Epilepsia, **44**(Suppl. 10):34–39, 2003
Blackwell Publishing, Inc.

An In Vitro Study of New Antiepileptic Drugs and Astrocytes

*Antonino Pavone and †Venera Cardile

*Department of Neurology, Garibaldi Hospital, and †Department of Physiological Sciences, University of Catania, Catania, Italy

Summary: *Purpose:* The aim of our research was to study some biochemical modifications elicited in primary rat astrocyte cultures by treatment with gabapentin (GBP), carbamazepine (CBZ), lamotrigine (LTG), topiramate (TPM), oxcarbazepine (OXC), tiagabine (TGB), and levetiracetam (LEV), commonly used in the treatment of epilepsy. We investigated the biologic effects of these anticonvulsants (AEDs) at concentrations of 1, 10, 50, and 100 μg/ml.

Methods: The study was performed by examining cell viability (MTT assay), cell toxicity [lactate dehydrogenase (LDH) release in the medium], glutamine synthetase (GS) activity, reactive oxygen species (ROS) production, lipoperoxidation level (malondialdehyde; MDA), and DNA fragmentation (COMET assay). The level of the expression of 70-kDa heat-shock protein (HSP70) and inducible nitric oxide synthase (iNOS) as oxidative stress–modulated genes also was determined.

Results: Our experiments indicate that CBZ, TPM, and OXC induce stress on astrocytes at all concentrations. GBP, LTG, TGB, and LEV, at low concentrations, do not significantly change the metabolic activities examined and do not demonstrate toxic actions on astrocytes. They do so at higher concentrations.

Conclusions: Most AEDs have effects on glial cells and, when used at an appropriate cell-specific concentrations, may be well tolerated by cortical astrocytes. However, at higher concentrations, GBP, LTG, TGB, and LEV seem to be better tolerated than are CBZ, TPM, and OXC. These findings may reveal novel ways of producing large numbers of new AEDs capable of reducing the extent of inflammation, neuronal damage, and death under pathological conditions such as epilepsy and/or traumatic brain injury. **Key Words:** AEDs—Epilepsy—Glial cells—Rat.

Glial cells comprising astrocytes and microglia constitute >90% of the total cell population in the adult brain, and support neurons by providing trophic factors and the regulation of inflammatory/immunologic processes (1–3). For a long time, these nonneuronal cells were largely ignored or believed to play roles in the nervous system that were subservient to those of neurons. During the initial period of documentation of the structural features of the nervous system, significant focus turned to neurons as a result of the recognition that their electrical excitability permitted them to convey relevant information in the nervous system. Only during the past 10–15 years, with the advent of the available techniques, have the potentially diverse dynamic roles of glial cells begun to be appreciated. Recently Araque et al. (4) focused attention on one of the most identified roles of glial cells, which are strategically positioned, as integrators and modulators of neuronal activity and synaptic transmission. This capability, now demonstrated in several studies, raises the untested possibility that astrocytes are an integral element of the circuitry for synaptic plasticity.

Address correspondence and reprint requests to Dr. V. Cardile at Department of Physiological Sciences, University of Catania, V.le A. Doria 6, 95125 Catania, Italy. E-mail: cardile@mbox.unict.it

The term *epilepsies* is a collective designation for a group of chronic central nervous system (CNS) disorders having in common the spontaneous occurrence of brief episodes (seizures) associated with loss or disturbance of consciousness, usually but not always with characteristic body movements (convulsions) and sometimes autonomic hyperactivity, and always correlated with abnormal and excessive EEG discharges (5).

The prime objective of therapy for epilepsy is complete suppression of all seizures without impairment of CNS functions. It is commonly accepted that, with patience and persistence, 70–80% of all epilepsy patients are significantly benefited by currently available pharmacotherapy (6). Unfortunately, this estimate of efficacy seems to be based largely on clinical impression (7). Recently Sisodiya et al. (8) reported that epilepsy is resistant to drug treatment in about one third of cases, but the mechanisms underlying this drug resistance are not understood.

This uncertainly may be a reflection of the complicated etiology of epilepsy or the pleiotropic nature of the antiepileptic drugs (AEDs). We think that the new theory of neuron–astrocyte interaction might be useful in provoking a reformulation of concept of the disorders affecting the CNS, including epilepsy, and that the study of the possible effects of AEDs on astrocytes, much less

investigated and largely unknown, might be a helpful tool in the therapeutic use of these drugs.

To better understand the involved cellular mechanisms, we therefore planned a systematic investigation on rat cortical astrocytes, used as an in vitro model, exposed to some new AEDs at different (but within the recommended therapeutic range) concentrations (9). Under these experimental conditions, several significant biochemical parameters were examined, analyzing the induced biologic effects as well as the possible neurotoxic actions to evaluate the resistance of astrocytes to addition of AEDs at increasing concentrations and to see whether these cells are capable of protecting themselves against the treatment with different concentrations of AEDs.

The tested AEDs were the most commonly used in the treatment of epilepsy: gabapentin (GBP), carbamazepine (CBZ), lamotrigine (LTG), topiramate (TPM), oxcarbazepine (OXC), tiagabine (TGB), and levetiracetam (LEV).

We chose to perform tetrazolium salt assay and to measure lactic dehydrogenase (LDH) release as markers of cell viability (10,11). Tetrazolium salts are metabolized by various mitochondrial dehydrogenase enzymes and reduced to a blue formazan by living cells only, whereas the presence of LDH release in the culture medium is a marker of membrane breakdown.

Maturation and differentiation of astrocytes were tested by measuring glutamine synthetase activity because it is well documented that some AEDs change the expression and the activity of this enzyme (9).

To verify the possible induction of stress condition in the cells after AED treatment, we performed assays to show reactive oxygen species production and lipoperoxidation by fluorescent quantitative analysis with fluorescent probe 5 and 6-carboxy-2′7′-dichlorodihydrofluorescine diacetate-bis-acetomethyl ester (DCFH-DA) and by thiobarbituric acid reactive substances (TBARS) analysis. In addition, we evaluated the amount of nitrite/nitrate in the culture medium and by immunoblotting the changes in the cytosolic proteins of the level of inducible nitric oxide synthetase (iNOS) and, on only TGB-treated astrocytes, 70-kDa heat-shock proteins (HSPs70), a family of evolutionary conserved constitutive and/or inducible proteins acting as molecular chaperons in the cells and having a well-known protective and stabilizing effect on stress-induced cell injury (12). In addition, to study possible AED-induced DNA damage, we chose to perform single-cell gel electrophoresis or Comet assay, which, within the last decade, has been used with increasing popularity to investigate the level of DNA damage in terms of strand breaks and alkaline labile sites (13,14).

Although the experiments are in progress and not complete for all these AEDs, the first findings were consistent enough to convince us that they should be reported. These findings may open different and interesting ways both for the investigation of the activity of new AEDs and for the possibility of developing new pharmacologic approaches.

MATERIALS AND METHODS

Drugs

GBP, CBZ, LTG, TPM, OXC, TGB, and LEV were dissolved in culture medium (DMEM/F12) and added to the medium of 12-day-old cultured astrocytes to obtain final concentrations of 1, 10, 50, and 100 μg/ml, respectively. After 48 h, the cells were harvested and submitted to different assays.

Astrocyte primary cultures

Astrocyte primary cultures were prepared from 1- to 2-day-old Wistar rats, decapitated, and the cells obtained as described by Cardile et al. (15). In brief, the removed cerebral cortex was mechanically dissociated, centrifuged, and washed. The cells were seeded in DMEM/F12 medium containing 10% fetal calf serum, 1 m*M* L-glutamine, and antibiotics, and incubated at 37°C in a humidified 5% CO_2/95% atmosphere. Typically, astrocytes reached confluence ~7–10 days after seeding; at 12 days, the experiments were performed. To assess the astroglial nature of the cells, the test for glial fibrillary acidic protein (Kit SIH908; Sigma Diagnostic, U.S.A.) was performed before the beginning of the treatments (15).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) proliferation assay is based on conversion by mitochondrial dehydrogenases of the substrate containing a tetrazolium ring into blue formazan, detectable spectrophotometrically (10). The level of blue formazan is then used as indirect index of cell density. In brief, the astrocytes were set up in flat-bottomed 200-μl microplates, incubated at 37°C in a humidified 5% CO_2/95% air mixture, and treated with 1, 10, 50, and 100 μg/ml of AEDs for 48 h. Four hours before the end of the culture, 20 μl of 0.5% 3-(4,5-dimethylthiazol-2-yl)diphenyltetrazolium bromide in phosphate-buffered saline (PBS) was added to each microwell. After the incubation with the reagent, the supernatant was removed and replaced with 100 μl of acidified isopropanol and 20 μl of 3% (wt/vol) sodium dodecylsulfate (SDS) in water. The optical density of each sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories) at 570 nm, and four replicates were performed for each sample.

Lactic dehydrogenase release

The enzymatic activity of LDH was measured spectrophotometrically in culture medium and in the cellular lysates at λ_{max} 340 nm, analyzing the NADH reduction during the pyruvate-lactate transformation (11). The untreated control and AED-treated cells were harvested, washed in PBS, added to lysis buffer (Tris–HCl, 50 m*M,* + 20 m*M* EDTA, pH 7.4 + 0.5% SDS), further submitted to sonication, and then centrifuged at 10,000 *g* for 15 min.

The supernatant was harvested and analyzed for its protein content, according to Bradford (16) and for enzymatic activity measurement. The incubation mixture (1 ml final volume) for enzymatic analysis was composed of: 33 μl of sample in 48 m*M* PBS, pH 7.5, plus 1 m*M* pyruvate, and 0.2 m*M* reduced pyridinic coenzyme. The release of LDH was calculated as a percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that of the culture medium.

Determination of glutamine synthetase activity

At 48 h after the addition of different AED concentrations, the astrocytes (12 days in culture) were harvested, homogenated, and the supernatant was spectrophotometrically assayed for glutamine synthetase activity, as described in Renis et al. (11); the enzyme activity was expressed as μM of L-α-glutamyl hydroxamate 30 min/mg protein.

Reactive oxygen species analysis

Reactive oxygen species (ROS) formation was estimated by using DCFH-DA as a fluorescent probe (Molecular Probes, Eugene, OR, U.S.A.). DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to monofluorescent DCFH in the presence of ROS (17). The intensity of fluorescence is parallel to the levels of intracellular ROS. Monolayer astrocytes (control or differently treated with AEDs) were added with 5 μM DCFH-DA for 30 min before the end of the treatment time. They were maintained in a humidified atmosphere (5% CO_2 and 95% air) at 37°C. Dye-loaded cells were washed with PBS, and the fluorescence was monitored in scraped and resuspended cells by using a luminescence spectrometer (Perkin-Elmer). The excitation was 475 nm, and the emission, 525 nm.

Lipid peroxidation

Malonyldialdehyde (MDA) was measured to evaluate the extent of lipid peroxidation in the AED-treated astrocytes by monitoring the formation of TBARS (18). In brief, 2 ml of each sample treated with trichloracetic acid (15% wt/vol) containing 1 m*M* EDTA was centrifuged at 1,000 *g* for 10 min. The supernatant was heated at 100°C with an equal volume of thiobarbituric acid (0.7% wt/vol) for 20 min, and after cooling, the absorbance at 532 nm was monitored. The values were expressed as nmol of malonylaldehyde formed per milligram of protein, calculated as a percentage of the control.

Determination of NO_3^- release

Nitrite concentration in the supernatant was quantified by colorimetric assay based on the Griess reaction (19), as described by Ding et al. (20). In brief, 0.1 ml of supernatant from untreated or treated culture was mixed with an equal volume of Griess reagent at room temperature for 10 min. The absorbance was measured at 570 nm in a Titertek Multiskan microplate reader. Sodium nitrite was used as standard.

Western blotting

For immunostaining of the Western blotting, both for iNOS and HSP70, the treated and harvested cells were washed twice with ice-cold PBS, collected with lysing buffer (50 m*M* Tris-HCl, pH 7.5, plus 20 m*M* EDTA and 0.5% SDS), and, after 30 min on ice, homogenized and centrifuged at 13,000 *g* for 15 min. Twenty micrograms of total protein, present in the supernatant, was fractionated by electrophoresis on 8% SDS-polyacrylamide gel, according to Laemmli (21), by using Bio-Rad miniprotein apparatus. After electrophoresis, at constant current of 30 mA, samples were semidry transferred to nylon membranes. The blots were blocked for 1–2 h in PBS, and then incubated overnight with a monoclonal antibody (1:3,000) anti-HSP70 (Santa Cruz, U.S.A.) that recognized the constitutive and the inducible forms of the HSP70 family and with a monoclonal antibody (1:500) for iNOS (Santa Cruz, U.S.A.). Blots were visualized according to the manufacturer's instructions (ELC Plus; Amersham, Denmark).

The levels of HSP70 and iNOS were quantified by densitometric analysis of the autoradiographies, and the values were expressed as densitometric arbitrary units corresponding to signal intensity.

COMET assay

The Comet assay was applied according to Singh et al. (22). In brief, 10 μl of untreated control and differently AED-treated astrocytes (0.8–1 $\times$ 10^5 cells) were mixed with 75 μl of 0.5% low-melting-point agarose (LMA) and spotted on microscope slides previously rinsed with a 1% solution of standard-melting-point agarose (NMA). A third layer of 85 μl LMA was then added. The "minigels" were immersed for 1 h at 4°C in ice-cold lysis solution (*N*-laurosil-sarcosine, 1%; NaCl, 2.5 *M*; Na_2EDTA, 10 m*M*; Triton $\times$100, 1%; DMSO, 10%; pH 10), denatured in a high-pH buffer (NaOH, 300 m*M*; Na_2EDTA, 1 m*M*) for 20 min, and run in the same buffer for 25 min at 25 V. At the end of the electrophoresis, the slides were washed in a neutralization buffer (0.4 *M* Tris-HCl, pH 7.5), stained with 100 μl of ethidium bromide (2 $\mu\mu$g/ml), covered with an 18 $\times$ 18-mm cover slip, and scored by using a Leitz fluorescence microscope interfaced with a computer. Software (Cayman Sarin, Florence, Italy) allowed us to analyze and quantify DNA damage by measuring (a) tail length (TL), tail intensity (TI), and tail area (TA); and (b) head length (HT), head intensity (HI), and head area (HA). These parameters are used by the software to determine the level of DNA damage as (a) percentage of fragmented DNA (TDNA), and (b) tail moment (TMOM), expressed as product of the tail/head distance (TD) and TDNA.

TABLE 1. *Effects of AEDs on primary rat astrocytes in vitro*

	MTT (Optic density; n = 4)	GS (μM/mg prot; n = 4)	LDH (% release; n = 4)	NO (nM/10^6 cells; n = 4)	ROS DCF (Fluorescence intensity; n = 4)	MDA TBARS (Formation % of control; n = 4)
Control	1.385 ± 0.02	27 ± 1.3	10 ± 0.9	3 ± 0.2	29 ± 2	100
Gabapentin						
1 μg/ml	1.380 ± 0.05	26 ± 0.4	11 ± 0.8	15 ± 1.3[a]	30 ± 4	106 ± 3
10 μg/ml	1.376 ± 0.09	26 ± 0.2	13 ± 0.9	89 ± 2.1[a]	35 ± 6	109 ± 8[a]
50 μg/ml	1.280 ± 0.05[a]	19 ± 0.5[a]	33 ± 3.3[a]	90 ± 2.6[a]	80 ± 3[a]	184 ± 6[a]
100 μg/ml	0.990 ± 0.08[a]	17 ± 0.3[a]	53 ± 2.5[a]	92 ± 3.4[a]	94 ± 2[a]	196 ± 13[a]
Topiramate						
1 μg/ml	1.310 ± 0.05[a]	25 ± 0.3[a]	12 ± 0.7[a]	30 ± 2.3[a]	50 ± 4[a]	121 ± 5[a]
10 μg/ml	1.250 ± 0.02[a]	20 ± 0.4[a]	30 ± 0.5[a]	80 ± 3.6[a]	81 ± 8[a]	173 ± 9[a]
50 μg/ml	1.117 ± 0.08[a]	18 ± 0.5[a]	41 ± 3.3[a]	96 ± 1.4[a]	128 ± 6[a]	192 ± 7[a]
100 μg/ml	0.780 ± 0.09[a]	16 ± 0.2[a]	64 ± 4.8[a]	106 ± 3.8[a]	139 ± 2[a]	213 ± 12
Carbamazepine						
1 μg/ml	1.172 ± 0.03[a]	21 ± 0.6[a]	15 ± 1.2[a]	38 ± 2.6[a]	49 ± 5[a]	134 ± 11[a]
10 μg/ml	1.028 ± 0.06[a]	18 ± 0.6[a]	22 ± 3.1[a]	108 ± 4.2[a]	91 ± 7[a]	168 ± 8[a]
50 μg/ml	0.968 ± 0.05[a]	17 ± 0.9[a]	49 ± 6.1[a]	112 ± 3.8[a]	147 ± 8[a]	193 ± 5[a]
100 μg/ml	0.680 ± 0.08[a]	14 ± 0.7[a]	70 ± 8.1[a]	126 ± 2.3[a]	167 ± 11[a]	208 ± 9[a]
Lamotrigine						
1 μg/ml	1.378 ± 0.09	27 ± 0.5	11 ± 0.3	12 ± 1.6[a]	33 ± 4	113 ± 5
10 μg/ml	1.381 ± 0.07	26 ± 0.9	13 ± 3.4	62 ± 2.5[a]	39 ± 2[a]	114 ± 9[a]
50 μg/ml	1.310 ± 0.05[a]	20 ± 0.3[a]	28 ± 4.6[a]	78 ± 3.6[a]	39 ± 1[a]	124 ± 7[a]
100 μg/ml	1.118 ± 0.03[a]	19 ± 0.5[a]	44 ± 3.7[a]	108 ± 3.1[a]	51 ± 4[a]	153 ± 5[a]
Oxcarbazepine						
1 μg/ml	1.287 ± 0.08[a]	22 ± 0.6[a]	17 ± 1.9[a]	35 ± 1.7[a]	38 ± 4[a]	136 ± 5[a]
10 μg/ml	1.180 ± 0.06[a]	21 ± 0.2[a]	29 ± 0.9[a]	135 ± 3.9[a]	119 ± 13[a]	143 ± 8[a]
50 μg/ml	0.933 ± 0.09[a]	17 ± 0.9[a]	47 ± 2.9[a]	135 ± 4.8[a]	164 ± 9[a]	187 ± 11[a]
100 μg/ml	0.652 ± 0.02[a]	14 ± 0.4[a]	73 ± 6.8[a]	144 ± 2.1[a]	199 ± 12[a]	203 ± 14[a]
Tiagabine						
1 μg/ml	1.382 ± 0.04	24 ± 0.9	10 ± 0.3	4 ± 0.3	31 ± 4	118 ± 10
10 μg/ml	1.376 ± 0.07	23 ± 0.7	12 ± 2.8	3 ± 0.9	43 ± 5[a]	129 ± 11[a]
50 μg/ml	1.328 ± 0.09[a]	22 ± 0.3[a]	19 ± 1.2[a]	18 ± 1.1[a]	53 ± 8[a]	131 ± 5[a]
100 μg/ml	1.118 ± 0.05[a]	20 ± 0.6[a]	21 ± 3.5[a]	28 ± 3.4[a]	61 ± 10[a]	143 ± 15[a]
Levetiracetam						
1 μg/ml	1.379 ± 0.09	27 ± 0.6	10 ± 0.3	3 ± 0.1	33 ± 4	103 ± 3
10 μg/ml	1.352 ± 0.06	27 ± 0.5	13 ± 3.1	5 ± 0.6[a]	39 ± 1[a]	109 ± 6
50 μg/ml	1.335 ± 0.08[a]	24 ± 0.9[a]	15 ± 2.3[a]	11 ± 2.0[a]	49 ± 5[a]	113 ± 9[a]
100 μg/ml	1.291 ± 0.05[a]	22 ± 0.2[a]	17 ± 0.9[a]	19 ± 4.2[a]	54 ± 8[a]	138 ± 15[a]

The values are representative of results ± SEM.

DCF, dichlorodihydrofluoresceine; GS, glutamine synthetase; LDH, lactic dehydrogenase; MDA, malonyldialdehyde; MTT, tetrazolium salt test; NO, nitric oxide; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

[a]Significant vs. controls: $p < 0.01$.

Statistical analysis

Each experiment was repeated ≥3 times in quadruplicate, and the mean ± SEM for each value was calculated. All the statistical analyses were performed by using the statistical software package SYSTAT. A difference was considered significant at $p < 0.01$.

RESULTS

In the present study, only data were considered from cultures in which immunostaining for GFAP showed that 90–95% of cells were astrocytes.

Cell viability, evaluated by MTT assay and LDH release, is reported in Table 1. We observed that GBP, CBZ, LTG, TPM, and OXC, but not TGB and LEV, were capable of interfering with cell viability at all concentrations of drug tested. Specifically, the MTT assay showed that GBP and LTG did not interfere with cell viability at lower concentrations (1 and 10 μg/ml), but at 50 and 100 μg/ml, reduced the astrocytes' ability to metabolize tetrazolium salts. Concerning CBZ, TPM, and OXC, we observed reduction in capability to metabolize tetrazolium salts also at 1 and 10 μg/ml. No reduction was present in astrocytes treated with 50 μg/ml TGB or LEV.

Similar differences were observed for LDH release. In GBP- or LTG-treated astrocytes, LDH release did not differ from control after 48-h exposure at 1 and 10 μg/ml, but it was increased at 50 and 100 μg/ml. With CBZ, TPM, and OXC, LDH release was seen to increase significantly in treated astrocytes at 10 μg/ml, whereas TGB or LEV interfered with LDH release only at 100 μg/ml.

To verify the possible AED-related induction of oxidative stress leading to tissue injury, we measured glutamine synthetase activity, MDH levels, and ROS production. The results demonstrated no significant difference

TABLE 2. *Comet assay of genomic DNA of rat astrocytes untreated or treated for 48 h with different concentrations of tiagabine*

	TD	TL	TDNA	TMOM
Control	1.5 ± 0.3	4.2 ± 1.1	20 ± 3	30 ± 8
Tiagabine 1 μg/ml	1.6 ± 0.8	4.8 ± 0.9	20 ± 2	32 ± 6
Tiagabine 10 μg/ml	1.8 ± 0.5	5 ± 0.5	23 ± 2	41 ± 10
Tiagabine 20 μg/ml	3 ± 1[a]	10 ± 2[a]	41 ± 6[a]	123 ± 15[a]
Tiagabine 50 μg/ml	11 ± 2[a]	32 ± 5[a]	81 ± 5[a]	891 ± 18[a]
Tiagabine 100 μg/ml	15 ± 0.7[a]	46 ± 3[a]	97 ± 4[a]	1,455 ± 32[a]

Values are expressed as the mean ± SEM of three experiments performed in duplicate.

TD, head/tail distance; TDNA, percentage of fragmented DNA; TL, tail length; TMOM, tail moment (product of TD and TDNA).

[a]Significant vs. control untreated astrocytes; $p < 0.01$.

in glutamine synthetase activity, MDH levels, and ROS production between untreated controls and low concentrations of GBP-, LTG-, TGB-, or LEV-treated astrocytes (1 and 10 μg/ml), whereas 50 and 100 μg/ml and 1 and 10 μg/ml CBZ, TPM, and OXC increased these parameters (Table 1). In this scenario, we investigated iNOS levels in AED- treated astrocytes. The results indicated that TGB or LEV did not induce NO synthesis. All other AEDs tested produced a dose-related increase in the biosynthesis of NO, compared with the controls. The greatest effect was observed with CBZ (Table 1). We previously investigated TGB (data published) on modulation of HSP70 and evaluated the amount of genomic DNA damage. At dosages of 1 and 10 μg/ml, a significant increase of HSP70 levels (23) and no DNA damage were noted (24). The results of TL, TDNA, and TMOM showed more evident DNA damage after treatment with 50 and 100 μg/ml of TGB (Table 2).

DISCUSSION

We studied the in vitro effects of GBP, CBZ, LTG, TPM, OXC, TGB, and LEV on cell viability (MTT assay and LDH release), maturation and differentiation (GS activity), presence of stress conditions (ROS formation, iNOS expression, and HSP70 production) in primary cultures of rat astrocytes, which are intimately involved in the normal functioning of neurons. We investigated the effects of these AEDs at concentrations of 1, 10, 50, and 100 μg/ml.

Our results suggest that the tested AEDs induce functional modifications and affect some biochemical events in cortical rat astrocytes. The biologic effects of these AEDs are dose related, seem to be differently exerted, and are not uniform for all tested drugs. Our experiments indicate that GBP, LTG, TGB, and LEV, at low doses (1 and 10 μg/ml) do not change the examined metabolic activities and do not exhibit toxic actions on astrocytes. However, significant changes in NO production and enzymatic activity occurred after the addition of GBP and LTG at 50 and 100 μg/ml, or of CBZ, TPM, and OXC at lower concentrations (1 and 10 μg/ml).

We performed the MTT assay and LDH-release analyses to study the cytotoxic effect of AEDs on both cell viability and cell-membrane breakdown, such systems being useful for detecting the cell death process. CBZ, TPM, and OXC exerted their activity at lower concentrations than did GBP, LTG, TGB, and LEV.

Our results, obtained by studying ROS production and MDH levels, confirm that AEDs are capable of initiating an oxidative process in astrocytes. These drugs increase ROS generation, which in turn induces lipoperoxidation. Among the radical species present in the treated cells, NO seems to be well represented. Our results suggest that when the concentrations of AEDs are increased, concomitant with an increase in ROS production, a decrease in cell metabolism and an increase in LDH release are observed. It could be inferred that only high concentrations might generate, probably in a NO-mediated way, neurotoxic molecules in rat astrocyte cultures. These molecules, interacting with different cellular targets, including lipids, proteins (particularly heme-containing proteins), thiols, and DNA, could elicit negative effects on astrocytes.

Among the tested AEDs, TGB and LEV do not appear to influence negatively the astrocyte metabolism, because at low concentrations, they do not interfere with the cell viability, do not induce NOS synthesis, increase the HSP70 levels, and induce DNA fragmentation only at very high concentrations.

How these experimental findings are interpreted and translated into clinical practice remains to be determined. It is still unclear whether such reactions are beneficial or detrimental. It was believed that reactive astrogliosis, observed in most neurologic disorders, may regulate the removal of toxic compounds produced by damaged neurons and support neuronal growth by releasing trophic factors. Further investigations are needed to explore the possible roles of astroglia, not only in the maintenance of homeostasis, but also as protective agents against neuronal injury as well as contributors to the epileptogenic process. It is well demonstrated that brain injury elicits a sequence of cellular responses leading to neuronal death, but little is known about how the same processes affect the surrounding astrocytes. As we know more about the properties of the epileptic neurons, we may better understand the mechanisms of action of drugs that prevent neuronal firing. Exposing primary cultures of astrocytes to agents known to affect the neurons and recording their cellular responses in a controlled environment is the first step toward a better understanding of how glia may participate in pathological processes of primary importance for neuronal firing, survival, or death. It is clear that further investigations are warranted before a plausible explanation of the phenomena we have described can be offered. One speculation is that AEDs, which must be gradually titrated

to avoid symptoms of toxicity during the initial phase of treatment, may require metabolic adjustments and modifications in the extracellular environment before they can be administered in the full dose. Other speculations can be advanced to explain the "toxic" response of glia when exposed to effective AEDs. From the work of Aghdasi et al. (25), evidence suggests that the overstress and "accelerated death" of glial cells may produce substances that are "neuroprotective" or protective against seizures. Thus astrocytes may respond to the initial injury with "stress," which shortens their life, but with a protective effect. Finally, Sills et al. (26) described the differential effect of AEDs on γ-aminobutyric acid (GABA) and glutamate uptake in primary cultures of rat astrocytes. They suggested that such effects may contribute to clinical action.

The results of our study suggest that most AEDs have effects on glial cells. More studies on both components of the neuropil, particularly the astroglia, are warranted to unravel the complexity of mechanisms of action of these drugs. Primary cultures of cortical astrocytes provide a suitable environment for such investigations.

CONCLUSION

These results indicate that most AEDs, when used at an appropriate cell-specific concentrations, may be well tolerated by cortical astrocytes. However, at higher concentrations, GBP, LTG, TGB, and LEV seem to be better tolerated than TPM, CBZ, and OXC. We think that these data could be taken into account in the therapeutic use of these drugs.

Acknowledgment: We thank Prof. Vincenzo Perciavalle, Head of Department of Physiological Sciences, University of Catania, and Prof. Marcella Renis, Department of Biol.Chemistry, Medical Chemistry, and Molecular Biology, University of Catania, for their supervision of our work and for all the suggestions given.

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