UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, DC 20460

OFFICE OF PREVENTION, PESTICIDES AND TOXIC SUBSTANCES

April 22, 2003
TXR 0051647

MEMORANDUM

SUBJECT: D293798 Fosthiazate (PC Code 129022)
Revised Developmental Neurotoxicity Study Protocol and
Comparative Cholinesterase Study Protocol

TO: Meredith Laws (PM 04) and Rita Kumar
Registration Division (7505C)
and
Anna Lowit
Reregistration Branch 2
Health Effects Division (7509C)

FROM: Susan L. Makris 4/22/03
Toxicology Branch
Health Effects Division (7509C)

THRU: Alberto Protzel, Branch Senior Scientist 4/25/03
Toxicology Branch
Health Effects Division (7509C)

Executive summary

The purpose of this memorandum is to review the revised draft protocols for a dietary developmental neurotoxicity study and a comparative cholinesterase study with fosthiazate (PC Code 129022), which were submitted to the Agency by ISK Biosciences Corporation, 7470 Auburn Road, Suite A, Concord, Ohio 44077:

1. A Developmental Neurotoxicity Study in Rats with Fosthiazate Technical by Oral Gavage Administration
2. A Sensitivity Comparison Study Among Dam, Fetus/Pup and Young-Adult Rats with Fosthiazate Technical

These protocols are considered partially adequate for the assessment of developmental neurotoxicity and comparative cholinesterase activity data as specified in the EPA Data-Call-In (DCI) for adult and developmental neurotoxicity (DNT) studies on the organophosphate (OP) pesticides (issued September 10, 1999). For the draft developmental neurotoxicity protocol, the major deficiencies noted were that 1) adequacy of exposure of the offspring to fosthiazate was not demonstrated, 2) the motor activity procedures require refinement, specifically in regard to

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number and duration of testing subsessions, and 3) cholinesterase activity measurements during the course of the DNT study (e.g., in culled pups at PND 4 and in sacrificed dams and pups at PND 21) are not included in the protocol. For the draft comparative cholinesterase study protocol, the major deficiencies noted were 1) the time-to-peak cholinesterase effect in immature animals was not addressed and 2) for the repeated-dose cholinesterase assessments, the proposed duration of treatment (i.e., 7 days) would result in an inability to compare cholinesterase measures in the immature rats following acute or repeated doses, since the pups would be of different ages (PND 21 versus PND 17, respectively) at the time of assessment.

Introduction

Fosthiazate Technical is an organophosphate pesticide currently under consideration as a methyl bromide alternative. In the spirit of compliance with the EPA Data-Call-In (DCI) for adult and developmental neurotoxicity (DNT) studies on the organophosphate (OP) pesticides (issued September 10, 1999), the registrant, ISK Biosciences Corporation, previously submitted a draft developmental neurotoxicity study protocol for fosthiazate, dated February 14, 2001. This protocol was reviewed by the Agency, and comments were provided in a memo from S. Makris to M. Laws/R. Kumar, dated May 30, 2001. In response to the comments received, and in an attempt to address issues raised in additional instructions provided by the Agency in a document entitled *Guidance on Cholinesterase Measures in DNT and Related Studies (10/29/01)*, the registrant has submitted two protocols, dated February 5, 2002, for a revised draft developmental neurotoxicity study and for a comparative cholinesterase study.

The submitted protocols do not specify the laboratory that will perform the studies. The study sponsor is listed as Ishihara Sangyo Kaisha, Ltd., 3-15, 1-chome, Edobori, Nishi-ku, Osaka 550-0002, Japan.

The following discussion presents the Agency response to the contents of the draft protocols for the proposed studies.

DEVELOPMENTAL NEUROTOXICITY STUDY

Adequacy of the study design to address the additional issues/concerns specified in the DCI

1. Increase dosing period to: Gestation Day 6 through Postnatal Day 21

The study design specifies that the test substance will be administered by gavage to dams from gestation day 6 through lactation day 21. This is considered to be an acceptable dosing duration that meets the specification of the DCI.

2. Determine adequacy of postnatal dosing for substances not present to any significant extent in the milk of dams (including assessment of the need for direct exposure of pups to test substance)

The specifications of the DCI are not adequately addressed by the submitted protocol. In the proposed study design, fosthiazate will be administered by gavage to the maternal animals only. Thus, the dams will be exposed during gestation and lactation, with exposure to the offspring assumed to occur *in utero* and postnatally via the milk. No evidence was provided to confirm this assumption, e.g., no measurement is made to determine the presence of test substance and/or

metabolite in the milk (i.e., to determine pup exposure during lactation). In the absence of information documenting pup exposure levels, it will not be possible to evaluate the adequacy of postnatal dosing in this study.

It is recommended that compound and important metabolites be measured in the blood of dams and pups at the end of gestation, and in the milk during early, mid, and late lactation. If test substance is not present in the milk, or if levels in the milk are minimal during some periods of lactation, direct exposure to the pup during lactation (e.g., gavage administration to the pups) may be needed in order to ensure adequacy of dosing.

3. Measure brain, RBC, and plasma cholinesterase in dams and pups: characterize comparative levels of inhibition and both time of peak effect and recovery

This aspect of the DCI requirements is addressed in the review of the draft comparative cholinesterase study protocol, below. It is noted that the measurement of cholinesterase activity during the course of the DNT study is recommended, as a tool in assessing the adequacy of postnatal dosing. Animals should be available for these cholinesterase assessments at PND 4 (culled pups) and at PND 21 (dams and extra weanlings). The draft DNT study protocol does not include cholinesterase measurements at these ages.

4. Increase number of offspring examined for neuropathology to10/sex/dose

The draft protocol states that 10 offspring/sex/dose level will be killed and their brains preserved for neuropathology on postnatal days 11 and 65±2. This meets the specifications of the DCI.

Additional comments and recommendations

Developmental landmarks

It is recommended that individual body weight measurements be recorded on the day of vaginal opening or preputial separation for each F1 weanling. These data can be very useful in the interpretation of any observed alterations in the age of sexual maturation.

Assignment of pups to testing

The assignment of pups to observational groups is described in the draft protocol (p 17-18). The procedures as described appear to be adequate.

Behavioral evaluations

The study protocol indicates that detailed clinical observations (i.e., a functional observation battery) will be conducted outside the home cage on dams twice during gestation and twice during lactation, and on offspring (10/sex/dose) at six specified postnatal time points (PND 4, 11, 21, 35, 45, and 60). Offspring that are assigned to the FOB testing will also be evaluated for motor activity levels (all time points); this is considered acceptable. The FOB assessments will include measures of autonomic function, convulsions, tremors or abnormal movements, posture and gait abnormalities, and unusual/abnormal behaviors. The study report should clearly specify the manner in which the detailed clinical observations are altered to render them age-appropriate

for preweaning rats.

Motor activity measurements will be conducted in 10 offspring/sex/dose on PND 13, 17, 21, and 60±2. The protocol states that "measurements will be conducted every minute during a 6-minute session" (p 18). The developmental neurotoxicity study guideline (OPPTS 870.6300) specifies that "the test session should be long enough for motor activity to approach asymptotic levels by the last 20 percent of the session for nontreated control animals." It is unlikely that asymptotic levels will be achieved in a 6-minute session. Measurements of activity counts in test sessions with durations of 40-90 minutes have been typically used in developmental neurotoxicity testing [Raffaele, KC, WF Sette, SL Makris, VC Moser, and KM Crofton. (2003) Motor activity in developmental neurotoxicity testing: a cross-laboratory comparison of control data. *The Toxicologist* 72(S-1):123]. The duration of motor activity testing sessions should be revised in the DNT protocol for fosthiazate.

Auditory startle response will be evaluated in 1 male or 1 female pup per litter (10 offspring/sex/dose) on PNDs 21±2 and 60±2. It is stated that peak amplitude will be measured and that average response magnitude and the pattern of responses over 10 trial blocks (habituation) will be compared among dose groups. It is recommended that latency of response also be measured; habituation should be demonstrated.

The protocol specifies that learning will be assessed utilizing a water-filled swimming maze on PND 21±2 and 60±2. Memory will be evaluated in 1 male or 1 female pup per litter (10 offspring/sex/dose) at 1 and 5 days after each initial test. Apparently, different animals will be used for testing of weanlings and adults; this methods of assignment to testing is acceptable.

Sample collection and processing

This protocol specifies immersion fixation of brain tissue at PND 11 and *in situ* perfusion fixation at PND 65±2, which is acceptable. The protocol does not specify whether brain weights will be recorded before or after fixation (p 21). Particularly for PND 11 pups, some laboratories have found that fixing the brains prior to removal from the cranial vault has been helpful in minimizing tissue damage.

It is noted that peripheral tissues will be frozen and retained for possible future analysis.

Histopathological evaluation

The draft protocol indicates that tissue sections will be prepared "from all animals terminated" (p 24). This is acceptable.

The draft protocol includes measurements of the thickness of the neocortex, corpus callosum, hippocampus, and folia of cerebellum (pyramis) for offspring on PND 11 and at termination. Behavioral findings or qualitative histopathological findings may guide the selection of additional brain regions for morphometric assessment.

Positive control data

The protocol states that appropriate, adequate positive control data from the laboratories that

performed the DNT studies will be provided to the Agency at the time of study submission.

Dose levels

The protocol indicates (p 12) that the dose levels to be used in the DNT study are: 0, 0.2, 1, and 5 mg/kg/day. The test substance will be administered by gavage at a dose volume of 1ml/100g body weight. The draft protocol states that these dose levels were based upon the results of the 28-day and 90-day studies in rats, as well as the prenatal developmental and two-generation reproduction studies in rats. These studies have been previous submitted to the Agency; summaries of the study results are presented below [memo from Christina Jarvis/Diana Locke (HED) to Rita Kumar (RD), November 15, 2002]. Although the dose selection rationale for the DNT study appears to be supported by these data, no information is available that would predict whether age-related sensitivity to fosthiazate will define a lower NOAEL/LOAEL for immature rats. Since the DNT study and the comparative cholinesterase studies should be conducted at the same dose levels, final dose selection for the DNT may need to be adjusted, based upon range-finding data or the results of the comparative cholinesterase studies.

Guideline No./ Study Type	MRID No. (year)/ Classification /Doses	Results
870.3100 13-Week Feeding Study-Rat	41347632 (1989) Acceptable/Guideline 10 rats/sex/dose at 0, 1.07, 10.7, 53.6 and 429 ppm (0.08, 0.77, 4.12 and 36.37 mg/kg/day for males; 0, 0.09, 0.89, 4.74, and 41.03 mg/kg/day for females).	**Systemic Toxicity LOAEL:** 0.08 and 0.09 mg/kg/day for males and females, respectively, based on microscopic lesions in the adrenals (males) and increased ALT (females) levels. **No NOAEL** was established. At higher doses, the severity of vacuolation of cells in zona fasciculata (≥1.07 ppm) and zona glomerulosa (≥53.6 ppm) of the adrenals increased in a dose-dependent manner; at ≥53.6 ppm, the brain ChEI was also noted. In addition, there was increase in adrenal gland weight at 429 ppm **LOAELfor ChEI:** 10.7 ppm (0.77 and 0.89 mg/kg/day for males and females, respectively) based on plasma and RBC ChEI. **NOAEL:** 1.07 ppm (0.08 and 0.09 mg/kg/day for males and females, respectively).
4-Week Range-Finding Feeding Study-Rat	44269905 (1989) Acceptable/Non-guideline 10/sex/dose at 0, 0.5, 1, 5, 10, 100 and 400 ppm (equivalent to 0, 0.05, 0.10, 0.48, 0.97, 9.69 and 40.87 mg/kg/day in males and 0, 0.05, 0.10, 0.50, 1.00, 10.67 and 43.52 mg/kg/day in females).	**Systemic LOAEL:** 400 ppm (equivalent to 40.87 mg/kg/day in males and 43.52 mg/kg/day in females) based on fur loss, muscle tremor, enlarged pale spongiocytes in the adrenals, increased adrenal weights, and increased alkaline phosphatase and alanine aminotransferase levels. **Systemic Systemic NOAEL**: 100 ppm (equivalent to 9.69 mg/kg/day in males and 10.67 mg/kg/day in females). **LOAEL for ChEI:** 5 ppm (equivalent to 0.48 mg/kg/day in males and 0.5 mg/kg/day in females) based on decreased plasma butyryl- and acetyl-cholinesterase, and brain acetyl-cholinesterase in females, and erythrocyte acetyl-cholinesterase in males. **NOAEL:** 1 ppm (equivalent to 0.10 mg/kg/day in males and females.
870.3700 Developmental Toxicity-Rat	43534505 (1990) Acceptable/Guideline 24 dams/dose at 0, 3, 5, 10 mg/kg/day from GD 6-15	**Maternal Toxicity LOAEL:** 10 mg/kg/day, based on reduced body weight gain. **NOAEL:** 5 mg/kg/day **Developmental Toxicity LOAEL:** Not determined. **NOAEL:** 10 mg/kg/day Although data were not provided on clinical signs in the dams during or after dosing, no cholinergic signs were seen in neurotoxicity studies at the same dose. Therefore, the study classification is upgraded to acceptable/ guideline.

Guideline No./ Study Type	MRID No. (year)/ Classification /Doses	Results
870.3800 2-Generation reproduction--Rat	41381113; 44414501 (1989) Acceptable/Guideline 25 rats/sex/dose at 0, 3, 10, 30, or 100 ppm [approx. 0.21, 0.69, 2.09, or 7.21 mg/kg/day for males, and 0, 0.25, 0.91, 2.62, or 9.32 mg/kg/day for females, respectively]	**Parental Toxicity LOAEL:** 100 ppm (equivalent to 9.32 and 7.21 mg/kg/day in females, and males, respectively) based on increased incidences of adrenal zona glomerulosa hypertrophy, centriacinar hepatocytic vacuolation and liver inflammation in F_0 females and periacinar hepatocytic hypertrophy in F_0 males. **NOAEL:** 30 ppm (equivalent to 2.6 and 2.09 mg/kg/day) in females and males, respectively). in F_0 females and in males. **Reproductive Toxicity LOAEL:** >100 ppm. **NOAEL:** 100 ppm. **Offspring Toxicity LOAEL:** 30 ppm based on decreased litter size and decreased pup weight and viability index during lactation. **NOAEL:** 10 ppm

Conclusion (developmental neurotoxicity study protocol)

The protocol submitted by the registrant is considered partially adequate for the assessment of developmental neurotoxicity as specified in the EPA Data-Call-In (DCI) for adult and developmental neurotoxicity (DNT) studies on the organophosphate (OP) pesticides (issued September 10, 1999). The major deficiencies noted were that 1) adequacy of exposure of the offspring to fosthiazate was not demonstrated, 2) the motor activity procedures require refinement, specifically in regard to the number and duration of testing subsessions, and 3) cholinesterase activity measurements during the course of the DNT study (e.g., in culled pups at PND 4 and in sacrificed dams and pups at PND 21) are not included in the protocol.

COMPARATIVE CHOLINESTERASE STUDY

In accordance with the *Guidance on Cholinesterase Measures in DNT and Related Studies (10/29/01)*, the EPA position regarding the optimal schedule for measurement of cholinesterase activity is summarized in the following table:

Summary of EPA Guidance on Required Cholinesterase Measures	
Study	Populations
Main DNT study	1. PND 4 (pups) 2. PND 21 (pups and dams)
Maternal GD 6-20 study	1. GD 20 dams 2. GD 20 fetuses
Sensitivity study	Acute doses: 1. Pre-weaning pups (both sexes); a) Early-Mid lactation [no later than PND11]; b) Late lactation [7-10 days after first time point, no later than PND 21]; 2. Young adults (both sexes).

	Repeated doses: 1. Pre-weaning pups -- exposure beginning during early lactation, with a duration of 7-10 days (starting no later than PND 11, e.g., PND 11-21), with ChE evaluations after dosing on last day of exposure; 2. Young adults (both sexes) -- repeated dose exposure using duration and doses as for pre-weaning.

In addition, as described in the guidance, 1) the time of peak effect should be determined for each age group and should be based upon cholinesterase inhibition and 2) it is important that doses be selected in a manner that allows characterization of the dose effect curves for all 3 compartments (i.e., plasma, erythrocyte, and brain).

The following discussion presents the Agency response to the draft comparative cholinesterase study protocol for fosthiazate.

Proposed study design

The draft comparative cholinesterase protocol includes three segments:

1. Comparison between pregnant females and fetus
2. Comparisons between pups on PND 11, pups on PND 21, and young adults
3. Comparison between pups exposed from PND 11 to PND 17 and young adult rats dosed 7 consecutive days

Each comparison segment will include a vehicle control group and dose groups of 10 animals per sex at each age. Dose levels will be the same as the main DNT study. Whole brain, plasma, and erythrocyte samples will be collected for cholinesterase analysis.

Cholinesterase measures following acute exposure to adult and immature rats

In the study segment listed as #2 above, a single gavage dose of fosthiazate will be administered to PND 11 pups, PND 21 pups, and 8-week old adult rats. The animals will be terminated three hours after the dose is administered, blood and brain samples will be collected, and cholinesterase activity will be measured.

The time-to-peak effect in immature rats is not provided. The draft protocol cites a previous study (Document No. 5994-94-0096-TX-002) which determined that the peak effect for cholinesterase inhibition in rats occurred at three hours post-dose. The age of the animals is not indicated; however, it is most likely that this study was performed in adult animals. The time of peak response in immature rats could be quite different from adults and must be evaluated.

Cholinesterase measures following repeated dose exposures to adult and immature rats

GD 20 dams and fetuses - In the study segment listed as #1 above, fosthiazate will be administered daily by gavage to pregnant rats from gestation day 6 through gestation day 20. Three hours after the last administered dose, the dams will be terminated, blood and brain samples will be collected from dams and fetuses, and cholinesterase will be measured. Samples for fetuses will be pooled for each sex per litter.

Immature rats versus young adults - In the study segment listed as #3 above, fosthiazate will be administered to pups from PND 11 to PND 17 and to young adult rats for 7 consecutive days. Three hours after the last administration, the pups and young adult rats will be terminated, and brain and blood samples will be collected for cholinesterase analysis, separately by sex. Again, the time to peak effect for immature rats is not provided.

While the Agency guidance on comparative cholinesterase assessments indicates that 7 days is an adequate dosing period for the assessment of differences in cholinesterase activity following repeated doses, it is noted that extending the dosing duration to PND 21 for pups and extending the adult dosing duration to 11 days would provide a comparison of response to both acute and repeated doses for weanlings of the same age. Conversely, the response of PND 17 pups that had been treated for 7 days could not be compared to the response of PND 21 pups that received only a single dose, since the metabolic capacity of the rats is maturing rapidly during that time frame. An alternative approach would be to evaluate acute doses to PND 17 pups rather than PND 21 pups.

Cholinesterase measures in the main DNT study

Agency comments on the draft protocol for the main DNT study are presented earlier in this memorandum. The registrant is reminded that the current Agency guidance (10/29/01) recommends the measurement of cholinesterase activity during the course of the DNT study, as a tool in assessing the adequacy of postnatal dosing. Animals should be available for these cholinesterase assessments at PND 4 (culled pups) and at PND 21 (dams and extra weanlings).

Additional comments and recommendations

Animal source: The protocol indicates that the source of the rats will be "Charles River Breeding Laboratory Inc or other acceptable source" (p 12). The animals used in the comparative sensitivity study should be from the same source as those used in the DNT study.

Conclusion (comparative cholinesterase protocol)

The protocol submitted by the registrant to assess cholinesterase activity in adult and immature rats following acute or repeated exposures is considered partially adequate for the assessment of comparative cholinesterase activity data as specified in the EPA Data-Call-In (DCI) for adult and developmental neurotoxicity (DNT) studies on the organophosphate (OP) pesticides (issued September 10, 1999). The major deficiencies noted were 1) the time-to-peak cholinesterase effect in immature animals was not addressed and 2) for the repeated-dose cholinesterase assessments, the proposed duration of treatment (i.e., 7 days) would result in an inability to compare cholinesterase measures in the immature rats following acute or repeated doses, since the pups would be of different ages (PND 21 versus PND 17, respectively) at the time of assessment.

ELECTRONIC SUBMISSION OF REPORTS AND DATA

As part of a voluntary pilot program intended to develop greater efficiency in study submission and review processes, Registrants are invited to submit developmental neurotoxicity study reports to the Agency in electronic format. **Attachment 1** contains information on electronic submission of reports and data.

Attachment 1

Electronic submission of reports and data

The Office of Pesticide Programs (OPP) has been working to establish a process for accepting and reviewing electronic submission of study reports and related data for pesticide registration. The goals of this effort are to facilitate the assembly and submission of studies to the Agency by registrants, provide tools to OPP reviewers that make their work more efficient and effective, improve information archiving to ensure future accessibility of submissions, and enhance communications between OPP and registrants. Through discussions with other US and multinational regulatory agencies, and in consultation with the American Crop Protection Association, significant progress has been made in developing guidance for implementing electronic data submission.

Electronic submission and review of study reports is in pilot testing stages in OPP, and industry participation in these pilot efforts is optional and voluntary. Due to the overall number and complexity of developmental neurotoxicity studies that are expected to be submitted to the Agency in the near future under the directive of the DCI, the submission of these reports in electronic format is considered a unique opportunity to test the efficiency of the system and to gain valuable experience with this medium. We invite you to submit your study report in electronic format.

Adobe Acrobat Portable Document Format (PDF) has been selected as the software tool for formatting electronic submissions of study report text and tables. Supplemental files of individual animal data could also be included electronically, for example in a spreadsheet or some other negotiated format. It may not be necessary to submit all study data in supplemental file format; Agency scientists welcome the opportunity to discuss the need for supplemental files of specific study data on a case-by-case basis.

Contact Kate Bouvé (703-305-5032) or Teresa Downs (703-305-5363) in the Information Resources and Services Division of OPP for detailed information regarding document specifications, suggested formats for bookmarking and hyperlinks in PDF, and submission procedures, including data integrity certification. Contact Susan Makris (703-305-5222) or William Sette (703-305-6375) regarding the submission of supplemental files of textual or numerical animal data.