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SYNERGISTIC EFFECT OF FLUNARIZINE AND SODIUM VALPROATE ON SEIZURE THRESHOLDS ELICITED BY CORTICAL STIMULATION IN CONSCIOUS RATS

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Abstract : The effect of flunarizine (FLU) and sodium valproate (SV) alone and in combination were examined for their effects on seizure thresholds elicited by cortical stimulation in conscious rats. Two different pharmacodynamic parameters could be distinguished viz, the threshold for localised seizures (TLS) defined as the current (μ A) required to elicit forelimb clonus and the threshold for generalised seizure (TGS), defined as the current (μ A) required to elicit vigorous clonic activity without a tonic component. In preliminary neuro-behavioral studies on rats, the most favourable combination was FLU 10 mg/kg ip and SV 200 mg/kg ip, which produced anticonvulsant efficacy with minimal neurotoxicity. With FLU alone, SV alone and the combination of FLU and SV, the mean % change ± SEM from baseline values over a period of 6 h were for TLS : 3.8 ± 0.8, 23.9 ± 3.7, and 29.8 ± 2.1; and for TGS 5.5 ± 0.7, 15.6 ± 2.7 and 190.9 ± 22.7 respectively, indicating that FLU alone had no effect on TLS or TGS, SV significantly elevated TLS but had no effect on TGS and the combination of FLU plus SV produced a synergistic elevation of both TLS and TGS - the intensity of effect being more on TGS than on TLS. This model provides a new dimension to the profiling of two anticonvulsant agents with different mechanisms of anticonvulsant activity and offers predictive criteria for protective effects on clinical manifestations of partial or generalised tonic clonic seizure.

Key words : threshold for localised seizures, threshold for generalised seizure, sodium valproate, flunarizine

INTRODUCTION

Treatment of epilepsy is unsuccessful in approximately 25% of epileptic patients, hence, there is a need for novel approaches in the management of resistant epilepsy based on meaningful pharmacodynamic experiments, supported by clinical validation.

The influx of Ca^{++} into neurones is an important factor in triggering epileptic activity (1, 2) and consequently, the prevention of cellular Ca^{++} overload may have anticonvulsant effects. Flunarizine a calcium channel blocker, has protective effects in experimental seizure models (3, 4).

Flunarizine at low doses, enhanced the anticonvulsant effect of carbamazepine as well as diphenylhydantoin in the

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pentylenetetrazol (chemoshock) induced seizure test in mice (5). Results obtained in our laboratory, showed that ED_{50} doses of a combination of sodium valproate and flunarizine had complete seizure protection against electro and chemoshock induced seizures in mice, without any evidence of associated adverse effects. The electro and chemoshock models have the inherent limitation that only additive, but not supra-additive (synergistic) effects can be demonstrated.

This investigation was undertaken to further evaluate the anticonvulsant efficacy of flunarizine and sodium valproate alone and in combination, on seizure thresholds elicited by direct cortical stimulation, in conscious rats, using ramp generated pulse trains, a method by which synergistic effects can be detected.

The aim of this study, therefore is to provide pharmacological evidence for a new approach in the management of refractory epilepsy.

METHODS

Male Wistar rats (240–270 g), were obtained from the Indian Institute of Science, Bangalore, India. Animals were kept under regulated conditions of temperature (28°C ± 2°C) and lighting (lights on 7:00–19:00) with food and water supplied *ad libitum* except during drug testing.

Electrode implantation : For implantation, the rats were placed in a stereotaxic instrument under pentobarbitone anesthesia (35 mg/kg, ip). Two stimulation stainless steel screws, were implanted bilaterally in the skull over the motor area of the frontoparietal cortex, 1 to 1.5 mm posterior to bregma and 3 mm lateral to the midline. The reference electrode was placed on the nasion. The electrodes were fixed to the skull with dental cement. Following surgery, the rats were housed individually and stimulation was started one week after recovery.

Cortical stimulation : For stimulation, the rat was placed freely on a table in order to observe post stimulatory behaviour and nature of the induced seizure activity. Rats were connected to the ramp generated stimulator via appropriate leads, that permitted the animal free movement. The method described by Voskuyl et al (6) has been validated in our laboratory (7). Cortical stimulation was carried out and a single train of bipolar pulses, pulse duration 2 msec, was delivered at a rate of 50 pulses/sec, with a ramp time of 15 secs, increasing linearly in amplitude with time. This was achieved by modulating the stimulator output with an externally applied ramp voltage. Digital read out of the applied current required to attain either a localized or a generalised seizure were obtained.

Routinely, stimulation was interrupted at the start of the first clonic movements viz. of a single forelimb. The strength of the current read out, in μA, indicated the threshold localized seizure (TLS). When stimulation was stopped at this stage, seizure activity was immediately aborted and the animal resumed its normal pattern of activity. When the stimulation was continued, more severe and forceful clonic jerks of the forelimbs progressing to vigorous clonic activity occurred, the

current read out at this event being the threshold for the generalised seizure (TGS). When stimulation was terminated at TGS, only post-threshold clonic activity lasting for a very short period of about 10 secs was seen. (In contrast to maximal electroshock induced seizures, tonic components or extension of the hind limbs were never seen). Thus, different thresholds for localized (TLS) and generalized (TGS) seizure activity were differentiated, the stimulation being done repeatedly at appropriate intervals, on the same animal, enabling the correlation of drug dose, specific elevation of threshold of either TLS or TGS, or of both together. The time course of action of the test drugs could also be profiled simultaneously. TLS and TGS were established in daily sessions for each rat, twice per session for about fourteen sessions, until the thresholds remained stable for at least three consecutive sessions, prior of drugging.

Drug studies : Six rats/group were employed for vehicle/drug treatment and for establishing TLS and TGS. The following drugs administered intraperitoneally, were tested, flunarizine (FLU) at doses of 10, 20 and 40 mg/kg, ip and sodium valproate (SV) 200 and 300 mg/kg, ip. FLU was dissolved in 50% polyethyleneglycol and 50% distilled water, while SV was dissolved in distilled water. Controls were given corresponding vehicles. The behavioural effects seen after drugging were quantitatively evaluated according to Desmedt et al (4) and the appropriate dose for the combination therapy was ascertained to be FLU 10 mg/kg together with SV 200 mg/kg, ip (hereinafter called the combination). The drugs were injected independently in right and left lower abdominal regions. In view of the long half life of flunarizine (8), the combination was given after a washout period of 2 weeks, following SV/FLU. Prior to drug/vehicle treatment, each animal was tested 3 times and the mean was used to calculate the baseline, or "O" time TLS/TGS for that animal. Postdrug TLS/TGS testing was done at 10, 20, 30, 40 mins, 1, 1½, 2h and thereafter, at hourly intervals till 6 h and was repeated at 24 h.

Statistical analyses : All data are expressed as means±SEM. Changes in the measured parameters (TLS and TGS) between the different treatments were tested for significance using a repeated measures analyses of variance (ANOVA). A paired t-test with Bonferonni correction was applied to determine which time points were significant in relation to basal measures, and at which time point the various treatment groups differed from each other (9). Data were also analysed by using appropriate summary statistics, as has been recommended for serial measurements (10). The one way ANOVA was applied to test the significant differences of summary statistics followed by Fishers test of least significance to test the differences between each of three treatment groups. All data were analysed using SPSS/PC statistical software programme. The null hypothesis were rejected at $P<0.05$. (As the measured parameters returned to baseline in 24 h, the latter values are not represented in the figs and have been omitted from statistical analyses).

RESULTS

Behavioural effects : FLU 10 mg/kg, showed no behavioral changes. At 20 to 40

mg/kg, dose dependent reduction in motor activity and hypotonia were observed. Mild ataxia was seen at 40 mg/kg.

SV 200 and 300 mg/kg, showed mild ataxia, hypotonia and "wet dog shakes". These effects appeared 5 mins following administration and lasted for about 1½ h.

At the highest doses of the combinations tested, FLU 40 and SV 300 mg/kg, produced marked ataxia, sedation, hypotonia and reduced motor activity and partial loss of the righting reflex. Though the animals appeared sedated they could be aroused by touch or sound. These effects reached a maximum at 1 to 1½ h declined by 6 h and normal behavior was seen at 24 h. FLU 20 and SV 300 mg/kg and FLU 20 and SV 200 mg/kg produced the same symptoms in a dose dependent fashion, though to a lesser extent, and the rats were normal at 6 h. FLU 10 and SV 200 mg/kg produced mild sedation and reduced motor activity, without ataxia, for about 3 h and thereafter, normal behaviour was seen and as this combination had the least side effects, it was selected for testing on TLS and TGS. No immediate or delayed mortality occurred with any of the dose combinations employed. No behavioral or other changes were observed in the corresponding vehicle treated controls.

Effect on TLS and TGS : In untreated animals, TLS and TGS remained stable over the test period. Mean threshold values ± SEM were 679 ± 101.45 μA for TLS and 1013 ± 144.92 μA for TGS. The TLS and TGS after FLU alone, SV alone and in combination over a period of 360 mins in comparison with their respective controls are shown in Figs. 1 and 2. Repeat measures ANOVA revealed that while the main effects of FLU or SV given alone were not significantly different from their respective controls (F df 1,8 = 0.72 for FLU-TLS; = 0 for SV-TLS; = 0.87 for FLU-TGS and =2.55 for ST-TGS), when given in combination there was a significant difference from controls (F df 1,10 = 15.82 P< 0.003 for TLS; = 841.3 P<0.001 for TGS) indicating that neither FLU nor SV given alone in the dosages employed influenced TLS or TGS, but the combination significantly elevated both TLS and TGS as compared to controls.

The main effect of time with FLU or SV alone and in combination were significant for TLS and TGS (F df 12,96 = 8.59 for FLU-TLS; = 16.3 for SV-TLS; = 20.08 for FLU-TGS; and = 9.82 for SV-TGS; F df 12, 120 = 11.78 for FLU + SV-TLS and = 274.24 for FLU + SV-TGS P<0.001) indicating that both TLS and TGS changed across time in all three treatment groups. The treatment × time interactions were also highly significant for both TLS & TGS in all three treatment groups as compared to their respective controls (F df 12,96 = 2.1, P<0.02 for FLU-TLS, = 13.64 for SV-TLS, = 19.5 for FLU-TGS, = 7.76 for SV-TGS, F df 12,120 = 11.78 for FLU+SV-TLS, = 240.26 for FLU+SV-TGS P<0.001) indicating that FLU or SV alone or in combination produced significantly different effects on TLS and TGS as compared to corresponding controls across time.

A paired t-test with Bonferroni correction showed that FLU alone did not alter the TLS or TGS when compared to baseline values (Fig 1A and 2A). SV alone, significantly (P<0.05) elevated the TLS

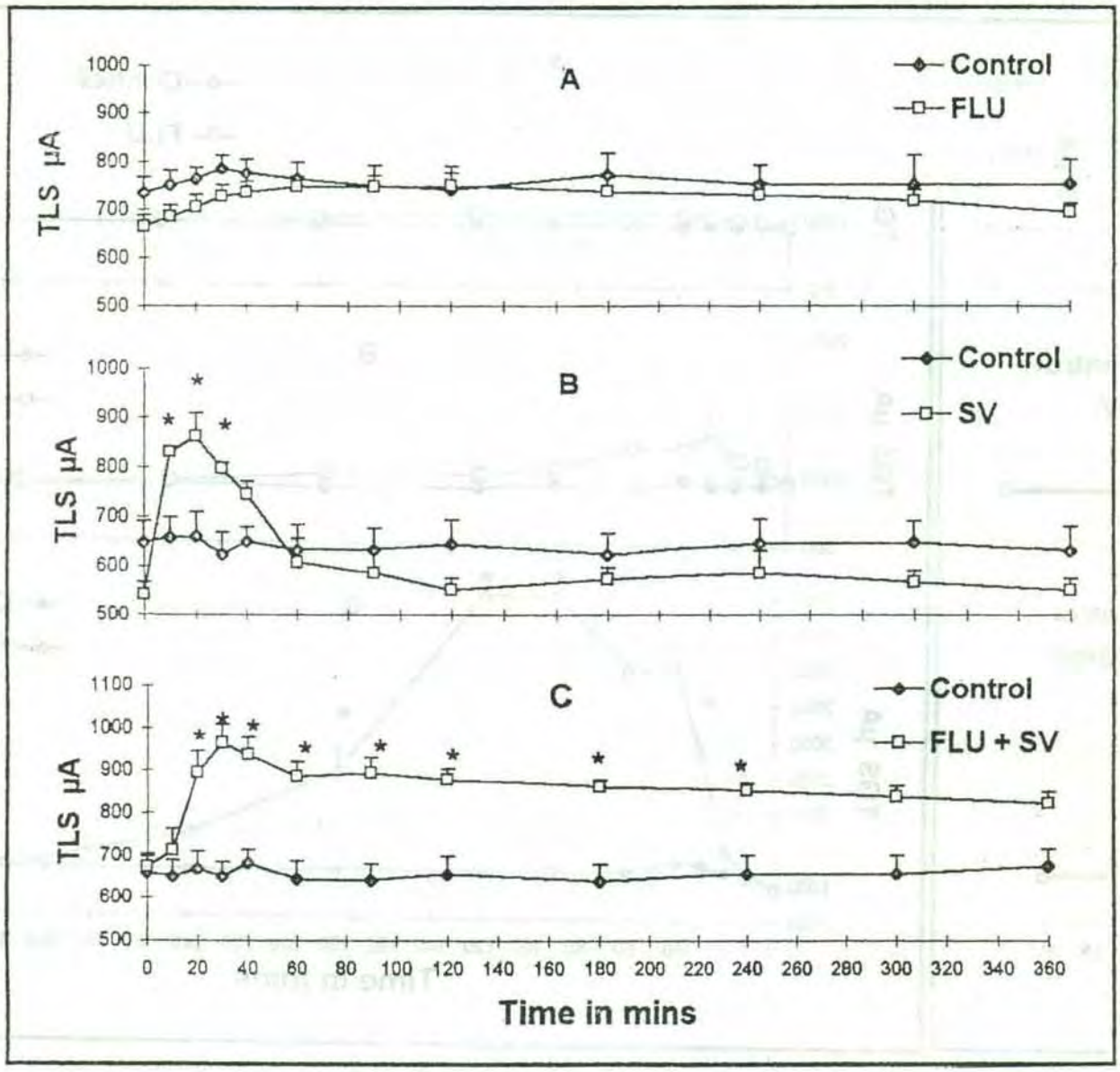

Fig. 1 : Threshold for localised seizures (TLS) after A. Flunarizine (FLU) 10 mg/kg alone, B. Sodium Valproate (SV) 200 mg/kg, ip alone and C. Combination of FLU and SV in the above dosages compared to controls treated with corresponding vehicle. Repeated measures ANOVA revealed no significant effects for FLU or SV alone, but a significant effect with the combination ($P<0.001$). Time and time x treatment interations were significant in all three groups ($P<0.001$).

*Indicates significance from baseline, i.e. time 'O' using t-tests with the Bonferroni correction.

between 10 to 30 mins, peak effect being at 20 min (Fig. 1B), SV however had no significant effect on TGS (Fig. 2B). When the two drugs were given in combination,

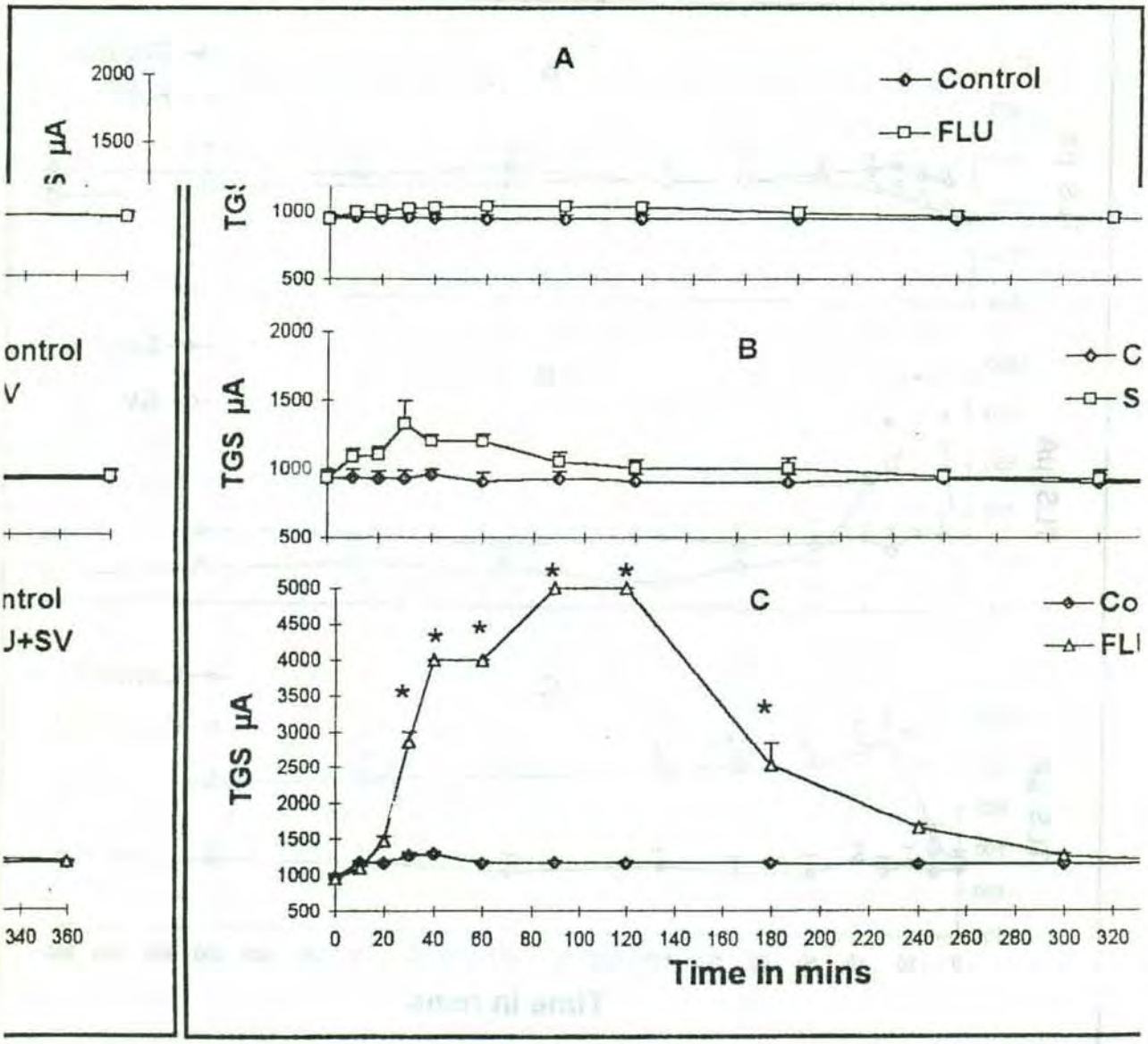

Fig. 2: Threshold for generalised seizures (TGS) after A. Flunarizine (FLU) 10 mg/kg alone, B. Sodium Valproate (SV) 200 mg/kg, ip alone and C. Combination of FLU and SV in the above dosages compared to controls treated with corresponding vehicle. Repeated measures ANOVA revealed no significant effects for FLU or SV alone, but a significant effect with the combination ($P<0.001$). Time and time x treatment interactions were significant in all three groups ($P<0.001$).

*Indicates significance from baseline, i.e. time '0' using t-tests with the Bonferroni correction.

TLS was significantly ($P<0.05$) elevated between 20 to 240 mins, peak effect was seen at 30 min (Fig. 1C). On TGS significant ($P<0.05$) elevation was observed from 30 mins, which was sustained upto 180 min peak effects were between 90–120 min (Fig.

2C). Both TLS and TGS returned to baseline by 360 min. It may be noted that with the combination the intensity of action was more on TGS than on TLS whereas, the duration of action was more prolonged on TLS than on TGS.

In Figs. 3 and 4 are depicted the percentage change in TLS and TGS from baseline over a period of 360 mins in the three treatment groups. Repeat measures ANOVA showed highly significant differences between the treatment groups for both TLS and TGS (F df 2,13 = 7.2 for TLS P<0.02; and = 476.01 for TGS P<0.001) indicating that all treatments produced different effects on TLS and TGS. Both TLS and TGS increased significantly over time in all groups (F df 12, 156 = 19.72 for TLS; and 183.8 for TGS P<0.001). The treatment × time interactions were also highly significant for TLS and TGS. (F df 24, 156 = 10.14 for TLS; and = 155.41 for TGS P<0.001) indicating that all three treatments produced significantly different effects across time.

A paired t-test with Bonferroni correction showed that on TLS (Fig. 3) FLU by itself had no significant effect. The effect

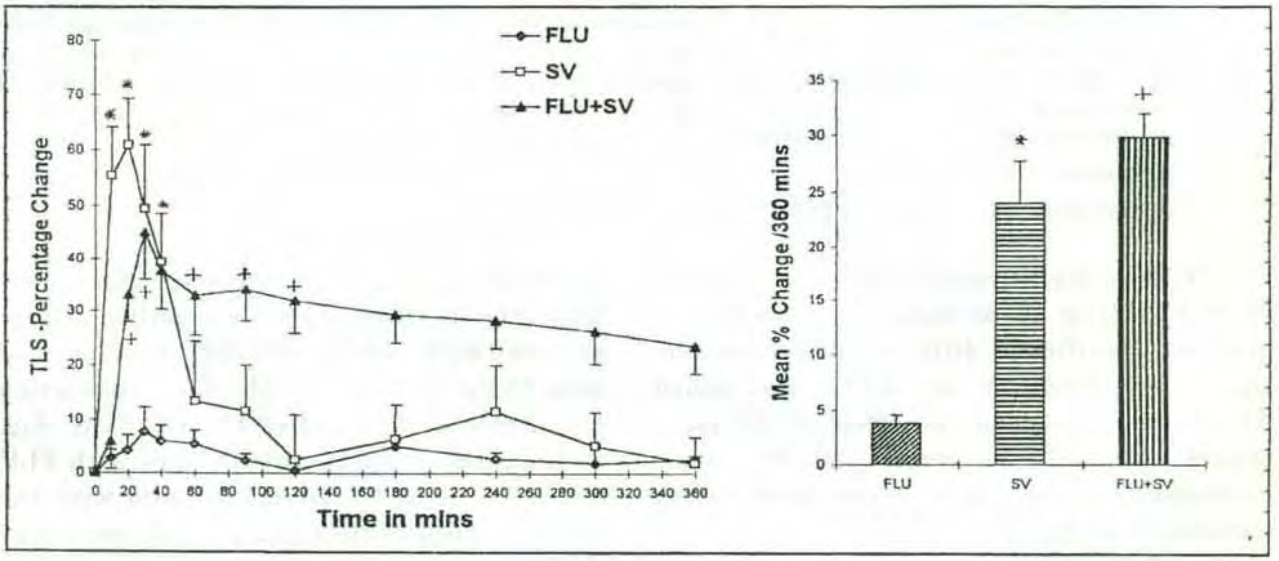

Fig. 3 : Percentage change in threshold for localised seizures (TLS) and after flunarizine (FLU) 10 mg/kg, ip. Sodium Valproate (SV) 200 mg/kg, ip given alone and combination of FLU + SV in the above dosages as compared to baseline ie time '0'. Repeat measures ANOVA analyses reveals that there were significant changes between treatments P<0.001, across time P<0.001 and a significant treatement x time interaction P<0.02. Significant differences between the treatment groups at each time points after the Bonferroni correction is also indicated (paired t-test – P<0.05). Bar diagrams depict the summary statistics for graph on left (significance one way ANOVA).

*Significance SV vs FLU

·Significance Combination vs FLU / SV

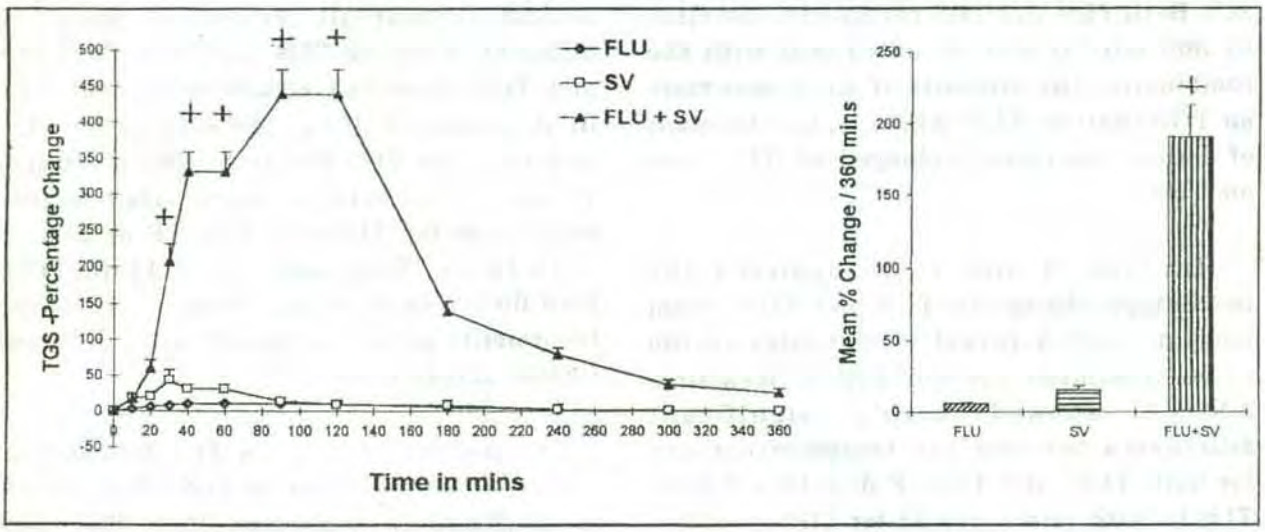

Fig. 4 : Percentage change in threshold for generalised seizures (TGS) after flunarizine (FLU) 10 mg/kg, ip. Sodium Valproate (SV) 200 mg/kg, ip given alone and combination of FLU + SV in the above dosages as compared to baseline ie time '0'. Repeat measures ANOVA analyses reveals that there were significant changes between treatments P<0.001, across time P<0.001 and a significant treatment x time interaction P<0.001. Significant differences between the treatment groups at each time point after the Bonferroni correction is also indicated (paired t-test – P<0.05). Bar diagrams depict the summary statistics for graph on left (significance one way ANOVA).

*Significance SV vs FLU

+Significance Combination vs FLU/SV

of SV was significantly (P<0.05) greater than FLU from 10–40 mins, thereafter there was no significant difference between the two drugs. However, when FLU was added to SV, FLU reduced the effect of SV for a short period between 20–30 min, subsequently the effect of the combination remained higher than either drugs alone upto 360 mins, though statistical significance was evident only upto 120 mins. On TGS (Fig. 4) there was no difference between the effects of FLU and SV, however with the combination, there was a significant (P<0.05) elevation from 30–120 mins.

The mean % change ± SEM in TLS and TGS from time 0 to 360 mins between the three treatment groups are depicted as bar diagrams in Fig. 3 and 4 respectively. On TLS (Fig. 3) effect of FLU was only a change of 3.8±0.81%, while with SV the increase was 23.9±3.7%, and with the combination the increase was 29.8±2.1%. On TGS (Fig. 4) the corresponding values were with FLU 5.5±0.7%, with SV 15.6±2.7% and with the combination 190.9±22.7%. A one way ANOVA revealed that the mean % increase in TLS and TGS between the 3 groups were significant (P<0.001). Fishers t-test revealed that on TLS, SV produced significantly elevated (P<0.001) thresholds as compared to FLU, whereas on TGS there was no significant difference between the two drugs. The combination of FLU and SV produced significantly much higher thresholds for both TLS and TGS as

compared to either FLU or SV alone indicating a superadditive effect or synergism. This synergistic effect was more prominent on TGS than on TLS.

DISCUSSION

Combining drugs with different pharmacological actions may result in additive, sub-additive (antagonism) or supra-additive (synergism) effects (11, 12). The results obtained with the combination of FLU and SV in this study, suggests that the combination exhibits a pharmacological synergistic effect on both cortical seizure thresholds, TLS and TGS with the effect on TGS being more prominent. Apparently, different mechanisms of antiepileptic action of FLU and SV may converge to a common pathway. The most favourable dosage combination of FLU 10 and SV 200 mg/kg ip, showed side effects that appeared to be minimal or additive, whereas anticonvulsant efficacy on TLS/TGS was supra-additive. In previous studies, we have found that a low dose of FLU augmented the anticonvulsant effect of ED_{50} doses of phenytoin, carbamazepine and SV in the maximal electroshock seizure test in mice, 100% seizure control occurring only with the combination of FLU and SV (*authors data in publication*). Anticonvulsant efficacy on thresholds for localised or generalised seizures in rats may be predictive of a protective effect on clinical manifestations of partial or generalized tonic clonic seizures and hence, it can be speculated that the combination of FLU and SV may be effective in similar conditions (13–17)

An interesting finding was that the percentage change obtained with SV alone on TLS from 20–40 mins was greater than that of the combination. This can probably be explained by the rapid anticonvulsant effect of SV occuring within minutes (18) FLU on the other hand is characterised by a slow onset and a long duration of action (4).

Voskuyl et al., (6, 18) quantified the anticonvulsant effects of SV and FLU administered as zero order infusions in cortically stimulated conscious rats. SV 690 $mg/kg^{-1}.3hr^{-1}$ had a concentration range of 200 to 600 mg/l and elevated both TLS and TGS from 100 to 1200 mA up to 2 h. FLU 40 $mg/kg^{-1},5hr^{-1}$, showed a 3 to 5 fold elevation of TGS alone, without a change in TLS (concentration levels were not indicated). In this study, we had opted for a combination of FLU and SV at a dose level that had submaximal anticonvulsant efficacy that was devoid of adverse effects. Presumably sufficiently high plasma concentrations were not attained in our studies to show similar effects on TLS or TGS.

In the case of the widely used antiepileptic, SV, two general hypotheses have been proposed to explain its activity, viz. blocking of voltage-dependent Na^{++} channels and enhancing GABA mediated inhibition as evidenced by increased brain GABA levels (16). On the other hand, FLU possesses an anticonvulsant profile similar to that of phenytoin and carbamazepine (but not of SV), suggesting the possibility of an analogous mechanism of action on voltage-sensitive sodium channels. However, in spite of some evidence for implicating FLU in binding site II of the sodium channel (13), it remains uncertain whether it owes its anticonvulsant action to an effect on Na^{++}

channels or even on neuronal Ca^{++} channels (20). FLU does not increase GABAergic inhibition and its other attributes are that it readily crosses the blood brain barrier and may block T-type calcium channels (13). For the present, the pharmacological mechanism underlying the interaction of these two drugs must await further elucidation, but it must be stressed that an unique characteristic of FLU is that it prevent pathological calcium overload without affecting the normal function of voltage dependent calcium channels, and hence synaptic transmission.

The concept of rational polytherapy in refractory epilepsy has been gaining acceptance (17). Binnie (21) reports that the long term safety of flunarizine is good, side effects are mild and when used as add-on therapy, produces a clinically significant reduction in seizure frequency in a third of therapy-resistant patients with epilepsy. The results obtained in this study provide supporting pharmacological evidence of the efficacy, safety and possible potential benefit of combining FLU with SV in refractory epilepsy.

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REFERENCES

1. Prince DA. Physiological mechanisms of focal epileptogenesis. *Epilepsia* 1985; 26: S3–14.
2. Schwartzkroin PA, Wyler AR. Mechanisms underlying epileptiform burst discharge. *Ann Neurol* 1980; 7: 95–107.
3. De Sarro GB, Meldrum BS, Nistico G. Anticonvulsant effects of some calcium entry blockers in DBA/2 mice. *Br J Pharmacol* 1988; 93: 247–256.
4. Desmedt LKC, Niemegeers CJE, Janssen PAJ. Anticonvulsive properties of cinnarizine and flunarizine in rats and mice. *Arzneim forsch Drug Res* 1975; 25: 1408–1413.
5. Rodger C, Pleuvry BJ. Protective effect of flunarizine and nifedipine alone and in combination with anticonvulsant drugs agains PTZ–induced seizures in mice. *Neuropharmacology* 1993; 32: 257–263.
6. Voskuyl RA, Dingemanse J, Danhof M. Determination of threshold for convulsions by direct cortical stimulation. *Epilepsy Res* 1989; 3: 111–120.
7. Joseph S, David J, Joseph T. Determination of anticonvuslant effects in seizure htresholds using ramp gererated cortical stimulation in conscious rats. *Ind J Expt Biol* 1997; 35: 933–940.
8. Holmes B, Brogden RN, Heel RC, Speight TM, Avery GS. Flunarizine A review of its pharmacodynamic and pharmacokinetic properties and therapeutic use. *Drugs* 1984; 27: 6–44.
9. Everitt BS, Hay DF. The analyses of repeated measures designs in Talking about statistics, A Psychologist's Guide to Design & Analysis, Ed E. Arnold, 1992, pp 85–90.
10. Mathews JNS, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medicinal research. *Br Med J* 1990; 300: 30–35.
11. Plummer JL, Short TE. Statistical modelling of the effects of drugs combinations. *J Pharmacological Methods* 1990; 23: 297–309.
12. Smith NT. Use of isobolograms in anaesthesia. *Anaes Analg* 1966; 45: 467–473.
13. Binnie CD. Potential antiepileptic drugs. Flunarizine and other calcium entry blockers. In : R. Levy, R. Mattson, B. Meldrum, J.K. Penry. F.E. Dreifuss (Eds), *Antiepilieptic Drugs,* Third Edition, Raven Press Ltd., New York, 1989, pp. 971–982.

14. Overweg J, Binnie CD. Clinical treatment of epilepsy with calcium entry blokcers. *Funct Neurol* 1986; 1: 539–541.

15. Overweg J, Binnie CD, Meijer JWA, Meinardi H, Nuijten STM, Schmaltz S, Wauquier A. Double-blind placebo-controlled trial of flunarizine as add-on therapy in epilepsy. *Epilepsia* 1984; 25: 217–222.

16. Porter RJ. General principles how to use antiepileptic drugs. In : R. Levy, F. Dreifuss, R. Mattson, B. Meldrum, J.K. Penry (Eds). *Antiepileptic Drugs,* Third Edition, Raven Press Ltd., New York, 1989, pp. 126.

17. Sabers A, Gram L. Drug treatment of epilepsy in the 1990s. *Drugs* 1996; 52: 483–493.

18. Kervin RW, Olpe HR, Schmutz M. The effect of sodium-n-dipropyl acetate on γ-aminobutyric acid - dependent inhibition in the rat cortex and substantia nigra in relation to its anticonvulsant activity. *Br J Pharmac* 1980; 71: 545–551.

19. Voskyl RA, Hoogerkamp A, Danhof M. Properties of the convulsive threshold determined by direct cortical stimulation in rats. *Epilepsy Res* 1992; 12: 111–120.

20. Bebin M, Bleck TP. New anticonvulsant drugs. Focus on funarizine. fosphenytoin, midazolam and stiripentol. *Drugs* 1994; 48: 153–171.

21. Binnie CD, Beukelaar F, Meijer JWA, Meinardi H, Overweg J, Wauquier A, Van Wieringen A. Open dose-ranging trial of flunarizine as add-on therapy in epilepsy. *Epilepsia* 1985; 26: 424–428.