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Fundamental and Molecular Mechanisms of Mutagenesis

Genetic toxicities of human teratogens

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Abstract

Birth defects cause a myriad of societal problems and place tremendous anguish on the affected individual and his or her family. Current estimates categorize about 3% of all newborn infants as having some form of birth defect or congenital anomaly. As more precise means of detecting subtle anomalies become available this estimate, no doubt, will increase. Even though birth defects have been observed in newborns throughout history, our knowledge about the causes and mechanisms through which these defects are manifested is limited. For example, it has been estimated that around 20% of all birth defects are due to gene mutations, 5–10% to chromosomal abnormalities, and another 5–l0% to exposure to a known teratogenic agent or maternal factor [D.A. Beckman, R.L. Brent, Mechanisms of teratogenesis, Ann. Rev. Pharmacol. Toxicol. 24 (1984) 483–500; K. Nelson, L.B. Holmes, Malformations due to presumed spontaneous mutations in newborn infants, N. Engl. J. Med. 320 (1989) 19–23.]. Together, these percentages account for only 30–40%, leaving the etiology of more than half of all human birth defects unexplained. It has been speculated that environmental factors account for no more than one-tenth of all congenital anomalies [D.A. Beckman, R.L. Brent, Mechanisms of teratogenesis, Ann. Rev. Pharmacol. Toxicol. 24 (1984) 483–500]. Furthermore, since 'there is no evidence in humans that the exposure of an individual to any mutagen measurably increases the risk of congenital anomalies in his or her offspring' [J.F. Crow, C. Denniston, Mutation in human populations, Adv. Human Genet. 14 (1985) 59–121; J.M. Friedman, J.E. Polifka, Teratogenic Effects of Drugs: A Resource for Clinicians (TERIS), The John Hopkins University Press, Baltimore, 1994], the mutagenic activity of environmental agents and drugs as a factor in teratogenesis has been given very little attention. Epigenetic activity has also been given only limited consideration as a mechanism for teratogenesis. As new molecular methods are developed for assessing processes associated with teratogenesis, especially those with a genetic or an epigenetic basis, additional environmental factors may be identified. These are especially important because they are potentially preventable. This paper examines the relationships between chemicals identified as human teratogens (agents that cause birth defects) and their mutagenic activity as evaluated in one or more of the established short-term bioassays currently used to measure such damage. Those agents lacking mutagenic activity but with published evidence that they may otherwise alter the expressions or regulate interactions of the genetic material, i.e. exhibit epigenetic activity, have likewise been identified. The information used in making these comparisons comes from the published literature as well as from unpublished data of the U.S. National Toxicology Program (NTP). © 1997 Elsevier Science B.V.

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1. Introduction

1.1. Overlaps of teratogenesis and mutagenesis

Teratogenesis comes from the Greek word terata, meaning monster, and relates to monstrosities or malformations produced during development. It is a component of developmental toxicology, which encompasses, in addition to malformations, embryo lethality, growth retardation and functional impairment. Teratogenesis and mutagenesis are both processes and may involve overlapping targets, but they differ in many ways. For example, we know that all mutagenesis, which is the production of sudden heritable changes in the genetic material or mutations, ultimately involves alterations in either the primary message, structure or quantity of a molecularly-identifiable target DNA. In contrast, there is no similar term 'teratation' to describe the result of teratogenesis and no specific molecular target has been identified with certainty for even the most thoroughly studied of human teratogens, such as thalidomide and ethanol. Indeed, it seems certain that no one molecular target will ever be identified for a particular type of terata, much less all types. On the other hand, while teratologists can claim a list of two dozen or more demonstrable environmental teratogens, genetic toxicologists have yet to prove the existence of even one germ cell mutagen, with the possible recent exception of radiation exposures at Chernobyl [1]. Perhaps the fact that the teratologist's endpoint, as well as target, is defined at the organismic rather than the molecular level has its advantages.

For chemicals to be mutagens they, or their metabolites, must be able to interact with DNA or with some other molecule which has a direct impact on the integrity of DNA. Windows of mutagenic susceptibility are generally delimited by specific DNA-associated processes like accessibility and replication or repair. Once it is established that a mutagenic chemical can reach the DNA interactive target, no threshold is assumed for its effect. On the other hand, virtually any chemical may be considered a potential teratogen at some dose level and a threshold response is generally assumed. It is the presence of the teratogen during a specific sensitive period in development, defined at the organismic–morphologic rather than the molecular level, that is critical. Furthermore, whether a chemical is likely to pose a teratogenic hazard may also be delimited by its relative maternal toxicity.

The most susceptible period for induction of malformations is the period of organogenesis, which begins with gastrulation just after implantation, during the second week of gestation in humans, and continues through about the 14th week of gestation. As the name implies, it is the time when all of the major organ systems are developed. Until recently [2], pre-gastrulation stages of development were thought to be susceptible only to induction of embryo lethality, not malformations. No human teratogens have yet been identified as specifically being active in the pregastrulation period of development.

In defining a teratogenic agent as one that acts during pregnancy to produce a physical or functional defect in the conceptus or offspring, Shepard [3] suggested a modification of Koch's postulates for application to teratology to make this definition more specific. (1) The agent must be present during the critical periods of development. (2) The agent should produce congenital defects in an experimental animal with the rate of defects being statistically higher in the treated group than in controls receiving the same vehicle or sham procedure. (3) Proof should be obtained that the agent in an unaltered state acts on the embryo–fetus either directly or indirectly through the placenta.

Distinguishing congenital defects from normal variation can be a challenge in identifying teratogenic agents, especially human teratogens. A congenital defect has its genesis during embryonic or fetal development and consists of a major or minor deviation from normal morphology or function. The borderline between a minor congenital defect and normal variation is sometimes difficult to define. In general, however, a minor defect should be present in less than 2–3% of the population.

Schardein [4] estimated that over 3300 chemicals have been tested for teratogenicity with about 37% exhibiting some evidence of teratogenicity. Likewise, Shepard [3] lists more than 2500 agents in his catalog, of which about 1200 can produce congenital anomalies in experimental animals, but only about 40 are known to cause defects in humans. These 40 include various infectious agents, physical factors,

maternal metabolic imbalances, drugs and environmental chemicals.

As criteria for selecting those he considered to be 'teratogenic agents in human beings' from among the more than 2500 agents listed in his catalogue, Shepard [3] looked for one or more of the following. (1) Proven exposure to an agent at critical time(s) in prenatal development (prescriptions, physician's records, dates). (2) Consistent findings by two or more epidemiological studies of high quality that included (a) control of confounding factors, (b) sufficient numbers, (c) exclusion of positive and negative bias factors, (d) prospective studies, if possible, and (e) a relative risk of six or more. (3) Careful delin-

Table 1
Teratogenic agents

Teratogenic agents in human beings [a]	
Radiations	Drugs and environmental chemicals
Atomic weapons	Aminopterin and methylaminopetrin
Radioiodine	Androgenic hormones
Therapeutics	Busulfan
	Captopril (renal failure)
Infections	Chlorobiphenyls
Cytomegalovirus (CMV)	Cocaine
Herpes virus hominis? I and II	Coumarin anticoagulants
Parvovirus B-19 (Erthema infectiosum)	Cyclophosphamide
Rubella virus	Diethylstilbestrol
Syphilis	Diphenylhydantoin
Toxoplasmosis	Enalapril (renal failure)
Varicella virus	Etretinate
Venezuelan equine encephalitis virus	Iodides and goiter
	Lithium
Maternal metabolic imbalances	Mercury, organic
Alcoholism	Methimazole and scalp defects
Chorionic villus sampling (before day (60)	Methylene blue via intraamniotic injection
Cretinism, endemic	Penicillamine
Diabetes	13-*cis*-Retinoic acid (isotretinoin and accutane)
Folic acid deficiency	Tetracyclines
Hyperthermia	Thalidomide
Phenylketonuria and Sjogren's syndrome	Toluene abuse
Rheumatic disease and congenital heart block	Trimethadione
Virilizing tumors	Valproic acid

Possible and unlikely teratogens	
Possible teratogens	Unlikely teratogens
Binge drinking	Agent orange
Carbamazepine	Anesthetics
Cigarette smoking	Aspartame
Colchicine	Aspirin (in second half of pregnancy may increase cerebral hemorrhage during delivery)
Disulfiram	Bendectin (antinauseants)
High vitamin A	Illicit drugs (marihuana, LSD)
Lead	Metronidazole (flagyl)
Primidone	Oral contraceptives
Quinine, suicidal doses	Progesterone (hydroxyprogesterone and medroxyprogesterone)
Streptomycin	Rubella vaccine
Zinc deficiency	Spermicides
	Video display terminals and electromagnetic waves
	Ultrasound

[a] Adapted from table 2 of Shepard [3].

eation of the clinical cases (a specific defect or syndrome, if present, is very helpful). (4) Rare environmental exposure associated with a rare defect (see examples of oral anticoagulants and nasal hypoplasia and methimazole and scalp defects in Table 1). (5) Teratogenicity in experimental animals (important but not essential). (6) An association that makes biological sense. (7) Proof in an experimental system that the agent acts in an unaltered state (important information for prevention). These criteria represent an amalgamation from writings by Wilson [5], Brent [6], Stein et al. [7], Hemminki and Vineis [8], and Shepard [9]. Shepard noted that items (1), (2) and (3) or (1), (3) and (4) are essential criteria and that items (5), (6) and (7) are helpful but not essential.

We have selected for elaboration of their genetic toxicities in this paper, the list of 'Drugs and Environmental Chemicals' which Shepard [3] considered to be proven human teratogens (Table 1), as well as a few of those he considered as possible human teratogens (binge drinking, carbamazepine, cigarette smoking, primidone and quinine). 'Radiations' are unquestionably mutagenic, and some of the noted effects of radiation on the human conceptus, such as early embryonic death, segmental and complete aneuploidies, and long-term carcinogenicity, must certainly be the direct result of induced gene mutations or chromosomal damage, either in the parental germ cells and/or the somatic cells of the early embryo or developing fetus. It is unclear whether microcephaly, mental retardation and other neurological abnormalities associated with radiation are the direct result of mutagenic, epigenetic or non-genetic damage. Mole [10] discussed mechanisms by which ionizing radiation could produce malformations. Radiations are not included in our subsequent elaborations. We have also excluded from our subsequent elaborations the list of 'infections' and 'maternal metabolic imbalances' included in Shepard's list, since a reasonable theoretical discussion of mutagenic potential would not be possible for most of these factors.

In addition to the human teratogens from Shepard's list indicated above, we have also chosen to include for discussion of their mutagenicity those drugs (excluding biologics) from *Teratogenic Effects of Drugs: A Resource for Clinicians* (*TERIS*; [11]) whose 'magnitude of teratogenic risk' is categorized as 'moderate or high'. The list of summaries on the teratogenicity of agents in *TERIS* provide aphorisms in which the risks of teratogenic effects in the children of women exposed to the agent during pregnancy are categorized as 'none, minimal, small, moderate, high or undetermined' based upon the available data which are rated in quality and quantity as 'none, poor, fair, good or excellent'. The analyses of each agent's teratogenicity is based upon the reproducibility, consistency, and biological plausibility of available clinical epidemiological and experimental data. In regard to biological plausibility, however, it is emphasized that exposures which produce malformations in the embryo or fetus should do so only during organogenesis or histogenesis, and affected structures should be susceptible to the teratogenic action of an agent only at specific gestational times. Furthermore, it is noted that the risk ratings refer only to the risk of teratogenic effects after maternal exposures to commonly encountered doses and that exposures to unusually high doses, especially to doses that are toxic to the mother, may be associated with higher risk. Thus, the categorizations are conservative and more limited than one might encounter in assessments of mutagenic risks.

Shepard's list includes all of the drugs in *TERIS* whose magnitude of teratogenic risk is moderate or high except for cytarabine, daunorubicin, indomethacin and mercaptopurine. Other than these, most of the entries in the *TERIS* list are categorized as undetermined due to none to poor quality and quantity of the data. However, there are some non-teratogenic agents with good quality and quantity of data, such as over-the-counter medications like nasal decongestants and antihistamines, spermicide contraceptives, and various vaccines, to which large numbers of pregnant women are exposed with no reports of associated abnormal births. Information on *TERIS* can be obtained from the World Wide Web at: http://weber.u.washington.edu/~terisweb/teris/.

Most of the information on the use and teratogenicity, including the types of malformations and the magnitude of risk of each of the chemicals discussed in this paper, is excerpted directly from either Shepard [3] or Friedman and Polifka [11].

Information on the mutagenicity of each chemical was obtained from the available literature, and for some, from the NTP data base.

Listing of a human teratogen as one with evidence of mutagenic or epigenetic activity is not meant to imply that this has been established as a mechanism for its teratogenic activity. In some instances (e.g., cigarette smoking), we have expressly stated we do not think an agent's mutagenicity is related to its teratogenicity. In other cases (e.g., cyclophosphamide), we have suggested that both activities may be related. Obviously, if an agent has been thoroughly tested for mutagenic activity and none has been found, it is reasonable to conclude its teratogenic activity is unrelated to mutation induction. There are no established tests specifically for epigenetic activity and agents are included in this list on the basis of some reported observation of their altering transcriptional, translational or other gene interactive processes, generally unrelated to evaluation of their teratogenicity.

2. Human teratogens with evidence of mutagenic activity

Agents with mutagenic activity are those which produce sudden heritable changes in the DNA or mutations. These mutations include both structural changes, such as base additions, deletions and substitutions, chromosome rearrangements like translocations and inversions, and even small deletions that do not lead to immediate cell death, as well as numerical changes such as aneuploidy. We consider mutagenicity to be a subcategory of genotoxicity which, in addition to heritable effects, includes lethal and other toxic effects upon the nuclear or extranuclear genetic material [12]. Mutagenic or genotoxic agents can act by both direct and indirect mechanisms. Agents with evidence for both mutagenic and epigenetic activity could be listed in either category and it is mostly a judgment-call as to where to put them. Diethylstilbesterol (DES), for example, has been included under the latter category primarily because its epigenetic activity seems more certain and better defined than its mutagenic activity, and because its mutagenic activity may be due to an indirect epigenetic mechanism. Others like ethanol and penicillamine are included under the mutagenic rather than the epigenetic category, but might better belong in the former.

2.1. Aminopterin (4-aminopteroylglutamic acid, CAS 54-62-6) and methotrexate (CAS 59-05-2)

Aminopterin and its methyl derivative, methotrexate, are folic acid antagonists. Both have been used to induce therapeutic abortions. Methotrexate is also used in the treatment of neoplastic and rheumatic diseases. A number of children with a very uncommon and characteristic pattern of congenital anomalies have been born to women treated with aminopterin or methotrexate during the first trimester of pregnancy. These anomalies include abnormal cranial ossifications resulting in large fontanelles and craniosynostosis, abnormal auricles, ocular hypertelorism, cleft palate, micrognathia, hydrocephalus and other head abnormalities, as well as limb, digital and skeletal defects with resultant short stature. The teratogenic risk associated with both aminopterin and methotrexate is moderate to high based on a quality and quantity of data which are fair to good. Methotrexate and aminopterin have been used frequently as selective agents in studies involving induction of MTX- or Ami-resistance mutations in cultured cells by other agents [13], but by themselves have limited mutagenic activity. Although no published data were located specifically on the mutagenicity or genotoxicity of aminopterin, results of numerous mutagenicity studies with methotrexate indicate it is primarily a clastogen rather than a gene mutagen [14]. It failed to induce gene mutations either in Salmonella [15,16] or in cultured mammalian cells [17], but it has been shown to induce chromosomal aberrations (CA) in cultured mammalian cells [18] as well as in vivo in mouse bone marrow cells [19]. It also induced micronuclei (MN) in mouse bone marrow cells [20–22] and peripheral blood lymphocytes [23], with multiple treatments significantly increasing the frequency of MN [19]. Significant increases in the frequency of sister chromatid exchange (SCE) in human lymphocytes have been reported for oncologic nurses occupationally exposed to methotrexate [24] and for 1 of 8 patients treated with methotrexate for psoriasis [25]. SCE induction was also observed in fetal-mouse cultures

exposed to methotrexate at toxic levels [26]. Results of tests for germ cell mutagenicity in male ([27]; NTP unpublished results) and female ([28]; NTP unpublished results) mice are inconclusive, based on a mix of weak positive and negative results. Methotrexate is known to induce fragile sites in certain chromosomal regions which may be related to its ability to induce chromosome damage in vitro [29]. In in vitro studies of chromosome breakage and chromosome fragile sites, a significantly higher level of damage was seen when the cells were cultured in medium lacking folic acid [30]. Folate deficiency has also been associated with increased incidences of MN in peripheral blood erythrocytes of mice [31]. Methotrexate's inhibition of dihydrofolate reductase (DHFR) can result in a depletion of tetrahydrofolate-dependent one-carbon-transfer reactions in both amino acid and nucleic acid biosynthesis. The requirement for folic acid in DNA synthesis and DNA methylation provides a plausible mechanism for mutagenic, and perhaps epigenetic, effects associated with its depletion by agents such as methotrexate. It may also account for its teratogenic effects, as folic acid deficiencies have been found to be associated with congenital malformations, especially neural tube defects. There is considerable individual variability in DHFR levels and this may contribute to the varied results seen in human as well as in animal model studies of methotrexate.

2.2. Busulfan (Myleran®, CAS 55-98-1)

Busulfan is a bifunctional alkylating agent which was, for many years, the drug of preference in treatment of chronic myelogenous or granulocytic leukemia [32]. In a review of the toxicology data on busulfan, Bishop and Wassom [33] summarized 40 published case reports involving busulfan treatment of pregnant women. Of the 37 full-term or near full-term infants in this summary, 33 were classified as 'normal' at birth. Of the four abnormal infants, three were malformed and one with 'no specific lesions' died of hyaline membrane disease [34] two days after delivery. Two of the three malformed infants had been exposed to other chemotherapeutics in addition to busulfan, but the defects of one of the infants [35], which included small birth weight, cleft palate, bilateral ocular defects, thyroid hypoplasia and disintegrating ovarian follicles, were attributed to the busulfan therapy. The third infant [36], who received only busulfan chemotherapy, had reduced birth weight, partial mongoloid features, microcephaly, epicanthal folds, high-arched palate, incisor abnormality, undescended testes and cytogenetic abnormalities persisting at 5 years of age. Although the specific molecular and cellular mechanisms of busulfan's toxicities are unknown, it appears to be cytotoxic to slowly proliferating or non-proliferating stem cell compartments. Busulfan has been shown to be mutagenic to microorganisms, mammalian cells in culture, Drosophila and rodents. It is positive in the Salmonella assay [37] and the Drosophila sex-linked recessive lethal and reciprocal translocation assays [38]. It was shown to induce HPRT mutations and MN in CHO cells, CA in mammalian cells in vitro as well as in mouse bone marrow cells in vivo, and SCE in human peripheral blood lymphocytes treated in vitro. It is a confirmed inducer of dominant lethal mutations in testicular spermatozoa [39,40] and maturing oocytes of mice [41]. Specific locus mutations were induced in spermatids and spermatozoa of mice exposed to busulfan; extensive killing of differentiating and stem cell spermatogonia was also noted [42]. In addition, severe toxicity to developing gonocytes was reported in male and female rat fetuses exposed transplacentally to busulfan [43–45].

2.3. Cigarette smoking

Cigarette smoke contains more than 3500 different chemicals, many of which are mutagenic, such as benzo[*a*]pyrene, ethylene oxide and coal tars. But the most abundant chemicals are nicotine and carbon monoxide and it is probably these, especially the latter, which most likely account for the adverse developmental effects associated with cigarette smoking. The risk of malformations associated with cigarette smoking appears to be minimal-to-none, but the risk of fetal growth retardation is moderate-to-high. The quality and quantity of the data associated with these risks are good-to-excellent. Although many of the chemicals in cigarette smoke are extremely mutagenic [46] the observed growth retardation is probably not the result of mutagenic events. There is a strong association between cigarette smoking and reduced fecundity, reduced fertility and early

mean age of menopause, suggesting that smoking may impair oocyte function and viability [47]. Several of the components of cigarette smoke, including benzo[*a*]pyrene and coal tars, have been shown to induce P450 metabolizing enzymes and CYPE2 expression. Cigarette smoke has been shown to induce MN in a variety of human somatic cells including lymphocytes [48,49], exfoliated cells of the buccal mucosa [50] and exfoliated urothelial cells [51], as well as in the livers of newborn and fetal mice exposed to tobacco smoke in utero [52]. Increases in SCE frequency have also been demonstrated in lymphocytes of smokers [53] and liver cells of mice exposed in utero [54], but not in newborns of smoking mothers [55]. Cigarette smoke condensates and/or urinary extracts from smokers have been shown to be mutagenic in bacteria, to induce SCE and CA in cultured CHO cells, and to induce gene mutations and SCE in cultured rodent cells [46].

2.4. Cyclophosphamide (Endoxan®, CAS 50-18-0)

Cyclophosphamide is an anticancer chemotherapeutic and immunosuppressive agent used to treat a wide range of neoplastic diseases, as well as some autoimmune diseases like rheumatoid arthritis. It is also used to suppress organ rejection after transplant. Multiple anomalies, including flattening of the nasal bridge, missing and hypoplastic digits, cleft palate, and eye defects, have been reported among offspring of women treated at various gestational days ranging from 15 to 82. Cyclophosphamide is a DNA-damaging agent that is metabolized by liver monofunctional oxygenases to a phosphoramide mustard and acrolein. The parent molecule is both genetically inactive and non-teratogenic. Spielmann and Jacob-Muller [56] concluded that phosphoramide mustard was the active teratogenic metabolite in a mouse blastocyst system. Further, phosphoramide mustard in equimolar doses caused effects similar to those of bioactivated cyclophosphamide in rat embryos treated in vitro [57] and when given intraamniotically [57]. Mirkes et al. [58] demonstrated that phosphoramide mustard lengthened S-phase in neuroepithelial cells and slowed or arrested their G_2-phase. There is a very extensive data base documenting the mutagenicity of cyclophosphamide which was recently reviewed by Anderson et al. [59]. The mutagenicity of cyclophosphamide extends from the molecular level through the microbial and insect systems to somatic mammalian cells in various cell lines and primary cells in vitro, somatic cells in vivo, and germ cells of rodents. Cyclophosphamide has been reported to increase the incidence of developmental anomalies among progeny of male mice and rats treated prior to mating [60,61]. Growth retardation was the predominant malformation seen in both species. Exencephaly was the second most common malformation seen in mice while hydropia/edema was the second most common malformation seen in rats. Over $\frac{1}{3}$ of the abnormal rat fetuses which were successfully karyotyped carried various chromosomal abnormalities such as translocations, trisomies and deletions. It is likely that most of cyclophosphamide's teratogenic effects are related to its ability to induce chromosomal damage, resulting in genetically defined morphological changes and apoptotic and/or necrotic cell death.

2.5. Cytarabine (CAS 147-94-4)

Cytarabine, also known as cytosine arabinoside or Ara-C, is a nucleoside analog that inhibits DNA synthesis and is used in the treatment of leukemia. Although no controlled epidemiological studies have been conducted, there are anecdotal descriptions of more than 50 pregnancies in which the mother was treated with cytarabine, usually in combination with other antineoplastic agents. Although most of the infants from these reported cases were normal, major limb malformations, including ectrodactyly and longitudinal hemimelia, were observed in at least two infants whose mothers were treated with cytarabine alone or with one other antineoplastic agent early in pregnancy. Unexplained fetal death has also been observed several times in pregnancies of women who were being treated with cytarabine and other antineoplastic agents. Cytarabine is frequently used as a selective agent in studies involving induction of Ara-C resistance-mutations in cultured cells by other agents [62]. Cytarabine has been shown to induce gene mutations in Chinese hamster V79 [17] and mouse L5178Y [63] cells in vitro, and CA in CHO cells [64] and human leukocytes [65] in vitro. In in vivo studies in mice, it has been shown to induce MN in splenocytes [66] and bone marrow cells [67],

CA in bone marrow cells, and sperm head abnormalities [65].

2.6. Daunorubicin (acetyladriamycin, CAS 20830-81-3) and doxorubicin (adriamycin, CAS 23214-92-8)

Daunorubicin and doxorubicin are anthracycline glycoside antibiotics that are administered intravenously in the treatment of leukemia and other neoplasms. These drugs are generally administered to humans in chemotherapeutic regimens involving other mutagenic teratogens and, thus, it is almost always uncertain whether the effects seen can be attributed solely to daunorubicin and doxorubicin versus the other antineoplastic agents, combinations of the drugs, or other factors. The teratogenic risk associated with daunorubicin is small-to-moderate and that for doxorubicin is undetermined, with both based on poor-to-fair data. Congenital anomalies reported from pregnant mothers treated with daunorubicin in combination with other cancer chemotherapeutic agents include polydactyly, optic lens defects, and spontaneous abortions. An infant born to a woman who received daunorubicin therapy in the third trimester of pregnancy exhibited diffuse myocardial necrosis. This observation is of particular concern since daunorubicin is known to cause cardiac damage in children and adults. Structural chromosome abnormalities have been observed in the cord blood of a clinically normal infant whose mother was treated with daunorubicin and other antineoplastic agents throughout the last half of pregnancy [68]. An increased frequency of micronuclei was induced in blastocysts of pregnant rats treated with 10 times the human dose of daunorubicin [69]. Both daunorubicin and doxorubicin have been shown to induce gene mutations in Salmonella [15,70,71], and gene mutations, SCE and chromosome aberrations in cultured mammalian cells [17,64,72–75]. In regards to germ cell mutagenesis by anthracyclines, Toppari et al. [76] reported adriamycin induction of micronuclei in germ cells of male rats while Katoh et al. [77] found adriamycin to be a female-specific dominant lethal mutagen in mice. At least part of the mutagenic activity of these two anthracyclines has been reported to be mediated through the generation of free radicals [70]. Doxorubicin is also known to be an inhibitor of topoisomerase enzymes. As such, it has the capability of interfering with the replication and segregation of chromatin which could result in production of mutations, as well as alterations in gene expression, through indirect epigenetic activity.

2.7. Ethanol (CAS 64-17-5)

Fetal alcohol syndrome (FAS), characterized by small birth weight resulting from growth retardation in the prenatal period, retardation of physical development postnatally, skeletal–facial anomalies such as microcephaly, reduction in width of the palpebral fissures and maxillary hypoplasia, and CNS lesions often resulting in mental deficits, is one of the most amply described effects from exposure to a human teratogen. Furthermore, similar craniofacial defects have been induced in mice exposed to ethanol in utero [78]. Factors such as poor protein intake, pyridoxine or other vitamin B deficiency, alcohol contaminants such as lead, and genetic predisposition, may play an important etiologic role in FAS in humans. Acetaldehyde has been hypothesized by some as the ultimate or proximate metabolite responsible for the toxic effects of ethanol [79], but the teratogenic effects of acetaldehyde per se have not been investigated in humans. Although the data are insufficient to draw any conclusion, the risk of FAS appears to be unusually high in women who drink heavily despite taking disulfiram (antabuse – inhibitor of acetaldehyde dehydrogenase; [80]). *TERIS* considers the teratogenic risk associated with heavy drinking during pregnancy to be moderate-to-high based upon data of good-to-excellent quality [11]. The mechanism by which alcohol induces FAS remains unknown. It is unclear whether the equivocal mutagenic activity of ethanol might play a role in its teratogenic activity. Evidence for the mutagenicity of ethanol is limited to certain test systems and/or test organisms [81]. Ethanol is generally not mutagenic in Salmonella [82,83]; however, it has been reported as weakly positive in certain strains [84,85] in which results suggest an indirect mutagenic activity of the oxidant type [86]. Both positive and negative effects of ethanol have also been reported for induction of CA [87,88] and SCE [89,90] in mammalian cells in vitro. Induction of SCE in CHO cells was found to require S9 and NADP co-factor or *Vicia-faba*-root S10, S9 and NAD co-factor [91]. In a review of the

genetic effects of ethanol, Obe and Anderson [92] noted that it generally does not induce genetic damage in mammalian cells in vitro, except SCE in the presence of metabolic activation, but it induces a variety of genetic effects in animal model studies in vivo including SCE, MN and dominant lethal mutations. It certainly appears to be able to consistently produce a weak induction of SCE in vivo [93,94] in mouse bone marrow, but many studies of in vivo mutagenicity by ethanol, such as induction of MN in rat bone marrow [95,96] and dominant lethal mutations in germ cells ([96,97]; NTP unpublished results), have produced contradictory results. Ethanol did not induce hprt mutations in T-lymphocytes of humans in vivo [98], but it has been reported to increase SCE (see table 3, [92]). Acetaldehyde, the initial metabolite of ethanol and a highly reactive compound which plays a central role in the most adverse effects of alcohol, is mutagenic in a variety of eukaryotic cells and mammals [92]. Some, if not all, of the mutagenicity exhibited by ethanol is probably related to its metabolism to acetaldehyde. There is also ample evidence of enzyme inductions and alterations in the expression of various genes by ethanol. Ethanol induces cytochrome P450 enzymes [99,100], which can enhance in vitro mutagenicity of chemicals requiring P450 metabolism for their action, but its ability to enhance mutagenicity in vivo is variable [101,102]. Induction of the P450(CYP)2E1 isozyme has been found to be post-translational, whereas its transcriptional activation requires a long-term presence of highly intoxicating levels of ethanol and may occur via indirect physiological responses related to those triggered by starvation [100]. It has been suggested that ethanol might also interfere with histone regulation of transcription [103]. Further, ethanol has been shown to enhance responses to iontophoretically applied GABA, perhaps by affecting a GABAA receptor [104]. It has also been demonstrated that ethanol administration to pregnant rats can influence the long-term regulation of insulin-like growth factor messenger RNA levels in their pups examined postnatally [105]. Studies with rat hypothalamic cells in culture have shown that ethanol reduces intracellular levels of Bt2cAMP and increases DNA degradation, suggesting that the neurotoxicity for beta-endorphin neurons induced by ethanol during early differentiation involves an apoptotic process [106]. It has been speculated that the sites of ethanol action could range from transcription of the apoptosis genes to translation and post-translational modification of their protein products [107]. Alterations in expressions of c-fos and c-jun were detected in rats undergoing ethanol withdrawal [108]. These alterations of various expressions, including inductions of P450 and other enzymes, however, may all be secondary or indirect results of other toxic actions rather than due to any direct epigenetic activity of ethanol.

2.8. 6-Mercaptopurine (CAS 50-44-2)

6-Mercaptopurine is a purine analog that interferes with nucleic acid synthesis and is used in the treatment of neoplastic diseases. The magnitude of teratogenic risk with 6-mercaptopurine is small-to-moderate based on a quantity and quality of data which are poor-to-fair. Transient neonatal anemia and pancytopenia have been observed among the infants of women treated with 6-mercaptopurine and other chemotherapeutic agents during pregnancy. Microphthalmia, cleft palate and hypospadias have also been observed following these combined therapy regimens. Some of the pregnancies end with still birth or neonatal death. Low birth weight may also be unusually frequent among such infants. However, as with most chemotherapies, the number of reported births from pregnant women treated with regimens that included 6-mercaptopurine that have no congenital malformations are greater than the number reported with malformations. Reproductive failure in surviving offspring of female mice treated with 6-mercaptopurine during pregnancy, including increased mortality among embryos and fetuses of the second and third generations, have been attributed to genetic damage of primordial oocytes of the first generation females exposed in utero [109]. It seems likely that the teratological effects of 6-mercaptopurine are directly and/or indirectly related to its mutagenic activity. 6-Mercaptopurine has been demonstrated to be mutagenic in a variety of assays, both in vitro and in vivo [110]. Positive results were obtained in the Salmonella assay [15,83], and in assays with cultured mammalian cells for induction of gene mutations [17,111], chromosome aberrations [21] and SCE [112]. In vivo, 6-mercaptopurine has been shown to induce chromosome aberrations [113],

MN [21] and SCE [114] in mouse bone marrow cells, and dominant lethal mutations in early meiotic and premeiotic stages of male mouse germ cells [115]. No increases in specific locus mutations [116] or heritable translocations [117] were observed in any stage of spermatogenesis in mice treated with 6-mercaptopurine.

2.9. Penicillamine (CAS 52-67-5)

Penicillamine is a thiol (sulfhydryl) chelating agent used in the treatment of rheumatoid arthritis and cystinuria. Striking and unusual connective tissue abnormalities resembling cutis laxa have been reported in some infants born to women receiving penicillamine therapy during pregnancy. In addition to lax skin, some of the infants also had inguinal hernias, loose joints, flat faces, small jaw, or vascular or tissue fragility. As with virtually all human teratogens, there appears to be considerable individual variation in susceptibility and/or maternal metabolism as there are more unaffected infants born to women treated with penicillamine than there are affected ones. But the unusual nature of the abnormalities associated with the unusual exposure suggests a causal relationship. Penicillamine has been shown to have some mutagenic activity, and perhaps anti-mutagenic activity as well. In Salmonella, its mutagenicity was found to be oxidative in nature and involve H2O2 and possibly hydroxyl radicals [118]. Earlier studies by Stark et al. [119] indicated that a thiolate anion rather than a free thiol is required. Penicillamine also induced SCEs and CA [120] but not gene mutations [121] in cultured V79 Chinese hamster cells. However, penicillamine failed to induce MN in vivo in mouse bone marrow cells [122], and it has actually been shown to protect against acrylonitrile-induced unscheduled DNA repair synthesis in vivo in rats in association with glutathione modulation [123]. Penicillamine also exhibits non-genetic or indirect-epigenetic activity. It has been reported to modulate (dose dependent increase or decrease) interleukin-8 mRNA synthesis and protein secretion from explanted human endothelial cells [124], and, as an inhibitor of heme synthesis, to reduce expression for ferrochelatase in erythroleukemic cells of mice induced with hexamethylenebisacetamide [125]. Penicillamine is also recognized as an inducer of the autoimmune disease lupus [126].

3. Human teratogens with evidence of epigenetic activity

Epigenetic does not mean non-genetic or non-mutagenic. Rieger, et al. [12] define epigenetic as 'all processes relating to the expression (transcription and translation) and the interaction of the genetic material'. An agent that acts epigenetically can alter the epigenotype; which comprises the totality of interactions among genes as well as between genes and the non-genetic environment, and which produces the phenotype. The epigenotype of a cell is a stable, heritable (at least during many cell generations) character whose mode of impression is over and above, or in addition to, the classical genotype (base sequence).

Epigenetic mechanisms may act at 3 levels of cell organization: (1) direct regulation of gene function, involving the turning-on and -off of genes or modulation of the synthesis of specific kinds of proteins; (2) regulation of cell differentiation by modifying the translation of RNA into proteins; and (3) regulation of the topographic distribution and function of proteins. Agents can induce mutations (i.e., heritable alterations in DNA) indirectly via their epigenetic actions (e.g., something that shuts off transcription or translation of a replication or repair enzyme). An agent that alters the topographic function of proteins could also produce mutations epigenetically.

It is difficult, and often impossible, to determine whether a reported alteration of gene expression induced by a chemical is actually a direct result of epigenetic activity by that chemical or just some indirect effect resulting from that chemical's non-epigenetic action upon another target. Thus, identification of epigenetic activity is tentative and dependent on further determination of pathway specificity for regulated expression of the gene in question. Inclusion of a chemical in this category is not meant to imply necessarily that the described epigenetic activity is the mechanism for its teratogenicity. The agents listed below, however, have exhibited some evidence that they have some epigenetic activity for some expression pathway. In some cases, like that of DES, where there is evidence of both mutagenic and epi-

genetic activity, we have chosen to group the chemical in this category rather than the one above because its epigenetic activity seems more certain than its mutagenic activity, or more likely to be the teratogenic mechanism or, as in the case of DES, there is some indication that even its mutagenic activity may involve an epigenetic mechanism.

3.1. Androgens (testosterone, CAS 58-22-0; methyltestosterone CAS 58-18-4; danazol, CAS 1723-88-5; and 17å-ethinyl-testosterone, CAS 434-03-7)

Testosterone and methyltestosterone are used medically to treat postpartum breast engorgement and as a palliative therapy for breast cancer. Danazol is an anabolic steroid with both androgenic and antiestrogenic activity that is used in the treatment of endometriosis, fibrocystic breast disease and hereditary angioedema. Treatment of pregnant females with androgenic steroid hormones results in virilization or masculinization of female genitalia. Affected girls have varying degrees of phallic enlargement and labioscrotal fusion, depending on the time of gestation the exposure occurs. Labioscrotal fusion is only produced by exposures during the 8th to 13th week of gestation. The magnitude of teratogenic risk for virilization is moderate based upon data which are fair-to-good. The risk for congenital malformations is undetermined as there are no epidemiological studies of congenital anomalies among infants born to women treated with androgens during pregnancy. The mechanism of androgen action varies in different tissues, but in the majority of androgen target tissues either testosterone or 5 alpha-dihydrotestosterone (DHT) binds to a specific androgen receptor to form a complex that can regulate gene expression [127]. DHT is considered the active metabolite of testosterone and methyltestosterone which reacts with the androgen receptor to produce the masculization effects seen with these androgens. Yalcinkaya et al. [128] found that ovarian tissues of spotted hyenas, which exhibit male-like genitalia and dominance over males, produced large quantities of the steroid hormone precursor androstenedione but their placentas produced only a fraction the human-equivalent amount of aromatase that would convert it to estrogen. Since the level of 17 beta-hydroxysteroid dehydrogenase, which would convert the androstenedione to testosterone, was the same in these female hyenas as in humans, they concluded that the androstenedione production by residual ovarian stromal cells during reproductive life accounts for the epigenetic transmission of this virilization. Androgens are also implicated as responsible for altering gene expression in the endogenous regulation of programmed cell death for both normal and neoplastic prostatic cells [129,130]. Strain and sex differences to chemical carcinogens have been shown to be mediated through androgen control of beta-glucuronidase [131] and cytochrome P450 inductions [132], but it is not clear whether this involves a true epigenetic regulation or just an indirect association. No studies on the mutagenicity or genotoxicity of testosterone, methyl-testosterone, danzol or 17å-ethinyl-testosterone were located. However, a synthetic androgen, trenbolone, was found to be non-genotoxic in the Ames Salmonella assay, in a variety of in vitro mammalian cell cytogenetic assays and in vivo in the mouse MN assay [133].

3.2. Cocaine (Cas No. 50-36-2)

Cocaine is a topical anesthetic, local vasoconstrictor and central nervous system (CNS) stimulant that is widely abused recreationally. Caution is urged in interpretation of effects attributed to cocaine because it is often abused along with other substances. However, a variety of malformations, including exencephaly, microcephaly, low nasal bridges with transverse nasal creases, renal defects such as hydronephrosis and renal agenesis, ventricular septal heart defects and limb reduction, have been reported among the offspring of mothers known to have used cocaine during pregnancy. Abruptio placenta is increased ten-fold to about 10% of all exposed pregnancies, and this along with other vascular effects, such as cocaine-induced CNS hemorrhage or ischemia, may account for a major portion of the damage to the central nervous system associated with in utero cocaine exposures. The magnitude of teratogenic risk is moderate for placental abruption and other serious pregnancy complications and small-to-moderate for congenital anomalies. The quality and quantity of the data are fair-to-good. No studies on the mutagenicity of cocaine were located in the published literature, except for a dubious report of a male-mediated effect

for increased-hyperactivity among offspring of cocaine-treated male rats [134]. However, there were numerous studies indicating that cocaine may have epigenetic activity. Weaver et al. [135] reported increases in c-fos and jun-B mRNA levels in fetal rats whose mothers were injected with cocaine. Cocaine-binge induction in rat brain of mRNA for a nuclear transcription factor, zif/268, has also been reported [136]. A cocaine- and amphetamine-regulated cDNA and genomic DNA transcript has been identified in rats and humans [137]. Cocaine is purported, from in vitro studies, to be a ligand for the cocaine- and antidepressant-sensitive human norepinephrine transporter (hNET) [138].

3.3. Diethylstilbestrol (DES, CAS 56-53-1)

Diethylstilbesterol (DES) is a nonsteroidal synthetic estrogen that is used in treatment of ovarian insufficiency and as a postcoital contraceptive. It has also been used to inhibit lactation and in palliative treatment of breast carcinoma. Adenocarcinoma of the vagina was found to occur in a high proportion of daughters of women treated with DES during pregnancy. The median age at which malignancy was diagnosed was 19 years. The overall risk that a women whose mother took DES during pregnancy will develop clear cell adenocarcinoma of the vagina or cervix by age 34 is estimated to be about 1/1000. However, studies have found that more than 70 percent of the women who were exposed in utero before the 9th week of gestation exhibited precancerous vaginal adenosis, and the risk of vaginal or cervical squamous cell intraepithelial neoplasia is about twice as great in exposed versus non-exposed women. Miscarriages and absence of full-term infants seem to be more common in women exposed to DES in utero. Malformations, such as T-shaped uterus, constricting bands of the uterine cavity, uterine hypoplasia, or para-ovarian cysts, also occur with increased frequency. Clitoromegaly has also been observed. The duration and amount of menstrual bleeding has been found to be decreased in women exposed to DES in utero, but the length and variability of their menstrual cycle, as well as the prevalence of menopausal symptoms and age at menarche, was found to be unaffected [139,140]. Males exposed in utero have been found to have genital lesions such as epididymal cysts, hypotrophic testes or capsular induration of the testes. Subsequent follow-up of males with genital malformations have found no impairment of their fertility or sexual function [141], and no signs of malignancy. The magnitude of teratogenic risk for genital tract anomalies in females is considered to be small-to-moderate based on fair-to-good quality and quantity of data. The magnitude of teratogenic risk is minimal-to-small for clear cell carcinoma of the cervix, minimal for genital tract anomalies in males, and none-to-minimal for non-genital congenital anomalies, based upon data which are good, poor-to-fair and fair-to-good, respectively. DES has been shown by ^{32}P-postlabeling/TLC to produce DNA adducts on cell-free calf-thymus DNA, but it did not induce single-strand breaks in lambda DNA [142]. In vivo studies in mice by Moorthy et al. [143] suggested that the adduct formation first involved hydroxylation of the allylic alpha-carbon of the ethyl side chain(s) followed by formation of DNA-reactive sulfuric acid esters. Studies of DNA adducts produced by DES in maternal and fetal hamster tissues have implicated diethylstilbestrol-4′,4h′-quinone (DES-Q) and other unknown quinones as the active intermediate metabolites [144]. DES was negative for mutation induction in Salmonella [145], but positive in mouse lymphoma L5178Y cells [146]. In CHO cells, DES was concluded to be a weak inducer of chromosomal damage, including polyploidy and aneuploidy, but it did not induce SCE [147]. DES induction of aneuploidy has also been demonstrated in cultured human lymphocytes using fluorescence in situ hybridization (FISH) techniques with chromosome specific DNA probes [148,149]. DES induced MN in mouse germ cells in vivo with detection in round spermatids following treatment of late differentiating spermatogonia or early spermatocytes; MN were also seen following in vitro exposure of isolated tubules [150]. The mechanism(s) by which DES produces clastogenic and/or aneuploidigenic effects are unknown, but could include epigenetic actions. DES has been reported to affect microtubule polymerization and depolymerization by preferential covalent binding to tubulin [151–153], and to alter the expression of lactoferrin and epidermal growth factor genes [154–156], perhaps through alteration of methylation patterns [157].

3.4. Hydantoin anticonvulsants (phenytoin / diphenylhydantoin, CAS 57-41-0; mephenytoin; and ethotoin)

Hydantoin anticonvulsants are used in the treatment of seizure disorders. A characteristic pattern of congenital anomalies including congenital heart disease, facial clefts and other malformations, and referred to as the 'fetal hydantoin syndrome', has been observed among children of epileptic women treated with hydantoin anticonvulsants during pregnancy. For phenytoin, the magnitude of teratogenic risk is considered small-to-moderate based on a quality and quantity of data which are fair-to-good. There is much less data for mephenytoin and ethotoin. More recent studies have suggested that some of the anomalies associated with fetal hydantoin syndrome may be due to the epilepsy per se rather than to anticonvulsant therapy, and indicate that further studies are needed to understand better the role of folic acid deficiency and genetic predisposition relative to this syndrome. Phenytoin produced a significant down-regulation in the level of expression of 6 or 7 of 20 genes studied in mouse embryos following chronic in utero exposure [158], and significantly altered (some up- and some down-regulated) the expression of one or more of 10 genes examined in neural tubes of mice similarly treated killed at various time-points throughout neural tube closure [159]. Phenytoin is also associated with alterations in expression of other genes such as interleukin-1 beta, prostaglandin E2 [160] and those involved in the cytochrome P450 pathway [161,162]. Phenytoin was positive in a few short-term tests for mutagenic activity but the overall impression is that it is not mutagenic, although it may have some weak genotoxic activity. The chemical did not induce mutations in Salmonella [163], mouse lymphoma L5178Y cells [164] or germ cells of male *Drosophila melanogaster* [165], nor did it induce CA in CHO cells in vitro [166]. Small but significant increases in SCE were induced in CHO cells treated with phenytoin in vitro in the presence of S9 [166]. McFee et al. [167] observed no induction of MN or CA in bone marrow of mice treated with phenytoin. In earlier studies, Montes-de-Oca-Luna et al. [168] reported significant increases in MN in bone marrow cells of mice given single or multiple injections of various doses of phenytoin and Barcellona et al. [169] reported weak induction of MN in mouse fetuses exposed in utero to phenytoin. Similar to the in vitro CHO results above, weakly positive responses were observed in vivo in the mouse bone marrow SCE test at both 23 and 42 hours post-treatment [167]. Kindig et al. [170], however, reported negative results with phenytoin for three different cytogenetic assays, including CA in vitro in CHO, and in vivo induction of SCE and MN in mice. When SCE frequencies were examined in peripheral blood lymphocytes of phenytoin treated and untreated epileptic patients and in normal controls, Taneja et al. [171] found that both groups of epileptic patients had significantly increased SCE compared to normal controls but the frequencies of SCE in phenytoin treated and untreated patients were similar. No mutagenicity data were located in the published literature on mephenytoin or ethotoin.

3.5. Lithium (CAS 7439-93-2)

Lithium salts, especially lithium carbonate, are used for the prevention and treatment of affective mental illness (mood disorders) such as manic (or bipolar) depression. An association has been observed between maternal treatment with lithium carbonate during pregnancy and the occurrence of cardiovascular malformations, especially Ebstein's anomaly, in children. The magnitude of teratogenic risk is considered to be small based on a quality and quantity of data which are fair-to-good. If congenital heart disease is increased by in utero exposure to lithium, the risk is probably only doubled. Abnormalities associated with lithium toxicity, such as neurological, cardiac and hepatic dysfunction, may also occur in infants born to women who are receiving treatment with lithium salts near term. Lithium chloride was negative for gene mutation induction in Salmonella [163]. Lithium carbonicum failed to induce 6-thioguanine resistant and HGPRT mutants in mammalian cells [172]. Lithium hypochlorite was not mutagenic in Salmonella and did not induce HGPRT mutations in CHO cells; although it did induce CA in vitro in CHO cells it did not induce them in rat bone marrow cells in vivo [173]. Leonard et al. [174], in a recent review of the mutagenicity of lithium compounds, concluded that they have no significant clastogenic activity and that mutagenic

activity is doubtful. Lithium has been shown to induce homeotic changes in developing zebrafish [175,176] and frogs [177–179] that are associated with alterations in gene expressions; and in frog embryos, lithium treatment is also associated with abnormal heart development [180].

3.6. Methylene blue (CAS 61-73-4)

Methylene blue is used in the treatment of methemoglobinemia and as a marker after injection into amniotic fluid. Hemolytic anemia is a recognized complication of methylene blue therapy in children and hemolytic anemia and jaundice have been observed in several infants born after intra-amniotic injection of methylene blue late in pregnancy. There are also several reports of discordant multiple intestinal atresia in twins where one received methylene blue and the other did not. In some instances, the affected twin was known to have been the one receiving the dye. The magnitude of teratogenic risk associated with intraamniotic injection is considered to be minimal-to-small based on poor-to-fair data. The risks associated with topical or intravenous use are unknown. Methylene blue inhibits soluble guanylate cyclase activity [181], which is involved in the production of guanosine monophosphate (GMP) that directly affects transcriptional processes and the control of cell cycling. This epigenetic effect could be responsible for the general pharmacologic properties of methylene blue as well as its teratogenicity. Methylene blue has also given positive results in several genotoxicity tests, predominantly due to singlet oxygen radicals generated in the presence of visible light [182]. Studies in E. coli suggest that the major promutagenic lesion induced by methylene blue photosensitized by visible light is 8-oxo-7,8-dihydroguanine, which, through SOS dependent repair processes, results in the production of primarily G–C transversions [183,184]. Methylene blue induced gene mutations in Salmonella ([182,185–187]; NTP unpublished data) and cultured mouse lymphoma cells [188], and CA and SCE in cultured CHO cells (NTP, unpublished data). In contrast to the positive in vitro results, no induction of MN was observed in polychromatic erythrocytes of male mice treated with a single intravenous [188] or intraperitoneal (NTP, unpublished data) injection of methylene blue.

3.7. Primidone (CAS 125-33-7) and its metabolite phenobarbital (CAS 50-06-6)

Primidone is an anticonvulsant used to treat grand mal, psychomotor and focal motor seizures, and whose major metabolite is phenobarbital, a barbiturate. It has a small-to-moderate teratogenic risk based on a quality and quantity of data which are fair-to-good. A pattern of minor dysmorphic features has been observed among a few children born to women treated with primidone for epilepsy during pregnancy. This pattern, which may represent a 'fetal primidone syndrome', includes poor growth, especially of the head, and clefts of the palate and lip. Heart defects, low nasal bridges, ocular hypertelorism and retardation have also been reported. The teratogenic risk associated with phenobarbital, the metabolite of primidone, is minimal-to-small based on data which are fair-to-good. Children of women taking phenobarbital for treatment of seizure disorder during pregnancy were also reported to have increased frequencies of congenital anomalies, especially facial clefts and congenital heart disease. As with primidone, however, many of these women were also taking other anticonvulsants. Furthermore, there is always the question of whether some or all of the malformations may be associated with the mother's seizure disorder rather than the anticonvulsants. Prospective studies of the effect of barbiturates in general on pregnancy outcomes, in conjunction with their use as anti-epileptics and as tranquilizers, reveal no increase in congenital defects. Primidone has very limited mutagenic activity. It was found to be mutagenic in strain TA1535 of Salmonella in the absence of S9 exogenous activation but was not mutagenic in any of the other tester strains or in the presence of S9 [189,190]. Primidone failed to induce sex-linked recessive lethal mutations in Drosophila [191], SCE or CA in CHO with or without S9 [190,192], CA in human peripheral lymphocytes treated in vitro in the absence of S9 [193,194], CA in peripheral blood lymphocytes of children on primidone therapy [54], CA in mouse bone marrow cells [191] or dominant lethal mutations in mouse germ cells [191,195]. There is one dubious report of an increased frequency of MN in mouse bone marrow cells induced by primidone [196] that was not confirmed by a subsequent study of MN in mouse

peripheral blood [190]. Like primidone, phenobarbital also has minimal mutagenic activity. It was found to be weakly active in Salmonella strain TA1535 [197] and for induction of CA in CHO [198]. It was negative for induction of SCE and CA in CHO [192] and in human fetal fibroblasts [199], with and without S9. One report [200] claimed induction of dominant lethal mutations in mouse germ cells, but these results appear suspect in that most of the effect was pre-implantation loss with an increase in dead implants primarily from exposed pre-meiotic stages of spermatogenesis, which are generally insensitive to dominant lethal induction. Both primidone and phenobarbital induce P450 enzymes and are known to enhance the mutagenicity of a variety of mutagens dependent upon P450 enzyme metabolism. Phenobarbital is reported to also stimulate cell proliferation and inhibit apoptosis of neoplastic foci in rodent livers [201]. The molecular response 'triggered' by phenobarbital exposure is reported to be uncoordinated in that P450IIB1 mRNA expression is enhanced or up-regulated while P450IIC7 is repressed or down regulated [202]. Phenobarbital has also been shown to induce transcription of the alpha-1-acid glycoprotein (AGP) gene in rats, the expression of which is controlled by a specific combination glucocorticoids and cytokines [203], and phenobarbital has been shown to alter chromatin structure of the CYP2B1/2 rat liver gene in association with induction of its transcription [204]. Phenobarbital also induced expression of P450 gene CYP6A2 in Drosophila [205], and was shown to induce hepatic bilirubin in vitro by increased transcription from the UGT-1 gene [206]. It is unclear from the available literature whether these inductions actually represent true epigenetic alterations of transcription and/or translation by phenobarbital directly or an indirect association through its interactions with some other epigenetic actor.

3.8. Retinoids (tretinoin, isotretinoin, CAS 302-79-4; and etretinate, CAS 54350-48-0)

These retinoids are the acid form of vitamin A or its derivatives, and are used primarily in the treatment of dermatologic diseases such as acne and psoriasis. Some have recently found limited use as anticancer therapeutics. Isotretinoin, which is the 13-*cis* form of retinoic or vitamin A acid, and etretinate are both administered orally. The magnitude of teratogenic risk associated with these two retinoids is considered to be high, based upon qualities and quantities of data which are excellent and fair, respectively. A very uncommon but strikingly similar pattern of anomalies has been observed in children exposed to isotretinoin during embryonic development. This pattern includes microtia/anotia, micrognathia, cleft palate, conotruncal heart and great vessel defects, thymic abnormalities, eye anomalies, and central nervous system malformations. Limb reduction defects may occasionally occur. Affected children often perform in the subnormal range on standard intelligence tests. The number of cases of documented exposure to etretinate are much fewer than with isotretinoin, but a similar embryopathy involving neural tube and other CNS defects and craniofacial and skeletal abnormalities has been associated with its use during pregnancy. Furthermore, some malformed offspring have been reported from mothers who ended etretinate therapy months before conception. The mean serum half-life for isotretinoin is less than 24 hours, but etretinate persists in the body for an extremely long time and has been detected in serum one to two years or more after cessation of therapy. The magnitude of teratogenic risk associated with tretinoin, or all-*trans* retinoic acid, is undetermined based on poor data because it is generally used only as a topical application where the exposure levels from cutaneous absorption is low. No studies on the mutagenic activity of retinoids were located in the published literature. However, both vitamin A and retinoic acid (tretinoin) have been shown to reduce the frequency of mutations induced in Salmonella by a variety of mutagenic carcinogens through their ability to inhibit certain forms of cytochrome P450 enzymes [207]. Inhibition of heterocyclic amine (Trp-1)-induced umu gene expression in Salmonella by vitamin A and retinoic acid appeared to be due to inhibition of P450-mediated metabolic activation of Trp-1 [208]. Isotretinoin, tretinoin, and some of their metabolites have been shown to induce homeotic limb malformations in mice exposed on gestation day 5 suggesting that alteration of gene expression in pre-embryonic cell lineages may be involved [209] (NTP unpublished data). The role of retinoids in regulation of the

expression of Hox and numerous other genes in the signalling cascade involved in limb patterning is well established [210–212]. An important role for retinoids in gene expressions associated with neural patterning and craniofacial development has also been established [213,214]. Most important to the unequivocal establishment of the direct epigenetic activity of retinoids has been the identification of the various components of the signalling pathways including cellular retinoic acid binding proteins (CRABP I and II), the retinoid receptors (RAR and RXR), their subfamilies, regulation of their expression and most, if not all, of the orchestrated steps in the cascade [215–217].

4. Human teratogens with unknown or equivocal evidence of mutagenic or epigenetic activity

Inclusion in this category is based upon our inability to locate, through search of both the Medline and NTP data bases, significant hits under the terms mutagen*, genotoxicity, epigenetic or gene-expression when combined with the chemical name and CAS registry number. In some cases, these human teratogens have either not been tested or not tested adequately and/or gave weak, mixed or equivocal results in the mutagenicity tests to which they were subjected. Information about the epigenetic activity of these chemicals either is lacking or reported alterations of gene-expression appear to be the indirect result of non-genetic action(s) of the chemical upon other pathways.

4.1. ACE inhibitors (Captopril, CAS 62571-86-2; and Enalapril MK-42)

Angiotensin converting enzyme (ACE) inhibitors are oral medications used in the treatment of hypertension. *TERIS* indicates that, based upon a quality and quantity of data which are fair-to-good, there is a moderate teratogenic risk from exposures to ACE inhibitors after the first trimester. Adverse effects include oligohydramnios and fetal anuria, pulmonary hypoplasia, renal failure, persistent patent ductus arteriosus, mild or severe intrauterine growth failure, and fetal death. Hypoplasia of the skull bones and other skeletal anomalies are also a frequent finding. Although the risk of congenital anomalies is considered unlikely, there is substantial risk of oligohydramnios and fetal distress or death associated with captropril or enalapril treatment of pregnant hypertensive women. Alterations of gene expressions are observed with treatments involving ACE Inhibitors; however, it is not clear whether these effects are due to epigenetic activity of the ACE Inhibitor or are simply the indirect result of angiotensin-converting enzyme inhibition, subsequent changes in the vascular muscle or effects on blood pressure. Captopril was found to cause dose-dependent suppression of the interleukin 1 (IL-1) beta-induced synthesis of tumor necrosis factor alpha (TNF) and the IL-1 alpha cytokine [218]. Since accumulation of mRNA for IL-1 and TNF were not affected by captopril, it was concluded that suppression of IL-1 and TNF synthesis was at the post-transcriptional level and that it might influence cytokine-mediated vascular cell growth. Captopril analogs/derivatives, alaceprila and imidapril, were found to suppress the enhanced gene expression of skeletal alpha actin and atrial natriuretic polypeptide in rat cardiac muscle [219]. No evidence of direct epigenetic activity by enalapril was located from the published literature. The only reference to mutagenic activity with either captopril or enalapril found in the published literature was a report of antimutagenic activity in which the frequency of micronuclei induced by adriamycin in mouse bone marrow was reduced by captopril pretreatment [220].

4.2. Carbamazepine (CAS 298-46-4)

Carbamazepine, an anticonvulsant agent used to prevent grand mal, psychomotor and partial seizures, is indicated as having a small teratogenic risk based on fair-to-good data. Reported anomalies include an increased frequency of neural tube defects such as spina bifida and microcephaly, and growth and developmental delay associated with minor facial and other anomalies such as up-slanting eyes, hypoplastic nasal bridge, short upturned nose and variable nail hypoplasia. Based upon a very limited amount of genetic toxicity information, carbamazepine does not appear to be mutagenic. Carbamazepine was reported to bind DNA in vitro after incubation with liver microsomes [221]. It also induced SCE and changes

in proliferation indices in lymphocytes of patients on carbamazepine therapy, but it did not induce CA or MN in this same cell population [222]. A Salmonella assay conducted on the urines of these patients was also negative [222]. Schaumann et al. [223] had previously reported induction of SCE and CA in human lymphocytes treated in vitro but not those exposed in vivo. Carbamazepine-epoxide is suspected as the cause of the teratogenicity and other side-effects of carbamazepine [221] but the 10,11-oxide metabolite was not mutagenic in Salmonella [224]. No information was located regarding potential epigenetic activity by carbamazepine.

4.3. Coumarin anticoagulants (wafarin, CAS 81-81-2; and dicumarol, CAS 66-76-2)

Warfarin and dicumarol are members of the coumarin family. They are anticoagulants used in treatment of a variety of thromboembolic disorders. Their anticoagulating activity is associated with their ability to depress synthesis of vitamin K-dependent clotting factors through their inhibition of DT-diaphorase [NAD(P)H dehydrogenase: (quinone acceptor) oxidoreductase], a flavoprotein that reversibly catalyzes the oxidation of NADH or NADPH by various quinones like phylloquinone (a.k.a., vitamin K). Both warfarin and dicumarol are considered to have a moderate teratogenic risk based on fair-to-good data. There is a syndrome of congenital anomalies associated with exposure to coumarin anticoagulants during pregnancy consisting of nasal hypoplasia, stippled epiphyses, growth retinal-optic atrophy and central nervous system anomalies. Similar anomalies have been reported in an infant with an inherited deficiency of multiple vitamin K-dependent coagulation factors, suggesting the vitamin K deficiency as the causal factor. Furthermore, DT-diaphorase is part of the mitochondrial respiratory chain and defects or deficiencies in this enzyme are a known cause of neurological diseases such as Parkinsonism; it is plausible that warfarin- or dicumarol-induced deficiencies of this enzyme may similarly produce neurological pathologies during development. No epidemiological studies were found on production of congenital anomalies by coumarin per se, which is a camphor found in tonka beans, sweet clover and some other plants and is used as a flavoring agent. Although coumarins in general modify many different biological processes, only a small number of them have been shown to be genetically active [225]. Coumarin induced gene mutations in *Salmonella typhimurium* [163,226,227] and SCE in CHO cells in vitro [166]. It did not induce CA in CHO cells [166] or sex-linked recessive lethal mutations in germ cells of male *Drosophila melanogaster* [228,229]. No induction of MN occurred in erythrocytes of mice administered coumarin for 13 weeks (NTP, unpublished data). Various other coumarins have also been shown to be genotoxic, inducing C-mitotic effects, chromosome breakage, various sorts of rearrangements and affecting DNA-repair and mutation frequency in bacterial, plant and animal cells [225]. Furthermore, a particular coumarin may be innocuous and metabolically stable in one species, yet in another it may be metabolically modified to a toxic derivative. Because of significant chemical and metabolic differences, the mutagenicity of these coumarins is probably not indicative of any potential for mutagenic activity by warfarin and dicumarol. No studies on the mutagenicity or epigenetic activity of either warfarin or dicumarol, specifically, were located in the published literature. Dicumerol, however, has been reported to both decrease [230,231] or increase [232,233] the mutagenicity of other agents depending on whether those agents are activated or inactivated by an NAD(P)H-dependent metabolism. It would appear unlikely that direct mutagenesis would play a role in the production of the types of developmental anomalies seen with warfarin compounds.

4.4. Indomethacin (CAS 53-86-1)

Indomethacin is a prostaglandin synthetase inhibitor that is used as an analgesic, anti-inflammatory, and antipyretic agent. It has been used to arrest premature labor, to treat polyhydramnios, and induce closure of the ductus arteriosus in premature infants. Treatment with indomethacin late in pregnancy may be associated with the development of fetal anuria, oligohydramnios, premature closure of the ductus arteriosus and consequent problems in prenatal adaptation (e.g., persistent pulmonary hypertension). The magnitude of teratogenic risk for premature closure of the ductus arteriosus is small-to-moderate based

on a quality and quantity of data which are fair-to-good. The magnitude of risk for malformations is minimal-to-small based on poor-to-fair data. Indomethacin is associated with alterations in the expression of numerous genes involved with the activities of prostaglandin synthetase [234–236]; however, these appear to be indirect effects of altered prostaglandin synthetase rather than direct epigenetic activity by indomethacin per se. There are also numerous examples of both co-mutagenic and anti-mutagenic activity for indomethacin associated with its inhibition of prostaglandin synthetase, but only a couple of reports indicating independent mutagenic activity. Indomethacin induced gene mutations in Salmonella [189]. Devi and Polasa [237] also reported evidence of genotoxicity by indomethacin in vivo in male mice through assays for induction of MN in bone marrow cells and chromosome anomalies in germ cells at meiotic metaphase, as well as assessment of abnormal sperm morphology. There is also a report of induction of SCE in peripheral blood of exposed humans. In association with its inhibition of prostaglandin endoperoxide synthetase (PES), indomethacin was shown to suppress polycyclic aromatic hydrocarbon (PAH) induction of SCE in cultured mammalian cells [238], and of gene mutations in Salmonella [239]. Conversely, Lee and Norppa [240] demonstrated indomethacin potentiation of SCEs in cultured human lymphocytes induced by styrene and styrene-7,8-oxide, which are inactivated by PES. Indomethacin was also shown to protect against macrophage-mediated induction of DNA damage in tumor cells associated with reactive oxygen and arachidonate [241] and to reduce ochratoxin DNA adducts in rodent bladder and kidney tissue [242]. Co-administration of indomethacin with benzene prevented induction of micronuclei in vivo in polychromatic erythrocytes of mice [243].

4.5. Iodide

Protracted ingestion of iodide-containing medications (expectorants) by the mother may result in fetal thyroid enlargement which can lead, in some cases, to tracheal compression and choking in the newborn. These goiters are due to fetal thyroid inhibition along with secondary compensatory hypertrophy. No information on either the mutagenic or epigenetic activity of Iodide was located in the published literature. One of the 'iodinated' expectorant medications, however, was found to contain, in addition to the expected ingredient iodinated glycerol, mutagenic agents such as 3-iodo-1,2-propanediol and dioxanes (NTP, unpublished results). It is not clear what relationship, if any, mutagenic activity of components of expectorants might have to production of the effects seen in exposed fetuses.

4.6. Methimazole (CAS 60-56-0) and propylthiouracil (CAS 51-52-5)

Methimazole and propylthiouracil are used to treat hyperthyroidism. They cross the placenta and can cause suppression of fetal thyroid function, resulting in transient neonatal hypothyroidism that may cause fetal thyroid hyperplasia and goiter. Methimazole, an imidazole derivative, has also been reported to produce ulcer-like midline scalp defects in the offspring of mothers under treatment for hyperthyroidism. Other congenital defects associated with treatment of pregnant mothers with methimazole and/or propylthiouracil include aortic atresia, hypospadias, and imperforate anus. Propylthiouracil is not associated with production of scalp defects. The magnitude of teratogenic risk associated with propylthiouracil is none for malformations, based upon a quality and quantity of data which are poor-to-fair, and small-to-moderate for goiter, based upon data which are good. No information on the mutagenic or epigenetic activity of propylthiouracil was found in the published literature. For methimazole, we found one negative mouse bone marrow MN study [244] and one negative mouse dominant lethal study [245]; there were no reports on its epigenetic activity. However, as an inhibitor of flavin-containing monooxygenases (e.g., thyroid peroxidase), methimazole has been reported to have anti-mutagenic activity in a Salmonella assay on urine from rats treated with ethylene dibromide [246] and in a plant cell assay of mutation induction by phenylenediamine [247]. Methimazole has also been reported to have enhanced mutagenic activity in a plant cell assay of mutation induction by 2-aminofluorene [248]. There are numerous examples in the literature of alterations in the expression of various genes regulated by thyroid-related hormones in association with both methima-

zole's and propylthiouracil's production of hypothyroidism [249–252]; however, none of these appear to specifically involve regulation of gene expression by methimazole or propylthiouracil and, thus, they are not considered to have epigenetic activity.

4.7. Methyl mercury

Ingestion of methyl mercury, through the eating of fish in Japan and bread in Iran, as well as a few other food sources in various sites, is associated with microcephaly, cerebral palsy and other impairments of motor and mental development in the offspring of pregnant women who consumed these contaminated food products. Some of the exposures have been documented in the third gestational month, while others were in the third trimester. Milder cases characterized by developmental retardation along with exaggerated tendon reflexes and pathological extensor plantar reflexes have also been described. Long-chain organic mercury compounds, which are widely used as preservatives in the paint industry and as agricultural fungicides, are believed to be less toxic than methyl mercury. The teratogenic risk for these long-chain organic mercury compounds is undetermined, but it may be substantial considering that at least one such compound, phenylmercuric acetate, has been shown to induce embryonic death, growth retardation and central nervous system malformations in mice. Inorganic mercury, to which pregnant females may be exposed in the workplace or through dental amalgams, is not converted to the organic form and is not considered to be teratogenic. The mutagenicity of organic mercury compounds was reviewed by Ramel [253] and Leonard et al. [254]. Methyl mercuric (II) chloride and methyl mercury hydroxide were not mutagenic in Salmonella [83]. Methyl mercuric (II) chloride did not induce dominant lethal mutations in germ cells of male *Drosophila melanogaster* [255], but positive results were reported in this assay with methyl mercury hydroxide [253] and ethyl mercury [256]. The most commonly reported effect of mercury exposure in cultured mammalian cells is the production of C-mitoses [254]. Experiments designed to measure the genetic effects of mercury in mammalian germ cells gave no consistent or clear results, perhaps due, at least in part, to the extremely poor uptake of mercury by male reproductive tissues [254].

4.8. Polychlorinated biphenyl (PCB)

Maternal ingestion of chlorobiphenyls from contaminated cooking oil in Japan was responsible for dark-brown staining of the skin of newborn babies and an associated intrauterine growth retardation. The skin discoloration faded in a few months. Similar reductions in birth weight were observed in offspring from mothers eating PCB contaminated fish from the Great Lakes as well as in offspring from mothers exposed to PCBs in the workplace. In postnatal functional studies, fetuses exposed to the higher levels of PCB contaminants exhibited hypotonicity and hyporeflexia. Effects on motor maturation and some evidence of impaired infant learning have also been detected. Although a locus encoding a biphenyl/polychlorinated biphenyl (PCB) degradation pathway has been exquisitely elucidated in Pseudomonas [257,258], and a PCB binding protein in mammalian lung Clara cells has been discovered, characterized as to structure and expression, and its gene even cloned [259], no regulated control of either gene's expression by PCB has been described. PCBs have also been implicated in the induction of cytochrome P450 metabolizing enzymes and CYP1A genes in rat liver [260] but, again, the relationship between these functions and any epigenetic activity is unclear. Although PCBs are clearly involved in the enhancement of the mutagenicity of compounds that are metabolically activated by P450 enzymes induced by PCBs, the amount of data specifically on the mutagenicity of PCBs is more limited. PCBs can covalently adduct DNA but they do not do so readily [261]. PCBs are classified by IARC as non-mutagenic in vivo; but, in spite of almost 20 years of research, their mutagenicity in vitro is still debated [262]. They have generally produced mixed or negative results for induction of gene mutations in Salmonella [263–265] as well as CA in cultured mammalian cells [266]. PCBs were found to be negative for induction of single strand breaks and MN in human lymphocytes [267] and negative in both the electrophoresis or comet assay and the MN assay in blood cells of fishes exposed to PCB-containing water [267]. Methylsulphonyl PCB congeners

also failed to induce MN in vivo in human lymphocytes [268].

4.9. Quinine (CAS 130-95-0)

Quinine is a cinchona alkaloid that is administered in small doses to treat night leg-cramps and in large doses to treat malaria. It has sometimes been taken illicitly to induce abortion. It has also been used as a cold remedy, to 'cut' narcotics and, in small amounts, as a flavoring agent. It is also an ingredient in some hair-care products. The magnitude of teratogenic risk for large doses of quinine taken to induce abortion are considered moderate based on data which are fair-to-good. Deafness is perhaps the most commonly reported malformation and ototoxicity is a known complication of quinine therapy in adults. Major malformations reported anecdotally among infants born to women who took large doses of quinine during the first trimester of pregnancy include central nervous system anomalies (especially hydrocephalus), limb defects, cardiac defects, and gastrointestinal tract anomalies. Sideropoulous et al. [269] reported that quinine enhanced UV mutagenicity in E. coli suggesting it might be an inhibitor of DNA repair. King et al. [270] reported a positive response for mutation induction by quinine dihydrochloride in Salmonella strain TA98, but quinine hydrochloride failed to induce gene mutations in any Salmonella strain [271]. Quinine hydrochloride was found to produce small but significant increases in the incidences of SCE, MN and chromosome breaks in bone marrow cells of inbred mice, but it did not produce any of these effects in Chinese hamsters [271]. No information on the epigenetic activity of quinine or its salts was found in the published literature.

4.10. Tetracyclines (tetracycline-HCl, CAS 60-54-8; oxytetracycline, CAS 79-57-2; doxycycline CAS 564-25-0; and demeclocycline)

Tetracyclines are broad-spectrum antibiotics which are frequently used in the treatment of respiratory tract or other infections. Demeclocycline is used to treat protozoal infections and to inhibit antidiuretic hormone-induced water resorption and inappropriate antidiuretic hormone secretion. The main effect of exposure to tetracyclines is staining of the primary dentition in fetuses exposed during the second or third trimesters of pregnancy. However, this staining appears to be only of cosmetic significance, and does not affect development of the enamel or the likelihood of forming caries. The magnitude of risk for dental staining in fetuses exposed during the second or third trimesters of pregnancy is high for both tetracycline and doxycycline based on excellent data, and small-to-moderate for both demeclocycline and oxytetracycline based on fair-to-good data. Where it has been examined, the frequencies of congenital anomalies in general, of major malformations, and of minor anomalies, among pregnant women exposed to tetracyclines are no greater than expected in the general population. The magnitude of teratogenic risk for malformations with tetracyclines is either undetermined because of a lack of data or none-to-minimal based on a quality and quantity of data which are fair. Tetracycline HCl was not mutagenic in Salmonella [197], and it did not induce SCE or CA in CHO cells [272]. No induction of sex-linked recessive lethal mutations was seen in male *Drosophila melanogaster* after exposure to tetracycline HCl [38]. Questionable results were obtained with this chemical in the mouse lymphoma assay [273]. Oxytetracycline HCl was not mutagenic in Salmonella [189,274], but after nitrosation, mutagenic activity was detected in several strains [274]. No increase in SCE or CA was seen in CHO cells exposed to oxytetracycline, with or without S9 [272], and no induction of sex-linked recessive lethal mutations was seen in Drosophila [38]. Oxytetracycline HCl induced mutations in mouse lymphoma L5178Y cells in the presence of induced rat liver S9 [273,275]. Oxytetracycline hydrochloride also induced MN in bone marrow cells of Swiss mice administered the compound by gavage [276]. The response was observed in both the presence and the absence of potassium nitrite. No mutagenicity information was found for either demeclocycline or doxycycline. The role of the bioengineered tetracycline transactivator–repressor–promotor–operator system in controlled expression of genes in a variety of cell types is well-established [277–281]. But there was no information located in the published literature specifically on the epigenetic activity of tetracycline in in vivo models that would pertain to its teratogenicity.

4.11. Thalidomide (CAS 50-35-1)

Thalidomide is probably the most notorious of all the human teratogens, essentially launching the field of teratology as a unique scientific discipline. A couple of very important teratologic principles were established by the observations with thalidomide: (1) there may be extreme variability in species susceptibility to teratogens, in that mouse and rat embryos were found to be relatively insensitive to thalidomide, while the rabbit, monkey and man were sensitive; and (2) there is often a very clear affiliation between the time of exposure and the presence and type of congenital defect produced by a teratogen. Prescribed as a sedative, primarily in Europe and Japan in the 1950s–60s, thalidomide was found to induce limb malformations when taken between the 27th and 40th day of pregnancy. The specific type of defect observed was well correlated with the specific time of treatment; exposure between the 27th to 30th days affecting mostly the arms while treatments between the 30th to 33rd days causing primarily leg malformations. Exposures prior to the 27th day sometimes produced malformations in the head and neck region. Approximately 20 percent of the mothers who ingested the drug during the sensitive period had defective children. There is currently a resurgent interest in the US in the use of thalidomide for treatments of AIDs-related oral lesions and in the treatment of leprosy, with preclinical trials and an application for FDA approval of its use being filed. Thalidomide is not a mutagen [282]. It does not induce mutations in Salmonella [283]. It gave both negative [284] and positive [285] results for chromosome aberrations induced in embryos exposed in utero. Relative to germ cell mutation tests, it was reported to be negative in the mouse dominant lethal assay [195] and the NTP (unpublished results) found it negative in the Drosophila test for induction of aneuploidy, sex-chromosome gain/loss. However, reports of offspring with limb deformities produced by fathers with limb deformities purportedly produced by their exposure to thalidomide [286,287], have raised the question of whether thalidomide might have acted in a way to produce a mutation in the fathers' germ cells in utero. This postulate has been challenged partially on the basis of mutagens not having specificity for site directed mutations [288,289], as well as on the lack of evidence for thalidomide's mutagenicity [290]. Although the arguments against thalidomide having mutagenic activity in germ cells, especially specific-site mutagenicity, appear to be correct, insufficient mechanistic knowledge on the action of germ cell mutagens, especially those which may act indirectly and/or epigenetically, continues to leave room for doubt. No information on the epigenetic activity of thalidomide was located in the published literature.

4.12. Toluene (CAS 108-88-3)

Toluene is a widely used organic solvent in paint, printing and adhesive industries that is sometimes abused by sniffing as a 'recreational drug'. There are also reports of excessive exposures of pregnant women in the work environment resulting in malformations. Congenital defects that have been associated with recreational toluene sniffing by pregnant mothers include developmental delay, central nervous system dysfunction, hydronephrosis, ventricular septal defects, and craniofacial and limb anomalies such as microcephaly, micrognathia, deformed ears and blunted fingernails. These facial features are frequently described as resembling those of fetal alcohol syndrome (FAS). *TERIS* classifies the magnitude of teratogenic risk for occupational exposure to toluene as none-to-minimal and for toluene-abuse as undetermined based on a quality and quantity of data which are poor-to-fair although the above described 'toluene embryopathy' is prevalent in all human studies of excessive in utero exposure [291]. The effects of toluene exposure are confounded by variables such as the general health of the pregnant mothers, especially those with toluene-induced renal tubular acidosis which can profoundly lower serum pH, and their exposure to other teratogens such as alcohol and recreational drugs. Genetic variations that result in deficiency of the aldehyde dehydrogenase enzymes involved in toluene metabolism may increase the risks of toluene teratogenicity at lower levels of exposure [291]. toluene has been studied extensively for genotoxic activity and the overwhelming weight of evidence indicates that the chemical is not mutagenic [292,293]. The purity of the toluene sample used in such testing is critical because non-reagent grade toluene is frequently con-

taminated with benzene, a known clastogen. A summary of the genotoxicity test results for toluene is also presented in Ref. [294]. It was not mutagenic in Salmonella [163] and it did not induce SCE or CA in CHO cells [294]. Questionable results were reported in a mouse lymphoma gene mutation assay [295]. No significant effects on SCEs or cell cycle delay were observed in human lymphocytes following short-term low-level exposures to toluene either in vivo or in vitro [296]. Results of a mouse bone marrow MN test were also negative [294]. No increase in the frequency of MN was detected in cultured lymphocytes of filling-station attendants exposed to petroleum derivatives containing toluene [297]. No information on the epigenetic activity of toluene was located in the published literature.

4.13. Trimethadione (CAS 127-48-0) and paramethadione (CAS 115-67-3)

Trimethadione and paramethadione are closely related oral anticonvulsant agents used to treat petit mal seizures. Their associated teratogenic risk is considered to be high based on data of good-to-excellent quality and quantity. Their use is associated with a characteristic pattern of malformations referred to as 'fetal trimethadione syndrome' [283,298]. Features of this syndrome include growth retardation, microcephaly, cleft lip and/or palate, and unusual facies with V-shaped eyebrows, broad nasal bridge, epicanthal folds, and anteverted nostrils. Cardiovascular malformations, especially ventricular septal defect, teratology of Fallot, patent ductus arteriosus, and transposition of the great vessels, are common. Genitourinary and gastrointestinal anomalies may also occur. Many affected pregnancies are miscarried, and still birth or death in infancy appears unusually frequent. Surviving affected children often are short and mentally retarded; speech difficulty and conductive hearing loss are common. As with all the other anticonvulsants, the standard caveats that diminish confidence about the perceived risks, such as confounding factors of multiple anticonvulsant drug exposures, the disease itself, and the lack of data from studies of women without seizures, are applicable here as well. No information regarding the epigenetic, mutagenic or other genotoxic activity of either trimethadione or paramethadione was located in the published literature.

4.14. Valproic acid (CAS 99-66-1)

Valproic acid is a commonly used oral anticonvulsant. The magnitude of teratogenic risk from exposure to valproic acid during pregnancy is small-to-moderate for neural tube defects and other congenital anomalies. The quality and quantity of the data for assessing the risk of neural tube defects are good, while those for assessing other congenital anomalies are poor-to-fair. A distinctive pattern of anomalies referred to as the fetal valproate syndrome, and involving postnatal growth retardation, developmental delay, anomalies of the head, face and digits such as microcephaly, midface hypoplasia, epicanthal folds, short nose, broad nasal bridge, thin upper lip, thick lower lip and micrognathia, has been described in infants born to women treated with valproic acid during pregnancy. Valproic acid did not induce mutations in Salmonella, with or without S9 (NTP, unpublished data). There is equivocal evidence for induction of SCE in lymphocytes obtained from children and exposed to valproic acid in vitro, as well in lymphocytes exposed in vivo that were obtained from epileptic children receiving valproic acid therapy; CAs were also slightly elevated following the in vivo exposure [299]. Conversely, with adult epileptic patients no statistically significant differences in SCE level were found between treated and untreated patients, but both groups had significantly higher SCE levels than healthy controls [299–301]. Preliminary data suggest that altered expression of neural cell adhesion molecules (NCAMs) may be involved in induction of neural tube defects by valproic acid [302].

5. Discussion

Transmitted gene mutations and chromosome aberrations, many of which are de novo, account for as much as 25% of all human birth defects while environmental teratogens account for less than 5%. But more than 40% of human birth defects are of unknown etiology. It seems likely that environmental

causes exist other than those already identified. However, developing causal associations between exposures and congenital defects, which have their genesis during embryonic or fetal development and consist of major or minor deviations from normal morphology or function, is not a simple task. For one thing, the borderline between normal variation and a minor congenital defect, defined as being present in less than 2–3% of the population, is often difficult to identify. Furthermore, we know that exposures to

Table 2
Genetic activities of human teratogens

Chemical name	Mutagenic activity				Epigenetic activity	Unknown or equivocal	
	In vitro		In vivo			mutagenic activity	epigenetic activity
	Gene mutation	Chromosome damage	Gene mutation	Chromosome damage			
Folic acid inhibitors aminopterin/methotrexate	−	+		+			i
Busulfan	+	+	+	+			
Cigarette smoking	+	+	+	+			i
Cyclophosphamide	+	+	+	+			✓
Cytarabine	+	+		+			✓
Daunorubicin/doxorubicin	*	*		*			?
Ethanol	*	*/−	−	*/−	*		i
6-Mercaptopurine	+	+	+	+			✓
Penicillamine	*/−	*		−	*		
Androgens					+	✓	
Cocaine					+	✓	
DES	+/−	*		*	+		
Hydantoin anticonvulsants	−	−		+/−	+	✓	
Lithium	−	+		−	+		
Methylene blue	*	*		−	*		i
Primidone/phenobarbital	+/−	−		+/−	*		i
Retinoids					+	✓	i
ACE inhibitors						✓	i
Carbamazepine	−	−				✓	✓
Coumarin anticoagulants						✓	i
Indomethacin				+	*	✓	
Iodide						✓	✓
Methimazole/propylthiouracil				−	*	✓	
Methyl mercury	−			+		✓	✓
Polychlorinated biphenyl (PCB)	+/−	+/−		−		✓	i
Quinine	+/−			+/−		✓	✓
Tetracyclines	+/−	−				✓	✓
Thalidomide	−			+/−		✓	✓
Toluene	−	−		−			✓
Trimethadione						✓	✓
Valproic acid	−			?	?	✓	

+ or − Indicates there is data supporting a positive or negative activity, respectively, for the endpoint.
* Indicates the appearance of activity for the endpoint that is through some indirect pathway or mechanism.
+/− and */− Indicates a mix of both positive and negative studies reported for the endpoint, frequently in different organisms, cell lines or strains.
i Indicates inhibition or induction of enzyme activity which may or may not involve epigenetic activity.
? Indicates the data for the endpoint is uncertain.
✓ Indicates there is insufficient data to judge whether, overall, the chemical has mutagenic or epigenetic activity.

teratogenic agents at different times of gestation often produce different spectrums of malformations; thus, establishing association with a specific syndrome can also be difficult.

Examination of the mechanisms of action for the known human teratogens should be helpful in directing our future studies of potential environmental teratogens. In this paper, we have examined the genetic toxicities of human teratogens. As indicated in the Section 1 we have not attempted to establish our own criteria for identifying human teratogens but have simply selected for discussion those agents listed by Shepard as 'Drugs and Environmental Chemicals' (table 1 of [3]), as well as a few of those he considered as possible human teratogens (table 2 of [3]), and those drugs (excluding biologics) in *Teratogenic Effects of Drugs: A Resource for Clinicians* (*TERIS*; [11]) whose 'magnitude of teratogenic risk' is categorized as 'moderate or high'. Wilson [5] suggested that there is no agent which lacks teratogenic potential if given at the proper dose and time of gestation; thus, Shepard has applied a rather stringent set of criteria to identify those agents he considers proven human teratogens. We have made no effort to judge the correctness of these criteria or to develop a set of our own criteria, but have simply described the criteria developed by others.

The mutagenic and epigenetic activities for each of the 40 or so recognized human teratogens, which collapse into 31 chemical or chemical category listings, are summarized in Table 2. These activities have been determined primarily on the basis of our review of data in the published literature and occasionally on the basis of unpublished NTP data. Of the 31 categories we have examined, there is insufficient information on almost half (14) of them to establish, with certainty, whether or not they have either mutagenic or epigenetic activity. Two of these 14, indomethacin and propylthiouracil, are readily associated with alterations in gene expressions, but it appears those are only secondary effects of an inhibition of prostaglandin synthetase and production of hypothyroidism, respectively. Sufficient information on mutagenicity was found for only one of the 14, toluene.

Epigenetic and mutagenic activity are not mutually exclusive, of course. Of the nine agents which are clearly mutagenic, sufficient information to establish their epigenetic activity was found for only two (ethanol and penicillamine), and it appears the epigenetic activities of both are indirectly mediated by oxidative-stress, signal-transduction pathways [303]. Two others, methotrexate and cigarette smoking, are associated with inhibition (dihydrofolate reductase) and induction (cytochrome P450) of enzymes, as is ethanol. This might be considered an indication of indirect epigenetic activity. Of the nine human teratogens with evidence of mutagenic activity, it would appear that the malformations induced by four of the five which are anti-cancer drugs (busulfan, cyclophosphamide, cytarabine and 6-mercaptopurine) are consistent with expectations of direct or indirect mutagenic damage. The fifth anti-cancer drug category (daunorubicin/doxorubicin) theoretically has the potential for indirect-epigenetic activity through its ability to disrupt transcription and translation via inhibition of topoisomerase activity. It may also exhibit indirect mutagenic activity by this mechanism. For cigarette smoking, on the other hand, the association with reduced fetal weight is probably related more to the effects of carbon monoxide than to its mutagenicity or induction of metabolizing enzymes. Likewise, the non-genetic, or indirect-epigenetic activity of penicillamine appears more likely to be responsible for its associated malformations than does its limited mutagenic activity. The teratogenic effects of both ethanol and the folic acid inhibitors appear to be more consistent with their epigenetic activity than their mutagenic activity, but too little is known about the cellular and molecular processes involved in the genesis of the developmental defects to justify further speculation about their etiology.

Eight chemicals were categorized as human teratogens with evidence of epigenetic activity. For most of them, there is information indicating that they directly affect a transcription or translation pathway. At least two, however, appear to act via an indirect pathway or mechanism. As indicated above, methylene blue inhibits soluble guanylate cyclase activity [181], which is involved in the production of guanosine monophosphate (GMP) that directly affects transcriptional processes and the control of cell cycling. And phenobarbital, which like ethanol somehow affects oxidative-stress, signal-transduction pathways, is reported to 'up-regulate, down-regulate,

induce expression and increase transcription' of genes [202–206]; although it is unclear whether this activity is occurring through a direct or indirect pathway. Nevertheless, one could argue there is little, if any, distinction between the indirect-epigenetic activity of these two agents categorized as human teratogens with evidence of epigenetic activity, and the activity of indomethacin and propylthiouracil, which are categorized as having unknown or equivocal epigenetic activity, except that these latter two are more clearly acting via an indirect mechanism. In fact, because of this 'gray area' of definition, three others in the unknown or equivocal epigenetic activity category indicated as inhibiting or inducing enzyme activity (ACE inhibitors, coumarin anticoagulants and PCB) may be similarly 'misclassified'.

Three of the human teratogens with evidence of epigenetic activity also have sufficient evidence of mutagenic activity. For two of these, DES and methylene blue, that mutagenic activity clearly appears to be indirect. DES has been reported to affect microtubular polymerization and depolymerization by preferential covalent binding to tubulin [151–153], and to alter the expression of lactoferrin and epidermal growth factor genes [154–156] perhaps through alteration of methylation patterns [157]. The mutagenic activity of methylene blue is reported to be due to a totally different type of indirect mechanism, the generation of singlet oxygen radicals in the presence of visible light [182]. Lithium, in general, does not appear to have significant mutagenic activity, and it would appear that the cardiovascular malformations associated with lithium exposures are more likely to be related to its epigenetic activity, especially in view of the fact that both alteration of gene expression and heart malformations are reported in frogs exposed to Lithium [177–180]. There is insufficient information to evaluate the mutagenic activity of the remaining five human teratogens with evidence of epigenetic activity; but for most of them, the malformations they produce are consistent with expectations of their epigenetic activity. This is especially true for the retinoids where there is considerable knowledge of the molecular and cellular pathways involved in their regulation of morphogenetic development, including developmental pathways for the body structures known to be affected by retinoids.

Based upon this review of the genetic toxicities of known human teratogens, a variety of cellular and molecular mechanisms appear to be responsible for the observed malformations, and very likely, a particular human teratogen may produce its effects by several different mechanisms. Mutagenic activity almost certainly plays a role in some of them, and its role in the teratogenicity of as-yet-undefined teratogens should perhaps be given greater consideration. Identification of teratogens which act epigenetically requires a much better understanding of the various molecular pathways in development and how toxicants can impact them. Obviously there is a need for additional research into the molecular and cellular mechanisms of abnormal development. More complete characterization of known human teratogens would be a good place to begin.

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