Developmental disorders induced by pesticide degradation products

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Publication date
2002

Citation for published version (APA):
Developmental Disorders in Embryos of the Frog *Xenopus laevis* Induced by Chloroacetanilide Herbicides and their Degradation Products

Abstract

Pesticides are known to transform in the environment, but so far the study of their effects in the environment has concentrated on the parent compounds thereby neglecting the effects of the degradation products. The embryotoxic, developmental and teratogenic effects of chloroacetanilide herbicides and their environmentally stable aniline degradation products were investigated in this study in view of the massive application of alachlor and metolachlor. Embryos at midblastula to early gastrula stages of a locally abundant African clawed frog *Xenopus laevis* were used as test organisms. The embryos were exposed to the test chemicals for 96 h in each experiment. Alachlor is more embryotoxic (the concentration causing 50% embryo lethality, 96-h LC50 = 23 µM [6.1 mg/L]) and teratogenic (teratogenic index, [TI] = 1.7) than metolachlor (96-h LC50 = 48 µM [13.6 mg/L], TI = 0.2). The degradation products of alachlor and metolachlor, respectively, 2,6-diethylaniline (96-h LC50 = 13 µM [19.4 mg/L], TI = 2.1) and 2-ethyl-6-methylaniline (96-h LC50 = 509 µM [68.8 mg/L], TI = 2.7) are less embryotoxic but more teratogenic than their parent compounds. The most common teratogenic effects observed were edema for alachlor as opposed to axial flexures and eye abnormalities for 2,6-diethylaniline and 2-ethyl-6-methylaniline. Metolachlor is found to be an example of a nonteratogenic herbicide that upon degradation loses toxicity but gains teratogenicity, and both the herbicides, metolachlor and alachlor, are potential sources of teratogenic transformation products.
Introduction

The chloroacetanilides (acetamides), alachlor (2-chloro-2',6'-diethyl-N-(methoxymethyl) acetanilide) and metolachlor (2-chloro-N-2 (ethyl-6-methylphenyl)-N-2-methoxy-1-methylethyl acetamide) are widely used herbicides in agriculture (Schottler and Eisenreich 1994; Partow 1995; Potter and Carpenter 1995; Galassi et al. 1996). They are used extensively in the cereal and sugarcane growing zones of the Lake Victoria basin in Kenya and are among the most detected herbicides in surface waters elsewhere (Gruessner and Watzin 1995; Müller and Buser 1995). Alachlor has a potential to induce cancer in laboratory animals (Kimmel et al. 1986) and is classified as group B2 carcinogen by U.S. Environmental Protection Agency (USEPA 2000). The herbicides, alachlor and metolachlor, and their stable aniline degradation product 2,6-diethylaniline and 2-ethyl-6-methylaniline, respectively, are converted in hepatic mixed oxidase systems of rats to the corresponding nitrosobenzenes, which are mutagenic in the Ames Assay (Kimmel et al. 1986). The compounds are genotoxic in Mutatox™ assay (Osano et al. 2002), which uses a dark mutant of *Vibrio fischeri* bacteria as the test organism. The bacteria revert to a fluorescent state when exposed to genotoxic compounds. The present study aims to extend the understanding of the role of the degradation products of the chloroacetanilides in embryonic development.

A genetic program guides early development of a fetus, entailing expression and repression of successive series of genes, and hence, genotoxic agents could be developmental toxicants as well (Bantle 1995). Developmental toxicants and teratogens producing developmental disorders have often been analyzed using embryos of *Xenopus laevis*, a clawed African frog. The frog embryo is an intact developing system, which undergoes events comparable to those of other vertebrates, including mammals. Validation studies using known human and mammalian developmental toxicants show that the predictive accuracy of tests with *Xenopus* embryos for developmental toxicant approaches or exceeds 85% (Courchesne and Bantle 1985; Dawson and Bantle 1987; Sabourin and Faulk 1987; Bantle et al. 1989). *Xenopus laevis* is a native species of the Lake Victoria basin and is therefore particularly suitable to analyze the potential risk of the widely used chloroacetanilides and their degradation products in this region.
In this article, we report on the comparative embryotoxic, developmental and teratogenic effects of two important chloroacetanilide pesticides and two of their stable aniline degradation products on the embryos of clawed frog *X. laevis*.

**Materials and Methods**

*Test chemicals*

The pesticides alachlor (99%) and metolachlor (99%) were obtained from Riedel-de Haën (Seelze, Germany). The pesticides’ degradation products, 2,6-diethylaniline (>98%) and 2-ethyl-6-methylaniline (>97%) were obtained from Fluka (Buchs, Switzerland). Analytical grade solvent dimethylsulfoxide (DMSO) was obtained from Fluka. Test medium, usually referred to as frog embryo teratogenesis assay-*Xenopus* (FETAX) solution, was prepared as 625 mg NaCl, 96 mg NaHCO₃, 30 mg KCl, 15 mg CaCl₂, 60 mg CaSO₄.2H₂O and 5 mg MgSO₄ all analytical grade per liter of distilled water (ASTM 1991).

*Test organisms*

Adult *X. laevis*, claw footed frogs, were obtained from the ponds at the shores of L. Victoria (Kenya) and raised at the School of Environmental Studies, Moi University. The frogs were acclimatized in the laboratory for 6 weeks before the first breeding. They were housed in glass aquaria with dechlorinated tap water and nourished on minced beef fillet fortified with Multivitamin® (Norbook, UK) and vitamin C supplements. They were fed twice a week and aquarium water was replaced at the time of feeding. The aquaria were maintained at a diurnal light:dark cycle of approximately 12:12 h, which is the natural equatorial cycle, and at a temperature of 22°C during the study period.

Embryos were obtained from at least three different male/female pairs for each bioassay. Human chorionic gonadotropin hormone, Pregnilet® (Organon, Oss, Netherlands), was injected into the dorsal lymph sac of each of the males (500 IU) and the females (1000 IU). Each male/female pair was placed in a different mating/laying cage (0.3 × 0.3 × 0.3 m) half filled with test medium. The bottoms of the cages were separated from the frogs.
about 3 cm by use of a perspex floor with 1-cm holes. The laid eggs sink to
the bottom through this floor out of reach from the adult frogs, who would
otherwise quickly feed on the eggs. After approximately 14 h the adult frogs
were removed and separately the embryos from each pair were de-jellied by
gentle swirling in 2% (w/v) L-cysteine (CAS 52-90-4, the pH of which had
been adjusted to 8.1 by use of 1 N NaOH) for 1 to 3 minutes. They were
then washed in copious amounts of test medium. Embryos of stage 8
(midblastula) to stage 11 (early gastrula) were selected for the tests.
Nieuwkoop and Faber's (1975) *Normal Tables* was used in staging of the
embryos.

*Test protocol*

The standard protocol for the FETAX (ASTM 1991) was used as a
guideline. The FETAX is a static-renewal assay, and each test medium is
renewed every 24 h. For each dilution two 60-mm diameter glass petri
dishes containing 10 ml test medium and 25 embryos were used.
Concurrently, four control dishes of which two had ≤1% (v/v) DMSO and
two were without, were tested in each experiment. Dishes were randomly
assigned to their positions in an incubator, and the incubation temperature
in all cases was set at 24°C (range of 22 - 26°C). At least three definitive
tests were conducted on each test chemical in a random block design.

Alachlor, metolachlor, 2,6-diethylaniline, and 2-ethyl-6-
methylaniline, were first dissolved in DMSO before being dispensed in
the test medium solution to make a stock solution of each of the test chemicals.
Nominal concentrations of 10.1, 20.1, 40.2, 80.4, 167.5, and 335.0 μM 2,6-
diethylaniline, 7.4, 37.0, 92.4, 184.9, 369.8, and 739.6 μM 2-ethyl-
methylaniline 3.9, 7.8 16.2 32.4, 37.1, and 64.9 μM alachlor and 3.5, 17.6,
35.2, 88.1, 176.2, and 352.4 μM metolachlor were tested. The final
concentration of DMSO was ≤1% (v/v) and was uniform at all the test
concentrations. The 25 (~1-mm diameter) eggs in each test were assumed
not to significantly sorb the dissolved chemical within the 24 h between the
renewals of the test solutions. This assumption was supported by earlier
toxicity tests in which 50 to 100% of the same test compounds were
recovered even after 4 d of incubation (Osano et al. 2002).

For each of the dishes the number of surviving tadpoles was
recorded daily. The dead ones were removed and the surviving larvae were
noted after every 24 h until 96 h of incubation. The embryos were then fixed in 3% formalin and examined. The 96-h 50% lethal concentration (LC50) and 96-h EC50 (malformation) defined as the concentration causing malformation in 50% of the surviving embryos, were determined by probit analysis (Wardlaw 1985) after Abbott's (1925) adjustment for mortalities and malformations in the control as \( Y = 100 \times (C - T)/C \), where \( Y \) = percent response, \( C \) = percent not responding in the DMSO control, \( T \) = percent not responding in the test dilution, and the response was considered as growth retardation, mortality, or malformation accordingly. The teratogenic index (TI) was deduced by dividing the LC50 by the EC50. Head to tail lengths of the tadpoles were measured at the end of each experiment under a binocular dissecting microscope at a magnification of \( 10 \times 2 \) by use of an ocular micrometer. Growth inhibition was deduced by determining whether growth at a particular concentration was significantly different from that of the control. Minimum concentration to inhibit growth (MCIG) was taken to be the minimum concentration of test chemical that significantly inhibited growth as determined by measurement of head (mouth)-to-tail length (end of the tail).

Data were statistically tested with the Students' t-test and ANOVA (with Bonferroni adjustment matrix of pairwise comparison probabilities) in the SYSTAT® (1996) statistical program at the \( \alpha = 0.05 \) level to determine any significant difference in growth of tadpoles.

All the tadpoles were examined for developmental aberrations under the dissecting microscope. The Atlas of Abnormalities (Bantle et al. 1990) was consulted to determine which embryos were normal, which were abnormal, and in the identification of the different kinds of aberration. An embryo was considered abnormal if it exhibited at least one of stunting, poor or incomplete gut coil, pericardial edema, abdominal edema, facial edema, cephalic edema, tail flexure, notochord flexure, undulating notochord, optic cap rupture, microphthalmia, poor heart development, or any other gross abnormality. An abnormality was scored regardless of the seriousness of the abnormality, e.g., a slight facial edema, large-gut edema, or extensive edema were all scored as edema and so on. The frequency of each type of malformation at each test concentration for each chemical was recorded. The reported incidence of each type of malformation was an Abbott's (1925) adjusted incidence for the similar type of malformation.
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occurring in the DMSO control. Tadpoles were preserved in rubber-sealed vials with 3% formalin.

**Results**

**Survival of embryos**

The parent compounds alachlor and metolachlor are more acutely toxic than their respective aniline degradation products 2,6-diethylaniline and 2-ethyl-6-methylaniline (Table 1). Alachlor and metolachlor are 5.7 and 10.6 times more embryotoxic than their stable aniline degradation products, respectively. The environmentally stable degradation product of alachlor 2,6-diethylaniline is 3.9 times more acutely toxic than 2-ethyl-6-methylaniline (metolachlor’s environmentally stable aniline degradation product). The EC50 and LC50 values are shown in the Table 1.

**Effects on growth**

Alachlor reduced the growth of the tadpoles in a dose dependent manner, while its degradation product 2,6-diethylaniline did not inhibit growth in the tadpoles at all the concentrations tested, not even in the highest tolerated concentration (Fig.1). Metolachlor and its stable aniline degradation product reduced growth at all the concentrations tested (Fig. 1). Bonferroni adjustment matrix of pairwise comparison probabilities showed no significant difference (p = 0.05) between growth in the concentrations 3.5, 17.6 and 44.0 for µM metolachlor and 7.4, 37.0, 92.4, 184.9 and 739.6 µM for 2-ethyl-6-methylaniline.

**Types of aberrations**

In alachlor-exposed embryos, the most frequent symptoms of developmental disorders were facial, pericardial, and gut edemas (46%) (Fig. 2). Gut malformation, axial flexures and eye abnormalities comprised 13, 29, and 17%, respectively, of all the observed abnormalities in the embryos exposed to alachlor. Similarly, in metolachlor-exposed embryos, edema (48%) was the most frequently observed disorder. Gut malformation,
<table>
<thead>
<tr>
<th>Chemical</th>
<th>EC50 (96 h)</th>
<th>IC50 (96 h)</th>
<th>95% CLs in μM</th>
<th>95% CLs in μM</th>
<th>Teratogenic</th>
<th>Xenopus Laxis</th>
<th>Xenopus Laxis</th>
<th>Teratogenic</th>
<th>Xenopus Laxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,7-ethyl-6-methylanthracene</td>
<td>188.6 (156.4 - 220.7)</td>
<td>135.2</td>
<td>47.92 (40.4 - 55.4)</td>
<td>268.15 (260.14 - 276.15)</td>
<td>Parent</td>
<td>Deletion</td>
<td>Parent</td>
<td>Deletion</td>
<td>Parent</td>
</tr>
<tr>
<td>2,7-ethyl-6-methylanthracene</td>
<td>115.58 (144.40 - 61.98)</td>
<td>129.9 (13.38 - 13.30)</td>
<td>13.30 (3.28 - 2.22)</td>
<td>22.6 (19.77)</td>
<td>Parent</td>
<td>Deletion</td>
<td>Parent</td>
<td>Deletion</td>
<td>Parent</td>
</tr>
</tbody>
</table>

Note: EC50 = 50% effective concentration; IC50 = 50% inhibitory concentration. CLs = confidence limits.
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Fig 1. Percent growth relative to the growth in the DMSO control of embryos of *Xenopus laevis* after 96-h exposure to different concentrations of herbicides and their degradation products. Panel A: filled symbol, Alachlor; open symbol, 2,6-diethylaniline. Panel B: filled symbol, Metolachlor; open symbol, 2,6-diethylaniline. Error bars show 95% confidence limits.

axial flexures, and eye abnormality comprised 19, 22, and 11% respectively, of the observed abnormalities in metolachlor-exposed embryos.

As opposed to the parent compounds, the degradation products 2,6-diethylaniline (6%) and 2-ethyl-6-methylaniline (7%) induced edema to a much lower extent (Fig. 2). The predominant aberrations induced by the degradation products were the axial flexures, which comprised 52 and 49% of all the observed abnormalities for 2,6-diethylaniline and 2-ethyl-6-methylaniline exposures, respectively. Gut and eye abnormalities comprised
3 and 39%, respectively, of the observed abnormalities for 2,6-diethylaniline exposures and 11 and 33%, respectively, of the observed abnormalities for 2-ethyl-6-methylaniline exposures.

**Incidence rates of the aberrations**

There was no significant difference between the frequency of the abnormalities reported in the control with (17.7%, 95% confidence limits [CL] = 12.5 - 22.9) and the control without (21.6%, 95% CL = 17.3 - 25.9) DMSO. In deducing the EC50 (malformation) Abbott’s adjustment (Abbott 1925) was used to account for the deformities that occurred in the concurrent control with DMSO. The abnormalities accounted for included minor changes like slight curvatures in the tail. Correction for the control deformities was further indicated since these were of different types than those induced by the test compounds. Alachlor induced higher frequency of aberrations for similar ranges of concentrations than metolachlor and the degradation products showed similar dose-response relationships at low concentrations (<70 μM). However, the tadpoles survived more in higher concentrations of 2-ethyl-6-methylaniline than 2,6-diethylaniline. The highest tolerated concentrations for the degradation products were 167.5 and 739.6 μM for 2,6-dithethylanilines and 2-ethyl-6-methylanilines, respectively. The character of our four test compounds was compared using their TIs. The TI of alachlor, metolachlor, 2,6-diethylaniline and 2-ethyl-6-methylaniline was 1.7, 0.2, 2.1, and 2.7, respectively. Thus, alachlor and its degradation product are teratogenic according to their TI values, whereas the teratogenincicity of metolachlor is only apparent after degradation to 2-ethyl-6-methylaniline.

**Discussion**

The embryonic TI, defined as LC50/EC50, has been developed to allow for comparison between known and suspected teratogens (Dumont et al. 1983). Based on the TIs derived from standard compounds, Dumont et al. (1983) have set TI value ≥2 as one that indicates the need for further testing of the chemical. The TI value of 1.5 - 2.0 indicates that the materials should be treated as potential teratogen and tested further in other screening systems, while TI value <1.5 reflects compounds that are more embryolethal, suggesting that they are co-effective teratogens (Johnson
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Their lethality may be more pertinent to risk assessment than teratogenicity. Therefore, alachlor (TI = 1.7) is a potential teratogen, while its stable aniline degradation product, 2,6-diethylaniline (TI = 2.1), is a teratogen that requires further testing in other assays to establish its teratogenicity. Metolachlor (TI = 0.2) is clearly not teratogenic, as opposed to its stable aniline degradation product, 2-ethyl-6-methylaniline, which is the most teratogenic of the compounds in this study with a TI of 2.7. Three-fold higher concentrations of 2-ethyl-6-methylaniline are needed to induce the 50% of malformation as in 2,6-diethylaniline, even though at lower concentrations (<60 μM) the two compounds induced similar percentages of aberrations.

![Graph showing the relative frequency of different types of malformations in Xenopus laevis after 96-h exposure to the chloroacetanilides and their stable aniline degradation products.](image)

**Fig 2.** The relative frequency of different types of malformations in *Xenopus laevis* after 96-h exposure to the chloroacetanilides and their stable aniline degradation products. The frequency is expressed as the percent of the total number of observed abnormalities in embryos accumulated for all test concentrations for each chemical. ALA: alachlor, DEA: 2,6-diethylaniline, MET: metolachlor, EMA: 2-ethyl-6-methylaniline.

Alachlor strongly induces excision repairable DNA lesions. Surralles *et al.* (1995) also found by use of fluorescence *in situ* hybridization and an antikinetochore antibody that alachlor is a clastogen.
acting in the S phase. It has been proposed that the genotoxicity of many organic pesticides could be mediated by their alkylating potentials, and since alachlor has alkylating radicals, it may act as a DNA alkylating agent (Surralles et al. 1995). It is oncogenic (Hoberg 1990), and it forms adducts with DNA (Nesnow et al. 1995). Consistent with earlier observations, the present study shows that alachlor is a suspect teratogen. Moreover, its stable degradation product, 2-6-diethylaniline, is similarly teratogenic.

Alachlor and metolachlor are structurally related, their major difference being the methoxy alkyl chain attached to the nitrogen atom of the basic structure. This relatively small difference in the molecular structure is apparently sufficient to impart some desirable properties to metolachlor as an herbicide. Some of these properties are high lipid (octanol-water partition coefficient) and water solubility for metolachlor (Chester et al. 1989). Lipophilicity is often associated with chemical persistence in the environment. Metolachlor is transformed in the soil to a lesser extent than alachlor (Konopka 1994). It is more effective than alachlor and other chloroacetanilides; therefore, its global demand has risen. Both alachlor and metolachlor revert to their intermediates of manufacture, 2,6-diethylaniline and 2-ethyl-6-methylaniline, respectively, in the mammalian metabolism (Kimmel et al. 1986) and upon environmental degradation (Tiedje and Hagedorn 1975; Wei and Vossbrinck 1992; Konopka 1994; Liu et al. 1995; Müller and Buser 1995). This study confirms that metolachlor has less adverse effects than alachlor, but caution in its use should be observed as its stable aniline degradation product, 2-ethyl-6-methylaniline, showed the most prominent teratogenic capacity in the present study.

Alachlor and metolachlor are some of the most widely used herbicides in agriculture in Kenya (Partow 1995) and worldwide (Tessier and Clark 1995), metolachlor being the most widely used herbicide in North America (Müller and Buser 1995; Surralles et al. 1995) after banning of alachlor in Canada (Hoberg 1990). We suggest that the criteria for establishment of safety of chloroacetanilides should include both acute and chronic effects of the parent compounds and their degradation products. So far, the present study indicates risk associated with the use of metolachlor, (i.e. teratogenicity after breakdown), which before have been associated with alachlor only and which led to banning of this latter product. *Xenopus*, which inhabits the fresh water ponds in the farming regions of Kenya, offer a potential as an ideal organism for the study of the effects of suspect
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This test organism is important because of the massive application of the chloroacetanilides in Kenya and the potential liberation of the degradation product. Furthermore, the present study and related ones may provide additional clues on factors causing the decline in the populations of amphibians worldwide (Wake 1991; Baustein and Wake 1995) and explain the increased incidence of abnormalities in the natural populations of frog species (Burkhart et al. 2000).

References


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