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A *DROSOPHILA* MODEL FOR METHOTREXATE DEVELOPMENTAL TOXICITY AND TERATOGENICITY

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ABSTRACT. MORE THAN 50 YEARS have elapsed since the discovery of antifolate therapies for the treatment of childhood leukemia, with the result that they have become one of the most useful family of pharmaceuticals in the physician's arsenal. Methotrexate (MTX), one member of this family, has become a commonly-prescribed chemotherapeutic agent, and used for a host of other diseases such as ectopic pregnancy, rheumatoid arthritis and asthma. However, caution must be exercised in MTX treatments since it is a potent teratogen. It is well known that MTX inhibits dihydrofolate reductase (DHFR), and is crucial for the synthesis of purines, thymidylate and certain amino acids, but it is less certain that birth defects can be attributed solely to DHFR inhibition. Clearly, more research is needed, but mammalian cell lines may not be ideal, and using substantial numbers of mammalian embryos for toxicity testing is not always held in high regard by the public. Recently, the fruit fly, *Drosophila melanogaster*, has been used to model human illnesses including cardiac disease, neurodegeneration, ageing and cancer. We have shown that MTX exposure of female *Drosophila* results in dose-dependent developmental abnormalities in progeny as well as changes in a number of ovarian transcripts, suggesting secondary drug targets. Since *Drosophila* DHFR has similar kinetic properties to the human enzyme, but the encoding gene is more compact, investigations to understand the molecular mechanisms that result in birth defects should include this model. In the future, such studies may also generate useful tools for mammalian antifolate "rescue" therapies.

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1. INTRODUCTION

Since the discovery that the synthetic antifolate aminopterin was beneficial for the treatment of children with leukemia in the 1940s by Dr. Sidney Farber [1], the use of aminopterin and other derived antifolates for the therapeutic care of patients with a variety of diseases has become widespread. Antifolates target enzymes involved in the folate pathway. The majority of these compounds have been shown to target the enzyme dihydrofolate reductase (DHFR; E.C. 1.5.1.3), which is essential for the synthesis of tetrahydrofolate (THF), a cofactor involved in the synthesis of purine, thymidylate, and certain amino acids [2]. By reducing THF levels, DNA synthesis is severely compromised, leading to the arrest of rapidly dividing cells. This antifolate-mediated inhibition explains the utility of these pharmaceuticals for a range of cancers and autoimmune diseases.

A second antifolate, methotrexate (MTX), was synthesized shortly after aminopterin and differed only by an additional methyl group at position N-10 (TABLE 1). It was discovered to be less toxic but still as effective as aminopterin in inhibiting cell proliferation [1]. Other antifolates have since been synthesized over the past half a century and are used in the treatment of breast cancer, psoriasis, HIV-AIDS, to name but a few conditions (TABLE 1). Nevertheless, since its initial discovery, MTX remains the most commonly used chemotherapeutic agent in leukemia, osteosarcoma, breast, head, neck and bladder cancers [3]. MTX is also used to treat of a variety of other diseases such as ectopic pregnancy [4], rheumatoid arthritis [5], inflammatory skin disease [6], Crohn's disease [7], asthma [8], and systemic lupus [9]. Although MTX is very effective and less toxic than aminopterin, side effects are commonly reported and its toxicity to non-target cells is often the limiting factor in chemotherapeutic treatment [10]. MTX is also a well known teratogen and must be prescribed with caution to women of reproductive age [11-14]. Due to the continued and even increased use of MTX, it is therefore essential that the pathway of events following this treatment be fully understood. This is crucial in the case of teratogenicity. Current study models include cell lines as well as mice, rats, cats and sheep but these models are not always ideal.

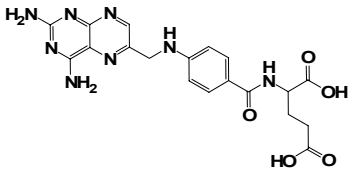
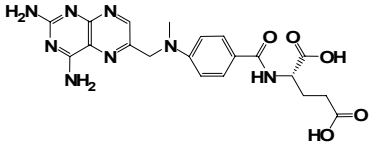
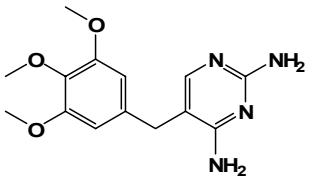
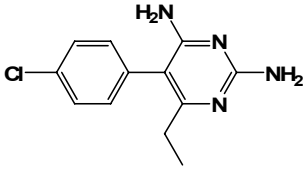
Perhaps surprisingly, the embryonic lethality and developmental abnormalities seen in these mammalian models after MTX administration have also been observed in the invertebrate *Drosophila melanogaster* [15]. This discovery introduces the possibility of using *D. melanogaster* as a model organism to investigate the toxic and teratogenic nature of MTX. Due to the many advantages of *Drosophila* as a model organism, this insect is used to study many human diseases such as cancer [16], cardiac disease [17], aging [18,19] and neurodegeneration [20]. Given *Drosophila's* proven utility as a model for a number of developmental disorders, the following is a comparison of the current mammalian model with a potential, alternative invertebrate model for the study of toxicity and teratogenicity following MTX administration.

2. DIHYDROFOLATE REDUCTASE (DHFR)

Folate, an important B vitamin, can be synthesized in plants and bacteria, however, for mammals as well as insects, folate is a dietary requirement [21]. Lack of folate in the diet of human adults can lead to anemia and accumulation of homocysteine which has been linked to heart disease [22] and cancer [23]. In pregnant mothers, deficiencies in folic acid are a well known cause of neural tube defects and anencephaly [24]. The recommended daily dose of folate for adults is 400 µg, 600 µg for pregnant women, and 500 µg during lactation [25]. Thus, it is not surprising folic acid is supplemented in certain grains [26,27] and additional folic acid is prescribed during and post pregnancy [25].

DHFR is an essential housekeeping enzyme involved in the conversion of folate to its active form. In most organisms, DHFR catalyzes the reduction of folate to dihydrofolate (DHF) followed by a second reduction that reduces DHF to THF. The molecular weight of mammalian DHFR is similar to that of *Drosophila* DHFR, ranging from 20 kD to 22.5 kD for the human and chicken protein, respectively [28,29]. *Drosophila* DHFR at 22 kD is within that range [30]. Mammalian DHFRs

TABLE 1. SELECTED ANTIFOLATES AND EXAMPLES OF THEIR THERAPEUTIC USE.

Antifolate	Structure	Examples of treatment use
Aminopterin		Previously used in childhood leukemia
Methotrexate		Breast, head, and neck cancers, leukemia, psoriasis, rheumatoid arthritis, ectopic pregnancy
Trimethoprim		Infections of *Myobacterium tuberculosis* (tuberculosis) and *Pneumocystis carnii* (commonly associated with immundeficiencies like HIV-AIDS)
Pyrimethamine		*Plasmodium falciparum* (malaria) and *Trypanosoma cruzi* (Chagas disease)

show two optimum pH values; for humans these values are 4.5 and 8.0 [31] and resemble those for *Drosophila* DHFR at 4.7 and 8.5 [30]. Both human and *Drosophila* DHFRs are monomers, unlike *Plasmodium falciparum*, the malarial parasite, which forms a dimer with thymidylate synthase [32]. Although the mammalian enzyme can convert folate to DHF at an acidic pH, this activity has not been observed with the *Drosophila* enzyme and it is currently unknown how this reduction is achieved in the fly. Similarly, prokaryote DHFR is unable to reduce folate [33,34]. The addition of urea, salt, and organic mercurials to vertebrate DHFR enhances its ability to reduce DHF [35]. The activity of *Drosophila* DHFR in contrast, is only slightly enhanced by urea, unaffected at low concentrations but inhibited at higher concentrations (>50 mM) of salt, as well as inhibited by organic mercurials [30]. The product of DHFR action, THF, is used as a cofactor to transfer one-carbon units and is essential for the biosynthesis of thymidylate, purines, and effects homeostatic levels of glycine, serine, homocysteine, and methionine [2]. DHFR also inhibits DNA methylation by the depletion of methionine, leading to the decreased abundance of a major methyl donor, S-adenosyl-L-methionine [36]. Therefore, it is surmised that this enzyme also plays a role in essential epigenetic mechanisms involved in correct genomic expression during embryogenesis. Given the crucial role of DHFR and folate in development, it is easy to see why the inhibition of DHFR activity or a reduction in folate levels can produce teratogenic effects.

DHFRs have been purified from a variety of organisms and models based on crystal coordinates have been elucidated. These structures, as well as

TABLE 2. COMPARISON OF THE HUMAN AND *DROSOPHILA DHFR* GENE CHARACTERISTICS.

***Dhfr* characteristic**	**Human**	***Drosophila***
Gene size	~ 30 kb	~ 1 kb
Number of introns	5 (ranging from 362 to 12000 bp)	1 (50 bp)
Number of promoters and directionality	2 bidirectional (major and minor)	1 unidirectional
5' region	Sp1 consensus sequences	TATA box

kinetic studies are used to determine the molecular mechanisms involved in DHFR catalysis [37]. There is no crystal structure for *Drosophila* DHFR at present but K_M values for NADPH and DHF for *Drosophila* DHFR are 5.2 μM and 0.3 μM, respectively [30], which are similar to that of human DHFR, at 7.1 μM for NADPH and 1.0 μM for DHF [31]. The comparable molecular weight, optimal pH range, and K_M values of human and *Drosophila* DHFR is also reflected in the sequence since *Drosophila* DFHR shares 17/24 of the residues involved in binding cofactor and substrates within the human enzyme's active site. Overall *Drosophila* DHFR shares a 49% identity to mammalian DHFRs [38]. *Drosophila* DHFR, like the mammalian enzyme, is inhibited by aminopterin and MTX but uninhibited by the bacterial DHFR inhibitor, trimethoprim and the plasmodium DHFR inhibitor, pyrimethamine (TABLE 1) [39]. MTX is a tight-binding competitive inhibitor of both *Drosophila* and mammalian DHFR, however, the K_d for the *Drosophila* DHFR is 860 pM, a value 10 to 1000 times higher than mammalian DHFRs [30]. Presumably, non-conserved residues involved in binding within the active site may contribute to this higher K_d. This property is also shared by the tobacco budworm moth, *Helicoverpa* (*Heliothis*) *virescens* [40]. Due to this slight "natural" resistance of the *Drosophila* DHFR to MTX, compared to the enzyme from some mammals, it would be worthwhile obtaining crystals for the *Drosophila* protein to investigate why MTX is unable to bind *Drosophila* DHFR as tightly as the vertebrate protein. This knowledge would be useful for future gene therapy research.

3. ANTIFOLATES

Antifolates are defined as any chemical that interferes with folic acid metabolism. Most antifolates are competitive inhibitors of DHFR and are grouped as either as classical or nonclassical inhibitors [3]. Classical antifolates are similar in structure; they are transported and polyglutamated as folate. Nonclassical antifolates are lipophilic and therefore, can cross the cell membrane by passive diffusion. Once inside the cell nonclassical antifolates are not polyglutamated.

MTX is a classical inhibitor of DHFR and therefore is similar in structure to folate. Folate structure is defined as having a pterin ring, aminobenzoic acid, and 1,6-glutamates. The fundamental difference between antifolates and folate is a substitution of the hydroxyl at the C4-position of the pterin ring for an amino group, as found in aminopterin (TABLE 1). MTX, along with the C4-amino group, has an additional methyl at N10 (TABLE 1). These small changes to folate structure are sufficient to make MTX a potent inhibitor of essential cellular processes, and thus, an excellent therapeutic drug [3].

Due to their structural similarity, MTX and folate not only compete for the active site in DHFR

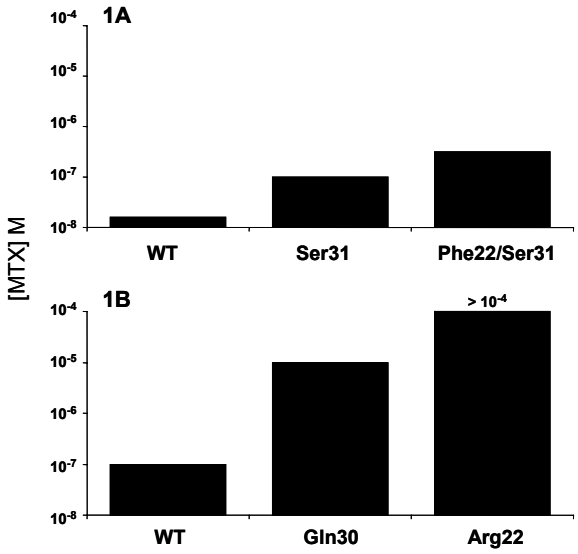


FIGURE 1. MTX RESISTANCE IN MAMMALIAN CELL LINES CARRYING HUMAN OR DROSOPHILA DHFRS. The MTX concentration at which 90% of cell growth was inhibited (IC_{10}) was determined for wild type (WT) and mutant DHFRs.

Panel 1A. Human hematopoietic cells expressing either WT, Ser31 or Phe22/Ser31human DHFRs (adapted from Meisel et al., 2003).

Panel 1B. Chinese hamster ovary (CHO) cells expressing either WT, Gln30 (equivalent to the mammalian residue 31) or Arg22 Drosophila DHFRs (adapted from Affleck et al., 2006). Note that cells expressing Drosophila Arg22 DHFR have not reached an IC_{10} value even at 10^{-4} M MTX.

but also utilize many of the same cellular factors. MTX and folate are both transported across the cell membrane in mammals by the reduced folate carrier (RFC) and polyglutamated by folylpolyglutamate synthetase (FPGS) [41]. Although there are no characterized RFC and FPGS proteins in *Drosophila*, putative genes have been described in FlyBase (http://flybase.bio.indiana.edu/) by sequence homology, and we presume that transport into the cell is similar in both groups. Once inside the cell, MTX competes with folate for the active site of DHFR. Since the K_d for MTX is much lower than either folic acid or DHF [42], when MTX is bound to DHFR it inhibits DHFR function, leading to partial or complete reduction in reduced folate levels and in turn, inhibition of processes involving folate derivatives. As the thymidylate synthase cycle requires reduced folate, 5,10-CH_2-THF, to donate a methyl group to dUMP for synthesis of *de novo* dTMP, depleted reduced folate pools reduce DNA synthesis. It is this inhibition that is thought to cause most of the cytotoxicity [3]. Reduction of reduced folates also affects the enzymes involved in purine synthesis (glycinamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleotide), thus further disrupting DNA synthesis as well as the synthesis of RNA. As previously mentioned, levels of certain amino acids are affected as well as synthesis of S-adenosyl-L-methionine, which is essential for correct gene expression [36].

Other antifolates, including trimethoprim (TMP), an inhibitor of bacterial DHFRs and pyrimethamine (PMA), an inhibitor of parasitic DHFRs, do not have a pterin ring characteristic of the mammalian inhibitors but instead both consist of a pyrimidine 2,4-diamine ring (TABLE 1). Although the folding of DHFR is thought to be similar across organisms, differences in amino acid composition of this enzyme allow selective targeting of bacteria or plasmodia DHFR by antifolates such as TMP and PMA. These then provide effective treatment of infectious agents without inhibiting the human host DHFR. TMP inhibits many bacterial DHFRs that cause human infection including; *Myobacterium tuberculosis* (tuberculosis) and *Pneumocystis carinii* (commonly associated with immunodeficiencies such as AIDS). PMA is used for the treatment of apicoplast parasites such as *P. falciparum* that cause malaria and kinetoplastid parasites such as *Trypanosoma cruzi* that cause Chagas disease.

4. THE DHFR GENE AND ITS REGULATION

In contrast to the broad similarity of the mammalian and *Drosophila* DHFRs, the genes that encode these proteins are very different (TABLE 2). The human *Dhfr* gene is approximately 30 kb and contains 5 introns, which range in size from 362 to 12,000 base pairs [43]. Other mammalian *Dhfr* genes from the mouse and hamster are similar in both size and in the number of introns [44-46]. *Drosophila Dhfr* is a more compact gene of ap-

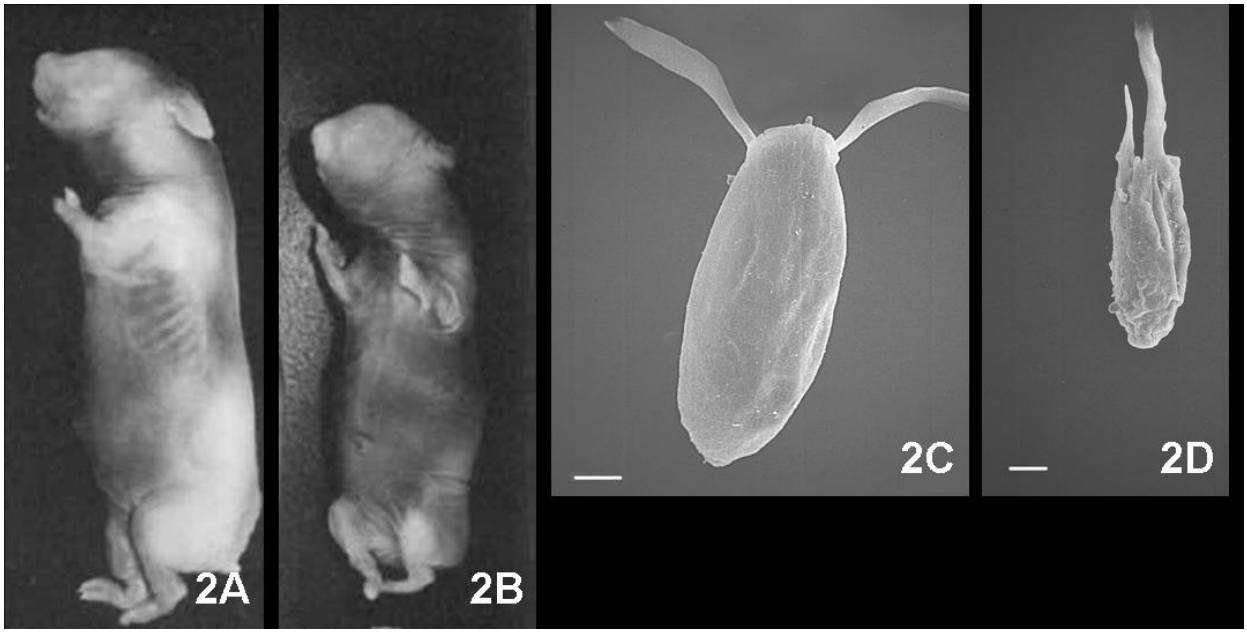


FIGURE 2. THE EFFECT OF MTX ON DEVELOPMENT IN MAMMALS AND *DROSOPHILA*. Normal development in a rabbit fetus (2A) and a *Drosophila* embryo (2C) is compared to abnormal development after exposure to 19.2 mg/kg of MTX on day 12 of gestation (2B) and in a *Drosophila* embryo from a mother exposed to 5 ppm MTX for 5 days (2D). The bar represents 50 µm. Photographs adapted from DeSesso and Goeringer, 1991 (2A, 2B) and Neumann (personal communication) (2C, 2D).

proximately 1 kb with a single 50 bp intron [38]. Similarly, the mosquito, *Aedes albopictus*, has a similar gene size and contains one 56 bp intron [47]. The single dipteran *Dhfr* intron is conserved with the first intron in mammalian *Dhfr*s. It should be noted that a single *Dhfr* intron is not generally characteristic of insects since the *Helicoverpa* gene has at least 3 introns (0.5, 1.5, and 1.0 kb) that correspond to the first, second and third introns found in the mammalian gene [40]. Thus the mammalian *Dhfr* and its corresponding pre-mRNAs present a more complex pattern of expression when compared to *Drosophila Dhfr*. This difference is not unique to this gene since the *Drosophila* genome is quite compact with fewer and smaller introns and a long-period interspersion pattern whereas, the mammalian genome has a short-interspersion pattern and multiple introns [48,49]. The simpler organization of the *Drosophila* genome may prove to be advantageous as complicated regulation and processing of mammalian genes, such as *Dhfr*, could complicate understanding of the underlying mechanisms of MTX-induced teratogenicity. Therefore, this may be another reason to study the effects of MTX in an organism with a less complex genomic organization of *Dhfr*, like that of *Drosophila*.

Similar to the contrast in the genome organization of mammalian and *Drosophila* DHFRs, regulation of *Dhfr* differs in these two classes of organisms (TABLE 2). The 5' region of *Drosophila Dhfr* contains a TATA sequence and a single transcription initiation site. In contrast, transcription of the mammalian *Dhfr* is not as simple. Mammalian *Dhfr* mRNA expression is controlled by major and minor promoter elements. The major promoter is located within a CpG island where a 48 bp sequence (repeated in some mammals) containing a Sp1 consensus sequence is responsible for 90% of transcription; the other 10% is transcribed by a minor promoter with several Sp1-binding sites [43,50]. Both major and minor promoters are bidirectional, and thus also transcribe the Repair-3 (*Rep-3*) gene. Transcription of mammalian *Dhfr* is induced by

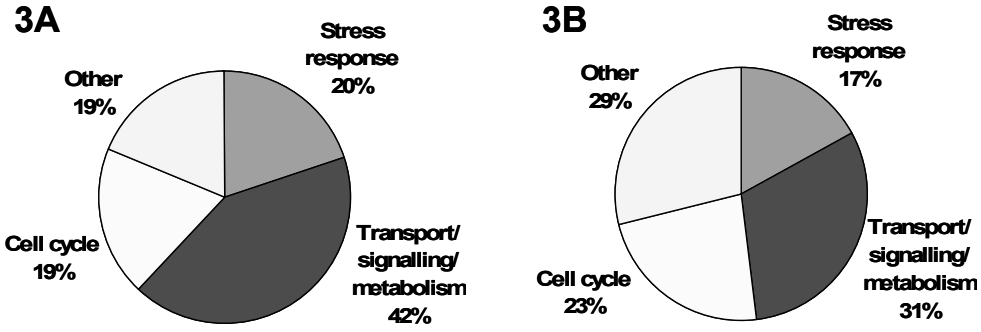


FIGURE 3. COMPARISON OF A SUBSET OF GENES SHOWING ALTERED TRANSCRIPT ABUNDANCE BY MICROARRAY ANALYSIS AFTER MTX EXPOSURE IN (3A) RAT LIVER (ADAPTED FROM HUANG *ET AL.*, 2004) AND IN (3B) *DROSOPHILA* OVARIES (adapted from Affleck et al., 2006).

binding of the transcriptional elongation factor, E2F, 3' to the major transcriptional start site [51]. A promoter lacking a TATA sequence and containing Sp1 and E2F binding sites has been reported in many genes such as thymidine kinase, human origin of replication 1 (*HsOrc1*), and DNA polymerase alpha (1-6) which are involved in DNA synthesis and are consistent with an increase in expression at the G1/S cell cycle boundary [52]. The cell cycle regulation of *Dhfr* transcripts has not been studied directly in *Drosophila*.

The 3' region in mammalian *Dhfr* contains multiple polyadenylation sites producing different length mRNA species [53,43] whereas, there seems to be only one such site in *Drosophila Dhfr* mRNA [38]. Mammalian DHFR has been shown to bind and regulate *Dhfr* translation [54] and in hamster, intron splicing was required for appropriate levels of translation and DHFR stability [55]. *Dhfr* mRNA from *Drosophila* has not been studied in such detail.

5. DHFR MUTATIONS, MTX AND TERATOGENICITY

Despite the striking difference in gene organization and regulation of *Dhfrs* in mammals and insects as mentioned, there is obvious conservation of DHFR function and the amino acid residues involved in catalysis [38]. This is dramatically demonstrated by the comparison of mutant DHFRs from different species that confer MTX resistance. The ability of equivalent "hot spot" residues to confer antifolate resistance in mammalian and *Drosophila* DHFRs appears to be well conserved. Mutations at either residue 22 or 31 (corresponding to residue 22 and 30 in *Drosophila*) are well known for their ability to alter the structure of DHFR in such a way as to decrease the binding affinity of MTX within the active site [56-58]. Studies of mutations (e.g., Phe31Ser, Leu22Phe/Phe31Ser, Leu22Tyr/Phe31Gly) (FIG. 1) in human DHFR have been shown to confer resistance to MTX in human cell lines [57]. The protective effect of Leu22Arg and Phe31Ser to antifolate exposure has also been demonstrated in whole animals. For example, constitutively expressed murine DHFR Leu22Arg in transgenic murine embryos and placental tissue ameliorated the teratogenic effects of MTX [59], and mice exposed to lethal doses of MTX were "rescued" by transplanting bone marrow transduced with mutant DHFR Phe31Ser [60]. Similarly, *Drosophila* DHFRs with altered residues at position 22 or 30 (Leu22Arg, Leu30Gln) allowed mammalian cell lines to continue to divide in con-

centrations of MTX that were 200-fold and 2-fold higher than the levels shown to inhibit division in control mammalian lines transfected with wild type Leu22 and Leu30 DHFRs (FIG. 1) [39].

As previously noted, MTX causes embryonic lethality in mammalian embryos [44]. *Drosophila* embryos from MTX-treated mothers also show abnormalities (FIG. 2). Flies exposed to increasing concentrations of MTX show a dose- and time-dependant reduction in egg production [15]. After only 2 days, egg production was reduced to ~61%, ~61%, ~35%, and ~22% at 4.4, 11, 22, and 44 μM MTX, respectively, when compared to eggs laid by non treated mothers. After 3 days, egg laying was reduced to ~17% by females on 4.4 μM MTX and ~9% on all remaining MTX concentrations. By the fourth day all females exposed to MTX were sterile. In contrast, when female flies were exposed to 1 μM PMA, they showed no significant reduction in egg production (unpublished observations). This is not surprising, as PMA is a plasmodium inhibitor and *Drosophila* DHFR is more similar to mammalian DHFR and therefore, more susceptible to mammalian antifolates. This result further suggests that DHFR inhibition is the major contributor to teratogenicity. As additional evidence, DHFR Leu30Gln in transgenic *Drosophila* females appears to confer some protection from MTX exposure (unpublished observations). It is important to note, however, that at high doses of MTX in both mammals and *Drosophila*, "rescue" is not complete; teratogenicity is not fully eliminated. This suggests that MTX may not only exert its toxicity by inhibition of DHFR but other factors may also play a role.

In an attempt to determine the contribution to toxicity exerted by targets other than DHFR, enzymes that represent putative alternative targets have been assayed in the presence of MTX and other antifolates [61]. With the demonstrated utility of microarray technology in gene expression profiling, recent studies have sought to create expression profiles in response to MTX. Microarray analyses (TABLE 3) have utilized mammalian tissues either directly from laboratory animals [62,63], from tissue biopsies [64], or cell lines [65]. These studies are limited however, either due to the inevitable sacrifice of large numbers of mammalian subjects or by the availability of tissue samples and impractability of many experimental manipulations. If *Drosophila* could be used as a model there would not be similar limits to sample availability or experimental design. Also direct comparison of tissues and cell line expression with the same drug treatment can be examined. Ovaries from *Drosophila* females treated with MTX as well as MTX-exposed cell lines have been compared using microarrays [15]. Similar to experiments using mammalian tissues, both revealed expression profiles with altered transcript abundance of many genes (~ 35 were further investigated in *Drosophila*) unrelated to folate biosynthesis (FIG. 3). The role of these genes in the MTX response is still not clear, but it is a step towards more fully understanding the toxicity of this antifolate.

Thus, direct and indirect inhibition of enzymes involved in the folate pathway and other potential unknown factors affected by MTX lead to cytotoxicity. This property makes MTX an excellent treatment for cancers and autoimmune diseases, but non-diseased cells are also targeted by MTX, producing common treatment side effects such as anemia, nausea and vomiting, mouth ulcers and reduced white blood cell counts, as well as long-term side effects such as liver damage [66]. When MTX is prescribed to women of child-bearing age, as mentioned, treatment can be complicated with pregnancy, as MTX is a well known teratogen [11]. Chemotherapy using MTX is not used for expectant mothers in the first trimester, and MTX therapy for other diseases is withdrawn well before pregnancy to prevent teratogenic effects [12-14]. Unplanned pregnancies are particularly worrisome since even low-dose MTX treatment in the first trimester can produce serious birth defects including craniofacial abnormalities; such as microcephaly, sloping forehead, low set and malformed ears, lack of auditory canals, flat nose, micrognathia, and skeletal abnormalities; such as lack of radius and metacarpals, as well as abnormalities in pulmonary, cardiac, and gastrointestinal organs [67,44], and in some cases mental retardation [69]. As well, persistence of MTX in the body (116 days in mammalian liver) [11], presumably due to the

tight binding to DHFR, further complicates treatment follow-up. In many cases, women of reproductive age who have been taking low-dose treatments over an extended period of time decide not to have children due to the increased risk [12].

Recently there has been an increased in the use of MTX for the treatment of ectopic pregnancy as well as for planned abortions since non surgical intervention in these cases may be easier and cheaper [70,71]. Ectopic pregnancies occur in 1.9% of reported pregnancies and if not spontaneously aborted are treated during the first trimester by either MTX or surgery [4]. It is of concern that MTX treatment of unwanted but otherwise normal pregnancies has a reported success rate of only 89% [4] or 94% [71]. As a result, teratogenic effects of failed abortions have been reported [67,72]. The tragic consequences resulting from of the use of antifolates to induce abortions in the 1950s is well documented in textbooks (e.g., ref [73]).

Along with case studies reviewing human teratology after MTX exposure, the effects of antifolates on developing embryos have been studied in many mammalian systems, including rat [74], mouse [75], rabbit (FIG. 2) [76] and cat [77]. Although it is essential to continue to study the teratogenic effects of antifolates in mammalian models, it is reasonable to consider alternate model organisms such as *Drosophila* for some of these studies.

Despite the call for toxicity testing on a wide range of chemicals including teratogens [78], there has been increased interest in reducing mammalian test subjects [79,80]. This, along with the elevated use of MTX for a variety of treatments, suggests a non-mammalian study organism is needed for both toxicity and teratogenic studies. Due to the conserved function of DHFR in reduced folate synthesis across organisms and the obvious advantages of using invertebrates such as short generation time, fully sequenced genome, as well as microarray and database technologies, we advocate the use of *Drosophila* as a model. As well, *Drosophila*, similar to mammals, have a pair of ovaries, and although the egg does not implant in the *Drosophila* uterus, mothers provide their offspring with all essential factors for development during oogenesis through follicle and nurse cells [81] and indeed, until the progeny are sufficiently developed in the larval stage to obtain their own nutrition. A *Drosophila* model is further supported by similar observations in mammals in response to MTX treatment including abnormalities in embryos that are so profound that complete lethality results (FIG. 2) and developmental abnormalities, which include abnormal tufts of bristles, limb curvature, and eye and wing deformities in *Drosophila* [15]. To our knowledge, the use of *Drosophila* as a model system for birth defects is not current practice. With increasing pressure to move towards non-mammalian testing, we propose the use of *Drosophila* to study toxicity and teratogenesis.

6. FUTURE DIRECTIONS

MTX is known to inhibit DHFR but it is still unclear if this is the only factor contributing to teratogenicity. Transgenic mammals or cell lines that express resistant forms of DHFR and are exposed to high concentrations of MTX do not show complete amelioration of cytotoxicity or teratogenic effects, suggesting there are other factors involved. The lack of a complete restoration of normal morphology was also observed in Leu30Gln transgenic flies exposed to the drug (unpublished observations) as well as CHO cells that expressed the same mutant *Drosophila* DHFR (FIG. 1) [39]. This was also true for CHO cells with the Leu22Arg variant, however, these cells were still able to divide at a considerably higher concentration of MTX than control cells [39]. Transgenic flies bearing a Leu22Arg mutation are being created and it will be interesting to see if these lines confer resistance to high MTX concentrations in a whole organism. More recently, specific double mutations at both positions 22 and 31 in human DHFR (United States Patent 6887467) have been shown to confer even higher resistance to MTX than the previously discussed human DHFR mutants. Double mutations in flies could also be created and would, presumably, also show higher tolerance to MTX. Lines of transgenic *Drosophila* with various DHFR mutations producing varying degrees of resistance to MTX

could be used for comparison of gene expression profiles using microarrays. Changes in these profiles would help elucidate targets of MTX other than those related to folate pathway inhibition and lead to the discovery of other genes that may be involved in cytotoxicity. A greater understanding of the complete mechanism of MTX action has great implications for "rescue" treatments after high-dose MTX exposure as well as making long-term, low-dose exposure to MTX less risky.

As mentioned, *Drosophila* DHFR has a slight "natural" resistance to MTX when compared to mammalian DHFRs, and mutations at certain "hot spot" residues have been observed to provide more resistance to MTX than similar mutations to mammalian DHFRs in mammalian cell lines (FIG. 1). Therefore, a *Drosophila* DHFR with its "natural" resistance combined with advantageous mutation(s) and engineered to more closely resemble the human DHFR enzyme could be very useful in gene therapies to provide partial protection to vulnerable non-diseased cells, especially during chemotherapeutic treatment. Additional targets identified by the use of microarrays could also allow for the supplementation of DHFR "rescue" therapies with additional beneficial adjuncts. It is our hope that the use of *Drosophila* as a model system will aid us in better understanding the mechanism of MTX teratogenicity and in the future, promote safer, more efficient use of this valuable, life-saving pharmaceutical.

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