

# Retinal Dehydrogenase-2 Is Inhibited by Compounds that Induce Congenital Diaphragmatic Hernias in Rodents

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**Currently, the etiology of the serious developmental anomaly congenital diaphragmatic hernia (CDH) is unknown. We have used an animal model of CDH to address this issue. We characterized four separate teratogens that produced diaphragmatic defects in embryonic rats that are similar to those in infants with CDH. We then tested the hypothesis that all these agents share the common mechanism of perturbing the retinoid-signaling pathway. Specifically, inhibition of retinal dehydrogenase-2 (RALDH2), a key enzyme necessary for the production of retinoic acid and that is expressed in the developing diaphragm, was assayed by measuring retinoic acid production in cytosolic extracts from an oligodendrocyte cell line. The following compounds all induce posterolateral defects in the rat diaphragm; nitrofen, 4-biphenyl carboxylic acid, bisdiazine, and SB-210661. Importantly, we demonstrate that they all share the common mechanism of inhibiting RALDH2. These data provide an important component of mounting evidence suggesting that the retinoid system warrants consideration in future studies of the etiology of CDH. (*Am J Pathol* 2003, 162:673–679)**

Congenital diaphragmatic hernia (CDH) is a serious developmental disorder occurring in ~1 in 3000 live births in which the diaphragm muscle fails to form completely, resulting in a hole in the diaphragm and incomplete separation of the thoracic and abdominal cavities. Lung hypoplasia and pulmonary hypertension are major pathological consequences that account for much of the morbidity and mortality of this problem.<sup>1,2</sup>

An animal model of CDH was developed, resulting from toxicological studies that showed that nitrofen, a herbicide, although relatively harmless to adult rodents, caused developmental anomalies in the lungs, hearts, diaphragms, and skeletal tissues of fetuses in pregnant rats.<sup>3,4</sup> Diaphragmatic defects resulting from a single 100-mg dose of nitrofen administered to pregnant rats on

day 8 of gestation are very similar to those documented in human CDH, with respect to the size and location of the defect and the accompanying intrusion of the abdominal viscera into the thoracic cavity. Further, the associated developmental defects observed with nitrofen-induced CDH such as skeletal and cardiac malformations are similar to those seen in a subpopulation of infants with CDH.<sup>5–7</sup>

Data derived from studies of the nitrofen model suggest that the pathogenesis of CDH is linked to a malformation of the primordial diaphragm, the pleuroperitoneal fold.<sup>8</sup> However, the etiology of CDH is completely unknown. Further, despite the fact that the nitrofen model of CDH has been used since the 1970's, a clear understanding of the mechanisms underlying the herbicide's teratogenicity is lacking. Given the striking similarities between the pathologies observed in CDH in the nitrofen-induced rat model and in infants with CDH, the possibility of a common underlying etiology certainly has to be considered. Therefore, we sought to delineate the biochemical mechanisms underlying the actions of nitrofen.

There are several pieces of data that provide a rationale for examining the role of the retinoic acid system in the etiology of CDH. Past studies examining the effects of vitamin A-deficient diets in rodents during pregnancy demonstrated that some of the offspring had diaphragmatic hernias.<sup>9,10</sup> In 1994, Mendelsohn and colleagues<sup>11</sup> published data showing that in a subset of double-retinoic acid receptor subtype knockouts, fetuses had diaphragmatic hernias. Major and colleagues<sup>12</sup> provided preliminary evidence supporting a role of vitamin A as a factor in human CDH. In a small study of human mothers and infants born with or without CDH, it was reported that the retinol levels in the maternal and infant plasma were abnormal when CDH was present. More recently, a direct

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interaction of nitrofen and the retinoid system arose from studies using transgenic mice with a lacZ reporter linked to a retinoid response element (RARE). The expression of the transgene was markedly reduced in response to nitrofen exposure.<sup>13</sup>

In this study, we take the next step by determining the specific stage in the retinoid cascade affected by nitrofen. Specifically, we test the hypothesis that nitrofen acts to inhibit retinal dehydrogenase-2 (RALDH2) and thus the formation of retinoic acid from retinaldehyde. Further, we characterize three other compounds that induce diaphragmatic defects. Past reports have indicated that 4-biphenyl carboxylic acid (BPCA),<sup>14</sup> bisdiamine [*N,N'*-octamethylenebis (dichloroacetamide)]<sup>15,16</sup> and SB-210661<sup>17</sup> induce diaphragmatic defects. BPCA is a breakdown product of a thromboxane-A<sub>2</sub> receptor antagonist, bisdiamine is a spermatogenesis inhibitor, and SB-210661 is a benzofuranyl urea derivative developed for inhibiting 5-lipoxygenase. We demonstrate that these compounds induce diaphragmatic defects characteristic of CDH. Importantly, they all share, along with nitrofen, the same common mechanism of inhibiting RALDH2 in a dose-dependent manner.

## Materials and Methods

### Animals

Timed pregnant Sprague-Dawley rats were used following procedures approved by the Animal Welfare Committee at the University of Alberta. The morning on which a sperm plug was observed in the breeding cage was designated as embryonic day (E) 0.

### Chemicals

SB-210661 was generously provided by Dr. H. M. Solomon (GlaxoSmithKline Pharmaceuticals, King of Prussia, PA). Bisdiamine [*N,N'*-octamethylenebis (dichloroacetamide)] was purchased from ACROS Organics (Fisher Scientific, Pittsburgh, PA) and 4-biphenyl carboxylic acid from Sigma (St. Louis, MO). Nitrofen was obtained from the United States Environmental Protection Agency (Bethesda, MD) and China National Chemical Construction Jiangsu Company (Nanjing, China). The dosages and timings of administration for each chemical are listed in Table 1. Each compound was dissolved in 1 ml of olive oil using sonication. On the appropriate day(s) of gestation (Table 1), pregnant rats were anesthetized with halothane temporarily (10 minutes) and the drug solutions were delivered via gavage feed. Rats were returned to the original cage and housed in the lab for further dosing where specified.

### Cesarean Section and Diaphragm Isolation

On E18, rats were anesthetized and cesarean sections performed to deliver the fetuses. The fetuses were euthanized, decapitated, and placed into 4% paraformaldehyde for 1 to 2 days of fixation. Using a dissecting micro-

**Table 1.** Dosages and Days of Administration of Teratogens and Corresponding Incidence and Location of Diaphragmatic Defects

Compound/ dose	Days of administration	Hernias/ number of fetuses	Location of hernia (left/right/ bilateral)
Nitrofen			
100 mg	E8	266/507	153L/86R/27B
BPCA			
100 mg	E8	0/10	—
50 mg/kg	E8–10	0/8	—
75 mg/kg	E8–10	0/12	—
50 mg/kg	E7–14	22/62	4L/16R/2B
SB-210661			
100 mg	E8	0/13	—
50 mg/kg	E7–14	23/30	1L/14R/8B
75 mg/kg	E7–14	11/13	5L/6R/0B
100 mg/kg	E7–14	26/26	0L/0R/26B
Bisdiamine			
100 mg	E8	0/12	—
50 mg/kg	E8–9	2/10	1L/1R/0B
100 mg/kg	E8–9	1/14	1L/0R/0B
75 mg/kg	E10–11	7/18	3L/3R/1B
100 mg/kg	E10–11	6/17	0L/0R/6B
50 mg/kg	E11–12	16/16	0L/6R/10B
100 mg/kg	E11–12	2/17	0L/2R/0B

scope, the diaphragms were then exposed and removed for subsequent assessment of defects. In some cases, immunolabeling for neural cell adhesion molecule was performed to delineate the orientation of myotubes and/or phrenic nerve intramuscular branches.<sup>18</sup> Photographs of the diaphragms were taken with a Nikon 990 digital camera mounted on a Leica research microscope.

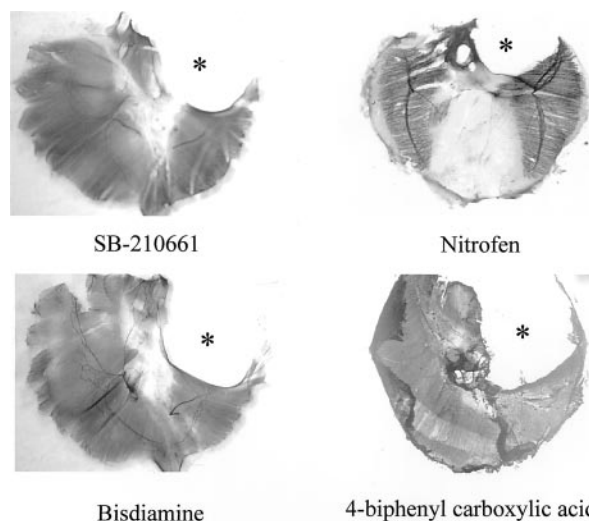
### Retinal Dehydrogenase Assay

To measure inhibitory effects on retinoic acid synthesis we used the RALDH2 isolated from an oligodendrocyte cell line.<sup>19,20</sup> Trypsinized oligodendrocyte cells were collected on ice, spun down, and triturated in an equal volume of 10 mmol/L of phosphate buffer, pH 7.4, with 30 mmol/L of NaCl containing 1 mmol/L of phenylmethyl sulfonyl fluoride, 1  $\mu$ mol/L of leupeptin, 1% aprotinin, and 1  $\mu$ mol/L of pepstatin as protease inhibitors. The homogenate was centrifuged for 15 minutes at 13,000  $\times g$  to obtain a supernatant containing the cytoplasmic proteins. Protein concentrations in these extracts were determined with the bicinchoninic acid protein assay (Sigma). Isoelectric focusing (IEF) of native proteins was performed in an IsoBox IEF apparatus (Hoefer Scientific/Pharmacia, Freiburg, Germany) with agarose gels using agarose-coated polyester film (GEL-Fix; Serva, Heidelberg, Germany), silanized glass plates and 1-mm-thick plastic spacers. The gel solution contained 0.8% agarose (11402, Serva), 2% sorbitol (Merck, Darmstadt, Germany), and 3% ampholytes pl 4 to 7 (42948, Serva). Electrode wicks were soaked in 0.5 mol/L of acetic acid and 0.5 mol/L of NaOH for anode and cathode, respectively. Samples were loaded with 10  $\mu$ g of protein per lane. Running conditions were: 10 minutes at 1 W; removal of the sample mask; 5 minutes, 5 W; 45 minutes, 15 W; all at 1200 V maximum. Internal protein standards with pl 3.6,

4.6, 5.1, 6.6, 8.2, 8.6, and 8.8 (Sigma) marked pH positions in the gel. Lanes with pI markers were fixed and stained with Coomassie G250. After IEF, parallel lanes of the gel were cut into 16 or 24 consecutive slices 2.25 mm apart. These IEF fractions were distributed into the wells of microtiter plates, where proteins were eluted and assayed for retinoic acid (RA) synthesis from 50 nmol/L all-*trans* retinaldehyde in the presence of 0.6 mg/ml dithiothreitol and 0.8 mg/ml NAD<sup>+</sup>.<sup>21</sup> After 4 hours of incubation at 37°C in darkness, 50 μl/well of reaction products were removed and tested with RA-sensitive reporter cells. The reporter cell line<sup>22</sup> consists of F9 teratocarcinoma cells transfected with the β-galactosidase gene under the control of the RA-responsive element from the RA receptor β. The cells were grown in CO<sub>2</sub>-buffered Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, penicillin/streptomycin (Sigma, P3539), and 0.8 g/L geneticin (Life Technologies, Inc., Grand Island, NY). For the RA assay, reporter cells were plated into poly-L-lysine-coated 96-well microtiter plates, grown to confluence, and cultured for ~12 hours with 150 μl of cell culture medium plus 50 μl of supernatant from the enzyme reaction. The RA-dependent induction of β-galactosidase was then visualized with the X-Gal staining procedure.<sup>20</sup> To measure the efficacy of enzyme inhibitors, IEF fractions were collected in medium that contained 0.1 μmol/L, 1 μmol/L, 10 μmol/L, or 100 μmol/L of SB-210661, bisdiamine, nitrofen, or BPCA. After 15 minutes of incubation at room temperature the NAD<sup>+</sup>/RAL solution was added for the aldehyde dehydrogenase reaction as described above. To ascertain that enzyme inhibitors did not interfere with RA detection, the reporter cells were incubated with medium containing inhibitors plus 0.1 nmol/L or 10 pmol/L of all-*trans* RA. Experimental procedures were identical to those when RA production was determined. Aldehyde dehydrogenase activity was also measured in cytosolic extracts without previous IEF separation. In this procedure cytosolic extracts containing 10 μg of protein were dissolved in 50 μl of medium with enzyme inhibitors and then processed as described. We chose concentrations of 1 pmol/L to 1 mmol/L for nitrofen and BPCA, 1 nmol/L to 1 mmol/L for SB-210661, and 0.1 pmol/L to 1 mmol/L for bisdiamine. Three independent experiments were performed.

### Immunohistochemistry

Paraffin-embedded embryos were cut with a microtome at 7 μm transversely and mounted. Slides were dewaxed, rehydrated, then incubated with 0.3% hydrogen peroxide in methanol for 30 minutes, and 0.4% Triton X-100/phosphate-buffered saline (PBS) for 1 hour. A blocking step was performed with 5% normal goat serum in 0.4% Triton X-100/PBS, followed by incubation with the primary antibody, rabbit anti-RALDH2 (1:1500; P. McCaffery, Waltham, MA) overnight at 4°C (antibody omitted for control sections). The slides were incubated in a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) secondary antibody at a dilution of 1:200 for 1



**Figure 1.** Photomicrographs of whole diaphragms labeled for neural cell adhesion molecule to demarcate myotubes. Representative examples of left-sided diaphragmatic defects (\*) induced by each of the four compounds. The posterolateral portion of the diaphragm was affected in all cases.

hour then washed in PBS. Slides were incubated with 1:100 avidin-biotinylated peroxidase complex (ABC kit PK4000, Vector). The antigen labeling was visualized by a 3,3-diaminobenzidine tetrahydrochloride product intensified by nickel (0.1 mol/L Tris buffer containing 0.04% 3,3-diaminobenzidine tetrahydrochloride with 0.04% hydrogen peroxide and 0.6% nickel ammonium sulfate). The slides were then counterstained with eosin and dehydrated in ethanol before being coverslipped.

## Results

### Diaphragmatic Defects

Fetuses removed from pregnant rats treated with nitrofen and 4-biphenyl carboxylic acid showed no outward abnormalities at the doses administered. A significant fraction (~50%) of the fetal rats from animals given high doses of SB-210661 (100 mg/kg/day, E7 to E14) or bisdiamine (100 mg/day on E10 to E11 or E11 to E12) displayed head abnormalities including blunt snouts and some skin defects (loose skin covering the trunk). Internal malformations were not systematically examined, although lung hypoplasia and retarded overall growth was evident in the majority of fetuses exposed to the teratogens regardless of CDH status.

Figure 1 shows representative examples of diaphragm defects induced by the four compounds. The number of diaphragmatic defects produced by each of the compounds and data regarding the location of the diaphragmatic defects are contained in Table 1. As reported previously,<sup>18</sup> a single dose of nitrofen administered on E8 results in ~50% of the embryos having hernias, with a predominance of left-sided defects. Past studies have also demonstrated that single doses of nitrofen administered beyond E11 induced solely right-sided defects. None of the other compounds induced diaphragmatic

**Table 2.** Interference of Enzyme Inhibitor with Retinal Dehydrogenase Detection Assay

Inhibitor	Bisdiamine	Nitrofen	SB-210661	BPCA
0.1 $\mu\text{mol/L}$	0 $\pm$ 0	0 $\pm$ 0	0.3 $\pm$ 0.5	9.8 $\pm$ 24.1
100 $\mu\text{mol/L}$	7.5 $\pm$ 10.6	0 $\pm$ 0	17.5 $\pm$ 17.7	48.0 $\pm$ 7.4

Complete inhibition of RA detection = 100, no inhibition = 0 (mean  $\pm$  SD).

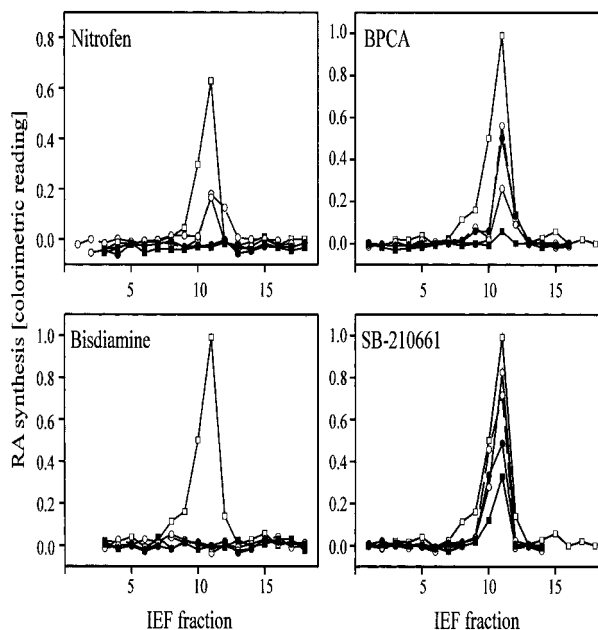
defects after a single administration on E8. Repeated doses were required.

Administration of 4-biphenyl carboxylic acid at a dosage of 50 mg/kg/day from E7 to E14 induced predominantly right-sided diaphragmatic defects. SB-210661, administered at a dose level of 100 mg/kg/day from E7 to E14 caused 100% large bilateral defects; with ~80% of the diaphragm tissue missing in some cases. Doses of 50 and 75 mg/kg/day from E7 to E14 induced defects of more moderate size in 74% and 85% of fetuses, respectively. Bisdiamine had a different dose dependency. A dose of 100 mg/day administered on E10 and E11 induced large bilateral defects. The same dosage administered on E11 and E12 induced only right-sided defects. Overall, the rank order of efficacy in producing diaphragmatic defects was bisdiamine > SB-210661 > nitrofen > BPCA.

### Measurements of Retinal Dehydrogenase Inhibition

Using the immortalized oligodendrocyte cell line OLN93 as a source of retinal dehydrogenase we investigated the inhibitory effect of nitrofen, bisdiamine, BPCA, and SB-210661 on this enzyme. Because RA synthesis was assessed by means of  $\beta$ -galactosidase expression in a bioassay we tested whether enzyme inhibitors interfered with RA detection in this system. At a concentration of 0.1  $\mu\text{mol/L}$  none of the inhibitors had a significant effect on RA detection (Table 2), nor did 100  $\mu\text{mol/L}$  of bisdiamine and nitrofen. SB-210661 reduced the reporter cell response at 100  $\mu\text{mol/L}$  to a small degree. At this concentration BPCA interfered with the zymography assay but not enough to render the RALDH inhibition data invalid (because BPCA inhibited RA synthesis completely and at much lower concentrations).

In zymography assays, IEF fractions were incubated with 0.1, 1, 10, and 100  $\mu\text{mol/L}$  of nitrofen, BPCA, bisdiamine, SB-210661, or no enzyme inhibitor (Figure 2). All compounds reduced RA synthesis in a dose-dependent manner. Complete inhibition of the aldehyde dehydrogenase activity was found with all concentrations of bisdiamine, 10  $\mu\text{mol/L}$  of nitrofen or higher, and 100  $\mu\text{mol/L}$  of BPCA. Because only one peak of RA-synthesizing activity was detected we then used cytosol homogenates without previous separation by IEF to determine dose-response functions for a broader range of concentrations (Figure 3). The ED<sub>50</sub> for suppression of RALDH activity were 10 to 100  $\mu\text{mol/L}$  for BPCA and SB-210661, ~10  $\mu\text{mol/L}$  for nitrofen, and less than 1 pmol/L for bisdiamine. In accordance with the zymography assays and the rank order of

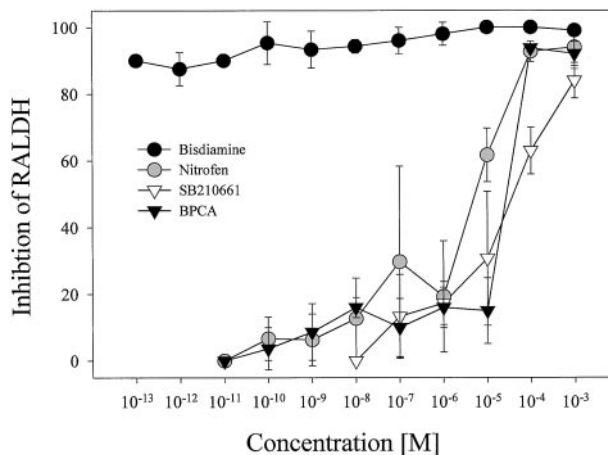


**Figure 2.** IEF fractions of cytosolic extracts from OLN93 cells exhibit RALDH activity. One peak of enzyme activity was detected in fractions with pI 5.0 to 5.6. Protein fractions were incubated with 0.1  $\mu\text{mol/L}$  (○), 1  $\mu\text{mol/L}$  (◻), 10  $\mu\text{mol/L}$  (●), or 100  $\mu\text{mol/L}$  (■) of each of the four compounds or no enzyme inhibitor (□).

efficacy in producing diaphragmatic defects, bisdiamine, which suppressed RALDH activity at all tested concentrations, was the most potent inhibitor, followed by nitrofen, BPCA, and SB-210661.

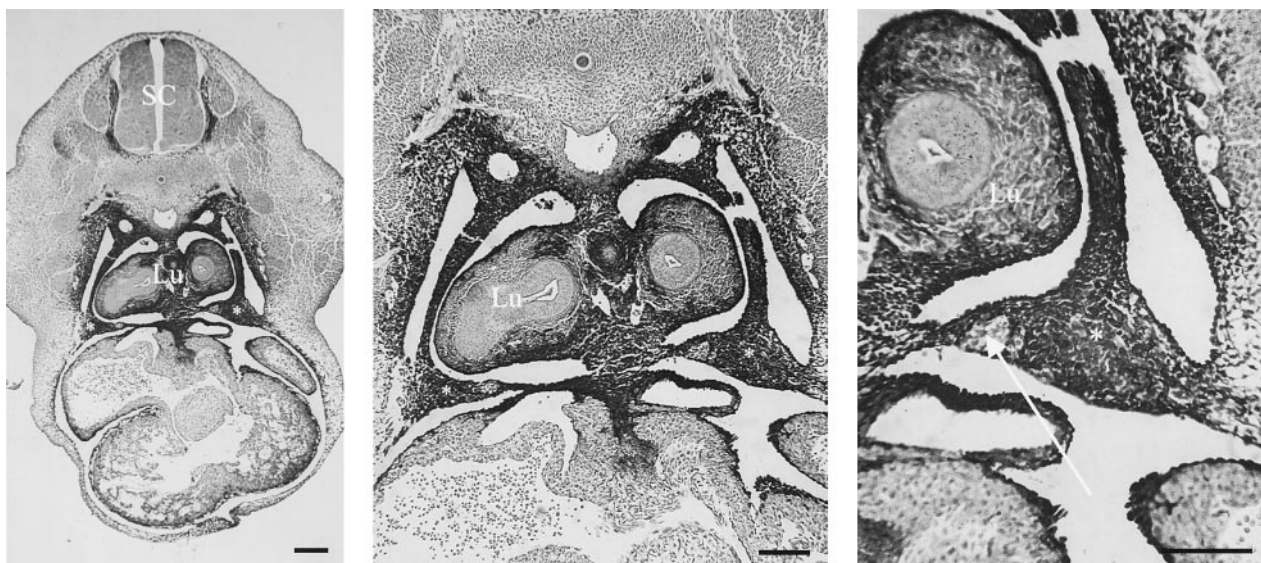
### RALDH2 Expression

Immunolabeling for RALDH2 was performed to demonstrate its expression in the developing diaphragm, the pleuroperitoneal fold. As shown in Figure 4 there is intense labeling within the pleuroperitoneal fold at E13, the age when the structure is well defined. As reported pre-



**Figure 3.** Dose-dependent effect of enzyme inhibitors on RALDH activity. Relative inhibition on RA synthesis is plotted against concentration for each of the four compounds. Error bars indicate SD of three independent experiments.





**Figure 4.** Immunolabeling for RALDH2 in the primordial diaphragm, the pleuroperitoneal fold at E13. There is intense RALDH2 expression (intense black 3,3-diaminobenzidine tetrahydrochloride deposits) in bilateral pleuroperitoneal folds (\*), as shown at different magnifications. Lu, lung; SC, spinal cord. **Arrow**, phrenic nerve. Scale bars, 100  $\mu\text{m}$ .

viously,<sup>23</sup> there is also RALDH2 expression in the developing lung.

### Discussion

We have demonstrated that nitrofen, bisdiamine, SB-210661, and 4-biphenyl carboxylic acid all induce diaphragmatic defects similar to those observed in infants with CDH. Importantly, all of these compounds inhibit RALDH2, a key enzyme responsible for the generation of retinoic acid from retinal. These data, in conjunction with that from past studies, support the hypothesis that perturbations of the retinoid system could be involved in the etiology of CDH.

Each of the four compounds induced diaphragmatic defects in the posterolateral corners of the diaphragm, similar to what is observed in infants with CDH. The differences in the number of treatments required for each compound is likely because of the length of time that each remains in the body before degradation or elimination. Nitrofen persists at elevated levels for at least 3 days,<sup>24</sup> which would explain why a single large dose is all that is needed to induce diaphragmatic hernias. Conversely, biphenyl carboxylic acid may be much more labile (the parent compound, AH23848, has a plasma half-life of 1 to 1.5 hours), so that supplemental doses are necessary to produce sufficiently high concentrations to be effective. SB-210661 and biphenyl carboxylic acid may have a comparable half-life within embryonic tissues because the same dosages administered throughout the identical period of gestation produce similar fetal consequences.

In addition to inducing diaphragmatic defects, all four compounds can produce cardiac defects. These include maldevelopment of the outflow tracts of the heart and septal defects. The cardiac defects may result from problems with neural crest proliferation and differentiation in

the fetuses.<sup>14,15,17,25,26</sup> There is no strong evidence, however, that diaphragm embryogenesis has any dependence on neural crest development. It may be that these compounds also interfere with the proliferation or differentiation of mesenchymal cells. We are currently examining the hypothesis that a defect in the formation of the mesenchymal substratum of the primordial diaphragm, the pleuroperitoneal fold, is associated with teratogen-induced CDH.<sup>8</sup>

Recent studies using mice with a lacZ reporter gene linked to the activation of RARE demonstrated that nitrofen was inhibiting some aspect of retinoid function.<sup>13</sup> The nitrofen-induced suppression of RARE was reversed by the addition of retinoic acid supplementation. Further, binding studies suggested that nitrofen was acting upstream of the binding of retinoic acid to its receptors. The preceding step in the retinoid cascade is the NAD-dependent oxidation of all-*trans*-retinal to all-*trans*-retinoic acid by RALDH2.<sup>27</sup> One of the CDH-inducing teratogens, bisdiamine, is a known inhibitor of alcohol dehydrogenase. Thus, we hypothesized that the compounds were acting by interfering with that aspect of retinoid function. The results demonstrating that all four CDH-inducing compounds inhibit RALDH2 in a dose-dependent manner strongly supports the hypothesis. Further, the most potent inhibitor of RALDH2, bisdiamine, was also the most effective at inducing diaphragmatic defects in embryonic rats. The other CDH-inducing compounds had similar dose-response curves for inhibition of RALDH2 activity. The concentration of nitrofen present in the embryo in response to gavage feed of 100 mg has been estimated to be in the  $\mu\text{mol}$  range. Similarly, exposure of fetal mouse embryos maintained in culture to  $\sim 15 \mu\text{mol}$  of nitrofen produced a pronounced suppression or RARE-lacZ activation.<sup>13</sup> The data in Figure 3 demonstrates that the ED<sub>50</sub> for nitrofen suppression of RALDH2 activity is within a similar range.

Collectively, the data support the hypothesis that the primordial diaphragm tissue, the pleuroperitoneal fold, is dependent on retinoid-mediated signaling for its proper formation. Retinoic acid receptors function as transcriptional activators that modulate the expression of developmentally regulated genes by binding as a ligand/receptor complex to DNA sequences designated as retinoic acid response elements.<sup>28</sup> Consistent with this notion is the fact that the cervical mesenchymal tissues and developing diaphragmatic tissue strongly express proteins associated with the metabolism and binding of retinoids.<sup>28–30</sup> Further, retinoic acid has been shown to be involved in regulating extracellular matrix formation, mesenchymal cell migration, and establishment of dorsoventral polarity, all of which could be key components of early diaphragm embryogenesis.<sup>31,32</sup> However, a determination of the specific cells responsible for the embryological origins of the pleuroperitoneal fold will be necessary before ascertaining the role of retinoid signaling and its perturbation by CDH-inducing teratogens.

In ~60 to 65% of cases in infants with CDH there are no obvious associated anomalies other than the diaphragm defect.<sup>33,34</sup> The reason that the diaphragm might be particularly susceptible to perturbations of the retinoid system is unclear. Immunolabeling for RALDH2 and HPLC analyses demonstrate that there are gradients of retinoic acid levels within the cervical mesenchyme.<sup>31,32</sup> Further, the levels of retinoic acid and associated activation of RAREs necessary for regulating retinoid-mediated transcription varies among genes in a dose-dependent manner.<sup>31</sup> Thus, it is conceivable that the safety margin for retinoic acid-mediated regulation of primordial diaphragm development is relatively low and thus more susceptible to perturbations compared with other tissues. Heart development is also particularly susceptible, as cardiac anomalies are observed in the rat model of nitrofen-induced CDH<sup>5,6</sup> and are the most commonly associated defect in infants with CDH.<sup>33</sup> RALDH2 is expressed in the developing lung<sup>28</sup> and its suppression by nitrofen could explain some of the abnormalities of lung development observed in the nitrofen model of CDH.<sup>35–38</sup>

One could formulate several hypotheses regarding retinoid system malfunction and the etiology of CDH in humans. Transient deficiencies or imbalances in the levels of retinoid metabolites, binding proteins, nuclear receptors, or retinoid-metabolizing enzymes within the developing primordial diaphragm at the time of initial malformation of the pleuroperitoneal fold (~4 to 5 weeks of gestation) could account for diaphragmatic defects. These defects, in theory, could be because of acute dietary deficiencies, impaired placental transport, spontaneous malregulation within the retinoid metabolic cascade, teratogenic-mediated insults, or chromosomal defects. Evidence for the latter arises from several references to an association of CDH with chromosome 15q defects.<sup>39–43</sup> A search of available databases found that some of the genes on chromosome 15 in the region of the deletion or translocation (15q24–26) encode for cellular retinoic acid-binding protein-1 (CRABP-1).

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