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Common Mechanism for Teratogenicity of Antiepileptic Drugs

Drug-Induced Embryonic Arrhythmia and Hypoxia-Reoxygenation Damage

BY

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ABSTRACT

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The Antiepilptic drugs (AEDs) phenytoin (PHT), carbamazepine (CBZ), phenobarbital (PB), tri- and dimethadione (TMD and DMD) are known teratogens having a common malformation pattern in human and animal studies. This thesis was designed chiefly to test a hypothesis correlating the teratogenicity of these AEDs to episodes of pharmacologically induced embryonic arrhythmia and hypoxia-reoxygenation damage.

Effects on the embryonic heart were studied both after maternal administration in mice and in mouse embryos cultured in vitro. Only AEDs, correlated with the same type of malformation as could be induced by episodes of interrupted oxygen supply to the embryo (e.g. cleft palate) caused concentration dependent bradycardia and arrhythmia. PHT and DMD had the highest potential and affected embryonic heart at clinically relevant concentration, followed by CBZ, TMD and PB. Valproate and vigabatrin not associated with hypoxia-related malformations caused neither arrhythmia nor severe bradycardia.

The results showed that the embryonic heart is extremely susceptible to PHT and DMD only during a restricted period of development, between gestational days 9-13 (weeks 5-9 of human pregnancy). An observed genetic susceptibility to react with arrhythmia at low concentrations when exposed to PHT or to external stress, could explain why A/J strain of mice is more susceptible to develop cleft palate compared to other strains. High activities of reactive oxygen species (ROS) capturing antioxidant enzymes observed in untreated A/J embryos supported this assumption. The potential to cause embryonic arrythmia by an AED was related to the potential to inhibit the rapid component of the delayed rectifier potassium channel (I_{kr}). A marked I_{kr} blocking activity (70%) of DMD in voltage clamping studies was observed. The I_{kr} inhibition occurred at similar concentrations, which causes severe arrhythmia.

The idea of a relation between teratogenicity and arrhythmia, resulting in ischemia followed by reperfusion and generation of ROS was supported by mechanistic studies. Pre-treatment with the spin-trapping agent PBN, which has the capacity to capture ROS, markedly reduced the incidence of PHT and DMD-induced cleft palate. In utero exposure to teratogenic doses of DMD and PHT resulted in hemorrhages in the embryonic palatal region. The same type of haemorrhage in the palatal region precedes orofacial clefts induced by episodic hypoxia.

Key words: Antiepileptic drugs, embryonic heart, arrhythmia, cleft palate, ROS, antioxidants, I_{kr}.

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PAPERS DISCUSSED

This thesis is based on the following publications/manuscript, which will be referred to by their Roman numbers:

I: Danielsson BR, Azarbayjani F, Sköld AC & Webster WS. 1997. Initiation of phenytoin teratogenesis: pharmacologically induced embryonic bradycardia and arrhythmia resulting in hypoxia and possible free radical damage at reoxygenation. Teratology 56:271-281.

II: Azarbayjani F, Danielsson BR. 1998. Pharmacologically induced embryonic dysrhythmia and episodes of hypoxia followed by reoxygenation: a common teratogenic mechanism for antiepileptic drugs? Teratology 57:117-126.

III: Azarbayjani F & Danielsson BR. 2001. Phenytoin-induced cleft plate: Evidence for embryonic cardiac bradyarrhythmia due to inhibition of delayed rectifier K^+ channels resulting in hypoxia-reoxygention damage. Teratology 63:152-160.

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V: Azarbayjani F, Borg LAH & Danielsson BR. Strain differences in susceptibility to phenytoin teratogenicity in relation to antioxidant enzyme activities. Manuscript.

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ABBREVIATIONS

AEDs	antiepileptic drugs
PB	phenobarbital
PHT	phenytoin
TMD	trimethadione
DMD	dimethadione
CBZ	carbamazepine
VPA	valproic acid
FH	fetal hydantoin syndrome
FADS	fetal antiepileptic drug syndrome
PGG2	prostaglandin G_2
PGH2	prostaglandin H
HERG	human ether-a-go-go-related gene
ROS	reactive oxygen species
I_k	delayed rectifier potassium channel
I_{kr}	rapid component of delayed rectifier potassium channel
I_{ks}	slow component of delayed rectifier potassium channel
GD	gestational day
RS	rat serum
TC	therapeutic concentration
SLB	slight bradycardia
SEB	severe bradycardia
a	arrhythmia
CA	cardiac arrest
PBN	α-phenyl-N-butyl-nitrone
FPIA	fluorescence polarization immunoassay
SOD	superoxide dismutase
CAT	catalase
GSH-px	glutathione peroxidase
NADPH	nicotinamide dinucleotide phosphate
MEM	minimal essential medium
O_2 .-	superoxide anion
OH.	hydroxyl radical

1. INTRODUCTION

1.1. Epilepsy

Epilepsy is one of the most common afflictions in man. With a prevalence of approximately 1%, it is estimated that 50 million persons worldwide have the disorder (Rogawski and Porter 1990). The term *epilepsy* is a collective designation for a group of central nervous system (CNS) disorders having in common the occurrence of recurrent unprovoked seizures[1]. The seizures are often correlated with abnormal and excessive discharges in the electroencephalogram (EEG). *Primary or idiopathic epilepsy* denotes those cases where no cause for the epilepsy can be identified. *Symptomatic epilepsy* designates the disorder having an identified underlying cause such as previous trauma, cerebrovascular and cardiovascular disease, neoplasm, infection and developmental abnormalities. The agreed clinical classification of seizures recognizes two major categories, namely partial[2] and generalized seizures[3], though there are many varieties of each (Rang and Dale 1995).

With optimal drug therapy, epilepsy is controlled completely in about 75% of patients whereas about 10% continue to have seizures at intervals of one month or less, which severely disrupt their life and work. There is therefore a need to improve the efficacy of therapy.

1.2. Epilepsy and pregnancy

Numerous epidemiological studies have shown that the offspring of epileptic mothers have a two to three fold higher risk of congenital malformations than the general population (Dansky and Finnell 1991). Each year 40000 infants are exposed to anticonvulsant drugs in *utero* worldwide, with the estimated birth of 1500-2000 infants having congenital malformations as a consequence of intra-uterine exposure to antiepileptic drugs (AEDs). Most studies indicate that the antiepileptic therapy rather than the maternal disease or convulsions is the major cause of malformations detected at birth. One example is an illustrative experimental study by Finnell and Chernoff (1982). In that study, they used an inbred strain of mice having a genetically determined spontaneous seizure disorder known as quaking (qk). The qk/qk mice had several seizures a day throughout gestation, yet produced normal healthy pups. Upon treatment with AEDs, the seizure frequency diminished as the malformation rate increased, showing that the AEDs were the cause of teratogenicity. Another example

[1] Term used to describe occurrence of episodic high-frequency discharges of impulses by a group of neurons in the brain.

[2] Focal, local seizures

[3] When the entire brain including the reticular system, is involve thus producing abnormal electrical activity throughout both hemispheres.

is a recent clinical study by Holmes et al. (2000), showing no increase in malformations in offspring of mothers with epilepsy, who were not treated with AEDs.

Although there is a risk of birth defects with AED therapy during pregnancy, the deleterious effects on the fetus of an uncontrolled seizure during pregnancy and at labour outweigh the risk of malformations for most epileptic women. About 30% of women with treated epilepsy will have an increase in seizure frequency during pregnancy (Schmidt et al. 1983). Several factors may contribute, among which hormonal (increase in serum estrogens), metabolic (increased sodium and water retention), psychological, pharmacokinetic (altered plasma protein binding) and poor compliance with therapy during pregnancy are the most frequent. The link between grand mal seizure[4] and fetal death (Hiilesmaa 1982), increased maternal and fetal mortality related to status epilepticus[5] and adverse effects of generalized seizure[6] on the fetus (hypoxia and acidosis), entails continued medication despite the awareness of the teratogenicity of AEDs (Orringer et al. 1977; Teramo et al. 1979).

1.3. Characteristics and malformation pattern of different AEDs

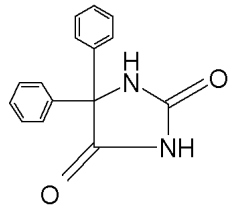
Several antiepileptic drugs, such as phenobarbital (PB), phenytoin (PHT), trimethadione (TMD), carbamazepine (CBZ) and valproic acid (VPA) drugs, have been shown to have teratogenic potential in studies in humans and other animals (Finnell and Dansky 1991). Although there is a wide variety of specific targets, all AEDs ultimately exert their actions by altering the activity of the basic mediators of neuronal excitability: voltage and neurotransmitter-gated ion channels. The chemical structure, pharmacological properties and pattern of malformation of each compound are discussed below.

[4] Frequently proceeded by an aura, in which a sudden loss of consciousness is immediately followed by generalized convulsions.

[5] An epileptic seizure that is sufficiently prolonged or repeated so as to produce a fixed and lasting epileptic condition.

[6] Epilepsy in which seizures are generalized; they may have a local onset or be generalized from the beginning.

Phenytoin



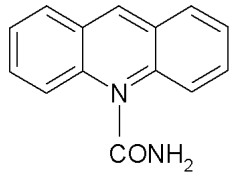
Pharmacological properties

Phenytoin (PHT) can interact with the voltage-dependent Na^+, Ca^{2+} and K^+ channels in a highly specific voltage and frequency dependent manner (Yaari et al. 1986; Twombly et al. 1988; Rogawski and Porter 1990; Nobile and Vercellino 1997). Voltage-dependent Na^+ channels are responsible for the action potential upstroke; L-type Ca^{2+} channels play a vital role in neurotransmitter release and in the control of neuronal excitation and finally; K^+ channels are responsible for the shape of the action potential and duration of the plateau. By interacting with these channels (mainly Na^+), phenytoin is capable of stabilizing the excitable cells and therefore has the ability to suppress seizure (Rogawski and Porter 1990).

Malformation pattern

PHT exposure in utero has been associated with a pattern of abnormalities known as fetal hydantoin syndrome (FHS), consisting of minor anomalies such as midfacial hypoplasia with a snub nose, a broad nasal bridge, ocular hypertelorism and an arched upper lip, and hypoplasia of distal phalanges and nails. Major anomalies such as cleft lip and palate and cardiac anomalies and pre and postnatal growth retardation (microcephaly and mental retardation) have also been associated with *in utero* exposure to PHT in humans (Hanson 1986; Dansky and Finnell 1991). The same pattern of malformations has been shown to be induced in mice after exposure to PHT by different routes of administration (Sullivan and McElhatton 1975; Vorhees 1983).

Carbamazepine



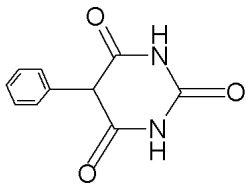
Pharmacological properties

Carbamazepine (CBZ) and phenytoin have a similar spectrum of anticonvulsant activity in animal seizure models. Like PHT, CBZ blocks voltage dependent Na^+ and K^+ channels, the former resulting in a limited sustained high-frequency repetitive firing of cultured mammalian central neurons at clinically relevant concentrations (McLean and McDonald 1986). CBZ has been shown to interact with excitatory glutamate receptors. The NMDA receptor is the focus of attention because its excessive activation has been implicated in the pathophysiology of epilepsy (Olney 1993).

Malformation pattern

Carbamazepine (CBZ) is also teratogenic causing an increased incidence of craniofacial and limb development in exposed children (Hiilesmaa et al. 1981; Bertollini et al. 1987 Jones et al. 1989) and even experimental animals (Sullivan and McElhatton 1977; Paulson et al. 1979; Vorhees et al. 1990). Buehler and co-workers (1987) report a striking similarity between the malformation pattern in children exposed to CBZ and fetal hydantoin syndrome, and concluded that they share the same teratogenic mechanism.

Phenobarbital



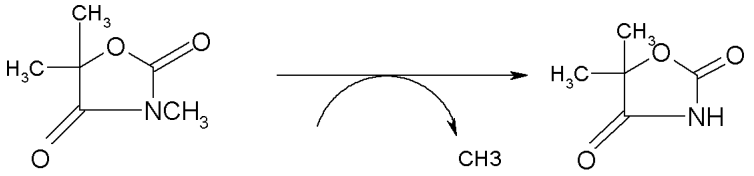
Pharmacological properties

Phenobarbital (PB) exerts its pharmacological action by blocking the voltage-dependent Ca^{2+} channels in nerve terminals, and it has also been shown that barbiturates can inhibit depolarization stimulated Ca^{2+} influx into synaptosomes, thus leading to reduced depolarization evoked neurotransmitter release in a wide variety of systems (Kalant and Grose 1967; Richter and Waller 1977; Coleman-Riese and Cutler 1978; Nicoll and Iwamoto 1978).

Malformation pattern

Barbiturates have also been associated with the same major and minor abnormalities and dysmorphic features as with PHT. These include congenital heart defects, facial clefts, craniofacial abnormalities and growth deficiency (Seip 1976; Rating et al. 1982; Kallén et al.1989). Due to the large overlap in the pattern of teratogenicity between PHT and PB, Majewski and co-workers (1981) proposed the term “Hydantoin-Barbiturate Embryopathy”.

Tri- and dimethadione



Trimethadione Dimethadione

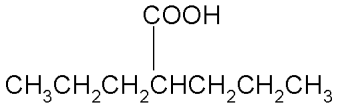
Pharmacological properties

Trimethadione (TMD) is demethylated by hepatic microsomal enzymes to its reactive metabolite dimethadione (DMD). Both drugs block the T-type Ca^{2+} and L-type Ca^{2+} currents (Coulter et al. 1989).

Malformation pattern

Due to the rapid and extensive metabolism of TMD to its demethylated, active metabolite, DMD, the latter has been regarded as the primary teratogen, the teratogenicity of which is well established in human and in experimental animals (Nichols 1973; Zackai et al.1975; Feldman et al. 1977; Rosen and Lightner 1978; Brown et al. 1979; Rischbieth 1979). The pattern of malformation induced by DMD resembles that of PHT, and many investigators have implied that in fact the fetal hydantoin and trimethadione syndrome could be used interchangeably. However, actually DMD has caused much more serious teratogenicity in both animals and clinical studies, and a higher Relative Teratogenic Index (RTI)[7] calculated for DMD, compared with PHT (4.0 versus 1.6 respectively) has been regarded as an indication of such in animal studies (Fabro et al. 1982). Due to the rapid and extensive metabolism of TMD to its demethylated, active metabolite DMD, the latter has been regarded as the primary teratogen.

Valproic acid



Pharmacological properties

Valproic acid (VPA) is a branched-chain fatty acid, structurally unrelated to any other antiepileptic drug. The anticonvulsant activity of VPA relies primarily on enhancing GABA levels in plasma and CSF, possibly by inhibiting GABA-T (Löscher 1980; Löscher and Siemes 1985). The effect on the GABA-T is however much weaker than that of the conventional inhibitors such as vigabatrin

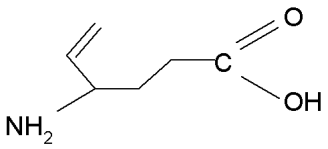
[7] RTI is the ratio of LD01 to tD50 and is a useful parameter in comparative studies as components of objective risk assessment.

(Larsson et al. 1986). Like PHT and CBZ, VPA limits the ability of neurons to fire Na^+-dependent action potentials at high frequency (McLean and McDonald 1986).

Malformation pattern

VPA has been reported to be teratogenic (Vorhees 1987a). A fetal valproate syndrome has been described which is characterized by posterior neural tube defects and a distinctive facial appearance featuring upward slanting palpebral fissures, epicanthic folds, and posteriorly rotated ears (DiLiberti et al. 1984).

Vigabatrin



Pharmacological properties

Vigabatrin, a γ-vinyl analogue of GABA, is designed to increase the GABA in brain by selectively and irreversibly inhibiting GABA-T, the enzyme that catabolizes GABA (Ben-Menachem et al. 1995; Meldrum 1996; Petroff et al. 1999). After treatment with VGB, patients demonstrate an increase in free GABA levels in the cerebrospinal fluid (CSF) (Petroff et al. 1999).

Malformation pattern

There is too little clinical and animal data available to assess the teratogenic potential of vigabatrin.

1.4. Mechanisms of teratogenicity of antiepileptic drugs

Despite the widespread use of AEDs and knowledge of their teratogenicity, the mechanism by which they damage the developing embryo is still not fully understood. Due to a great deal of overlap between the drug-specific syndromes for PB, PHT, TMD, DMD and CBZ, a term “fetal antiepileptic drug syndrome” (FADS) has been proposed to more appropriately describe these anomalies as opposed to a specific syndrome for each individual drug (Finnell et al. 1997). Due to the very similar pattern of malformations of these AED it has also been assumed that the malformations are caused by a common mechanism (Vorhees 1987b; Finnell 1991; Danielsson 1997). This idea is also supported by a significant increase in the risk of malformations if combination treatment is initiated (Lindhout 1992).

The malformation pattern of VPA is markedly different (Dansky and Finnell 1991; Lindhout and Omtzigt 1992), which suggests a different mechanism of teratogenicity for this compound.

The list of suggested mechanisms for AED teratogenicity is long and diverse, including alteration in thyroid status with lower levels of thyroxine and thyrotropine, interaction with glucocorticoid receptors (Goldman et al. 1987) and vitamin K metabolism (Yerby 1987; Ramsay and Slater 1991), disturbances in folate metabolism (Monie et al. 1961; Hansen and Billings 1985), bioactivation of PHT to a reactive toxic intermediate (epoxide) by cytochrome P450 (Martz et al. 1977) and cooxidation of phenytoin to free radical intermediates centered in the hydantoin nucleus (Wells et al. 1989a). The most recent theory is that the AEDs cause embryonic cardiac arrhythmia, resulting in hypoxia-reoxygenation damage (Danielsson et al. 1995). The four most important theories are discussed below.

Teratogenicity related to altered endogenous metabolism

Disturbances in folate metabolism

Folic acid is an essential vitamin used in single carbon metabolism, including nucleotide synthesis and DNA methylation (Hansen and Billings 1985). Since humans cannot synthesize folic acid, they are dependent on dietary sources. Dietary folate should be monoglutamated and reduced in order to be able to enter the circulation. Many enzymes are involved in this process, among which folylpolyglutamate hydrolase, 5,10-methylenetetrahydrofolate reductase and dihydrofolate reductase are the most important (Rosenberg and Selhub 1986; Hansen 1997). Rapidly growing and developing embryos have an increased requirement for folate. An abnormal pattern of folate metabolism would result in a decreased rate of DNA synthesis and gene methylation, with deleterious effects on the developing embryo (Finnell et al. 1991). Patients undergoing AED therapy usually develop folate deficiency (Pritchard et al. 1971; Reynolds 1974), and folate deficiency has been related to impaired development of offspring born by these mothers both in humans (Smithells et al. 1976) and in experimental animals (Tagbo and Hill 1977; Hansen and Billings 1985).

Exposure to PHT and folate deficiency

The similarity in the pattern of malformations caused by exposure to PHT and that after exposure to folate antagonists, such as aminopterin (Goetsch 1962) and methotrexate (Milunsky et al. 1968) has been the main reason why investigators have attributed that PHT-induced teratogenesis to disturbed folate metabolism (Meadow 1968; Hoffbrand and Necheles 1968; Kariks et al. 1971). However, data on the effect of dosing pregnant animals with a combination of the folate

or folinic acid (metabolically active formylated tertrahydrofolate) with PHT are inconclusive. Results have ranged from no change to a protective effect or enhancement of PHT teratogenicity (Schardein 1973; Sullivan and McElhatton 1975; Dansky et al. 1986).

Exposure to VPA and folate deficiency

Several studies have attributed to development of neural tube defects in humans to low blood folate levels (Smithells 1976; Dansky 1989, ph. D. thesis; Dansky et al. 1989). Neural tube defects have been reported to be a major teratogenic outcome after *in utero* exposure to VPA and CBZ and not PHT and PB. The risk of recurrence of neural tube defects in a mother who has a child with neural tube defects has been reduced by supplementation with folate prior to conception. However, there are some discrepancies in the importance of folate supplementation in preventing neural tube defects. PHT and PB, in particular, which cause a marked decrease in the folic acid levels, are not as strongly associated with neural tube defects as are VPA and CBZ, which have less influence on folic acid.

It remains to be established whether folate supplementation before and early in pregnancy is of any benefit to epileptic women undergoing AED therapy.

1.5. Teratogenicity related to chemical structure

Bioactivation to reactive intermediates

Epoxies

Numerous studies have suggested that the teratogenicity of AEDs might be dependent on bioactivation of these compounds to reactive epoxides. Bioactivation via the cytochrome P450 system to an arene oxide metabolite that is able to arylate various embryonic components, is the most credible. The theory relates the existence of a phenyl substituent, which is needed for the formation of epoxide, as a prerequisite for structurally related AEDs. However, there are some observations that cannot be readily explained by this hypothesis:

a) Inhibition and potentiation of PHT teratogenicity by pretreatment with Phenobarbital (cytochrome P-450 inducer) and SKF 525A (cytochrome P450 inhibitor) (Harbison and Becker 1971);
b) Teratogenicity of tri- and dimethadione, which do not possess the phenyl substituent required for the formation of arene oxide (Wells et al. 1989b);
c) L-isomers of nirvanol and mephenytoin, which primarily do not produce arene oxide, have been shown to be teratogenic (Wells et al. 1982).

Free radical formation via prostaglandin pathway

A more recent hypothesis, initially put forward by Wells and colleagues (1989b), attributes the teratogenicity of PHT and tri/dimethadione to the formation of free radicals of the drug during the normal metabolism of free arachidonic acid to prostaglandin G_2 (PGG_2) and H_2 (PGH_2). Two different enzymes are involved in this conversion, namely cyclooxygenase (conversion of arachidonic acid to PGG_2) and hydroperoxidase (conversion of PGG_2 to PGH_2). Based on their theory, the reduction step catalyzed by hydroperoxidase could utilize a number of drugs as co-factors and convert them to free radicals. The drug radical formed in this reaction could attack a wide range of targets in the developing organism and initiate oxidative stress (Liu and Wells 1994, 1995).

1.6. Teratogenicity related to pharmacological action of AEDs

Hypoxia due to pharmacological effects on the maternal heart

The first proposal in this direction was published in 1983, when Millicovsky and Millicovsky reported that A/J mice treated with PHT develop marked maternal bradycardia. They postulated that PHT teratogenicity might be related to the induced maternal hypoxia produced by the bradycardia (Watkinson and Millicovsky 1983). This idea was supported by results showing that maternal hyperoxia greatly reduced the incidence of PHT-induced malformations (Millicovsky and Johnston 1981). The human relevance of this theory can be questioned, as patients treated with PHT do not develop marked bradycardia.

Pharmacologically induced embryonic hypoxia mediated via induction of embryonic bradycardia and cardiac arrest

Interestingly, in the early 1990s, Danielsson and colleagues showed in a series of studies that exposure of pregnant rabbits to a teratogenic dose of PHT, resulting in free concentrations of the drug in the upper range of the recommended therapeutic interval, had little or no effect on maternal heart rate or blood pressure. Additionally, the induced malformations included distal digital reduction defects and orofacial cleft were preceded by edema, vascular disruption, hemorrhage and necrosis. These changes seen after PHT administration were almost the same as those seen after interrupted oxygen supply to the embryo. Hence it was concluded that embryonic (rather than maternal) ischemia /hypoxia plays an important role in PHT teratogenicity (Danielsson et al. 1991,1992; Danielson et al. 1992). It was further suggested that embryonic hypoxia was probably mediated via the pharmacological effects of PHT on the embryonic heart (Danielsson 1995).

At the same time, another class of drugs, selective I_{kr} blockers, were found in animal studies to cause very similar developmental defects to PHT. Reported major defects were orofacial clefts, distal digital reductions, cardiovascular defects and growth retardation (Ban et al. 1994; Webster et al. 1996; Marks and Terry 1996). Even more interesting, in relation to the proposed theory for teratogenicity of PHT, was that the teratogenicity was related to hypoxia secondary to the effect of these drugs on the embryonic heart, e.g. dose-dependent bradycardia and arrhythmia both in vitro and in vivo (Ban et al. 1992; Konishi et al. 1992; Abrahamsson et al. 1994; Webster et al. 1996). I_{kr} blockers have also been shown to increase the risk of lethal arrhythmia in patients. The risk is particularly higher in patients who have a defect (mutation) in the gene that encodes I_{kr}, e.g. Human ether a-go-go-related gene, HERG (Vincent et al. 1992; Curran et al. 1995).

In view of the suggested hypoxia-related teratogenicity, recent research indicates that adverse fetal effects after episodes of ischemia/hypoxia are caused by reperfusion/reoxygenation damage and generation of reactive oxygen species (ROS) (Fantel et al. 1992a). It has been shown that ROS are generated in cultured rat embryos exposed to episodes of hypoxia (30 min), followed by episodes of normoxia, during a stage when distal digital defects were induced after interrupted oxygen supply in live rats (Fantel et al. 1992b). It has also been shown that the generation of ROS is associated with vascular disruption in the distal part of embryo limbs.

1.7. Genetic susceptibility to teratogenicity of AEDs

Genetic susceptibility to the teratogenic effects of anticonvulsant drugs has been disputed for some decades. The fact that not all infants of epileptic mothers undergoing AED therapy are born with congenital birth defects emphasizes the importance of fetal genotype and its interaction with various environmental factors (Hansen and Hodes 1983b). In fact, genetic susceptibility to the teratogenicity of PHT has been demonstrated in different strains of mice. A difference in sensitivity to PHT-induced cleft palate between the A/J and C57BL/6 strains of mouse has been shown (Hansen och Hodes 1983a,b). Thus, A/J mice were susceptible to PHT-induced cleft lip/palate, whereas C57BL/6 mice were relatively resistant. Genetic predisposition in susceptibility of the embryonic heart to the arrhythmogenic effects of PHT is one suggested explanation (Danielsson et al. 1992).

Genetic predisposition might also be related to the content and potency of antioxidant enzymes (between the three strains of mice mentioned above) in capturing and detoxifying the harmful reactive species. As mentioned above, there is evidence that reactive ROS, generated in the embryo, may be directly responsible for certain teratogenic effects, such as cleft palate. Weak activity

of antioxidant enzymes such as superoxide dismutase (SOD), catalse (CAT) and glutathione peroxidase (GSH-px) in mitigating the toxicity of ROS in A/J embryos, may be an alternative explanation as to why the A/J strain is much more susceptible to the induction orofacial clefts after exposure to PHT.

2. AIMS OF THE STUDY

The overall aim of the study was to test the hypothesis that teratogenicity of PHT, TMD, PB and CBZ, which are known to cause the same types of malformation as hypoxia, is related to episodes of pharmacologically induced embryonic arrhythmia and hypoxia-reoxygen damage.

The specific aims of the study were:

1. To investigate the potential of different AEDs to induce embryonic arrhythmia. Both AEDs that have been associated with hypoxia-related malformations (PHT, TMD, CBZ and PB) and AEDs (valproate and vigabatrin), which have not been so associated, were studied.

2. To establish whether the embryonic arrhythmogenic potential of AEDs is related to their pharmacological effect on I_{kr}.

3. To ascertain whether PHT- and DMD-induced hypoxia-related cleft palate is preceded by the same early changes as reported after interruption of oxygen supply to the embryo.

4. To elucidate the role of hypoxia/reoxygenation damage and formation of reactive oxygen species (ROS) in arrhythmia-hypoxia related teratogenicity.

5. To study if genetically determined susceptibility to PHT teratogenicity might be related to (a) increased susceptibility to develop embryonic arrhythmia when exposed to PHT, (b) decreased endogenous antioxidant status in PHT in susceptible embryos.

3. MATERIALS AND METHODS

3.1. General aspects

Animal maintenance and housing

Mice were used for all the studies performed. The animals were allowed free access to water and a conventional pellet diet. The 24 h following detection of the vaginal plug was designated day 0 of gestation (GD 0) of pregnancy in all the studies. The Medical Ethics Committee of Uppsala University approved all animal work.

Whole embryo culture

In an attempt to study the effect of exposure to AEDs on the embryonic heart, New's modified culturing method (New et al. 1978) was employed. Embryos were explanted on different days of gestation, depending on the experiment (detailed information is shown in Table 1). The abdomen was opened, the uterus excised and decidual swellings removed under aseptic conditions. The decidua and Reichert's membrane were removed from each swelling and the embryos, with intact yolk sac (except for rat embryos on GD 13 which were cultured with an open yolk sac according to the method described by Spence et al. 1994) and ectoplacental cone, were placed in sterile culture bottles containing culture medium. This medium consisted of different compositions of rat serum (RS) and a buffer or physiological saline (for details see Table 1). The culture bottles were gassed and capped tightly and cultured in a rotating culture system at 37^{o}C and 40 rpm. Embryos with morphologically normal appearance, good yolk sac circulation and an adequate heartbeat were selected for each experiment. After the selection of embryos, test substances were added to each culture bottle. Embryos were then kept in culture for one and up to 22 h (depending on the experiment, see Table 1) after which the heart rate was measured and the incidence of arrhythmia and cardiac arrest was noted.

3.2. Determination of adverse effects on the embryonic heart

In-vitro studies

Effects on embryonic heart rate and heart rhythm (Papers I, II)

The heart rate of embryos cultured in vitro was counted for 15 sec on a heated stage that was used to maintain the culture bottles at 37-38°C. The embryos were examined for the existence of arrhythmia or cardiac arrest. The effects on the embryonic heart were videotaped for documentation and further investigation using a video camera connected to the microscope.

	Species	Strain	Culture day	Culture duration (h)	Culture medium	Post-dosing heart rate/rhythm examination (h)
Paper I	Mouse	CD-1	GD 9	19	20% saline in RS	1, $1^{1/2}$, 2
	Mouse	C57BL/6	GD 9	19	20% saline in RS	1, $1^{1/2}$, 2
	Mouse	A/J	GD 9.5	19	20% saline in RS	1, $1^{1/2}$, 2
	Rat	SD	GD 10	22	25% tyrode's in RS	22
	Rat	SD	GD 13	3	25% tyrode's in RS	2
	Rat	SD	GD 11	3	Eagle's medium	2
Paper II	Mouse	CD-1	GD10	4	20% RS in Tyrode's	$1^{1/2}$

Table 1.
RS= Rat serum, SD= Sprague-Dawley

Reversibility of effects on embryonic heart (Paper I)

In order to ascertain if the effects on the embryonic heart were reversible, the culture medium containing the drug was replaced with a fresh medium without the drug and the effect on heart function was measured after 90 min.

Ranking the drugs according to their potential to cause adverse cardiac effects in the embryo (Paper II)

The scoring system was designed to rank the drugs according to their potential to cause bradycardia, arrhythmia and cardiac arrest in relation to human therapeutic concentration. Scoring was based on the ratio between the concentration when adverse effects on the embryonic heart rate and rhythm were first discove-

red *in vitro* and the highest therapeutic concentration (TC) of the drug. The adverse effects examined were as follows:

1. Slight bradycardia (SLB); defined as a statistically significant decrease in heart rate compared with original heart rate (before adding the drug).
2. Severe bradycardia (SEB); defined as >40% decrease in heart rate, compared with that of unexposed embryos.
3. Irregular heart rate; arrhythmia or cardiac arrest (a/CA).

If the AED studied had the potential to induce a certain adverse effect (SLB, SEB or a/CA) at concentrations 1-4 times the highest therapeutic concentration (TC), it was given the highest score of 10; at 5-9 times TC, score of 5; at 10-15 times the TC, the score was 3; at 16-20 times the TC, the score was 2; and finally a score of 0 was given if the AED caused adverse cardiac effects at concentrations >20 times TC. Hence, the higher the score, the higher the potential of that AED to cause adverse effects on the embryonic heart.

Effects on embryonic heart rhythm following maternal exposure

The main purpose of this series of studies was to ascertain if maternal exposure to PHT and DMD cause embryonic heart rate and rhythm changes in the same way as observed in in vitro studies. Different procedures were followed, as described below.

Embryo heart rhythm after exteriorizing the embryos followed by WEC (Paper IV)

The pregnant mice were given an i.p. dose of either DMD (1000 mg/kg) or saline on GD 10. Three h later, laparotomy was performed, the uterine horns were exteriorized and the embryos were removed and cultured for 30 min. Their heart rate was examined for arrhythmia using a dissecting microscope.

Embryonic heart rhythm shortly after exposure to PHT and DMD (Papers III and IV)

The pregnant mice were dosed with DMD (1000 mg/kg) and /or PHT (85 mg/kg) or saline (controls) on GD 10 and 11. Pregnancy was terminated either on GD 12 (28 h after the last dose) or GD 13 (48 h after the last dose, only in DMD studies). The abdomen was opened and the embryos were examined to determine the heart rate and existence of arrhythmia or cardiac arrest immediately after their removal. The somites were counted as an indication of embryonic growth and development.

Period of sensitivity of embryonic heart (Papers III and IV)

PHT and DMD were administered as a single, maternal i.p. dose of 85 mg/kg and 1000 mg/kg for PHT and DMD respectively on GDs 9-16 (except for PHT which was not given on GD 11). The animals were sacrificed 4 h after dosing and the embryos were explanted without the extra-embryonic membranes. The embryonic heart rate and rhythm (arrhythmia, non-sinus rhythm) was examined under a dissecting microscope while keeping the embryos in a petri dish containing warm Tyrode's buffer, placed on a heated stage.

Dose and concentration dependence of effects on embryonic heart (Papers III and IV)

Pregnant animals on GD 12 were given an i.p. injection PHT (10, 25 and 85 mg/kg) or DMD (125, 250, 500 and 1000 mg/kg). The effect on the heart rate and rhythm was recorded as described earlier.

3.3. Morphological studies

Early pathological changes in the palatal region after exposure to PHT and DMD (Papers III and IV)

To detect early histological changes following an in vivo administration of a teratogenic dose of PHT (85 mg/kg i.p.), the following procedure was followed. On the morning of GD 10 and 11, a teratogenic dose of PHT (85 mg/kg i.p.), was given to the pregnant animal and the animal was sacrificed 28 h after the administration of the dose on GD 11. The embryos were freed from all the extra-embryonic membranes and examined for existence of hemorrhages in the palatal region. To examine the effects of maternal exposure to PHT and DMD on the embryonic heart at this stage, the heart rate and rhythm of the embryos were examined and videotaped prior to the procedure for detecting the hemorrhages.

Examination of developmental outcome at birth (Papers III and IV)

To study the effect of PHT and DMD exposure on the developing palate, PHT and DMD (85 and 1000 mg/kg respectively) were given to each experimental animal as single i.p. doses on GD 10 and 11. On GD 18 (168 h after the last dose), the animals were sacrificed and the incidence of early resorption, late fetal death, cleft palate and fetal weight was recorded using the litter as the experimental unit. Cleft palate and fetal weight were used as markers of developmental toxicity, as these endpoints have consistently been associated with intra-uterine exposure to both PHT and DMD in both human and animal studies.

3.4. Plasma concentration of PHT and DMO after maternal administration

Plasma concentration of PHT (Paper III)

Maternal blood was drawn from the abdominal aorta 4 h after PHT administration (doses of 85, 25 and 10 mg/kg). Plasma was immediately separated by centrifugation and stored at –20°C. The total and free fractions of the drug were separated by ultrafiltration and the plasma concentration of each was estimated using the fluorescence polarization immunoassay (FPIA) method in a commercially available kit (Wilson et al. 1978; Othman et al. 1987)

Plasma concentration of DMD (Paper IV)

Maternal blood was drawn from the abdominal aorta 4 h after DMD administration (doses of 1000, 500, 250 and 125 mg/kg). Plasma was immediately separated by centrifugation. The plasma concentration of the total drug (DMD is not bound to the plasma proteins) was analyzed using a modified method of Gazdzik and others (Gazdzik et al. 1987).

3.5. Possible role of ROS in teratogenicity (Papers IV and V)

To examine the role of reactive species in the teratogenicity of PHT (85 mg/kg) and DMD (1000 mg/kg), the spin trapping agent α-phenyl-N-butyl-nitrone (PBN) was administered to pregnant animals exposed to a teratogenic dose of PHT or DMD, and the effect on the incidence of cleft palate was studied. Due to the short half-life of PBN (3-4 h Chen et al. 1990) compared with PHT (42 h Mirkin 1971) and DMD (72 h Wells et al. 1989b) a repeated dosing regimen was also tested. Thus the pregnant animals were dosed with PBN (7.5 mg/kg or 75 mg/kg) alone or in combination with PHT and DMD (1h prior to and/or 1h prior and also 8 h after administration of PHT and DMD).

3.6. Genetic differences in teratogenicity

Genetic differences in susceptibility to PHT-induced embryonic heart rate and rhythm changes

Three mouse strains known for their differences in susceptibility to PHT teratogenicity viz. CD-1, C57BL/6J and A/J, were selected. The effects of an increasing concentration of PHT on the embryonic heart were studied by the techniques described earlier.

Levels of endogenous scavenging enzymes in three mouse strains

The procedure was arranged in order to measure the activity of the most important endogenous scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px) in the embryos and maternal liver of three strains of mice viz. A/J, C57BL/& and CD-1. These strains are known for their differences in susceptibility to the teratogenic effects of AEDs. Maternal liver and whole embryos without extra-embryonic membranes, on GD 10, were homogenized by sonication (20 KHz, 30 W) and dissolved in appropriate buffers (for details, see below).

Superoxide dismutase (SOD; E.C. 1.15.1.1)

The activity of SOD was measured by its inhibition of the chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which was induced by superoxide anions produced by the action of xanthine oxidase (E.C. 1.1.3.22) on xanthine, using a modified method by Puget and Michaelson (1974).

Catalase (CAT; E.C. 1.11.1.6)

The activity of CAT was measured by a sensitive spectrophotometric method for determination of catalase activity in small tissue samples (Johansson and Borg 1988).

Glutathione peroxidase (GSH-px; E.C. 1.11.1.9)

An enzymatic cycling method, initially described by Paglia and Valentine (1967) was used.Catalase activity were blocked by the presence of azide. The enzymatic cycling reaction gave a linear decrease in NADPH concentration for up to 10 min. The activity of GSH-px was determined by the hydroperoxide-specific oxidation of NADPH, and was calculated using a molar absorption coefficient for NADPH of 6200 $l \cdot mol^{-1} \cdot cm^{-1}$.

3.7. Effects on the rapid component of the delayed rectifier current (I_{kr})

Voltage clamp studies (Paper IV)

In HERG-transfected mouse Itk-fibroblasts (L-cells)

Preliminary studies on the effects of AEDs on the delayed rectifier potassium current were performed at Astra, Hässle, (Göteborg/Sweden). Mouse Itk-fibroblasts (L-cells) transfected with HERG-containing vector and selected for HERG expression using 0.5 mg/ml gentamicin G418, a neomycin antibiotic were utilized. HERG currents were measured using a modified method by Carlsson et

al. (1997).

Simply: HERG currents were measured using the single electrode whole cell voltage clamp technique. Voltage clamp was achieved using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Amplifier control and data acquisition were performed with Pclamp6 Clampex software (Axon Instruments) running on a Compaq Deskpro computer. The voltage clamp protocol is: holding potential -80 mV, activation clamp step to 0 mV for 2000 msec, tail currents elicited by returning the membrane potential to -60 mV for 4000 msec, frequency 0.1 Hz.

HERG-transfected Human Embryo Kidney (HEK) cells

Since a delayed rectifier current operates in the adult human heart, another more reliable cell line was utilized to study the effect of AEDs on this current. Thus, HEK293 cells transfected with HERG cDNA from the University of Wisconsin were utilized. The cells were maintained in minimal essential medium (MEM) supplemented with 10% calf serum and gentamicin. Membrane currents were recorded using an Axopatch 1D patch clamp amplifier. Once a stable patch was achieved, recording commenced in voltage clamping mode, with the cell initially clamped at –80 mV. Currents were elicited by stepping the membrane potential to +20mV (outward current, specific for I_{kr}), and then to –50 mV (tail current). Computer software (pCLAMP; Axon Instruments Foster City; CA) was used to generate voltage-clamp protocols, acquire data, and analyse voltage and current signals. Curve fitting was done using a non-linear least- square regression analysis.

4. RESULTS AND DISCUSSION

In this section an attempt is made to address the questions raised in the "Specific Aims". The overall hypothesis suggests a causal relationship between drug-induced embryonic arrhythmia (due to evidence of I_{kr} blocking potential for some AEDs) and reported hypoxia-related malformations. Special attention will be paid to comparing the results in the present study with those obtained after temporary interruption of oxygen supply to the embryo and after administration of selective I_{kr} blocking drugs.

4.1. Effects of AEDs on the embryonic heart

Effects on embryonic heart rate and rhythm

In-vitro studies

Exposure of cultured rodent embryos to PHT, PB, CBZ, TMD and DMD on GD 10 *in vitro* resulted in concentration dependent severe bradycardia (reduced heart rate compared with original heart rate), arrhythmia (defined as ineffective expulsion of blood from the ventricle) or cardiac arrest (no expulsion of blood from ventricle). Exposure to VPA caused neither arrhythmia nor severe bradycardia, but slight embryonic bradycardia at high concentrations. VGB did not show any potential whatsoever to affect the embryonic heart, even at concentrations 50-fold higher than TC (Papers I and II; Figure 1).

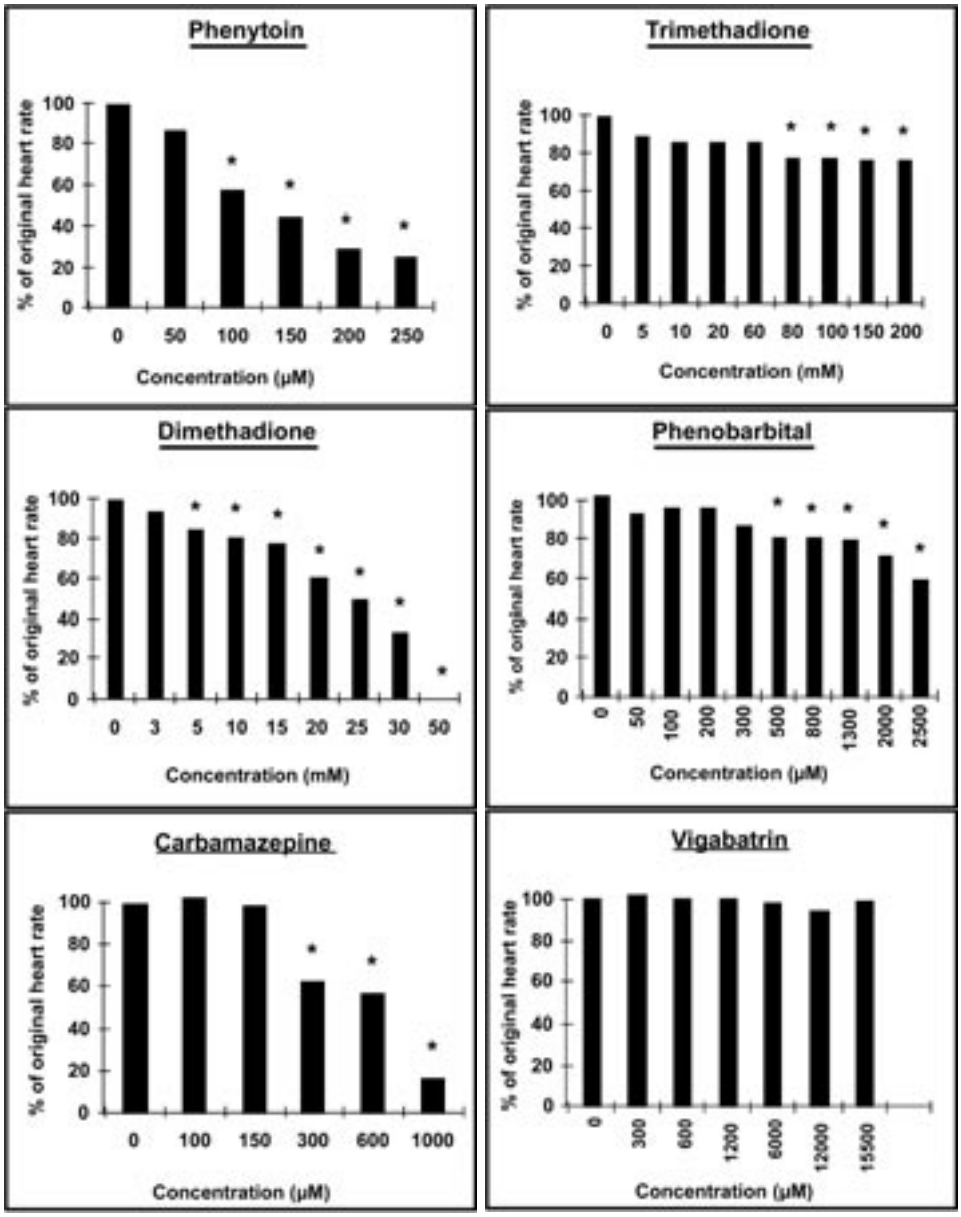


Figure 1. Effect of increasing concentrations of PHT, TMD, DMD, PB, CBZ, and VGB on embryonic heart rate of C57BL/6 mice on GD 10. *P< 0.05. ANOVA followed by Dunnett's test.

Ranking the drugs on the basis of their potential to cause adverse effects on the embryonic heart in relation to the highest TC gave the following order: DMD= PHT>> PB=CBZ> TMD= VPA >> VGB (no potential) (Paper II). The results show a close correlation between the potential to cause adverse effects on the embryonic heart and the ability to induce specific malformations (orofacial

cleft, cardiovascular abnormalities and distal digital defects). In both animal and human studies PHT and DMD have been suggested to be more teratogenic than the embryonic heart and the ability to induce specific malformations (orofacial cleft, cardiovascular abnormalities and distal digital defects). In both animal and human studies PHT and DMD have been suggested to be more teratogenic than PB and CBZ (Dansky and Finnell 1991; Sullivan and McElhatton 1977; Wells et al. 1989). Animal studies have even shown that DMD is a much more potent teratogen than TMD and that DMD is probably the proximate teratogen when animals are treated with TMD (Wells et al. 1989b). The relatively low score for VPA correlates well with the absence of potential of this drug to cause orofacial clefts and other hypoxia-related, defects and a different malformation pattern compared with PHT. Hence the results indicate that dysrhythmia is not involved in causing neural tube defects; malformations which have been closely highly associated with VPA (Ardinger et al. 1988; Källén et al. 1989; Lindhout 1993).

Effects on embryonic heart rate and rhythm in vivo

In order to ascertain if embryonic bradycardia/arrhythmia also occurs under *in vivo* conditions, embryos were excised their mothers 4 h after dosing with PHT or DMD, respectively, and the heart rate and rhythm were studied. A reduction in heart rate, and rhythm abnormalities were observed, which were correlated to maternal dose (Papers III, IV). At lower dose levels, with corresponding low plasma concentrations of free drug (for PHT) and total (for DMD since it is not protein bound), the embryo only experienced bradycardia. At higher doses and higher plasma concentrations, the heart responded with arrhythmia and cardiac arrest (Papers III, IV). The similarity in dose/concentration effects on the embryonic heart after maternal administration of PHT and DMD to pregnant mice thus confirmed that the bradycardia and arrhythmia observed *in vitro* also occur under in vivo conditions.

Comparison of effects between AEDs and selective I_{kr}-blockers on embryonic heart rate and rhythm

The propensity to affect the embryonic heart *in vitro*, by lowering the heart rate and causing rhythm abnormalities, is very similar to what has been shown to be a class effect for selective I_{kr} blockers (class III antiarrhythmic drugs-Abrahamsson et al. 1994; Ban et al. 1994; Spence et al. 1994). After treating the pregnant mother with a class III antiarrhythmic drug and excising the embryos out 4 h after maternal dosing, embryos showed bradycardia and arrhythmia (Ban et al. 1994). In support of these observations, in situ recordings of the action potential, after exposure of embryos to the drugs of this family, have revealed concentration dependent prolongation (associated with reduced in heart rate) and early after depolarization[8] (associated with rhythm abnormalities) (Carlsson

[8] Is a depolarisation that interrupts or retards normal repolarisation and may follow an excessive lengthening of the action potential duration

1991; Abrahamsson, 1994). The same type of changes in the heart rate and rhythm has been observed *in vivo*. Consequently, Several investigators have suggested that the teratogenicity of class III antiarrhythmic drugs is related to hypoxia secondary to concentration/dose dependent bradycardia and is arrhythmic both *in vitro* and *in vivo* (Ban et al. 1992; Konishi et al. 1992; Ban et al. 1994; Abrahamsson et al. 1994, Webster et al. 1996). The existence of embryonic bradycardia and arrhythmia, shortly after dosing the mother with PHT and DMD, suggests that the severe embryonic bradycardia/arrhythmia is the cause of embryo lethality and teratogenicity for these drugs as well.

4.2. Reversibility of adverse effects on the embryonic heart

In washout experiments, the replenishment of the culture medium containing PHT, with a fresh medium without PHT, caused the heart rate to return to baseline values 90 min post-washout. This would imply that the effects on the embryonic heart are pharmacologically reversible (Paper I). In view of this, it is therefore conceivable that the washout situation may resemble the conditions in vivo in that exposure to a moderate concentration of the drug might result in periods of bradycardia and episodic exposure to high concentrations in arrhythmia/severe bradycardia. Hence, a short period of severe hypoxia would occur due to embryonic arrhythmia, followed by reoxygenation when the heart starts to beat again.

Reversibility of the effects on the embryonic heart for selective I_{kr} blockers (class III antiarrhythmic drugs) has been demonstrated in same way as for PHT in washout experiments (Ban et al. 1994).

4.3. Stage specific cardiac embryonic adverse effects

PHT and DMD (in current study)

The sensitivity of the embryonic heart exposed to PHT and DMD in utero (after maternal administration) varied with the GD in an identical manner for both drugs. A decreased embryonic heart rate was observed on GD 9-13. The greatest decrease was seen on GD12 and the sensitivity decreased on GD 13, with no suppressive effect on the embryonic heart thereafter (Papers III, IV). Thus, the embryonic heart of embryos exposed to PHT and DMD *in utero* on GD 14-16 was indistinguishable from that of control embryos (Fig. 2 A, B). The results demonstrate that the susceptibility of the mouse embryonic heart to react with arrhythmia when exposed to PHT and DMD is high when the heart starts to beat and remains high for a restricted period of gestation; when this period ends, the embryonic heart no longer responds with bradycardia, arrhythmia, or cardiac arrest, despite the exposure to a high concentration of PHT.

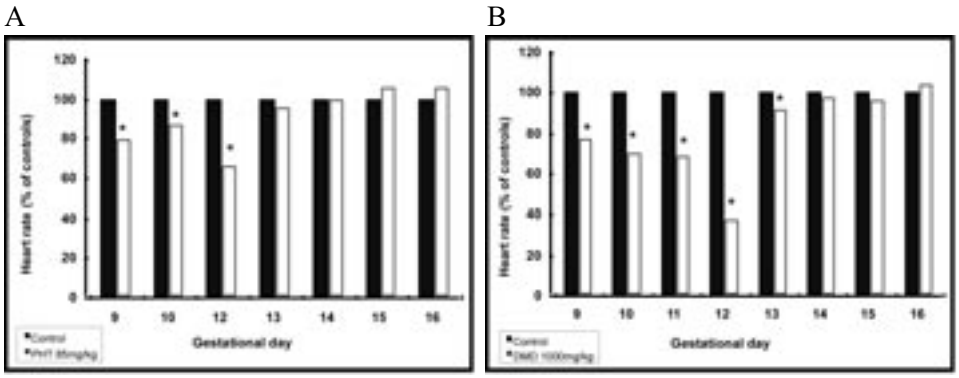


Figure 2. Effect on mean litter embryonic/fetal heart rate after maternal i.p. administration of saline (control), PHT (A), and DMD (B) on different gestational days. *p<0.05. ANOVA followed by Dunnett's test.

Selective I_{kr} blockers (in published studies)

A period of sensitivity of the embryonic heart to the effects of class III antiarrhythmic drugs has also been shown. The critical period is shown to range between GD 10 –14 for the rat embryos, after which the embryos become insensitive to the bradyarrhythmogenic effects of these drugs (Ban et al. 1994). This period in the rat corresponds to approximately. GD 9-13 in the mouse, the days when the mouse embryonic heart was susceptible in the present study. Selective I_{kr} blockers have also been shown to affect heart rhythm in the early mouse embryo, in the same way as observed in rat embryos (Sköld and Danielsson, 2000). This susceptible period, when maturation of the embryonic heart occurs (as was described earlier), has been shown to be the critical period for induction of embryo lethality and teratogenicity after exposure to class III antiarrhythmic drugs.

Observed stage-specific embryonic cardiac adverse effects in relation to maturation of embryonic heart

The induction of adverse effects on the embryonic heart in response to PHT and DMD in this study occurred in the same way as reported for I_{kr} blockers during a limited period of embryonic development. It is during this period that neural and physiological maturation of the heart occurs. The heart starts to beat at about GD 9 in the mouse embryo and day 10 in the rat embryo (Goss 1938; Gomez 1958). At this stage of development, the heart has no autonomic innervation and pumps the blood without the vagal reflex. Adrenergic and cholinergic receptors appear to be present at approximately. day 10, but do not seem to be functional until a much later stage. By GD 10 to 13, the peristaltic-like contractions of the primitive heart tube change to more sequential contractions of the atria and ventricles, indicating initial development of some form of organized conduction system (Davis et al. 1996). Atria and ventricles are not completely

separated by day 13 to 15. As the sinoatrial and atrioventricular nodes are first established on GD 13, no specific conduction tracts exist before this stage.

The deleterious effects of these drugs on the embryonic heart appear to be obtained during a restricted period that coincides with the period when I_{kr} are expressed in the embryo. An observation by Davis and co-workers (Davis et al.1996) indicate that:

1) The expression of delayed rectifier channels is subject to marked changes during early organogenesis.
2) I_{kr} has been shown to be the dominant delayed rectifier K^+ channel current during early embryonic development, (GD 10 and 13) in mice
3) Neither atrial nor ventricular cells express the slowly activating component of delayed rectification (I_{ks}) until just before birth.

Therefore, it is conceivable that, during the susceptible period for induction of embryonic arrhythmia and teratogenicity, the non-innervated embryonic heart is dependent on spontaneously beating myocytes of ventricular myocytes for the generation of heartbeats and regulation of heart rhythm. I_{kr} is therefore a highly suitable candidate by virtue of its important role as the dominant rectifier current during the refractory phase of the action potential in early development (Wang and Duff 1996; Wang et al. 1996).

Interestingly, in contrast to humans and species such as dogs monkeys and rabbits, I_{kr} is not expressed during adult life in rodents. The suggested important role of I_{kr} in early embryonic life is supported by the fact that I_{kr} is expressed and functional from the moment the heart starts beating and during a restricted period in both mouse and rats, despite its suppression by other depolarization channels in these species during adult life.

4.4. Role of I_{kr}-inhibition in induction of embryonic arrhythmia

The susceptible period when selective I_{kr} blockers induce embryonic arrhythmia and teratogenicity (Danielsson and Webster, 1996) was the same as for PHT and DMD (Papers III, IV). This period correlates to the period when I_{kr} is expressed and exercises a dominant functional role in regulation of heart rhythm in the early mouse embryo, as discussed above. In view of the suggested importance of I_{kr} for the induction of embryonic arrhythmia and AED teratogenicity, a review of the role of I_{kr} is presented below:

Role of established I_{kr} blockers in inhibiting development of arrhythmia

Adult heart

Selective I_{kr} blockers, such as the class III antiarrhythmic drugs almokalant and dofetilide are the able to prolong the depolarisation phase of action potential and increase the refractory period of the myocardium. This property is the basis for their use as antiarrhythmic drugs. The electrocardiogram shows a prolonged QT interval, which spans the period from the onset of ventricular depolarisation (the Q wave) to completion of depolarisation (the end of T wave). Drugs of this family have paradoxically also been associated with an increased risk of fatal ventricular arrhythmia in humans (Moss 1993). Recently, much attention has been paid to non-cardiac drugs, which primarily have other pharmacological effects but, which block I_{kr} and prolong the QT interval as a side effect. Examples of such drugs are the gastro-antimotility drug cisapride, and the antihistamines terfenadine and metiazol. These drugs have also been shown to induce an increased risk of lethal arrhythmia in patients (Viskin 1999). Other animals, e.g. rabbits, which just as humans express I_{kr} in the adult life, also develop arrhythmia of the same type when exposed to the class III antiarrhythmic drugs almokalant, dofetilide and sotalol (Carlsson et al. 1993; Sköld et al. 2000). At the same time, the QT interval of the adult rodent heart, with suppressed I_{kr} current, is not affected by selective I_{kr} (Abrahamsson et al. 1994; Wang et al. 1996).

Embryonic heart

The early embryonic heart of all tested species (including mice and rats) appears to react with bradycardia and arrhythmia following exposure to selective I_{kr} blocking drugs (Ban et al. 1994; Abrahamsson et al. 1994; Webster et al. 1996; Sköld and Danielsson 2000). As in the adult heart, non-selective I_{kr} blocker e.g. cisapride, induce embryonic arrhythmia and similar phase-specific malformations such as class III antiarrhythmic (Sköld and Danielsson 2000). However, the embryonic heart seems more susceptible (reacts with arrhythmia at lower concentrations) than the adult heart in species where I_{kr} is expressed and functional during both embryonal and adult life (Sköld and Danielsson 2000).

Interestingly, action potential recordings from rat embryonic cardiac cells during the susceptible period, following exposure to the selective I_{kr}-blocker almokalant, show concentration dependent prolongation, associated with a reduced heart rate. Exposure to higher concentrations results in early after depolarizations (associated with development with arrhythmia) (Abrahamsson 1994) in the same way as in susceptible patients, who develop arrhythmia following exposure to selective I_{kr} blockers.

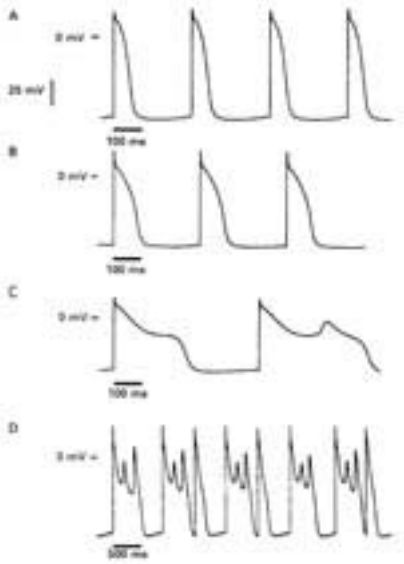


Figure 3. Transmembrane recordings of action potentials from the atrium of the spontaneously beating fetal rat heart at gestational day 13. (A) Control; (B) After 0.1 μM almokalant; (C) After 1 μM almokalant (prolongation); (D) After 10 μM almokalant (early after depolarisations).

Effects of different AEDs on I_{kr}

Effects of TMD, DMD and VGB on I_{kr} in present study

The primary pharmacological effect of TMD, mediated via its pharmacologically active metabolite DMD, is postulated to be exerted via inhibition of voltage dependent T and L-type calcium channels. This inhibition occurs at concentrations around 4-8 mM (Rogawski and Porter 1990).

In this study, a concentration of 10 mM TMD inhibited HERG outward and tail currents by approx. 30%, with smaller decreases being observed at the lower concentrations for tail currents, and similar sized decreases for outward currents. This result suggests that TMD interacts with HERG channels in a non-specific manner, which affects the outward current to a greater extent than the tail current. DMD caused a marked decrease by 65% and 73% in HERG outward and tail currents, respectively, at a concentration of 10 mM, at the same time as the tail current deactivation became much more rapid. This blockade was of the same magnitude as the selective I_{kr} blocker -E 4391. VGB had no IK- blocking potential, when tested in concentrations several times as high as the therapeutic concentrations.

Reported effects of PHT and PB on I_{kr} in other studies

The primary pharmacological effects of PHT and PB are postulated to be exerted via blocking of Na^+ and Ca^{2+} ion channels. This occurs at concentrations of around 40-80 mM (PHT) and 40-130 mM (PB), respectively. In 1997, it was shown that PHT has the capacity to block delayed rectifier potassium channels (I_k) in neurons at 100 μM. This property was suggested to be related to the anticonvulsant properties of (Nobile and Vercellino 1997). In very recent studies, we have shown that both PHT and PB (but not VPA) inhibit I_{kr} in voltage clamping experiments at concentrations similar to those exerting their postulated effects on Na^+ and Ca^{2+} ions, respectively, whereas valproate has no such I_{kr} blocking potential (Danielsson et al unpublished).

Correlation between I_{kr} blocking potential and embryonic arrhythmia

The results indicate that DMD, PHT and PB inhibit I_{kr} at concentrations similar to those exerting their postulated antiepileptic effects on Na^+ and Ca^{2+}channels. The observed I_{kr} blocking potential of DMD, PHT and PB may therefore be of significant importance in explaining their potential to cause embryonic arrhythmia and hypoxia-related malformations. The I_{kr} blocking potential of these AED also tallies with results in a previous study, where we attributed the hypoxia-related teratogenicity of different AED to their deleterious effects (bradycardia and arrhythmia) on the heart of embryos cultured *in vitro*. In that study, DMD and PHT had the highest potential to cause embryonic arrhythmia (occurred at clinical relevant concentrations), followed by PB. VPA and VGB had no potential at all to cause severe cardiac effects (only slight bradycardia by VPA), even despite tested at high concentrations.

4.5. Embryo/fetal morphological changes caused by PHT and DMD

Examination of external morphological changes at birth

In utero exposure of CD-1 mouse embryos on gestational days 10 and 11 to teratogenic doses of PHT (85 mg/kg i.p.) and DMD (1000 mg/kg i.p.) resulted in a high incidence of cleft palate in the embryos when examined at birth (Papers III and IV). Hence confirming the results of a previous study by Wells and colleagues (Wells et al. 1989). The fetuses showed signs of growth retardation and developmental delay (Papers III, IV). Cleft palate was not observed in any of the control fetuses. Interestingly, upon measurement of drug concentrations in maternal plasma of exposed animals in this study, it was evident that embryonic arrhythmia and cleft palate occurred at plasma concentrations above the human therapeutic concentrations for both DMD and PHT.

Pathological changes shortly after exposure to AEDs and their relation to embryonic arrhythmia/hypoxia

Pathological changes caused by PHT and DMD

Examination of embryos 28 h after the last dose of PHT or DMD, showed a significantly lower embryonic heart rate and increased incidence of irregular heart rhythm. Hemorrhages were also observed in the palatal region at this time after administration of both PHT and DMD (Papers III, IV). In addition, some PHT embryos showed widespread hemorrhage in the orofacial region (Paper III). The occurrence of hemorrhage in the same region in the embryo (palate) where tissue deficiency (cleft palate) was visible in the fetus at term suggests a causal relationship. No such hemorrhages were observed in any of the control fetuses (Papers III, IV)

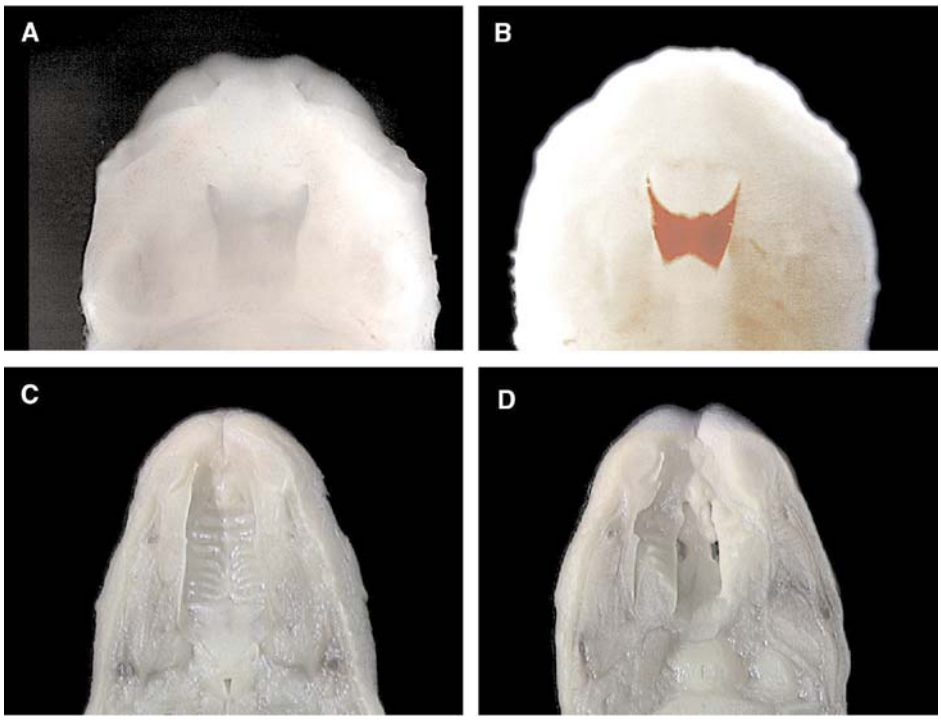


Figure 4. Effect of exposure to DMD (1g/kg on GD 10 and 11) on orofacial development in fetuses examined on GD 12, (A) normal (B) with hemorrhage in the palatal region, and GD 18 (C) normal and (D) cleft palate.

Pathological changes caused by selective I_{kr} blockers

As was mentioned previously, the adverse effects of selective I_{kr} blockers on the embryonic heart have been attributed to their teratogenic mechanism as well (Ban et al. 1994; Abrahamsson et al. 1994; Webster et al. 1996; Sköld and Danielsson 2001). The same stage-dependent malformations, e.g. cleft palate and distal digital amputations are preceded by the same early changes as those mentioned for PHT and DMD (edema, hemorrhage and necrosis) (Webster et al. 1996). These similarities indicate that adverse cardiac effects on the embryonic heart are the common denominator for the teratogenicity of these drugs.

Changes caused by temporary interruption of oxygen supply to the embryo

High incidences of cleft palate, preceded by hemorrhage in the palate of the early embryo, have been reported after exposure of experimental animals to hypoxic episodes, ranging from 30 min up to 90 min (Franklin and Brent 1964; Leist and Grauwiler 1974). The stage dependency, malformation pattern, distal digital amputation defects and orofacial cleft, and early changes preceding the malformations, e.g. edema, hemorrhage and necrosis, all resemble those observed after impairment of uterine blood supply by various means (Franklin and Brent 1964; Brent and Franklin 1969; Webster et al. 1986; Fawcett et al. 1998). The data suggest that episodes of hypoxia/anoxia, secondarily to PHT- and DMD-induced embryonic arrhythmia, are the cause of the teratogenicity of these drugs (Papers III and IV).

The restricted period when PHT and DMD induce embryonic arrhythmia (Papers III and IV) is closely correlated with the period when PHT induces adverse developmental effects. This period also overlaps with period when the embryo is very sensitive to interrupted oxygen supply (Leist and Grauwiler 1974, Danielsson and Webster 1997). This period appears to begin when the embryo changes to aerobic life, and ends in late organogenesis. Studies have shown that the early post-implantation rat embryos (GD 6-9 in rat) are rather resistant to the effect of hypoxia and that they can survive for periods of up to 2 h of hypoxia, with low incidences of death and congenital malformations (Grabowski 1970; Leist and Grauwiler 1973). Sensitivity toward hypoxia then escalates and peaks between GD 13 and 16. During this period even brief episodes of hypoxia (30 min) can induce a high incidence of defects and increased incidence of embryonic death (Leist and Grauwiler 1974; Webster 1986).

4.6. Arrhythmia, hypoxia and role of reactive oxygen species (ROS) in AED teratogenicity

There is considerable evidence that some malformations (e.g. orofacial clefts and distal digital reductions) induced by interrupted oxygen supply to the embryo, are not directly related to the induced hypoxia, but rather to the formation of Reactive Oxygen Species (ROS) during the reoxygenation phase (review Fantel 1996; 1999). In support of this hypothesis, it has been shown that the highest concentrations of ROS are generated when the hypoxia is episodic (Clark and Gewertz 1991). This type of reoxygenation damage probably occurred in our model after an episode of transient arrhythmia, when embryonic oxygen supply was completely interrupted for as long as the arrhythmia lasted. The proposed mechanism for ROS generation during reperfusion is shown in the following figure.

4.7. Ischemia/reperfusion injury

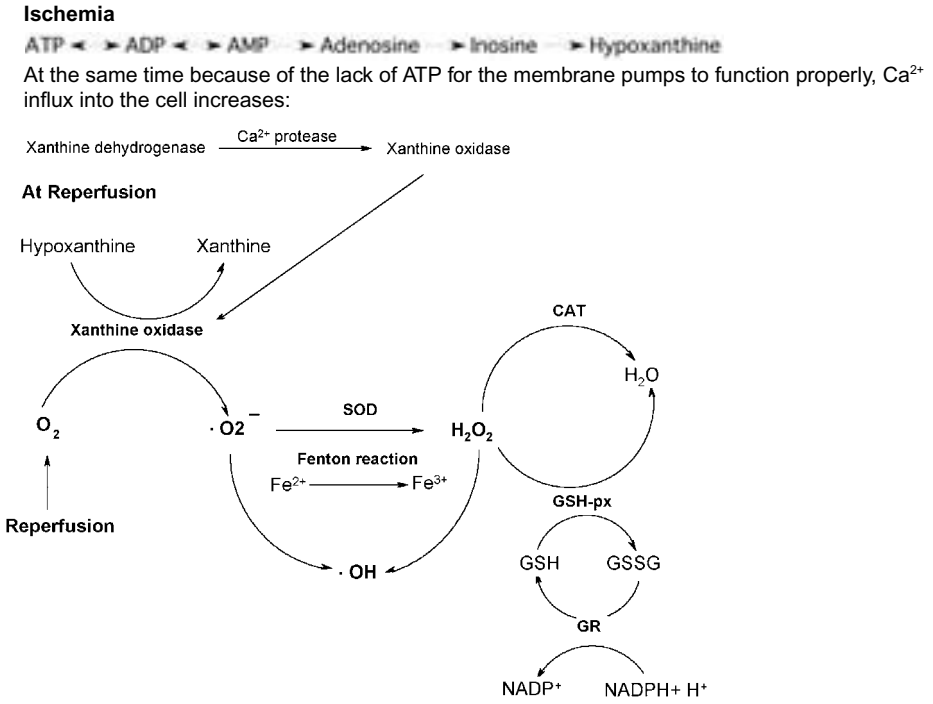


Figure 5. Schematic illustration of the cascade of events induced by ischemia and reperfusion

Effects of PBN on PHT- and DMD-induced cleft palate

PHT- and DMD-induced cleft palate was significantly reduced by concurrent treatment with the ROS trapping agent PBN. PBN has been utilized in order to elucidate the role of free radicals in developmental toxicity of almokalant (after doses known to cause embryonic arrhythmia) (Wellfelt 1999). Pre-treatment with PBN completely excluded the almokalant-induced orofacial clefts, distal digital defects and ventricular septal defects (Wellfelt 1999).

However, in the current study, repeated treatment with PBN was necessary in order to markedly reduce the teratogenicity. The disparity between the present and those of Wellfelt and co-workers is most likely related to differences in kinetics between PHT and almokalant. The half-life in rodents for both PHT (24 h) and DMD (72 h) is much longer than for both almokalant (1-2 h Abrahamsson et al. 1994) and PBN (1-2 h). Hence, repeated PBN dosage was needed to protect against the ROS generated during the reperfusion phase after PHT and DMD exposure.

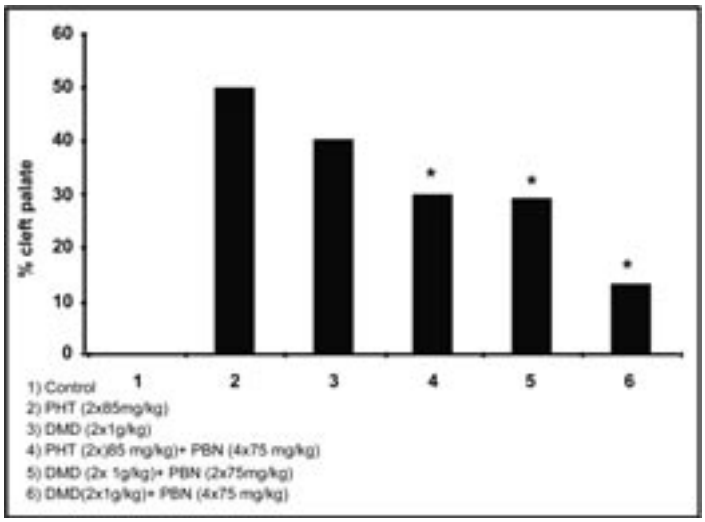


Figure 6. The effect of exposing pregnant CD-1 mice on GD 10 and 11 to PHT (85 mg/kg), DMD (1g/kg) alone or in combination with PBN, on the incidence of cleft palate observed at GD 18. $p<0.05$. Kruskal-Wallis test preceded by Wilcoxon-Mann-Whitney.

Mechanisms for the developmental toxicity resulting from AED-induced embryonic dysrhythmia

Hypoxia-reoxygenation damage resulting in ROS generation

The protective effects of PBN support the idea that orofacial clefts following exposure to PHT and DMD are related to drug induced embryonic episodes of arrhythmia, resulting in episodes of severe hypoxia/anoxia followed by formation of reactive oxygen species (ROS) during reoxygenation. In these studies (PHT - Paper III and DMD-Paper IV) there were a definite relationship between dosing regimens/plasma concentrations which caused arrhythmia and drug induced orofacial cleft.

Administration of selective I_{kr} blocker almokalant during the same stage of the susceptible period also caused orofacial clefts, which were preceded by vascular disruption and hemorrhage in embryonic tissues but could be prevented by PBN (Wellfelt 1999).

Exposure to PHT during a later stage of the susceptible period caused distal digital defects, which were preceded by vascular disruption and hemorrhage in distal parts of the embryonic limb (similar to those preceding cleft palate) within 24h of administration of PHT (Danielson et al. 1992).

These data suggest that major malformations caused by I_{kr} blockers which are preceded by hemorrhage, such as orofacial clefts and distal digital reductions, are linked to embryonic arrhythmia and reoxygenation damage. This also seems be the case for ventricular septal defects (Wellfelt 1999).

Developmental toxicity, a direct consequence of hypoxia

Less severe manifestations of developmental toxicity than orofacial clefts have been induced by dosing regimens/plasma concentrations that induce bradycardia, but not arrhythmia (Danielsson et al. 2000). These manifestations include growth retardation, microcephaly and craniofacial defects. Microcephaly and craniofacial defects have been associated both in Man (Van Lang et al. 1984) and in animal studies (Lorente et al. 1981) with retarded formation of skull bones, e.g. decreased size of the cranial base and maxilla with concomitant decrease in nasal and mandibular dimensions. Hence, the mechanism for PHT and DMD, like growth retardation, could be a direct consequence of longer periods of hypoxia due to mild/moderate bradycardia and reduced cardiac output (Paper III, Azarbayjani and Danielsson 2001).

Fluctuations and misdirection of embryonic blood flow

There is also evidence (Wellfelt et al. 1999) that some cardiovascular defects associated with exposure to I_{kr} blockers, such as transposition and absence of major vessels, could be a direct consequence the induced arrhythmia itself. Both PHT and TMD have been associated with such defects in both clinical and animal studies. Fluctuations and misdirection of embryonic blood flow and reduced embryonic cardiac output (induced by mechanical means or by drugs affecting embryonic heart rhythm) could be the cause of such cardiovascular anomalies (Rajala et al. 1984; Gilbert et al. 1980). This supports the idea that vascular defects are caused by the PHT itself rather than by hypoxia-related damage (Paper III)

4.8. Genetic susceptibility to toxic developmental effects of PHT

A genetic susceptibility to PHT-induced isolated cleft palate has been shown for different mouse strains (Hansen and Hodes 1983a,b). The A/J strain is the most susceptible, while the C57BL/6 and CD-1 strains are more resistant. According to our hypothesis, the increased susceptibility of the A/J strain could be a result of increased susceptibility to develop embryonic arrhythmia when exposed to PHT. This would result in an increased generation of teratogenic ROS in the A/J embryo, compared with the other strains.

An alternative explanation is that A/J mice are less able to detoxify generated ROS/possibly toxic reactive metabolites of PHT. Multiple endogenous defense mechanisms exist in the cells for coping with ROS. There is experimental evidence that ROS-induced teratogenicity is augmented by lower enzyme levels/activities of ROS detoxifying enzymes, resulting in an increased risk of malformations (Cederberg and Eriksson 1997).

Effects on embryonic heart rhythm in A/J, C57BL/6 and CD-1 mice

In the studies on the effects of PHT on the embryonic heart, all three strains of mice developed bardycardia and arrhythmia. The A/J strain developed arrhythmia at much lower concentrations and higher incidences than the embryos of the other strains studied. Hence, at a concentration of 200 mM, the embryos of A/J (50%) strain had a significantly higher incidence of arrhythmia compared with the embryos of C57BL/6 (13%) and CD-1 (8%) mice. However, the decrease in the heart rate was similar at the concentrations studied, between the different strains of mice

Activities of detoxifying enzymes in A/J, C57BL/6 and CD-1 mice

Embryos of A/J mouse embryos had a higher activity of antioxidant enzymes, thus supporting the idea of an increased generation of ROS in this strain in consequence of embryonic arrhythmia. This situation resembles the scenario of patients with mutations in their HERG gene that are predisposed to react with arrhythmia when exposed to physiological and physical stress. Thus, it is plausible that the A/J mouse strain lives under a condition of oxidative stress[9] under normal conditions and struggles with generated ROS caused by stress-induced embryonic arrhythmia.

[9] which occurs when production of intracellular oxygen-free radicals exceeds the ability of the cell's antioxidant enzyme capacity to remove the oxidants and restore homeostasis.

5. CONCLUSIONS

Overall, the results support the hypothesis that the teratogenicity of PHT, PB, PB and CBZ is related to a common mechanism: pharmacologically induced episodes of embryonic hypoxia due to embryonic cardiac arrhythmia and generation of reactive oxygen species during the reperfusion phase. Referring to the specific aims of this thesis, the results showed that:

1. All AEDs having the potential to cause hypoxia-related malformations were able to cause severe embryonic bradycardia or arrhythmia/cardiac arrest. The AEDs (valproate and vigabatrin), which have not been associated with hypoxia-related defects, were unable to cause such severe cardiac adverse effects.

2. The embryonic arrhythmogenic potential of DMD, TMD and VGB showed a very close correlation to their potential to inhibit I_{kr}. Recent data (not reported in this thesis), showing I_{kr}-blocking potential by PHT and PB (but not by valproate) further strengthen a causal relationship between I_{kr} inhibition and embryonic arrhythmia.

3. PHT- and DMD-induced cleft palate (a hypoxia-related malformation) was preceded by the same early changes (vascular disruption and hemorrhage in the embryonic region) as reported after inducing cleft palate by interrupting of oxygen supply to the embryo.

4. Treatment with PBN, which can capture reactive oxygen species (ROS), significantly reduced the teratogenicity of DMD and PHT. The results support a role of hypoxia/reoxygenation damage and formation of ROS in arrhythmia-related teratogenicity

5. The results confirm that the genetically determined sensitivity in PHT teratogenicity in different mouse strains is related mainly to an increased susceptibility in the sensitive strain to react by developing embryonic arrhythmia when exposed to PHT.

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