Toxic and teratogenic effects of antiepileptic drugs in *Drosophila*

Toxicity and teratogenicity are considered as important side effects of the antiepileptic drugs (AEDs), phenobarbital (PB), phenytoin (PHT), carbamazepine (CBZ) and valproate (VAL)[1]. A syndrome consisting of facial dimorphism, cleft lip and palate, cardiac defects, digital hypoplasia and nail dysplasia has been found to be associated with retrospectively and prospectively identified prenatal exposure to these drugs[1]. The embryotoxic and teratogenic effects of PB, PHT, CBZ and VAL have also been experimentally demonstrated in rodents[2–10]. To understand the toxic and teratogenic potentials of the AEDs in *Drosophila*, we administered each of these drugs separately to both larvae and adults. Developmental toxicity was evaluated on the basis of the number of pupae formed and adults eclosed after treating larvae with the drugs. Adult toxicity was measured in terms of mortality in treated flies. Teratogenicity was evaluated by performing a broad survey of external morphology of adults resulting after drug treatment of larvae.

The experiments involved the wild-type strain Oregon-R (OR) unless otherwise noted. Compared to normal food, food containing PB, PHT and VAL gave rise to a decreased ($P < 0.05$) larvae to pupae transformation (Table 1). This indicated that these three drugs are toxic to larvae. The turning of pupae to adults registered a reduction ($P < 0.01$) in case of PB and CBZ (Table 1). These two drugs therefore acted as pupal toxicants. The transformation of larvae to adults showed a decrease in case of all the drugs (Table 1), PB ($P < 0.01$), PHT ($P < 0.05$), CBZ ($P < 0.01$) and VAL ($P < 0.05$). This showed that all the four AEDs are overall toxic to larval and pupal development. Figure 1 compares, as an example, the abnormal pupae, which never mature, resulting after CBZ treatment with the normal one. The details of the morphological defects of pupae are, however, not available at this time. In flies, all the drugs except CBZ were found to be toxic (Figure 2). PHT caused an increased ($P < 0.05$) mortality than VAL (Figure 2). The AEDs thus differed with respect to their adult toxicity. Larval feeding of the drugs resulted in production of abnormal adults in case of PB and VAL (Table 1), the two drugs inducing leg (Figures 3 and 4) and wing (Figure 5) abnormalities respectively. PB mainly induced malformation or absence of tarsus in the 2nd and 3rd leg pairs. The majority of the affected individuals had abnormalities in both the left and right legs of both the pairs. However, flies with defects in only the 2nd or 3rd pair or in only the 2nd or 3rd left legs as well as with complete absence of the 3rd left leg were also encountered. In brief, PB showed a teratogenic effect. In the experiments described here, PHT and CBZ did not produce any noticeable abnormality in flies. CBZ had, however, occasionally caused leg abnormalities such as malformation of the 3rd pair and absence of the 3rd left leg in similar experiments earlier (data not presented). This AED therefore seems to have some teratogenic potential. VAL induced unexpanded wings. In majority of the cases, both the wings were unexpanded. Flies with abnormality in only one wing were, however, also observed. Since details of wing abnormality is not available at present, the 'teratogenic' poten-

Table 1. Mean ± SEm of per cent of OR larvae turning into pupae, pupae emerging as adults, larvae giving rise to adults and adults having morphologically abnormal organs after various treatments

	NF	PB	PHT	CBZ	VAL
L–P	98 ± 1 (5)	70 ± 11 (5)	56 ± 15 (3)	85 ± 11 (3)	86 ± 5 (5)
P–A	99 ± 1 (5)	60 ± 11 (5)	95 ± 5 (3)	50 ± 11 (3)	91 ± 4 (5)
L–A	97 ± 1 (5)	46 ± 12 (5)	53 ± 17 (3)	40 ± 15 (3)	78 ± 8 (5)
Ab. A.	0 (3)	54 ± 5 (5)	0 (3)	0 (3)	86 ± 4 (5)

Actively feeding 3rd instar larvae, resulting from eggs collected over a period of 4–5 h, of either sex were shifted to vials containing either normal food (NF) or food containing 4 mg/ml PB (Rhone–Poulenc), 2.5 mg/ml PHT (Samarth), 2 mg/ml CBZ (Sarabhai) or 2 mg/ml VAL (Sanofi–Torrent). L, Larvae; P, Pupae; A, Adults; Ab. A, Abnormal adults. Flies were grown on a standard *Drosophila* medium containing maize powder, sugar, yeast and Nipagin. Larvae were raised and further treated at 20 ± 2°C. Standard *Drosophila* manipulation methods were followed. Twenty larvae were treated in a single vial. Numbers in parentheses indicate *n*, the number of vials examined. After emergence, flies were shifted from treatment vials to new vials containing normal food for a minimum of 3 h, before being examined for morphological abnormalities. Both male and female flies had emerged in all the vials and the same morphological abnormalities were present in both the sexes. The relative drug doses used were basically derived from the recommended doses for humans[1]. In children, the starting doses of 4 (PB), 5 (PHT and CBZ) and 10 (VAL) mg/kg/day are recommended[1]. Under normal culture conditions, we observed that the average weight of a male fly is 1 mg which, when starved for 1 day, gained an average of 0.2 mg in body weight after feeding for a day. On this basis, the drug concentrations used here were supposed to be equivalent to starting doses of 800 (PB), 500 (PHT) and 400 (CBZ and VAL) mg/kg/day. Although these relative doses of 8:5:4:4 (PB:PHT:CBZ:VAL) compare poorly with the one recommended for humans (4:5:5:10), a higher dose of PB and a lower dose of VAL was selected because our preliminary observation (data not presented) had suggested that when starved flies are fed separately on food containing similar concentrations of either of the four drugs, PB, PHT, CBZ and VAL, the body weight gain is relatively less in case of PB and more in case of VAL. A higher concentration of PB and a lower concentration of VAL was therefore used to compensate for the differences in body weight gain observed. In general, very high drug doses (100–200 fold excess of recommended doses for humans) were used because of two main reasons. First, the pharmacokinetics (absorption, bioavailability, elimination half-life, rout of elimination, etc.), of these drugs in *Drosophila* are unknown. Second, these AEDs, in general, caused death of only a small number of flies within one day of treatment. In course of the current study, it was felt that treatment of larvae with the AEDs perhaps slightly affect the size of some of the resulting pupae/flies. At present, however, there is no data to confirm this.

Figure 1. Morphological defects of pupae. Two to three-day-old pupae resulting after treatment of larvae with either normal food (*a*) and food containing CBZ (*b* and *c*). Note the 'hollow' appearance of pupae in case of CBZ treatment. The abnormality is more pronounced in *c*. Magnification × 90. Other details are as described in Table 1.

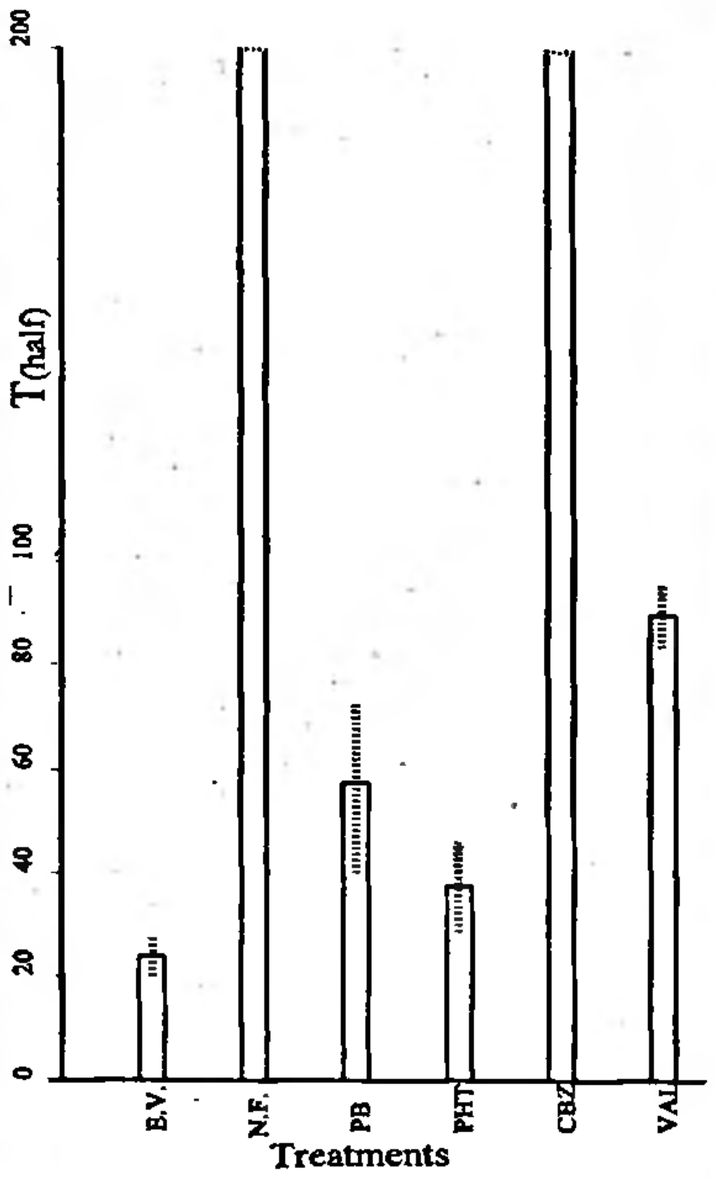

Figure 2. Mean ± SEm of $T_{(half)}$, the time (in h) at which half of the total number of OR flies died during various treatments. Up to 3–4-day-old female flies were first starved for 20–22 h and then 25–30 individuals were shifted to each of the following vials: empty vials (EV), vial containing normal food (NF) and vials containing normal food with either PB, PHT, CBZ or VAL. Number of live/dead flies were counted every 10–14 h. Loss of flies due to handling was corrected for. The experiment was repeated five times ($n = 5$). Broken lines indicate ± SE. Dotted top ends in NF and CBZ bars indicate no death during 8 days of observation. Other abbreviations, culture and treatment conditions, drug concentrations, *Drosophila* handling, etc. are as described in Table 1.

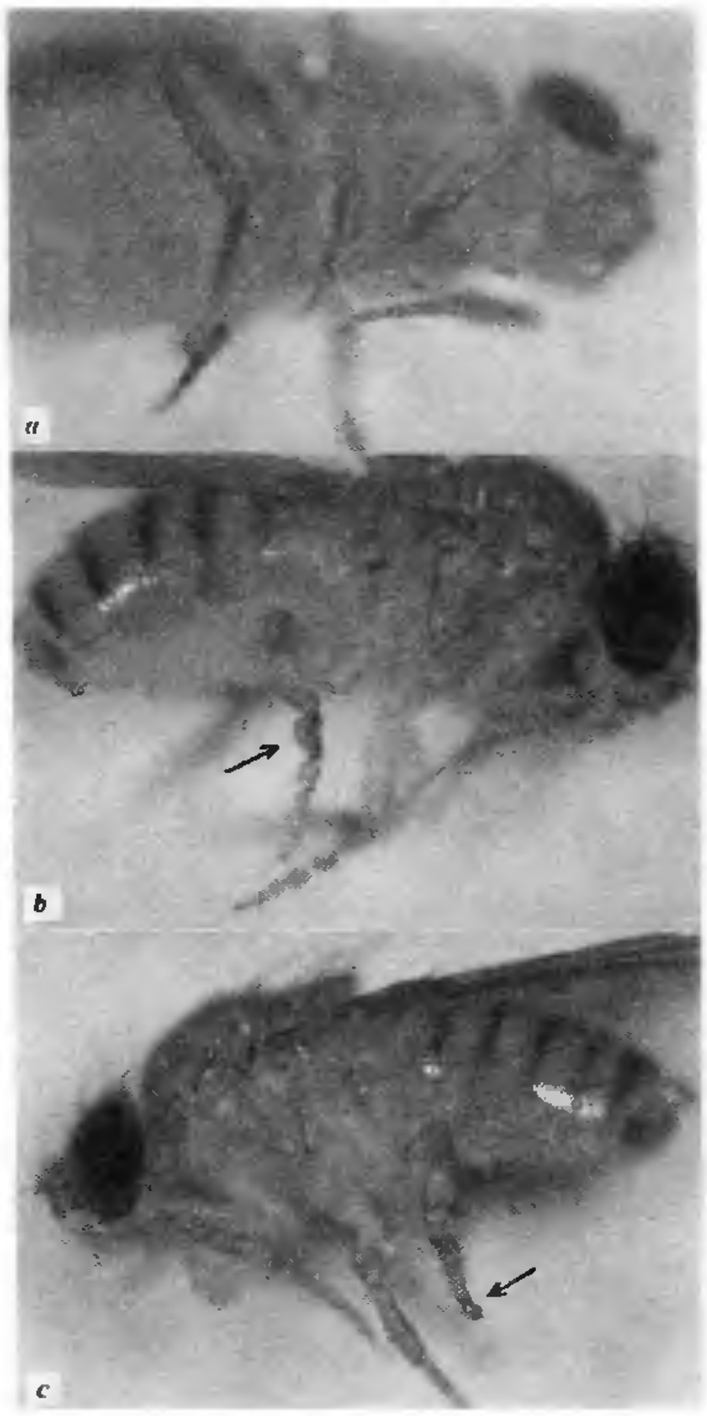

Figure 3 *a–c*. PB-induced leg abnormalities. Flies arising after treatment of larvae with normal food (*a*) and food containing PB (*b* and *c*). Note abnormal 3rd legs (arrows) in *b* and *c*. Magnification × 140. Other details are as described in Table 1.

tial of VAL is not yet clear. In the compound-X chromosome bearing paralytic mutant stock C(1) *DX yf/para*^{ts1}, only male, not female flies are paralytic[11,12]. Unlike OR, parallel VAL treatment, as described in Table 1, of larvae of the above *para* stock produced unexpanded wings only in females and not in males. In an experiment, 36 flies emerged out of 40 *para*^{ts1} larvae treated with VAL. Of this, 23 were females and 13 males. All females except one (~ 96%) had unexpanded wings. Whereas none of the males showed such an abnormality. *para*^{ts1} thus seems to suppress the production of wing abnormality induced by VAL.

Although any extrapolation of the *Drosophila* data to humans is inappropriate, our results may possibly serve as indicators of toxic and teratogenic potentials of the AEDs studied – more so because the 'relative' drug doses used here do not deviate much from that recommended for human therapy[1] (see Table 1). The following three parallelisms seem important in this regard. First, among the four AEDs, CBZ is considered to be most well tolerated and to cause least impairment in human epileptic's quality of life[13–15]. It is interesting to note here that CBZ, unlike PB, PHT and VAL, did not cause any mortality in *Drosophila* adults. Second, AEDs induce limb malformation in humans and rodents[3,4]. It is again interesting to note the apparent similarity in the pattern and diversity of drug-induced limb abnormalities between *Drosophila* and mammals. Third, alteration of osmotic fluid regulation or perturbation of physiology related to vascular fluid dynamics is one of the suggested effects of VAL in mammals[16]. It is tempting to speculate an analogous situation in *Drosophila*. The wings of newly hatched flies are initially tightly folded, which then slowly unfold after hemolymph is pumped into the wing veins[17]. It is possible that VAL interferes with pumping of hemolymph and thereby causes unexpanded wings. That *para*^{ts1} suppresses VAL-induced wing abnormality suggests an involvement of *para* Na^+ channel in the drug's action. *para*^{ts1} is a Na^+ channel insertional mutation that presumably results in reduced number of Na^+ channel[11]. *In vitro* cultured neurons from the mutant larvae are more resistant than that of wild-type larvae to the cytotoxic effects of veratridine, a neurotoxin that binds and stabilizes Na^+ channels in an open conformation[18]. VAL is believed to act through voltage-

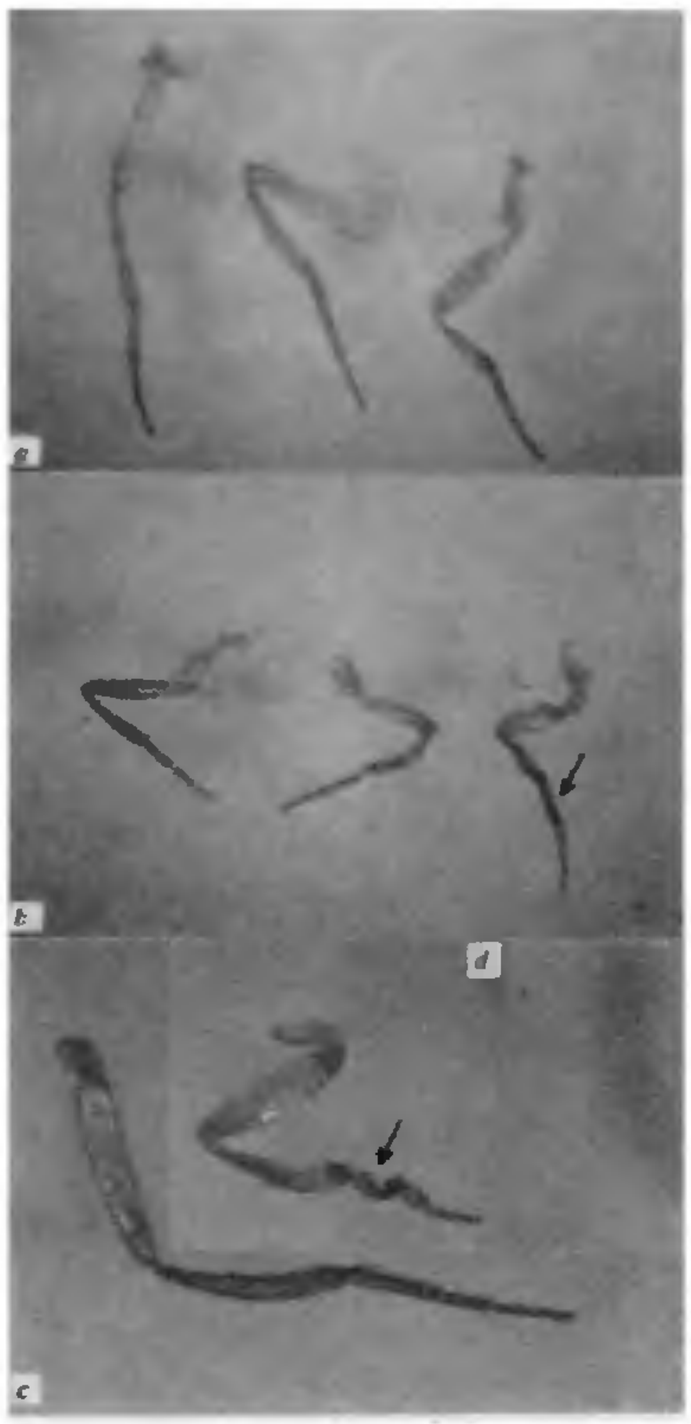

Figure 4 *a–d*. PB-induced leg abnormalities. Legs of flies resulting after treating larvae with normal food (*a* and *c*) and food containing PB (*b* and *d*). From left to right, 1st, 2nd and 3rd legs in *a* and *b*. 3rd legs in *c* and *d*. Note abnormal 3rd legs (arrows) in *b* and *d*. Magnification × 90 (*a* and *b*), × 140 (*c* and *d*). Other details are as described in Table 1.

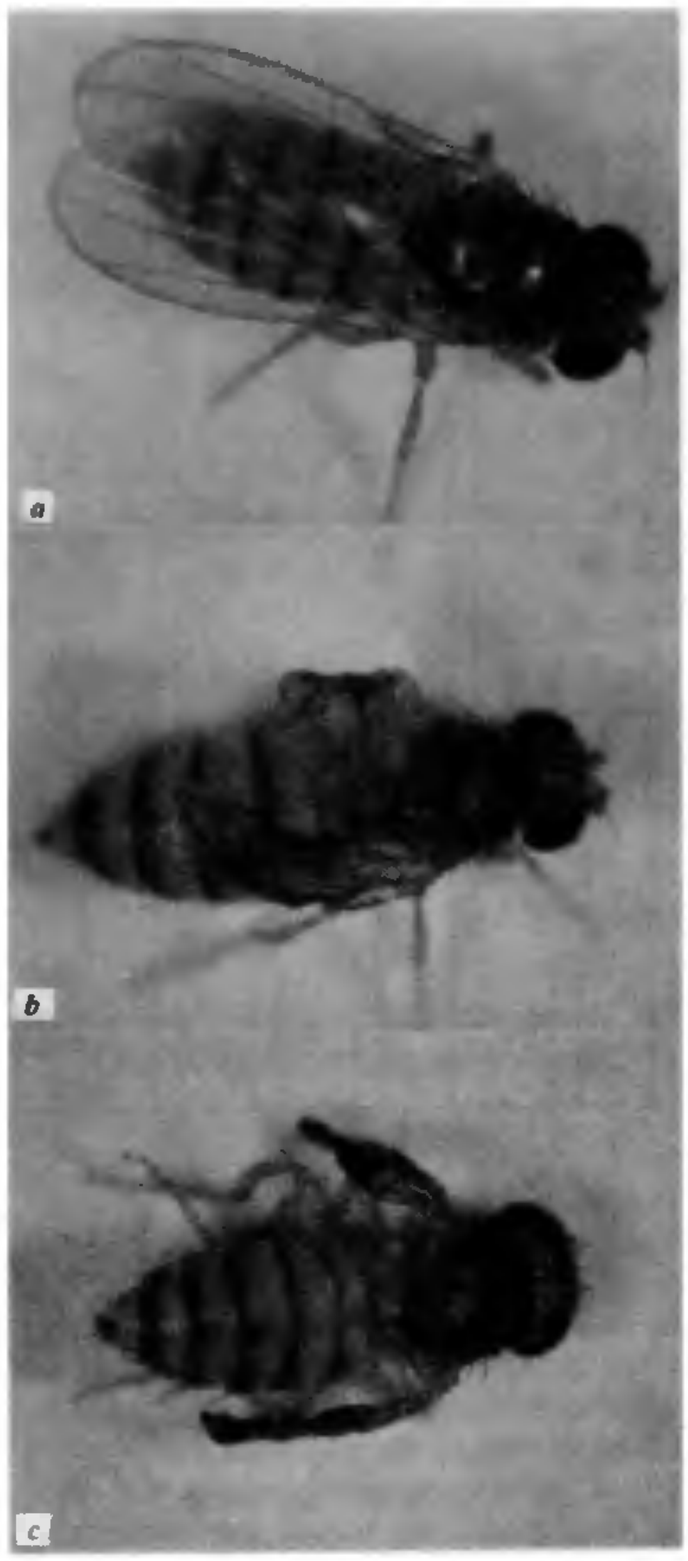

Figure 5 *a–c*. VAL-induced wing abnormalities. Flies resulting after treating larvae with normal food (*a*) and food containing VAL (*b* and *c*). Note unexpanded wings in *b* and *c*. Magnification × 90. Other details are as described in Table 1.

and use-dependent blockade of Na^+ channel[1]. The observed suppression of VAL-induced wing abnormality by *para*^{ts1} supports this hypothesis. In addition, it suggests that VAL also critically requires normal Na^+ channels for its deleterious effect on wing morphology.

Recently, the genetic, developmental and molecular analysis of a *D. melanogaster* gene, designated as *congested-like tracheae* or *colt*, have been described[19]. Although inactivation of *colt* does not result in any detectable effect on embryogenesis, its inactivation affects two physiological processes that occur during postembryonic development. The one which takes place during early larval development is the function of the tracheal epithelium. The absorption of the fluid contained in the tracheal system and its replacement by gas is found to be absent in numerous *colt* larvae and mutations in *colt* gene cause semi-lethality and death of a high proportion of first-instar larvae[19]. The other process affected by *colt* is the one which takes place at the time of eclosion and leads to the unfolding and expansion of the wings. This process is found to be severely impaired in *colt* adult escapers which are characterized by partially unfolded wings with loss of venation and reduced size, as well as poor viability and sterility. Since wing abnormalities observed in *colt* are, as reported here, partially mimicked by treating third instar larvae with VAL, studying the effect of the AED on *colt* expression during different developmental stages in both wild-type and *para*^{ts1} Na^+ channel mutant *Drosophila* would seem to be interesting. Genes involved in wing morphogenesis are known to encode diverse products such as mitochondrial metabolic enzymes, cell surface proteins and proteins involved in discrete signalling pathways[19,20]. *colt* encodes a putative mitochondrial carrier protein of unknown solute specificity. The predicted Colt protein shows a strong similarity with the Dif1 mitochondrial solute carrier of *Caenorhabditis elegans*[19]. The description of *colt* has thus indicated how a gene presumably involved in a mitochondrial metabolic process can specifically affect wing morphogenesis, by altering the differentiation of the wing epithelial cells and by blocking the unfolding and expansion of the wing after eclosion[19]. *Drosophila* larvae exposed to high levels of dietary ethanol is also known to cause unexpanded wings in flies[21]. It has been suggested that ethanol may produce toxic metabolites in mitochondria and thus affect normal *colt* function. The present observation of VAL causing unexpanded wings in flies may also similarly suggest that the drug produces toxic metabolites in mitochondria which in turn affect normal *colt* function. How *para*^{ts1}, a Na^+ channel mutation, suppresses VAL-induced non-expansion is however difficult to comprehend at this time. The antiepileptic effect of VAL is mediated by use- and voltage-dependent Na^+ channels. It is possible that normal Na^+ channels are also critically required for VAL-induced wing abnormality.

In vertebrates, a member of *Pax* family of pattern-forming genes has been recently suggested to be a molecular target in VAL axial skeletal teratogenicity[22]. Possibility has been raised that *Pax1* may help maintain borders between somites[23]. On this basis, *Pax1* is expected to share some functional commonality with the *Drosophila wingless* (*wg*), a segment polarity gene proposed to be involved in sealing of the borders between consecutive parasegments[23]. The possible targets of paired-box family of genes include genes encoding protein components of intercellular junctions involved in flow of molecules between cells[23]. In view of the above, investigating the effect of VAL on developmental expression of

wg in wild-type and *para*^{ts1} mutant would be perhaps valuable for further understanding of VAL teratogenicity.

In *Drosophila* imaginal leg discs, ectopic expression of *homothorax* (*hth*) or its murine homologue, *Meis1*, in the *Distal-less* (*Dll*) domain results in leg truncations[24]. Similar truncations are also generated by high levels of *extradenticles* (*exd*). It has been suggested that *hth/exd* act positively to maintain *Antennapedia* (*Antp*) expression in leg discs and that coexpression of *Antp* and *hth*, being incompatible with limb development, results in leg truncations[24]. The patterning of the proximal region of the leg requires *exd*, whereas that of the distal region requires signalling by Hedgehog (Hh) activated Wg and Decapentapelagic (Dpp)[25]. *Dll* is a Dpp/Wg response gene which prevents transport of Exd protein from cytoplasm to the nucleus; only nuclear Exd is functional. Recently, it has been found that the protein encoded by *PbxI*, a murine homologue of *exd*, is not only regulated at the subcellular level but also its pattern of nuclear localization in the mouse limb resembles that of the *exd* product in the *Drosophila* leg[25]. Moreover, *hh* mutant *Drosophila* and *Sonic hedgehog* (*Shh*) mutant mice show a similar limb phenotype, distal region lacking but proximal region unaltered. The above findings have indicated that the division of leg into two regions along the proximodistal axis, as defined by interactions between the *exd/Pbx* genes and the Hh pathway, is a general feature of leg development[25]. In addition to *Dll*, the expression of two other Dpp/Wg response genes, *dachshund* (*dac*) and *optomotor-blind* (*omb*) are also known to be confined within the region where *exd* is cytoplasmic. Recently, *hth* has been identified as a positive regulator of Exd nuclear translocation and it has been indicated that the role of *Dll*, and may be of other regulators, is likely to be mediated by repression of *hth*[25,26]. Keeping in view the above advancement in our understanding of leg development, it would be perhaps important to study the effect of PB on the expression of key genes involved in leg development in *Drosophila*.

The activity of nuclear factor regulating expression of kappa light-chain immunoglobulin (NF-kB), a well-known transcriptional regulator that was first identified in B cells, is mediated by a family of molecules, including *c-rel* gene product[27]. Recently, it has been reported that cells at the tip of chick limb buds express *c-rel* and that blocking NF-kB activity results in truncated or twisted limbs[28,29]. These results indicate a role of Rel/NF-kB transcription factors in vertebrate limb development. Moreover, mutations in *Twist* gene, one of the targets of NF-kB signalling, are also known to be responsible for the human Saethre–Chotzen syndrome in which limb defects are found[30]. Interestingly, the *Twist* expression is found to be reduced when NF-kB signalling is blocked[28,29]. The *Drosophila* homologue of NF-kB is the product of the gene *Dorsal*, which is known to activate and repress *Twist* and *dpp* expression respectively[27]. NF-kB signalling occurs by the same mechanism as *Dorsal* signalling. Its blockage leads to an increased expression of a gene encoding a bone morphogenetic protein, BMP-4, a vertebrate homologue of Dpp[29]. The bone morphogenetic proteins are multifunctional molecules implicated in skeletal differentiation, positional signalling and programmed cell death[31]. Considering the known teratogenic effect of PB in mammals, investigation of the effect of this drug, with respect to *Dorsal* signalling pathway, on embryonic development in *Drosophila* would seem to be informative.

It is currently unknown whether PB, PHT and CBZ themselves or their epoxide intermediates are toxic and teratogenic agents[5,10]. In case of alcohol teratogenicity, earlier work in *Drosophila* has supported, on the basis of known metabolism of ethanol to acetate without the accumulation of acetaldehyde in the fly, the contention that ethanol itself is primarily responsible for the 'fetal alcohol syndrome' in man[21]. The phenotypic features of children with prenatal exposure to AEDs are also shared by those with the fetal alcohol syndrome[32]. Interestingly, ethanol, like the AEDs reported here, has also been found to induce leg and wing abnormalities in *D. melanogaster*[21]. In addition, ethanol and anticonvulsants are found to produce similar effects on developing neurons *in vitro*[33]. As a common metabolic pathway cannot explain the above similarities, it has been argued that in teratogenesis it is perhaps the time of exposure to the agents during critical periods of fetal development which is more important than the teratogens or their metabolites[33]. In the current study, we have found the toxicity and teratogenicity profiles to be different among the AEDs. Although this may also be due simply to different effective doses of these drugs in flies, as nothing is known about their pharmacokinetics in *Drosophila*, it is noteworthy that their relative doses used here are more or less similar to those recommended for human therapy (Table 1). In view of the above, the AEDs seem to have drug-specific effects at least in *Drosophila*. Whether the AEDs act directly or through metabolites to produce these effects remains unclear. A knowledge of AEDs' metabolism in *Drosophila* would be required to understand this. Treatment of pregnant mice with 5-formyltetrahydrofolate *in vivo* has been reported to decrease the incidence of VAL-induced birth defects[34]. The protective effect of folinic acid however appeared to be due in part to the time of day of administration. Similarly, methionine is also reported to decrease embryotoxicity of VAL in rats[35]. Up to 70% of human neural tube defects (NTDs), one of the causative agents of which is considered to be VAL, can be prevented by administration of folic acid in the perioconceptual period[36]. The mechanism of the preventive action of folic acid is however unknown. Recently, disturbance of folate metabolism has been detected in *splotch* (*Pax3* gene is mutated in *splotch* mice) mouse embryos that develop NTDs *in vitro*[36]. A metabolic deficiency in the supply of folate for the biosynthesis of pyrimidine has been indicated in *splotch* embryos. It is reported that both exogenous folic acid and thymidine correct the biosynthetic defect and prevent some NTDs in *splotch* embryos, whereas methionine exhibit an exacerbating effect. In *Drosophila*, folic acid analogues aminopterin and amethopterin as well as 5-fluoro-2-deoxyuridine (FUdR) are known to cause some morphological aberrations[37]. The folic acid analogues inhibit the enzyme dihydrofolic acid reductase and prevent the synthesis of tetrahydrofolic acid, a cofactor of thymidylate synthase. Studies in different organisms have shown that FUdR

blocks the synthesis of thymidine by inhibition of thymidylate synthase. As expected, exogenous folic acid and thymidine prevent the morphological aberrations caused by folic acid analogues and FUdR in *Drosophila*[37]. Keeping the above findings in view, it would be perhaps interesting to investigate the effect of folic acid, thymidine and methionine on VAL-induced wing abnormality in *Drosophila*.

The mechanisms or sites of action, initial biochemical effects and cellular receptors involved in the toxicity and teratogenicity of the AEDs are largely unknown[2,5,7,38]. An increased understanding of the mechanisms underlying the toxic and teratogenic effects of these drugs could be gained by studying the molecular basis of direct effects of the parent compounds or their metabolites on specific cell types at various stages of development. Given the status of *Drosophila* as one of the most well studied eukaryotes, our results indicate that *Drosophila* might provide a better opportunity for the same. Earlier studies on the developmental toxicity of cycloheximide, vinblastine, ethanol and other teratogens have indeed suggested that the fruit fly is sensitive to mammalian teratogens and that it may be of value in the study of teratogenic effects[21]. The mechanism of teratogenesis is of fundamental importance to the fascinating new area of developmental ecology. Till recently, details about how teratogens work were lacking. We are now at least beginning to have some candidates such as alcohol and valproate[39]. *Drosophila* seems to hold a potential to serve as a model not only for studies involving known/suspected teratogens but also for testing novel compounds/drugs for teratogenic activities.

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Scent marking in the Asiatic lion

Schaller[1] first brought to the notice of scientists the scent marking of tiger. It is generally accepted that this fluid sprayed upward and backwards while in a standing posture and with the tail erect[1] is a source of pheromones[2]. This aspect has been later studied by McDougal[3], Jackson and Hillard[4] and, Schaller[5] in the tiger, snow-leopard and the African lion, respectively. Brahmachary *et al.* also studied the marking fluid of the tiger[6–9] and Poddar-