N-Demethylation of Methyl and Dimethyl Derivatives of Phenytoin and Their Anticonvulsant Activities in Mice

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Abstract—Anticonvulsant activities of 3-methylphenytoin (3-MP) and 1,3-dimethylphenytoin (1,3-DMP) were observed to peak 3 hr after i.p. administration of the drugs dissolved in dimethylsulphoxide (DMSO), while maximal activity was obtained within 15 min with phenytoin. HPLC was employed to monitor the plasma concentrations of all three compounds at various time intervals after injecting 3-MP or 1,3-DMP. In both cases, phenytoin appeared in the plasma, gradually reaching 14–15 μg/ml in 3 hr. The time course of increase in plasma phenytoin levels correlated with that of anticonvulsant activities. It was also found that 1,3-DMP gave rise to a major unidentified metabolite as well as 3-MP and phenytoin. This unidentified metabolite eluted only half a minute in front of 3-MP in the HPLC. Mice injected with high doses of 3-MP (100 mg/kg) in DMSO exhibited severe epileptiform activities. Phenobarbital, diazepam and clonazepam were found to protect against such seizures, but not phenytoin, carbamazepine and valproic acid. This shows that 3-MP is at least a pro-convulsant, taking into account that its effects might have been enhanced by DMSO. Unlike phenytoin, 3-MP lacked the ability to inhibit synaptosomal uptakes of both glutamate and GABA. This difference may be related to the fact that phenytoin, but not 3-MP, possesses potent anticonvulsant activity.

Phenytoin (5,5-diphenylhydantoin) is a widely used drug in the treatment of most forms of epilepsy. In laboratory animals, its anticonvulsant activity is characterized by the effectiveness against maximal electroshock seizure (MES) but not pentylenetetrazole-induced seizure. The ED50 is approx. 8 mg/kg in mice (1, 2). In our hands, the ED50 is 11 mg/kg (P.T.-H. Wong and S.-F. Tan, unpublished). Vida et al. (3) reported that two methyl derivatives of phenytoin, 3-methylphenytoin (3-MP) and 1,3-dimethylphenytoin (1,3-DMP), are effective against MES in mice with ED50 values of about 5 and 50 mg/kg, respectively. Peak activities for 3-MP, 1,3-DMP and phenytoin were obtained at 2–3 hr post-administration, probably because the drugs were administered by the oral route as suspensions in 10% aqueous acacia. It is well known that mephenytoin (3-methyl-5-ethyl-5-phenylhydantoin), a phenytoin analogue with clinical efficacy, is N-demethylated to an active metabolite 5-ethyl-5-phenylhydantoin (4). This means that there is a distinct possibility of phenytoin being formed from its methyl derivatives, particularly when there is a considerable time lapse of 2–3 hr between drug administration and the measurement of anticonvulsant activities. By using a suitable vehicle and route of administration to ensure rapid absorption and penetration into the brain, we reevaluated the actions of these phenytoin analogues.

Materials and Methods

Chemical synthesis: 3-MP was prepared by adding with continuous stirring 2 ml of NaOH (10%) followed by 1 ml of dimethyl sulphate to 10 ml of phenytoin sodium (0.2 g/ml). After standing for 30 min at room temp., the mixture was cooled in an ice bath before being acidified (tested with congo red) with HCl (5
M). The precipitate was filtered and washed thoroughly with distilled water and then recrystallized from 95% ethanol, yielding colorless shiny crystals with a melting point of 210–211°C. 1,3-DMP was prepared by the same procedure except for the use of larger quantities of NaOH (6 ml) and dimethyl sulphate (2 ml) and a longer reaction time of 1 hr. The final product was colorless, flaky and crystalline with a melting point of 184–185°C. Both derivatives were characterized by elemental analysis, infra-red and NMR spectroscopy and mass spectrometry.

Animals and drug administration: Swiss albino mice (male, 20–30 g) were obtained from the University Laboratory Animal Center at least two days before experimentation. They were kept under a natural light-dark cycle (approx. 12–12 hr) with food and water available ad lib. Phenytoin, 3-MP and 1,3-DMP were dissolved in dimethylsulphoxide (DMSO) and administered by i.p. injection at 5 ml/kg body wt. All other drugs were administered similarly in DMSO or in physiological saline. Phenytoin was obtained from Sigma, diazepam and clonazepam from Roche, phenobarbital sodium from Thornton and Ross, carbamazepine from Ciba-Geigy, and valproic acid from Tokyo Chemical Industry.

Maximal electroshock seizure (MES): Mice were shocked electrically with auricle electrodes at 40 mA for 0.2 sec. At this magnitude, all control (vehicle-injected) mice exhibited characteristic clonic-tonic convulsion which can be briefly described by four stages: clonus, tonic flexor, full tonic extensor (hindlimbs), and a pre-recovery period with loss of righting reflex. Under the influence of a drug with anticonvulsant activity, mice may show seizure activities up to different stages depending on the dose and the efficacy of the drug used. Therefore, mice that showed the full range of seizure activities were arbitrarily assigned an ‘anticonvulsant score’ of 1, while mice with seizure up to the flexor phase, 2; clonus only, 3; and no seizure, 4.

Measurement of phenytoin and derivatives by HPLC: Mice were injected with 3-MP (50 mg/kg) or 1,3-DMP (100 mg/kg) and sacrificed at various time intervals post-injection (0.5–3 hr). Heparinized blood samples were collected, centrifuged at 900 g for 10 min and the resultant plasma samples (0.1 ml) were treated with acetonitrile (0.15 ml) containing 0.75 μg of 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) as the internal standard. Precipitated protein was removed by centrifugation, and the supernatant (25 μl) was injected via a rheodyne injector into an isocratic HPLC system consisting of a Tracor pump, a Lichrosorb RP C18 column (10 μm, 250×4 mm), a Waters UV detector (254 nm wavelength) and a Hewlett-Packard integrator. The mobile phase was 35% acetonitrile in 10 mM potassium dihydrogen phosphate (pH 5.2). The flow rate was 1 ml/min. Retention times for the three analogues were 8.5, 16 and 30 min, respectively, while that for the internal standard MPPH was 13 min. Blank plasma gave a single peak at 10 min. For calibration, blank plasma were spiked with known quantities of phenytoin (2.5–15 μg/ml), 3-MP (3–20 μg/ml) and 1,3-DMP (3–20 μg/ml). The calibration graphs were linear with regression lines y=0.071x−0.02 (r=0.999) for phenytoin, y=0.073x (r=0.987) for 3-MP, and y=0.079x (r=0.999) for 1,3-DMP, where y is the peak area ratio and x, the concentration in μg/ml. For 3-MP in 1,3-DMP-injected mice, much less accurate estimation of 3-MP was obtained using peak height ratios because of interference from the unknown peak (see Fig. 3).

Uptake and binding assays: Uptakes of [3H]glutamic acid (GLU) and [3H]γ-aminobutyric acid (GABA) into rat cortical synaptosomes (P2) were measured as previously described (5) in the presence and absence of 3-MP (0.1–1 mM). [3H]-Flunitrazepam (FLN) binding was measured according to Braestrup and Squires (6) with modifications using washed crude synap- tosomal membrane fraction preincubated at 37°C for 20 min before the final wash. The final preparation was resuspended in 40 vol. of 50 mM Tris-HCl buffer, pH 7. [3H]GABA binding was measured with essentially the same procedure except that the membrane fraction was treated with Triton X-100 (0.05%) during preincubation at 37°C. Radioligands (0.1 μCi) were incubated with
the membrane preparation (0.3 ml) in a total volume of 1 ml of the same Tris-HCl buffer at 2°C for 30 min (GABA) or 1 hr (FLN). Nonspecific binding was obtained with either 3 μM diazepam or 0.1 mM GABA. Final concentration of 3-MP was 10–100 μM. Radioligand bound to the membrane was recovered by rapid filtration through Whatman GF/B filters and quantified by liquid scintillation counting. In all uptake and binding experiments, 3-MP was dissolved in DMSO at appropriate concentrations so that a fixed volume of 10 μl was used. In control experiments, only DMSO (10 μl) was added.

Protein was determined by the methods of Lowry et al. (7) with bovine serum albumin as the standard. All statistics were performed by a microcomputer according to Barlow (8).

Results

Intraperitoneal injection of phenytoin (30 mg/kg) in DMSO gave maximal anticonvulsant activity within 15 min postinjection. However, administration of 3-MP (30 mg/kg) or 1,3-DMP (100 mg/kg) in the same vehicle and by the same route caused anti-convulsant activities which increased with time and peak activities occurred about 3 hr postinjection. Moreover, their peak activities did not reach the maximal anticonvulsant score of 4 (Fig. 1).

When mice were injected with 3-MP (50 mg/kg), plasma concentrations of 3-MP were constantly about 14 μg/ml from 0.5–3 hr postinjection. However, phenytoin increased gradually from about 4 to 15 μg/ml during the same period of time, presumably being formed by N-demethylation of 3-MP. The time course of increase in plasma phenytoin coincided with the increase in anti-convulsant score (Figs. 1 and 2a). In contrast,
in mice injected with 1,3-DMP (100 mg/kg), the plasma concentration of the injected compound decreased from a low level of about 4 μg/ml at 30 min postinjection to a non-detectable level at 3 hr. This means that 1,3-DMP was metabolized extremely rapidly with the formation of at least three metabolites: 3-MP, phenytoin and an unidentified metabolite (Fig. 3). Plasma concentrations of 3-MP were low and constant at about 4 μg/ml throughout, while phenytoin increased from 1.6 to 14 μg/ml, again following a time course similar to that of the increase in anticonvulsant score (Figs. 1 and 2b). Since the same dose was used in this case, a direct correlation between the plasma phenytoin concentrations and the anticonvulsant score can be made by linear regression, which yielded a regression coefficient (r) of 0.91. An unidentified peak (X) with a retention time of 15.5 min appeared as soon as 30 min after injection of 1,3-DMP. The peak sizes were relatively large throughout (Fig. 3).

At the higher dose of 50 mg/kg, 3-MP was observed to induce very mild seizure-like activities in some mice. When the dose was increased to 100 mg/kg, all mice convulsed with an onset time of approx. 10 min. About 70% of the mice died within 1 hr with or without exhibiting full tonic extension of the hindlimbs (Table 1). Phenobarbital (30–40 mg/kg), diazepam (15–30 mg/kg) and clonazepam (30 mg/kg) were effective in preventing the seizures after injecting 3-MP. In contrast, carbamazepine (30 mg/kg), valproic acid (100 mg/kg) and phenytoin (30 mg/kg) were inef-
Phenytoin even appeared to have enhanced the 1-hr mortality rate to 100% (Table 1). No seizure activity was observed with either phenytoin (100–150 mg/kg) or 1,3-DMP (100 mg/kg). The latter is consistent with the observation that plasma concentrations of 3-MP remained low after 1,3-DMP injection (Fig. 2b).

3-MP (up to 1 mM) did not alter the synaptosomal uptakes of GLU and GABA, nor did it (up to 0.1 mM) affect GABA and FLN binding to synaptosomal membranes (Table 2).

### Table 2. Lack of effects of 3-methylphenytoin on various neurochemical parameters

| Parameter                  | Control       | With 3-MP*    |
|----------------------------|---------------|---------------|
| GLU uptake                 | $K_t$         | $V_{max}$     |
|                            | 10.1±1.8      | 11.6±3.1      |
| GABA uptake                | $K_t$         | $V_{max}$     |
|                            | 2.8±0.5       | 2.7±0.3       |
| GABA (1.3 nM) binding      |               |               |
|                            | 0.38±0.01     | 0.32±0.04     |
| FLN (1.2 nM) binding       |               |               |
|                            | 0.29±0.01     | 0.29±0.01     |

*1 mM for uptake experiments and 0.1 mM for binding experiments. $K_t$ values are expressed in $\mu$M, $V_{max}$ in pmol/hr/mg protein, and the binding values in pmol/mg protein.

**Discussion**

We have confirmed that both 3-MP (30 mg/kg) and 1,3-DMP (100 mg/kg) exhibit increasing anticonvulsant activities over 3 hr after intraperitoneal administration of the compounds completely dissolved in an organic vehicle. This delay in peak activities, therefore, did not result from a delay in absorption as evidenced by the maximal anticonvulsant activity of phenytoin only 15 min postinjection and by the rapid onset (approx. 10 min) of the seizure activities after 3-MP (100 mg/kg) injection. The present results strongly indicate that the anticonvulsant activities of 3-MP and 1,3-DMP are a direct result of the formation of phenytoin through N-demethylation as the time course of increase in plasma phenytoin concentrations correlated well with the anticonvulsant scores in both cases. For 3-MP, the process is a single N-demethylation step, while 1,3-DMP must be demethylated both at the 1- and 3-position to yield phenytoin. However, it is apparent that 1,3-DMP disappeared at a much faster rate despite the fact that a much larger dose was injected. 3-MP is found to be one of the two intermediates observed, occurring at a relatively low concentration throughout the 3-hr postinjection period. In contrast, the concentration of the unidentified metabolite X was much larger, judging from the relative peak sizes in the HPLC chromatograms. As the concentration of an intermediate is governed by the rates at which it is formed and further transformed, it means that either 1,3-DMP is converted predominantly to X or the rate of subsequent transformation of 3-MP is much faster than that of X, or both. Although it is tempting to speculate that X might be 1-methylphenytoin (1-MP), it must be emphasized that the present data do not offer any clues about the identity of X. We have attempted to synthesize 1-MP according to Cattelain and Chabrier (9), but the product obtained was identified to be 3-MP instead, confirming the previous findings (10). Alternatively, X could be the hydroxylated metabolite of 1,3-DMP. Attempts to identify samples of peak X collected from HPLC were also unsuccessful, presumably due to instability in the mobile phase after HPLC analysis.

In addition to the lack of anticonvulsant activity, 3-MP caused seizure activities when injected in DMSO at a dose of 100 mg/kg. Since DMSO can lower seizure threshold, there may be an added vehicle effect rather than simply facilitation of absorption. However, the observed severe seizure activities after the injection of 3-MP in DMSO, but not DMSO alone, indicated that 3-MP is at least a pro-convulsant even if the convulsion was, indeed, caused by a combined effect of drug and vehicle. It is not surprising that phenobarbital, diazepam and clonazepam were
effective in preventing 3-MP-induced seizures as the barbiturates and benzodiazepines provide good protection against various chemical convulsants such as pentylene-tetrazole and strychnine. N-demethylation of 3-MP would not attenuate its pro-convulsant effects as phenytoin appeared ineffective against the seizure activities. If anything, there might even be some potentiation of effects. On the other hand, 1,3-DMP was not observed to cause seizures at similar dosage levels. This may be explained by the observation that plasma 3-MP did not accumulate to any significant extent. Furthermore, since phenytoin, up to 150 mg/kg, was not observed to cause any seizure, this apparently eliminates any possibility that the seizure activities induced by 3-MP could be caused by the metabolically derived phenytoin.

3-MP was found to have no effects on several neurochemical parameters. Firstly, its lack of effects on GABA and FLN binding indicates that 3-MP has no affinity for both the GABA and benzodiazepine sites of the GABA-benzodiazepine-chloride ionophore complex. It is, therefore, unlikely to be an inverse (contra-) agonist for the benzodiazepine receptors. Secondly, its lack of effects on the synaptosomal transport of GLU and GABA is very interesting as phenytoin is able to inhibit both uptake processes (5). It may be inferred from this observation that the inhibitory action of phenytoin on the synaptosomal transport of GLU and GABA may well have a causal relationship with the anticonvulsant properties of this drug.

In conclusion, this report has shown that N-methyl substituted derivatives of phenytoin do not possess anticonvulsant activities. In fact, 3-MP is at least a pro-convulsant. These data support the idea that an unsubstituted hydantoin ring is an important feature in the structure of the phenytoin molecule for its anticonvulsant activity.

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