentrations. Preliminary results have been presented previously⁹.

Clinical therapeutic whole plasma levels of zonisamide are 15–20 $\mu\text{g/ml}$ and nearly all patients show marked neurological side effects at whole

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plasma levels above 40 $\mu\text{g/ml}$ (H. Bockbrader, personal communication). Since at these concentrations zonisamide is 50% bound to plasma proteins¹¹, we estimate that the free fraction of plasma zonisamide or the cerebrospinal fluid concentration of zonisamide is 7.5–12.5 $\mu\text{g/ml}$ (estimated therapeutic range) and that levels at and above 20 $\mu\text{g/ml}$ (unbound to plasma proteins) are thought to cause neurological side effects.

MATERIALS AND METHODS

Cell culture

Cultures of spinal cord neurons were prepared by standard methods⁸. Briefly, spinal cords and dorsal root ganglia were dissected from fetal (12–14 days gestation) mouse embryos. The tissue was minced, mechanically dissociated and plated in sterile collagen-coated 35 mm dishes. Growth media consisted of: 80% Eagle's minimal essential media supplemented with 5.5 g glucose and 1.5 g NaHCO_3/l , 10% heat-inactivated horse serum and 10% fetal calf serum adjusted to 325 mosM. The cultures were maintained in an incubator with an atmosphere of 90% room air and 10% CO_2 at 35 °C. The $\text{NaCO}_3/\text{CO}_2$ buffer maintained pH at 7.4. Growth of rapidly dividing non-neuronal cells was suppressed by treatment with uridine and 5-fluoro-2-deoxyuridine after 6–8 days in vitro. Medium was changed twice weekly thereafter and did not contain fetal calf serum. Cultures were maintained at least 4 weeks before electrophysiological experiments.

Experimental procedures

Cultures were placed on the heated stage of an inverted phase contrast microscope where intracellular impalements were obtained with high resistance glass micropipettes (20–60 $\text{M}\Omega$) filled with either 4 M KAc or 3 M KCl. Membrane potential was recorded with a high-impedance bridge amplifier (Dagan 8100) and recorded on analog magnetic tape. The experimental medium was Dulbecco's phosphate-buffered saline (DPBS; NaCl 137 mM, KCl 3 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM, MgCl_2 1 mM (spontaneous activity experiments) or 10 mM MgCl_2 (repetitive firing and iontophoretic experiments), CaCl_2 1

mM, glucose 10 mM, pH 7.3–7.4). Zonisamide was dissolved into dimethyl sulfoxide (DMSO) with a final concentration of DMSO not greater than 0.1% in drug solutions applied to neurons. The experiments were run at temperatures between 35 and 37 °C.

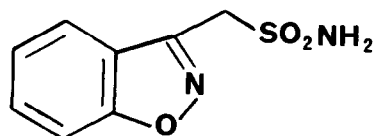
Tests were performed to determine the effect of zonisamide on (a) sustained repetitive firing of sodium action potentials, (b) spontaneous neuronal activity, and (c) iontophoretic GABA and glutamate responses.

Repetitive firing

Only cultures in which all cells exhibited sustained repetitive firing (3–5 neurons examined per culture) in control medium were used for experiments. To elicit repetitive firing, 450 msec depolarizing current pulses of varying amplitudes were used. If a neuron fired action potentials throughout any of the 450 msec pulses, firing was judged as sustained. In order to be included, resting membrane potential of a neuron had to be greater than -40 mV with overshooting action potentials. After addition of zonisamide to the bath, additional neurons were tested at membrane potentials of approximately -50 mV for the ability to fire sustained action potentials. Data are expressed as the percentage of cells showing sustained firing. The IC_{50} value for the blockade of repetitive firing was determined by probit analysis².

Spontaneous activity

Impaled cells were rated for the amount of (spontaneous) synaptically evoked action potentials (Fig. 4A). Neurons were judged on the following scale: grade 2 — normal activity — presence of excitatory and inhibitory postsynaptic po-



zonisamide
(CI-912, AD-810)

Fig. 1. Chemical structure of zonisamide. MW = 212.23.

tentials (EPSPs and IPSPs) measured indirectly by the frequency of action potentials; grade 1 — reduced activity — greatly decreased frequency of action potentials indicating fewer EPSPs; grade 0 — quiescence — no spontaneous action potentials or synaptic potentials. Resting membrane potential of all cells was greater than -40 mV. Only cultures exhibiting grade 2 spontaneous activity in control medium (3–5 cells examined per culture) were used. Significant reductions in spontaneous activity were determined by comparison of the scores from drug-treated neurons with those before drug addition using the Wilcoxon rank-sum test.

Iontophoretic experiments

GABA (0.5 M, pH 3.2, positive current) or glutamate (0.4 M, pH 10, negative current) was ejected by passing 400 msec current pulses every 5 sec from a high resistance micropipette placed approximately $2\ \mu\text{m}$ from the impaled cell. Retaining current (negative for GABA and positive for glutamate) was applied to prevent leakage between

pulses. Membrane potential was hyperpolarized from -60 to -80 mV by passing current through the recording micropipette. Since recording micropipettes in GABA experiments were KCl-filled, E_{Cl^-} was shifted to approximately -30 mV due to leakage of Cl^- into the cells. Thus, GABA application resulted in depolarizing responses. Recording micropipettes in glutamate experiments were filled with potassium acetate. Iontophoretic current was adjusted so that responses were approximately 10 mV in amplitude. Zonisamide was applied by pressure ejection (0.25–1.0 pounds/in.², 30 sec) from blunt tipped ($\approx 10\ \mu\text{m}$) micropipettes placed 30–100 μm from the cell. Data were expressed as percentage of control amplitude and were discarded if the response failed to return to control levels after drug application.

RESULTS

Effect of zonisamide on sustained repetitive firing

In control medium, neurons responded to 450

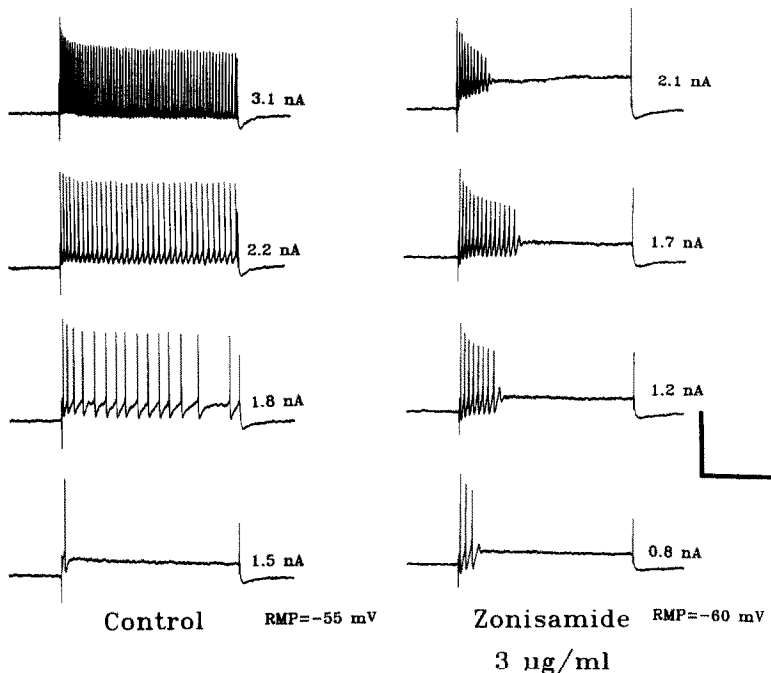


Fig. 2. Effect of zonisamide ($3\ \mu\text{g}/\text{ml}$) on sustained repetitive firing. In control media (left panel) increasing depolarizing current steps (nA current intensities at the right of each trace) increased number and frequency of action potentials. A different cell bathed in $3\ \mu\text{g}/\text{ml}$ zonisamide (right panel) no longer fired action potentials throughout the depolarizing step. Resting membrane potential (RMP) is indicated at the bottom of each panel. Calibration: vertical bar 50 mV, horizontal bar 200 msec.

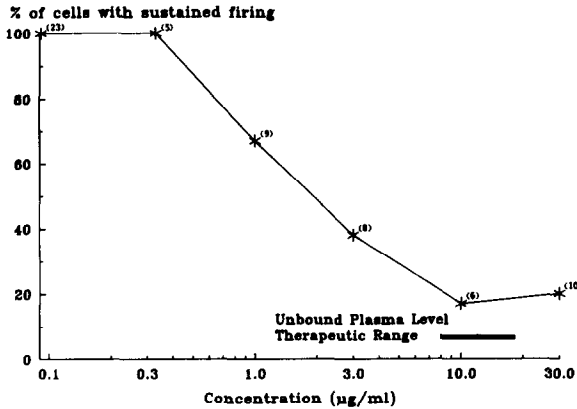


Fig. 3. Concentration dependence of zonisamide on sustained repetitive firing (SRF). The percentage of cell exhibiting sustained repetitive firing is plotted as a function of concentration of zonisamide. The estimated therapeutic range (7.5–12.5 µg/ml) is indicated by the black bar. Numbers in parentheses represent number of cells tested at each concentration.

msec depolarizing current steps with trains of action potentials; increasing the magnitude of depolarization resulted in increased number and frequency of action potentials (Fig. 2, left panel). Application of 3 µg/ml zonisamide (Fig. 2, right panel) blocked the ability of cells to respond with sustained firing throughout the depolarizing current pulse. Zonisamide blocked sustained repeti-

tive firing in a concentration-dependent manner (Fig. 3) and the concentration that blocked sustained repetitive firing in 50% of the cells (IC_{50}) was 3.7 µg/ml (1.8–7.5, 95% confidence interval).

Effect of zonisamide on spontaneous activity

Spinal cord cells in culture make functional synaptic connections, and therefore intracellular recordings from neurons in control medium exhibit spontaneous excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs) and action potentials. Cells were graded according to the frequency of occurrence of action potentials (an indirect measure of the frequency of synaptic potentials, see Fig. 4A). Zonisamide reduced spontaneous synaptic activity in a concentration-dependent manner with significant reductions from control at 10 and 30 µg/ml (Fig. 4B).

Effect of zonisamide on iontophoretic GABA and glutamate responses

High concentrations of zonisamide (30 µg/ml) had no significant effect on GABA responses (drug-treated GABA responses were a mean of $98\% \pm 3.5\%$ S.E.M. of control, $N = 6$) or glutamate responses ($103\% \pm 3.2\%$ of control, $N = 5$)

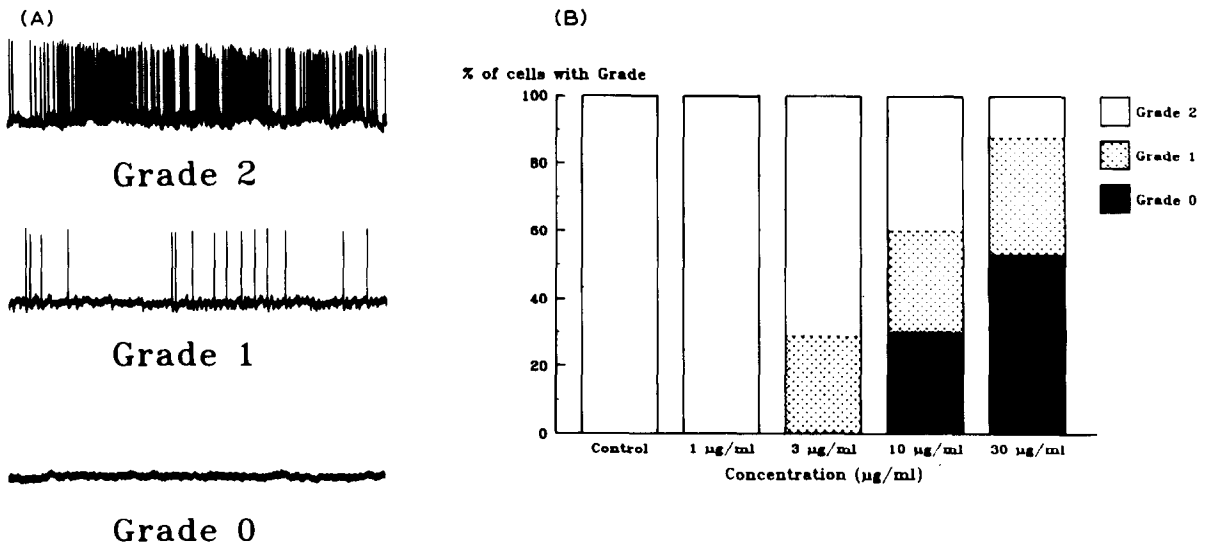


Fig. 4. Effect of zonisamide on spontaneous neuronal activity. A: rating scale for the measurement of spontaneous activity (see Methods). Grade 2, normal spontaneous activity; grade 1, reduced spontaneous activity; grade 0, quiescence, no spontaneous activity. Tracings are intracellular recordings from cells of each grade, resting membrane potentials were all greater than -40 mV. B: concentration-dependent reduction of spontaneous activity by zonisamide. Bars summarize data from 8 or more cells and are divided to represent the percentage of cells with each grade at a given concentration of zonisamide. Spontaneous activity was significantly reduced at both 10 and 30 µg/ml.

Zonisamide (CI-912)

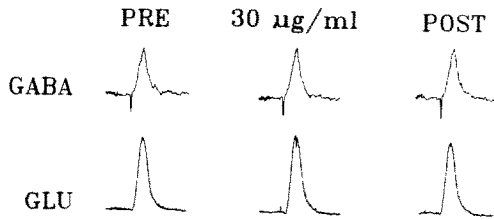


Fig. 5. Zonisamide (30 $\mu\text{g/ml}$) had no effect on either GABA or glutamate (GLU) responses. Tracings were from cells that were hyperpolarized to around -80 mV by injection of current.

Calibration: vertical bar 5 mV, horizontal bar 1 sec.

(Fig. 5). No change in membrane voltage or conductance was observed when zonisamide was applied to neurons from micropipettes at this concentration.

DISCUSSION

These experiments demonstrate that zonisamide selectively blocks sustained repetitive action potentials in mammalian neurons at concentrations that are expected to be present in the extracellular fluid of the brain during anticonvulsant treatment. Blockade of repetitive firing is dose related, with an IC_{50} of approximately 2 $\mu\text{g/ml}$. It is likely that zonisamide blocks repetitive firing by changing sodium current inactivation kinetics (similar to phenytoin and carbamazepine) since only the later action potentials of a train are blocked (Fig. 3).

These findings are compatible with the observations of Schauf¹⁰ who found that zonisamide also selectively alters the inactivation of voltage-sensitive sodium currents in voltage-clamped annelid worm axons *in vitro*. Steady-state fast inactivation in these axons occurs at more hyperpolarized membrane potentials in the presence of zonisamide. Such changes are similar to those reported in cultured spinal cord neurons³ or in voltage-clamped neuroblastoma cells^{4,14,15} for phenytoin

and carbamazepine. In Schauf's study, 2.6 $\mu\text{g/ml}$ zonisamide shifted inactivation by approximately 20 mV. This change in the voltage dependence of inactivation occurs without changes in sodium current activation. If excitability of neurons was decreased by zonisamide through an alteration of sodium current activation, one would expect a large increase in the voltage threshold for action potentials. Such a change was not observed in our study since low concentrations of zonisamide did not alter spontaneous neuronal activity (Fig. 4).

Zonisamide reduces spontaneous synaptically evoked neuronal firing in cultured neurons at concentrations of 10 $\mu\text{g/ml}$ and greater. This quiescence of normal neuronal excitability predicts that high doses of zonisamide would cause coma similar to that seen with high doses of barbiturates or phenytoin. A previous study with the anticonvulsant phenytoin⁵ correlated blockade of spontaneous activity with the reduction of calcium-dependent action potentials. Other studies with phenytoin have shown a reversible block of voltage-dependent calcium currents at concentrations that are slightly higher than those necessary to alter sodium currents¹². Further studies will be required to determine whether zonisamide reduces spontaneous synaptically evoked activity by blockade of voltage-dependent calcium currents.

At the concentrations of zonisamide tested (30 $\mu\text{g/ml}$ and lower, corresponding to free plasma concentrations of zonisamide higher than those producing significant neurological side effects), there were no changes in postsynaptic glutamate or GABA responses. Thus, the anticonvulsant and neurological side effects of zonisamide appear to be unrelated to modulation of GABA or glutamate receptors. The anticonvulsant action of zonisamide can be accounted for by a selective action on voltage-dependent sodium channels of neurons, as has been proposed for other anticonvulsants.

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