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Comparison of rat olfactory mucosal responses to carcinogenic and non-carcinogenic chloracetanilides

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Abstract

Alachlor and butachlor are chloracetanilide herbicides that induce olfactory tumors in rats, whereas propachlor does not. The mechanism by which alachlor induces tumors is distinct from many other nasal carcinogens, in that alachlor induces a gradual de-differentiation of the olfactory mucosa (OM) to a more respiratory-like epithelium, in contrast to other agents that induce cytotoxicity, followed by an aberrant regenerative response. We studied biochemical and genomic effects of these compounds to identify processes that occur in common between alachlor- and butachlor-treated rats. Because we have previously shown that matrix metalloproteinase-2 (MMP2) is activated in OM by alachlor, in the present studies we evaluated both MMP2 activation and changes in OM gene expression in response to carcinogenic and non-carcinogenic chloracetanilide treatments. All three chloracetanilides activated MMP2, and > 300 genes were significantly up- or downregulated between control and alachlor-treated rats. The most significantly regulated gene was vomeromodulin, which was dramatically upregulated by alachlor and butachlor treatment (>60-fold), but not by propachlor treatment. Except for similar gene responses in alachlor- and butachlor-treated rats, we did not identify clear-cut differences that would predict OM carcinogenicity in this study.

Keywords

alachlor; butachlor; propachlor; vomeromodulin; olfactory mucosa; gene expression

Introduction

The chloracetanilide herbicides, including acetochlor, alachlor, butachlor, metolachlor, and propachlor (Figure 1), are a structurally similar class of pesticides. Acetochlor, alachlor, and butachlor appear to be metabolized to a quinoneimine, which is proposed to be responsible for the olfactory mucosal carcinogenicity of these compounds (Mulkey, 2001). Acetochlor, alachlor, and butachlor induce glucuronidation activity, which is proposed as the basis for formation of thyroid follicular cell tumors in rats treated with these compounds. The chloracetanilide metolachlor, which is associated with neither olfactory nor thyroid tumors,

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similarly does not induce glucuronidation or thyroid cancer (Dalton et al., 2003). Other tumor sites in chloracetanilide-treated rats are summarized in Table 1.

A serendipitous finding of previous microarray analysis was that alachlor causes upregulation of matrix metalloproteinase-2 (MMP2) gene expression in OM, and MMP2 activation in OM was subsequently confirmed by zymography (Genter et al., 2002b; 2006). In addition, administration of an MMP2 inhibitor to alachlor-treated rats significantly decreased the number of rats with olfactory mucosal tumors and the number of tumors per rat (Genter et al., 2005). We therefore hypothesized that butachlor, which also causes OM tumors, would similarly stimulate MMP2 activity, but that propachlor would not. We further hypothesized that co-regulation of a signature set of genes by alachlor and butachlor would be predictive of a carcinogenic response, and that propachlor treatment would not similarly regulate this gene set.

Materials and methods

Chemicals

Alachlor, butachlor, and propachlor were purchased from Chem Services (West Chester, PA). All were >98% pure according to documentation furnished by the manufacturer. All other reagents were of the highest quality available commercially.

Rats

Male Long-Evans rats (Harlan, Indianapolis, IN) were used for all studies. Rats were obtained at 6–7 wk of age and acclimated for at least one wk prior to beginning feeding studies. Each chloracetanilide was mixed into powdered LM-485 rodent chow (Harlan/Teklad, Indianapolis, IN). We did not attempt to dose rats at equivalent dosage for all three compounds; instead, we provided chow containing the concentrations used in previous long-term feeding studies: alachlor (dietary equivalent of 126 mg/kg/d); butachlor (3,000 ppm in diet) and propachlor (3,000 ppm in diet). Control rats received powdered rodent chow only. Treatments were pre-approved by the University of Cincinnati Institutional Animal Care and Use Committee.

The present study was not designed to be a cancer study; rather, our goal was to identify endpoints that occur prior to histological changes that can predict a future carcinogenic response. Rats were treated for either 10 d or 2 mo (four rats per treatment per time point). The 10 d time point was chosen based on previous data that showed significant dysregulation of olfactory mucosal antioxidant levels with alachlor treatment at very early treatment time points (Burman et al., 2003); further, oxidative stress has been associated with upregulation of matrix metalloproteinases in other model systems (Siwik et al., 2001; Uemura et al., 2001). The 2 mo time point was selected based on prior data from our lab that the earliest histological changes seen in the OM of alachlor-treated rats occurred following 3 mo of treatment (Genter et al., 2002a). Euthanasia was via carbon dioxide asphyxiation, and tissues were snap frozen on dry ice and maintained at -80°C until analysis. OM was isolated by removing the ethmoid turbinates (including bone), as well as the caudal one-third of the nasal septum, using straight microscissors.

Zymography

Olfactory mucosal MMP2 activity was assessed by zymography (Leber and Balkwill, 1997; Porter et al., 1999; Roach et al., 2002). Tissue used for this analysis was the olfactory mucosa dissected from the caudal one third of the nasal septum from rats treated with the respective chloracetanilide compound for 10 d. Briefly, tissues were homogenized in lysis buffer (0.1 mM PMSF, 50mM Tris HCl (pH 7.6), 2M urea, 0.1% NaCl, 0.1% EDTA, 0.1% Brij 35 (Sigma Aldrich, St Louis, MO, USA)), centrifuged at $10,000 \times g$ for 15 min at 4°C , and the supernatant

dialyzed overnight at 4°C in Membra-Cel MD25-14 dialysis membrane (14 000 kDa cut-off) against 25mM Tris HCl (pH 8.5), 10mM CaCl₂, 0.1% Brij 35 and 0.1mM PMSF. Protein concentrations of the dialyzed samples were determined (Bradford assay; Bio-Rad), and 40 µg samples were loaded onto 10% polyacrylamide gels containing 0.1% gelatin. The sample buffer for loading samples contained no reducing agent (dithiothreitol or 2-mercaptoethanol) so that the proteins would run at their native sizes. Gels were run at 100V for approximately 2 h.

SDS was removed from the gels by washing in 2.5% Triton-X 100 (3 × 15 min) before incubation in zymography buffer (Tris HCl, pH 7.5, 200mM NaCl, 5mM CaCl₂, 0.02% Brij 35) at 37°C for 16 h for assessment of gelatinolytic activity. Gels were then washed in water and stained in 0.1% Coomassie blue, prepared in 50% methanol/30% acetic acid/20% water for 3 h. MMP activity was visually assessed by examination of the intensity of white bands on blue background of the stained gels.

Microarray analysis

The time point chosen, i.e. following 2 mo of chloracetanilide treatment, is prior to the development of any histological changes (Genter et al., 2002a), because we wanted to monitor very early changes in gene expression that might predict a carcinogenic response. Frozen olfactory tissue (50 – 100 mg) was homogenized in Tri Reagent (Molecular Research Center, Cincinnati, OH) and RNA was isolated from the homogenate per the manufacturer's protocol. RNA quality was assessed using an Agilent Bioanalyzer (Quantum Analytics, Foster City, CA). Each exposure group and control group consisted of 4 rats, hybridized separately (i.e. tissues were not pooled), using 20 µg total RNA per array. The co-hybridizations performed are summarized in Figure 2.

Microarray hybridizations

Microarray experiments were carried out essentially as described in published reports and references therein (Guo et al., 2004; Karylal et al., 2004; Sartor et al., 2006). The rat 70-mer Operon Oligonucleotide Library Version 3.0 (26, 962 oligos) (Operon Biotechnologies; Huntsville, AL) was suspended in 3× SSC at 30 µM and printed at 22°C and 65% relative humidity on aminosilane-coated slides (Cel Associates, Inc.; Pearland, TX) using a high-speed robotic Omnigrid machine (GeneMachines; San Carlos, CA) with Stealth SMP3 pins (Telechem; Sunnyvale, CA). The complete gene list can be viewed at <http://microarray.uc.edu>. Spot volumes were 0.5 nl and spot diameters are 75–85 µm. Oligonucleotides were crosslinked to the slide substrate by exposure to 600 mJ of ultraviolet light.

Fluorescence-labeled cDNAs were synthesized from total RNA using an indirect amino allyl labeling method via an oligo(dT)-primed, reverse transcriptase reaction. cDNAs were labeled with monofunctional reactive Cyanine-3 and Cyanine-5 dyes (Cy3 and Cy5; Amersham; Piscataway, NJ).

The microarrayed DNA probes were incubated in prehybridization buffer (5× SSC, 0.1% SDS, and 1% bovine serum albumin) at 48°C for up to 90 min. For hybridization, the microarray slides were spotted with 42 µl hybridization buffer (25% formamide, 5× SSC, 0.1% SDS, 5 µg/µl COT1-DNA, 5 µg/µl poly(A)-DNA, and 2µg/µl yeast tRNA) and the fluorescence-labeled target cDNA; covered with glass coverslips (Fisher; Pittsburgh, PA), and placed in humidified hybridization chambers (Corning; Acton, MA). Hybridization chambers were placed in a water bath at 48°C for 66 h. Slides were placed in a slide rack set in a staining dish and washed in 1× SSC with 0.2% SDS for 4 min at 48°C with agitation and transferred to a staining dish with 0.1× SSC and 0.2% SDS and washed with agitation for 4 min at room

temperature and two times for 4 min each in $0.1\times$ SSC at room temperature. Slides were spun-dried immediately after washing.

Imaging and data generation were carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments; Union City, CA) and associated software from Axon Instruments, Inc. (Foster City, CA). Microarray slides were scanned with dual lasers with wavelength frequencies to excite Cy3 (532 nm) and Cy5 (635 nm) fluorescence emittance. Images were captured in JPEG and TIFF files, and DNA spots captured by the adaptive circle segmentation method. Information extraction for a given spot is based on the median value for the signal pixels and the median value for the background pixels to produce a gene set data file for all the DNA spots. The Cy3 and Cy5 fluorescence signal intensities were normalized.

Data normalization and analysis

Data were analyzed to identify differentially expressed genes between control rats and rats treated for two months with alachlor, butachlor, or propachlor. Four biological replicate arrays, including two dye-flipped, were performed for each comparison. Analysis was performed using R statistical software and the *limma* Bioconductor package (Smyth et al., 2004). Data normalization was performed in two steps for each microarray separately (Guo et al., 2004; Karyal et al., 2004; Sartor et al., 2006). First, background adjusted intensities were log-transformed and the differences (M) and averages (A) of log-transformed values were calculated as $M = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy5 and Cy3 intensities, respectively. Second, normalization was performed by fitting the array-specific local regression model of M as a function of A. Normalized log-intensities for the two channels were then calculated by adding half of the normalized ratio to A for the Cy5 channel and subtracting half of the normalized ratio from A for the Cy3 channel. The statistical analysis was performed by first fitting the following Analysis of Variance model for each gene separately: $Y_{ijk} = \gamma + A_i + S_j + C_k + \Omega_{ijk}$, where Y_{ijk} corresponds to the normalized log-intensity on the i^{th} array, with the j^{th} treatment, and labeled with the k^{th} dye ($k = 1$ for Cy5, and 2 for Cy3). μ is the overall mean log-intensity, A_i is the effect of the i^{th} array, S_j is the effect of the j^{th} treatment and C_k is the gene-specific effect of the k^{th} dye. Estimated fold changes were calculated from the ANOVA models, and significance levels for each comparison were calculated using an intensity-based empirical Bayes method (IBMT) (Sartor et al., 2006). This method obtains precise estimates of variance by pooling information across genes and accounting for the dependency of variance on probe intensity level. Identification of significant genes was accomplished from two avenues. First, False Discovery Rates (*FDR*) were calculated (Benjamini and Hochberg, 1995), and genes with *FDR* < 0.15 were considered as differentially expressed. Next, discovery of gene categories enriched with differentially expressed genes was performed using DAVID (Dennis et al., 2003) with a *p*-value < 0.05 cutoff for genes, and using the *FDR* adjustment.

Quantitative real time polymerase chain reaction (q-PCR)

Six genes were selected for validation by q-PCR based on their significant regulation in the OM of control vs. alachlor samples or based on a difference in their regulation in butachlor vs. alachlor hybridizations compared to propachlor vs. alachlor hybridizations. Genes and the primers used are presented in Table 2. RNA (1 $\mu\text{g}/\text{sample}$) was treated with DNase-1 (Invitrogen), and reverse transcribed using the ABgene Revers-iT (Fisher Scientific) first strand synthesis kit using random decamers. PCR reactions were visualized using ethidium bromide-stained agarose gels to confirm the presence of a single band with the selected annealing temperature. Q-PCR reactions were performed using SYBRgreen (Bio-Rad). Reaction mixtures consisted of 1 μl of each primer (500 nM final concentration) and 1 μl of cDNA in a final reaction volume of 20 μl . PCR conditions were as follows: $95^\circ\text{C} \times 3 \text{ min}$, followed by 40 cycles of $95^\circ\text{C} \times 30 \text{ sec}$, $55^\circ\text{C} \times 45 \text{ sec}$, $72^\circ\text{C} \times 1 \text{ min}$. Extension was performed

at 72°C for 10 min, followed by melting curve analysis (60°C – 95°C every 0.2°C). Fold changes in gene expression were calculated by the delta delta C_t method (Borchers et al., 2006).

Results

Zymography

The zymography protocol employed in these studies can detect latent and active MMP2, as well as MMP9. As seen in Figure 3, active MMP2 is barely detected in the two control lanes, but is enhanced in the OM by all three chloracetanilide treatment groups. MMP9 activity does not appear to be different among the treatment groups.

Microarray analysis

A total of 365 genes were significantly changed in expression level in the control vs. alachlor comparison at a p-value of ≤ 0.05 . Genes that were significantly regulated in the control vs. alachlor comparison with a FDR of less than 0.15 are presented in Table 3, together with their responses in the alachlor vs. butachlor and alachlor vs. propachlor hybridizations. Raw data files and normalized data for all genes can be viewed at <http://eh3.uc.edu/supplements/genter-chloracetanilides/>. The larger issue that we wanted to address was whether we could identify genes that were similarly regulated in the OM of alachlor- and butachlor-treated rats (both olfactory carcinogens), but not so in propachlor-treated rats, given that propachlor does not cause OM tumors. We did this by identifying genes that were significantly up- or down- regulated by alachlor treatment (compared to controls), and then finding genes in which the ratio of expression was equivalent to 1.0 in the alachlor vs. butachlor comparisons, indicating that the gene was equivalently-expressed in both treatment conditions (Table 4). Examples of genes that met this criterion are the serine protease inhibitor *Spin2c*, the cytosolic sulfotransferase *Sult1c1*, transformation-related p53 inducible nuclear protein 1 (*Trp53inp1*), Ras-responsive element binding protein 1 (*Rreb1*), and sperm-associated antigen 6 (*Spag6*). Functional enrichment analysis using DAVID Bioinformatics Resource (Dennis et al., 2003) found that the only functional group that was significantly regulated was that for genes of olfactory function (FDR = 0.026; Table 5). Examination of the significantly regulated olfactory function genes reveals that nearly all are odorant receptor genes.

Quantitative PCR

The results of q-PCR analysis to confirm changes in gene expression that were identified by microarray analysis revealed that only vomeromodulin and RYF3 behaved similarly by microarray analysis and by q-PCR (Figure 4). Interestingly, significant regulation of beta actin, which is widely used as a reference gene in q-PCR experiments, was indicated by alachlor treatment both by microarray and q-PCR analysis (data not shown). Therefore, olfactory marker protein (*OMP*), the expression of which was not regulated by any of the chloracetanilide treatments, was used as the reference transcript. The changes in gene expression by microarray analysis for the remaining genes (Table 2) were not confirmed by q-PCR (not shown).

Discussion

Alachlor, butachlor, and acetochlor have previously been grouped together based on a common mechanism of toxicity for nasal turbinate tumors (Mulkey, 2001). The studies detailed herein were designed to investigate endpoints that might be predictive of the OM carcinogenic process. The results of these studies essentially reject our hypothesis that olfactory carcinogenic chloracetanilides are readily distinguished from a non-olfactory carcinogenic chloracetanilide

compound by their ability activate MMP2 and regulate a specific battery of genes. Instead, we found that all three chloracetanilides caused MMP2 activation in OM (Figure 3).

Several genes were selected for validation by quantitative RT-PCR (Table 2). Genes were chosen based on several criteria, including highly significant differences in expression by microarray analysis; biological significance of the respective genes; and availability of the complete rat gene sequence for primer design. Q-RT-PCR confirmed that vomeromodulin was highly upregulated by alachlor and butachlor (Figure 4). Vomeromodulin is a putative pheromone transporter (Khew-Goodall et al., 1991), and regulation by toxicant exposure has not previously been reported. Similarly, q-PCR results also confirmed microarray findings that RYF3 was highly upregulated by both alachlor and butachlor but not by propachlor (Figure 4). RYF3, (previously annotated as “*Rattus norvegicus* hypothetical protein LOC686891”) was characterized as a novel olfactory mucosal gene with high expression in the lateral nasal glands, and also as a transcript found in the rat incisor (Dear et al., 1991). Based on the similar, very high degree of upregulation of RYF3 and vomeromodulin by alachlor (14- and 63-fold, respectively), as well as previous reports that found expression of both vomeromodulin and RYF3 in lateral nasal glands (Dear et al., 1991; Khew-Goodall et al., 1991), we aligned the nucleotide sequences for these two transcripts (nucleotides 58 – 1302 of RYF3 and nucleotides 163 – 1257 of vomeromodulin) and found them to be 99% identical (1757/1765 nucleotides). Thus, it appears that vomeromodulin and RYF3 may in fact represent the same proteins.

Quantitative RT-PCR failed to confirm gene expression changes that were indicated by microarray analysis for other transcripts. We do not have historical data with this probe set concerning the rate at which microarray findings are ‘confirmed’ by q-PCR, but in at least one instance, the q-PCR findings confirm previous results and make biological sense. In our previous microarray analysis of alachlor-treated rat OM, we identified an expressed sequence tag ‘Deleted in malignant brain tumors’ (*DMBT1*) as a gene that was highly upregulated in the olfactory epithelium of alachlor-treated rats (this gene was previously annotated as ebnerin in the rat (Genter et al., 2002b)). The lack of upregulation of *DMBT1* after 2 mo of alachlor treatment actually makes biological sense, in light of our previous observation that DMBT (ebnerin) was expressed in areas of alachlor-induced respiratory metaplasia (Genter et al., 2002b); this histological change would not have been present in olfactory epithelium from rats receiving only 2 mo of treatment, as we did not observe any histological alterations until after 3 mo of treatment.

Other genes that were significantly up- or down- regulated in microarray results, but not confirmed by q-PCR, were the organic anion transporter *OAT6* (encoded by *SLC22A2*) and ‘spermatogenesis associated 1’ (*SPATA1*). *OAT6* is an organic anion transporter that transports various molecules such as propionate, 2- and 3-methylbutyrate, benzoate, heptanoate, *e*-ethylhexanoate, and estrone sulfate, and has recently been localized to non-neuronal cells of the OM. The role of *OAT6* in olfaction, if any, is unknown, but it is possible that odorants secreted into the urine are transported through the olfactory mucosa by *OAT6* (Kaler et al., 2006; Schnabolk et al., 2006).

We also attempted to confirm the high expression (>33-fold) of the transcript *SPATA1* in alachlor-treated rat olfactory epithelium OM. This transcript was of interest to us because the mouse ‘ciliome’ has recently been described (McClintock et al., 2008), and several sperm-related genes were found in olfactory mucosa and similarly regulated by alachlor and butachlor treatment (Table 4). This observation is not surprising, given the importance of cilia to both sperm motility and olfaction (odorant receptors are localized to the cilia on the apical surface of olfactory receptor neurons). The upregulation of this transcript would be consistent with the fact that we have observed de-differentiation of OM into a ciliated respiratory-like epithelium prior to the development of alachlor-induced OM tumors.

A final observation, for which there is not an obvious explanation, is that odorant/olfactory receptor transcripts were significantly regulated by chloracetanilide treatments. We have previously reported that the earliest histological change that can be observed in the olfactory mucosa of alachlor-treated rats is a gradual de-differentiation of the olfactory mucosa to a more respiratory-like epithelium (i.e. with loss of olfactory neurons and acquisition of ciliated respiratory epithelial cells) (Genter et al., 2002a). One might expect that, as the OM dedifferentiates in response to alachlor or butachlor treatment, odorant receptor expression would decrease, concomitant with histologically-detectable loss of olfactory receptor neurons (Genter et al., 2002a). However, we observed that for odorant/olfactory receptors, several of those showing significant regulation by alachlor were upregulated, rather than downregulated (Table 5; plus Olf94 in Table 3). This observation, coupled with the observed presence and regulation of odorant receptor transcripts in tissues other than OM, suggests that these proteins may function in roles distinct from their putative role in odorant detection (Feldmesser et al., 2006; also Genter, unpublished observation; D.W. Nebert and S. Schneider, personal communication). This idea is not without precedent; recently, *Drosophila* gustatory receptors have been localized to unexpected structures, including abdominal neurons, putative hygroreceptive neurons of the arista, peripheral proprioceptive neurons in the legs, and neurons in the larval and adult brain, suggesting that some gustatory receptor genes function in nongustatory roles in the nervous system and tissues involved in proprioception, hygroreception, and other sensory modalities (Thorne and Amrein, 2008). Further, an olfactory neuron was found to have temperature-sensing function in *C. elegans* (Kuhara et al., 2008).

In summary, we attempted to identify a biochemical or genomic predictor of the chloracetanilide-induced OM carcinogenic response following 2 mo of treatment, which is prior to the time at which we can observe histological changes or tumor formation (Genter et al., 2002a). Matrix metalloproteinase activation was observed with both carcinogenic and non-carcinogenic chloracetanilides, suggesting that MMP2 activation is not predictive of the OM carcinogenic response. This observation was somewhat surprising, as previous results from our lab revealed that administration of an MMP2 inhibitor reduced the carcinogenic response in alachlor-treated rats (Genter et al., 2005). Alterations in OM gene expression did not prove to be definitive in distinguishing olfactory carcinogenic chloracetanilides from propachlor, with the exception that selective regulation of vomeromodulin / RYF3 expression by alachlor and butachlor is among the most sensitive predictors identified in the current studies.

Abbreviations

Cy3	Cyanine-3
Cy5	Cyanine-5
d	days
FDR	false discovery rate
h	hour(s)
min	minute(s)
MMP	matrix metalloproteinase
mo	month(s)
OM	olfactory mucosa
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
SDS	sodium dodecyl sulfate

SSC Sodium chloride-sodium citrate buffer

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Figure 1.
Structures of commonly-used chloracetanilide herbicides.

Figure 2.
Hybridization scheme used in the present studies.

Figure 3.

Zymogram indicating MMP2 activation in olfactory mucosa by all three chloracetanilide compounds: Samples are indicated as follows:

Lanes 1 and 2: controls (powdered diet only);

Lanes 3 and 4: alachlor treatment (126 mg/kg/d);

Lanes 5 and 6: butachlor treatment (3000 ppm in diet);

Lanes 7 and 8: propachlor treatment (3000 ppm in diet). Control lanes show very weak 'active' MMP2 bands, whereas alachlor, butachlor, and propachlor treatments all resulted in MMP2 activation in OM, as indicated by the bright active MMP2 bands. This procedure also allows for detection of MMP9 activity, which does not appear to vary among the treated groups.

Figure 4.

Fold-increase in expression for vomeromodulin and RYF3, as assessed by q-PCR. Note that alachlor and butachlor caused marked increase in expression of these genes, whereas propachlor did not. Olfactory marker protein (*OMP*) was used as the reference gene, and fold differences in gene expression were calculated by the delta, delta C_t method (Borchers et al., 2006).

Table 1

Site of tumor formation in response to various chloracetanilide herbicides

Compound	Nasal tumors	Liver tumors	Thyroid tumors ^a	Gastric tumors
Acetochlor	+	+	+	+/-
Alachlor	+		+	+
Butachlor	+		+	+
Metolachlor		+		
Propachlor		+	+	

^a Acetochlor, alachlor, and butachlor are associated with thyroid follicular cell tumors, whereas propachlor-induced tumors are C-cell tumors.

Table 2**Quantitative PCR primers**

Solute carrier family 22 member 20	
(169 b.p.)	F: 5'-CCTGCTAGGAGATCCTGGTG-3'
	R: 5'-GCCCTGGACAGTAGTGCTTT-3'
Vomeromodulin	
(243 b.p.)	F: 5'-GCGTGCTTGGGGATATCTTA-3'
	R: 5'-TGCCTCCACCAAGGTTTAGT-3'
Human spermatogenesis associated 1	
(151 b.p.)	F: 5'-TTTGTAGATGAGCGGCAGACT-3'
	R: 5'-TCCTGATTCTTGTTAGGAAGCTC-3'
RYF3	
(163 b.p.)	F: 5'-ATCCTCAAAGCCCTCATCA-3'
	R: 5'-TTGATTTTGAGAGCCCATC-3'
Deleted in malignant brain tumors 1	
(99 b.p.)	F: 5'-TTTTTGCAGGCCTGTGTGTC-3'
	R: 5'-CATGTTTCTCCAGGATGCCA-3'
Olfactory marker protein	
(348 b.p.)	F: 5'-GACATTCTTCTAGCTGCTCC-3'
	R: 5'-CAGCTTTGGCCAGCAGGG-3'

Table 3

Genes regulated significantly by alachlor treatment (FDR < 15%)

Entrez Gene id	Gene name	fold change			p values		
		But_ala	Con_ala	Pro_ala	But_ala	Con_ala	Pro_ala
315686	similar to RIKEN cDN						
	C230081A13	-1.51	-4.23	1.16	0.032	8.02E-07	0.400
171178	alcohol dehydrogenase (Adh7)	1.02	3.78	1.73	0.921	1.12E-06	0.005
n/a	immunoglobulin heavy chain alpha peptide	1.97	-12.17	1.03	0.324	3.23E-06	0.934
n/a	similar to CYP2K6*	1.06	3.60	1.65	0.763	3.61E-06	0.013
381203 DCP1	decapping enzyme homolog*	-1.01	3.59	1.74	0.975	9.97E-06	0.007
n/a	significant alignment with rat BACs, including CH230-277M9, CH230-525H19	-1.22	-4.24	1.03	0.354	7.34E-06	0.880
79223	Glycerol kinase	-1.17	-2.90	-1.21	0.349	1.12E-05	0.268
23475	quinolinate phosphoribosyltransferase	1.15	2.74	-1.28	0.395	1.47E-05	0.132
25526	prostaglandin-H-2 D-isomerase	1.17	5.62	1.59	0.621	1.52E-05	0.100
171163	Solute carrier Slc6a13	-1.59	2.76		0.024	1.75E-05	0.065
10622	DNA-directed RNA polymerase III*	-1.21	-39.22 -3.43	-1.28	0.537	1.84E-05	0.268
333669	Sodium-glucose transporter (solute carrier family 5)*	1.46	-3.43	-1.01	0.037	1.90E-05	0.930
353317	olfactory specific medium-chain acyl Co A synthetase	1.41	4.29	1.47	0.167	2.29E-05	0.125
51444	Rattus norvegicus BAC CH230-195H16	-13.08	-39.22	-2.84	0.0005	2.81E-05	0.018
N/a	Deleted in malignant brain tumors*	1.03	3.46	1.09	0.889	3.40E-05	0.679
N/a	immunoglobulin variable Region	1.69	-3.61	1.17	0.012	4.0E-05	0.408
194357	dopamine-beta-hydroxylase*	4.34	5.10	-1.14	0.321	4.95E-05	0.656
9632	fucosyltransferase 11	-1.11	-3.09	-1.42	0.531	5.04E-04	0.050
11228	Ras association (RalGDS/AF-6) domain, family 8*	-1.40	-3.53	1.10	0.150	5.41E-05	0.676
399512	similar: solute carrier family 25 member 35	-1.15	2.38	1.36	0.379	5.48E-05	0.066
n/a	hypothetical LOC308768*	-1.33	-2.58	1.08	0.117	6.0E-05	0.638
221223	carboxylesterase 615**	1.17	-17.94	1.06	0.512	6.36E-05	0.817
66957	serine/cysteine peptidase inhibitor, clade B	-1.27	-3.58	1.20	0.304	6.36E-05	0.739
n/a	olfactory receptor 1393	-1.13	-2.35	1.01	0.463	8.01E-05	0.951
n/a	Ig heavy chain variable segment Q52 gene	2.35	-3.85	1.20	0.004	8.41E-05	0.471

Entrez Gene id	Gene name	fold change			p values		
		But_ala	Con_ala	Pro_ala	But_ala	Con_ala	Pro_ala
25239	apolipoprotein D	1.04	2.14	1.06	0.777	8.48E-05	0.698
3512	immunoglobulin J polypeptide	1.27	-2.99	1.19	0.268	0.0001	0.404
n/a	immunoglobulin, gamma 2a chain, variable	2.31	-3.48	1.03	0.003	0.0001	0.883
2090	glucocorticoid receptor DNA binding factor 1	-3.79	-6.34	1.25	0.001	0.0001	0.337
N/a	alkaline phosphatase	1.09	3.15	-1.19	0.656	0.0001	0.350
390882	olfactory receptor 1139*	1.47	2.18	-1.01	0.042	0.0001	0.927
24943	myelin proteolipid	-1.03	2.07	-1.09	0.825	0.0001	0.544
64782	stimulated exonuclease gene 20-like 1	1.13	-2.43	-1.32	0.494	0.0001	0.129
20646	small nuclear ribonucleoparticle-associated protein	1.22	1.94	-1.15	0.147	0.0002	0.290
n/a	immunoglobulin rearranged kappa-chain mRNA variable region	1.95	-3.07	1.31	0.009	0.0002	0.246
367586	immunoglobulin gamma2a constant region	2.69	-4.35	1.01	0.004	0.0002	0.976
283849	similar: RIKEN cDNA E430013E20	-1.33	-3.23	-1.09	0.237	0.0002	0.715
6949	BAC CH230-333G5	-1.03	6.60	1.01	0.867	0.0002	0.970
113938	small nuclear ribonucleoparticle associated protein*	1.20	2.19	-1.03	0.272	0.0002	0.876
163175	leucine-rich repeat LGI family, member 4	1.17	2.14	-1.08	0.318	0.0002	0.643
64458	scrapie responsive gene 1	1.16	3.21	-1.22	0.412	0.0002	0.329
231004	similar: hypothetical protein MGC45873*	1.64	-5.51	-1.26	0.160	0.0002	0.435
N/a	similar: olfactory receptor 01fr94	-1.90	-4.49	-1.02	0.56	0.0002	0.077
286987	transferrin	1.18	-3.34	-1.61	0.510	0.0002	0.077
9371	kinesin family member 3B	-1.37	-2.65	1.06	0.145	0.0002	0.778

* predicted

** also high identity with rat glutathione-S-transferase, alpha type2

Table 4

Transcripts similarly regulated by butachlor and alachlor

Gene i.d. #	Annotation	Symbol
24795	Serine protease inhibitor	Spin2c
171072	Sulfotransferase, cytosolic	Sult1c2
171178	Alcohol dehydrogenase 7	Adh7
381203	Similar to solute carrier family 22	Slc22a6
54942	hypothetical protein FLJ20457	FLJ20457
83643	hypothetical protein DKFZ761F241	DKFZp761F241
1770	dynein, axonemal, heavy polypeptide 9	DNAH9
6239	ras responsive element binding protein 1	RREB1
9576	sperm associated antigen 6	SPAG6
64782	interferon stimulated exonuclease gene 20-like 1	Isg2011 *
85438	testis development protein NYD-SP26	NYD-SP26
245955	lectin, galactoside-binding, soluble	Lgal3bp
1670	defensin alpha 5	DEFA5
313860	similar to AII15338, predicted	RGD1311939_predicted
25250	Cytochrome c oxidase subunit VIII-H	Cox8h
286987	hemiferrin, transferring-like protein	LOC286987
297822	transformation-related protein 53 inducible nuclear protein 1	TRP53inp1
59779	hypothetical protein FLJ11142	FLJ11142
25584	coagulation factor III	F3
64535	fibroblast growth factor binding protein 1	Fgfbp1
300438	low density lipoprotein receptor	Ldlr
25526	prostaglandin D2 synthase	Ptgds
50568	chondroitin sulfate proteoglycan 5	Cspg5
641173	spermatogenesis associated 1	SPATA1
6251	Ras suppressor protein 1	RSU1
64718	unkempt-like, human homolog	UNKL
228802	hypothetical protein LOC683134, rat	LOC683134 **
754	pituitary tumor-transforming 1 interacting protein	PTTG1IP
25206	guanylate cyclase 1, soluble, beta 2	Gucy1b2
n/a	vomeromodulin (fragment)	VOMERat
50624	deleted in malignant brain tumors	DMBT1 ***

* formerly hypothetical protein FLJ12484

** formerly cDNA sequence BC018465 (human)

*** formerly CUB and zona pellucida-like domains 1 (human)

Table 5

Significantly regulated olfactory pathway genes (* indicates predicted [NCBI])

Entrez Gene Id.	Gene name	fold change			p values		
		But_ala	Con_ala	Pro_ala	But_ala	Con_ala	Pro_ala
8388	Olr 1458*	1.51	-2.49	1.05	0.149	0.004	0.851
120066	Olr 245*	1.09	2.11	1.00	0.685	0.004	0.996
390197	Olr 323*	1.05	1.84	-1.02	0.766	0.019	0.893
390201	Olr 318*	1.40	1.67	-1.00	0.095	0.035	0.982
79345	Olr 131*	1.10	1.43	1.06	0.560	0.035	0.705
290050	Olr 1642*	1.54	2.33	1.14	0.180	0.034	0.665
196335	Olr 198*	-1.21	1.78	-1.04	0.441	0.014	0.810
26529	Olr 1729*	-1.15	2.06	1.39	0.470	0.007	0.921
341416	Olr 889*	-1.13	-2.46	-1.33	0.697	0.007	0.379
26212	Olr 1683	1.04	1.43	1.01	0.813	0.042	0.949
390882	Olr 1139*	1.47	2.18	-1.01	0.656	0.0001	0.927
29989	Vomeranasa1 secretory						
	protein	-4.19	-13.04	1.09	0.310	0.024	0.919
219858	Olr 1199*	-1.01	2.47	-1.14	0.986	0.035	0.683
387772	Olr 373*	-1.11	1.43	1.09	0.529	0.041	0.588
390437	Olr 1624*	-1.96	-5.15	-1.05	0.137	0.006	0.902
120775	Olr 222*	-1.07	2.15	1.27	0.731	0.016	0.239
81282	Olr 60*	1.79	2.02	1.24	0.015	0.005	0.246
171352	Cytochrome						
	P450 3A9	-1.44	2.00	1.76	0.105	0.019	0.979
346528	Olr 812*	1.23	1.63	-1.04	0.323	0.015	0.828
391191	Olr 1443	1.45	1.58	1.01	0.044	0.037	0.944
119692	Olr 49*	1.31	2.19	-1.08	0.220	0.010	0.670
170816	Olr 59	-1.16	1.47	-1.02	0.380	0.035	0.911
219959	Olr 376*	1.32	1.60	-1.10	0.117	0.033	0.586
4992	Olr 1373*	-1.52	-1.57	-1.21	0.056	0.042	0.370
390191	Olr 363*	1.05	2.36	1.23	0.842	0.004	0.427
119682	Olr 69*	-2.67	-5.29	-1.68	0.060	0.001	0.928
390067	Olr 152*	1.12	1.67	1.18	0.578	0.019	0.403

Entrez Gene i.d.	Gene name	fold change			p values		
		But_ala	Con_ala	Pro_ala	But_ala	Con_ala	Pro_ala
347168	Olr 409*	1.04	1.91	1.10	0.885	0.012	0.682
390078	Olr 184*	1.18	1.48	1.08	0.272	0.034	0.859
390174	Olr 455*	1.12	2.12	1.21	0.600	0.004	0.862
390155	Olr 520*	1.04	1.71	-1.28	0.856	0.031	0.222
121275	Olfir 287*	-1.48	-1.57	1.05	0.119	0.044	0.296
390442	Olr 1631*	1.06	1.64	1.06	0.710	0.018	0.723
120065	Olr 262*	-1.05	1.74	1.17	0.746	0.004	0.319
81300	Olr 630*	-1.23	-1.98	-1.13	0.225	0.0009	0.457
81168	Olr 484*	-1.02	1.65	1.02	0.950	0.037	0.937
401667	Olr 68*	-1.03	1.58	-1.10	0.922	0.041	0.567
119694	Olr 51*	1.13	1.99	1.05	0.562	0.005	0.834
144125	Olr 206*	-1.11	-3.26	1.07	0.669	0.022	0.733