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Introduction

The use of animals to address traditional endpoints in REACH legislation, such as repeated-dose toxicity, is tremendously high. Currently, there are no New Approach Methods (NAMs) that have achieved regulatory acceptance in REACH. This study was intended to develop an approach that combined traditional animal testing with a modern metabolomics component that could be used to support and reduce animal use to fulfil REACH information requirements.

Dicyclopentadiene (DCPD), CAS No 77–73-6, (3a,4,7,7a-tetrahydro-1H-4,7-methanoindene) is an olefinic hydrocarbon that is widely used in the production of various industrial products such as resins, adhesives, coatings, and elastomers and is registered in the EU according to the REACH legislation.

Methods

For this investigation, Wistar rats received DCPD daily for 14 days via gavage at dose levels of 0 (corn oil vehicle), 50 and 150 mg/kg bw (dose levels chosen based on a 7-day dose-range finding study).

Classical endpoints assessed based on OECD 407 test guideline:

Mortality, clinical signs of toxicity, body weight, food consumption, haematology, clinical chemistry, the weights of 14 organs, macroscopic-pathology, and histopathology for kidneys, liver, lungs, testes, and thyroid glands.

Metabolomics:

Blood samples were processed for metabolomic analysis using GC-MS and LC-MS/MS methods. The MetaMap®Tox¹ database was used to evaluate metabolome profiles of DCPD against the metabolome profile of >1000 substances present in the database as follows:

1. Analysis of specific metabolic changes for each dose group^{2,3}.
2. Similarity analysis of DCPD's metabolic profile with predefined patterns in MetaMap®Tox (> 110 patterns, currently covering 42 modes of action).

DCPD is a data rich substance with various toxicity studies readily available, including 90-day repeat dose toxicity studies (RDT). These data were compared to the results of this short-term “smart” study to assess NAMs suitability.

Results

Table 1: Summary of treatment-related, adverse findings
Treatment-related adverse findings based on observations made using classical toxicological parameters

	Dose (mg/kg bw/d)	Males	Females
Clinical toxicity	150	Body weight loss study day 3	Body weight loss study day 3
	50	No adverse finding	Body weight loss study day 3
Clinical pathology	150	Liver cell dysfunction CHOL increased 60.51 ± 2.35(co) vs 1.50 ± 0.24 * TPROT reduced, 1.76 ± 0.24 (co) vs 2.30 ± 0.16 ** GLUC (mmol/l): reduced 5.04 ± 0.75 (co) vs 3.81 ± 0.57 ** HTQ reduced 40.0 ± 2.5 (co) vs 34.9 ± 0.9 **	Liver cell dysfunction CHOL increased 1.06 ± 0.28 (co) vs 1.50 ± 0.24 * GLOB reduced 21.74 ± 1.59 (co) vs 19.61 ± 1.31 AST increased 1.75 ± 0.13 (co) vs 2.53 ± 0.96 ** ALP increased 1.37 ± 0.27 (co) vs 2.05 ± 0.28 ** INP increased 2.08 ± 0.20 (co) vs 2.48 ± 0.11 **
	50	No adverse finding	No adverse finding
Pathology	150	No adverse finding	Centrilobular liver cell hypertrophy Increased single cell necrosis (minimal) in the liver
	50	No adverse finding	No adverse finding

Chol: Cholesterol (mmol/l), HQT: clotting time (sec), GLUC (mmol/l): glucose, TPROT: total protein (g/l), GLOB: globulins, AST: aspartate amino transferase (µkat/l), ALP: alkaline phosphatase (µkat/l), INP: inorganic phosphate (mmol/l), co: control group * $p < 0.05$, ** $p < 0.01$

The highest dose induced transient clinical signs of toxicity (Table 1) and in males only reduced body weight gain. High-dose liver changes were characterized by increased liver weights, altered clinical chemistry parameters in both sexes and pathological changes in females (Table 1). In high-dose males, an accumulation of alpha-2u-globulin in the kidney was noted. Comparing the DCPD metabolome with previously established specific metabolite patterns in the MetaMap®Tox data base suggested that the high dose would result in liver enzyme induction leading to increased breakdown of thyroid hormones for males and females (Table 2 and Table 3). An indication for liver toxicity in males was also noted (Table 4). A match was observed for a pattern indicative for steroid hormone dysfunction and a weak match for effects caused by antiandrogens (Table 5) in high dose males. Previous studies observed increases in fatty droplets⁴ and cortical vacuolization⁵ in the fascicular zone of adrenals. Metabolomics also suggested an effect on the functionality of the adrenals in high dose males. This together with the published data is suggestive of a stress related effect in this organ, and accounts for the weak matches observed.

Results

Table 3: Metabolome pattern for indirect effects on the thyroid related to liver enzyme induction in high dose females

Values are fold changes relative to controls. Red indicates increase, yellow decrease with a statistical significance $p < 0.05$. HD: high dose, f14: females day 14.

Thyroid indirect through liver enzyme induction		Dicyclopentadiene HD			
Metabolite		f14	f14	f14	f14
Threonine	Amino acids	0.91	1.84	1.47	1.33
Palmitic acid (C16:0)	Complex lipids, fatty acids	1.32	1.27	1.30	1.40
Linoleic acid (C18:ci[9,12]2)	Complex lipids, fatty acids	1.47	1.34	1.37	1.73
Stearic acid (C18:0)	Complex lipids, fatty acids	1.35	1.34	1.02	1.40
Arachidonic acid (C20:ci[5,8,11,14]4)	Complex lipids, fatty acids	1.34	1.44	1.30	1.34
Docosahexaenoic acid (C22:ci[4,7,10,13,16,19]6)	Complex lipids, fatty acids	1.09	1.49	1.38	1.13
Cholesterol, total		1.29	1.45	1.41	1.73
Glycerol phosphate, lipid fraction	Complex lipids, fatty acids	1.27	1.44	1.29	1.39
Dodecanol	Complex lipids, fatty acids	1.30	1.13	1.11	1.28
Heptadecanoic acid (C17:0)	Complex lipids, fatty acids	1.36	1.07	1.18	1.23
Eicosanoic acid (C20:0)	Complex lipids, fatty acids	1.22	1.36	1.35	1.62
myo-Inositol-2-phosphate, fraction	lipid	1.62	1.71	1.63	1.25
4-Hydroxyphosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids	1.67	1.55	1.27	1.64
Behenic acid (C22:0)	Complex lipids, fatty acids	1.23	2.29	1.44	1.23
Phytosphingosine (d18:1)	Complex lipids, fatty acids	1.49	1.13	1.52	1.57
Nervonic acid (C24:ci[15]1)	Complex lipids, fatty acids	1.04	1.84	1.74	1.74
Unknown lipid (28000473)		1.45	1.34	1.35	1.68
gamma-Linolenic acid (C18:ci[6,9,12]3)	Complex lipids, fatty acids	1.34	1.34	1.42	1.34
3-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	1.78	2.10	1.63	1.78
threo-Sphingosine (d18:1)	Complex lipids, fatty acids	1.75	1.59	1.62	1.63
5-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	2.42	2.31	1.62	1.40
erythro-Sphingosine (d18:1)	Complex lipids, fatty acids	1.31	1.91	1.81	1.73
Cholesterol, total		1.27	1.34	1.33	1.63
Cholesterol, total		1.24	1.63	1.39	1.48
Threonine	Amino acids	0.86	1.81	1.40	1.44
Cysteine	Amino acids	0.97	1.09	0.63	0.79
Glycerol-3-phosphate, polar fraction	Complex lipids, fatty acids	1.09	1.08	1.83	1.13
Threonic acid	Complex lipids, fatty acids	1.73	1.87	1.26	1.56
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.27	1.57	1.69	1.28
Sphingomyelin (d18:2,C18:0)	Complex lipids, fatty acids	1.24	1.14	1.06	1.27
Choline plasmalogen (C36:2) (putative)	Unknown	1.29	1.46	1.43	1.34
DAG (C18:1,C18:2)	Complex lipids, fatty acids	1.05	1.07	1.13	1.35
Ceramide (d18:1,C24:0)	Complex lipids, fatty acids	1.49	1.24	1.33	1.36
Lysophosphatidylcholine (C17:0)	Complex lipids, fatty acids	1.13	1.11	1.11	1.11
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.17	1.15	1.23	1.13
Phosphatidylcholine (C18:0,C18:2)	Complex lipids, fatty acids	1.12	1.13	1.08	1.26
Phosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids	1.39	1.30	1.21	1.38
Sphingomyelin (d18:1,C24:0)	Complex lipids, fatty acids	1.37	1.33	1.37	1.62

Table 2: Metabolome pattern for indirect effects on the thyroid related to liver enzyme induction in high dose males

Values are fold changes relative to controls. Red indicates increase, yellow decrease with a statistical significance $p < 0.05$. HD: high dose, m14: males day 14.

Thyroid indirect through liver enzyme induction		Dicyclopentadiene HD			
Metabolite		m14	m14	m14	m14
Stearic acid (C18:0)	Complex lipids, fatty acids	1.33	1.65	1.25	1.19
Cholesterol, total		1.29	1.41	1.25	1.09
Glycerol phosphate, lipid fraction	Complex lipids, fatty acids	1.20	1.31	1.18	1.13
Galactose, lipid fraction	Complex lipids, fatty acids	1.62	1.45	1.07	1.15
Lignoceric acid (C24:0)	Complex lipids, fatty acids	1.39	1.29	1.35	1.18
myo-Inositol-2-phosphate, lipid fraction	Complex lipids, fatty acids	1.97	1.83	1.34	1.15
Behenic acid (C22:0)	Complex lipids, fatty acids	1.22	1.59	1.54	1.19
Nervonic acid (C24:ci[15]1)	Complex lipids, fatty acids	1.40	1.34	1.35	1.77
dihomo-gamma-Linolenic acid (C20:ci[8,11,14]3)	Complex lipids, fatty acids	1.52	1.51	1.77	1.02
3-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	1.63	1.99	1.69	1.62
threo-Sphingosine (d18:1)	Complex lipids, fatty acids	1.40	1.36	1.36	1.33
5-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	1.69	1.39	1.74	1.68
erythro-Sphingosine (d18:1)	Complex lipids, fatty acids	1.31	1.62	1.53	1.34
Cholesterol, total		1.48	1.74	1.23	1.51
Cholesterol, total		1.38	1.65	1.34	1.30
Glycine	Amino acids	1.10	1.69	1.23	1.18
Citrate	Energy metabolism and related	0.88	1.01	0.95	0.93
Mannose	Carbohydrates and related	0.92	1.18	1.17	1.03
Threonic acid	Complex lipids, fatty acids	1.31	1.69	1.69	1.69
Cytosine	Nucleobases and related	0.92	1.10	0.90	1.04
Lysophosphatidylcholine (C18:0)	Complex lipids, fatty acids	0.93	1.00	1.11	1.04
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.36	1.70	1.30	1.35
Choline plasmalogen (C36:2) (putative)	Unknown	1.46	1.60	1.30	1.28
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.49	1.10	1.15	1.11
Phosphatidylcholine (C18:0,C18:2)	Complex lipids, fatty acids	1.27	1.15	1.60	1.14
Phosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids	1.29	1.49	1.13	1.17

Table 4: Metabolome pattern for liver toxicity in high dose males

Values show fold changes relative to controls. Red indicates increase with a statistical significance $p < 0.05$. HD: high dose, m14: males day 14.

Liver Toxicity		Dicyclopentadiene HD			
Metabolite		m14	m14	m14	m14
Palmitic acid (C16:0)	Complex lipids, fatty acids	1.44	1.21	1.44	1.40
Linoleic acid (C18:ci[9,12]2)	Complex lipids, fatty acids	1.46	1.29	1.30	1.46
Stearic acid (C18:0)	Complex lipids, fatty acids	1.37	1.11	1.30	1.40
Arachidonic acid (C20:ci[5,8,11,14]6)	Complex lipids, fatty acids	1.43	1.34	1.34	1.44
Docosahexaenoic acid (C22:ci[4,7,10,13,16,19]6)	Complex lipids, fatty acids	1.48	1.34	1.34	1.44
alpha-Tocopherol	Vitamins, cofactors	1.40	1.34	1.46	1.43
Cholesterol, total		1.38	1.37	1.15	1.44
Glycerol phosphate, lipid fraction	Complex lipids, fatty acids	1.70	1.39	1.47	1.43
Galactose, lipid fraction	Complex lipids, fatty acids	1.62	1.29	1.70	1.46
Lignoceric acid (C24:0)	Complex lipids, fatty acids	1.38	1.10	1.23	1.71
Heptadecanoic acid (C17:0)	Complex lipids, fatty acids	1.37	1.39	1.45	1.37
Tricosanoic acid (C23:0)	Complex lipids, fatty acids	1.49	1.26	1.60	1.34
myo-Inositol-2-phosphate, lipid fraction	Complex lipids, fatty acids	1.57	1.19	1.54	1.43
Behenic acid (C22:0)	Complex lipids, fatty acids	1.40	1.49	1.60	1.34
4-Hydroxyphosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids	1.52	1.36	1.13	1.44
Nervonic acid (C24:ci[15]1)	Complex lipids, fatty acids	1.40	1.40	1.67	1.47
threo-Sphingosine (d18:1)	Complex lipids, fatty acids	1.52	1.21	1.30	1.74
5-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	1.43	1.47	1.11	1.71
erythro-Sphingosine (d18:1)	Complex lipids, fatty acids	1.40	1.49	1.44	1.43
Docosapentaenoic acid (C22:ci[7,10,13,16,19]5)	Complex lipids, fatty acids	1.40	1.31	1.34	1.14
5-O-Methylsphingosine (d18:1)	Complex lipids, fatty acids	1.46	1.49	1.49	1.47
Cholesterol, total		1.31	1.45	1.46	1.43
Cholesterol, total		1.48	1.46	1.40	1.43
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.46	1.13	1.40	1.17
Sphingomyelin (d18:2,C18:0)	Complex lipids, fatty acids	1.37	1.14	1.39	1.47
Phosphatidylcholine (C16:0,C22:6)	Complex lipids, fatty acids	1.13	1.13	1.17	1.46
Ceramide (d18:1,C24:1)	Complex lipids, fatty acids	1.13	1.14	1.19	1.11
Ceramide (d18:1,C24:0)	Complex lipids, fatty acids	1.17	1.46	1.46	1.38
Sphingomyelin (d18:1,C16:0)	Complex lipids, fatty acids	1.44	1.49	1.46	1.44
Phosphatidylcholine (C18:0,C18:2)	Complex lipids, fatty acids	1.27	1.11	1.12	1.45
Phosphatidylcholine (C18:0,C18:1)	Complex lipids, fatty acids	1.49	1.24	1.19	1.41
Sphingomyelin (d18:1,C24:0)	Complex lipids, fatty acids	1.29	1.14	1.11	1.74
Phosphatidylcholine (C18:0,C22:6)	Complex lipids, fatty acids	1.31	1.16	1.16	1.31

Table 5: Metabolome pattern for steroid hormone dysregulation in high and low dose males

Values show fold changes relative to controls. Red indicates increase, yellow decrease with a statistical significance $p < 0.05$. HD: high dose, m14: males day 14.

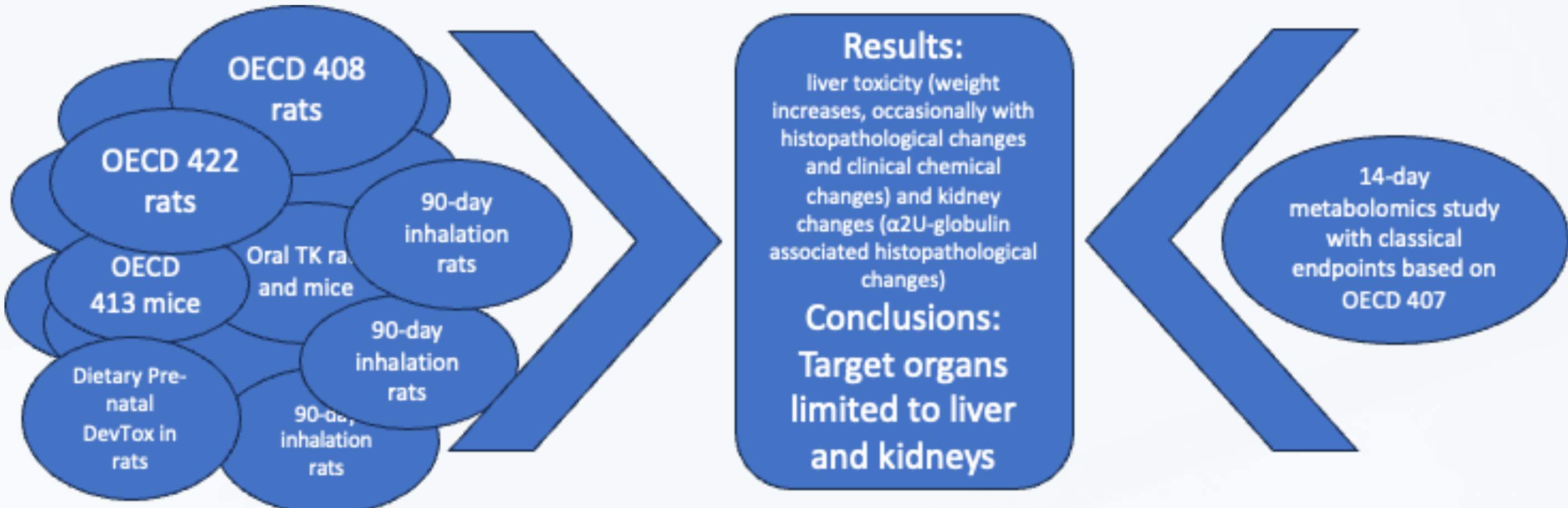
Steroid hormone dysregulation		Dicyclopentadiene HD & LD		Fenarimol	Flutamide	Vinclozolin
Metabolite		m14 HD	m14 - LD	m14	m14	m14
Androstenedione	Steroid Hormones	6.44	1.74	1.67	55.72	4.15
Testosterone	Steroid Hormones	2.75	1.32	1.80	19.61	2.83
Progesterone	Steroid Hormones	0.27	0.68	0.30	0.40	0.22
21-Hydroxyprogesterone (Deoxycorticosterone)	Steroid Hormones	0.53	1.27	0.31	0.43	0.12
18-Hydroxy-11-deoxycorticosterone	Steroid Hormones	0.48	0.96	0.17	0.68	0.17
Corticosterone	Steroid Hormones	0.45	1.10	0.24	0.46	0.16

Discussion & Conclusion

Short-term “smart” studies combining classical toxicology endpoints with modern “omics” technologies have produced data for DCPD:

- that is consistent with DCPD database; all known existing organ toxicity^{7,8} was identified using this approach. Importantly, no other types of organ toxicity, or hormone dysregulation was identified using metabolomics.
- that can provide a comprehensive identification of the toxicity of the test material
- that accurately represents the data obtained from classical toxicology studies on systemic, repeat dose organ toxicity (STOT) (Figure 1)
- that can be used in an integrated assessment of classical toxicology
- that can be used to reduce the number of animals required for REACH
- that can be used refine and reduce *in vivo* toxicity testing required to fulfil the information requirements of REACH

Figure 1: The short-term “smart” studies can be used to accurately represent repeat dose toxicity findings (STOT) and could contribute to a reduction in animal numbers required for REACH.



The physico-chemical properties of DCPD would predict the potential for accumulation in neural tissue and are important determinants for its specific effects on membrane geometry⁹. Evidence of this was observed in a 90-day repeat dose toxicity study in rats (OECD 408) where tremors and convulsions were observed during the last weeks of dosing¹⁰. The MetaMap®Tox database contains various patterns for neurotoxic endpoints, but these were not triggered. Therefore, a direct receptor or enzyme mediated effect of DCPD on neurons is less likely. The very late occurrence of these clinical signs of neurotoxicity suggest that they may be associated with a slow accumulation of DCPD in neural tissue. To predict such effects in a short-term “Smart” studies, attention should be given to potential accumulation of compounds.

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