AD_____

Award Number: DAMD17-02-1-0241

TITLE: Prostate Cancer Risk Through Exposure to Halogenated Hydrocarbons and Modulation by Dietary Supplementation

PRINCIPAL INVESTIGATOR: Gabriele Ludewig, Ph.D.
Larry W. Robertson, Ph.D.

CONTRACTING ORGANIZATION: University of Kentucky Research Foundation
Lexington, Kentucky 40506-0057

REPORT DATE: April 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2003	Annual (1 Apr 02 - 31 Mar 03)

4. TITLE AND SUBTITLE
Prostate Cancer Risk Through Exposure to Halogenated Hydrocarbons and Modulation by Dietary Supplementation

5. FUNDING NUMBERS
DAMD17-02-1-0241

6. AUTHOR(S):
Gabriele Ludewig, Ph.D.
Larry W. Robertson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Kentucky Research Foundation
Lexington, Kentucky 40506-0057

E-Mail: glude01@uky.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

A high androgen level, oxidative stress, and low levels of selenium were identified as possible risk factors in prostate cancer development. Halogenated compounds may produce all these effects. Our **hypothesis** is that *halogenated compounds may increase prostate cancer risk through these mechanisms.* To test this hypothesis and to develop protection for exposed men, we propose to a) measure enzyme activities and antioxidant levels in PCB-exposed rats, b) determine possible co-carcinogenic factors like low selenium or high fat diets, c) study the mechanisms involved with prostate cancer cells in culture, and d) develop chemopreventive strategies. During this first year of our studies we found that 1) in *in vivo* studies substituting bromine for chlorine makes biphenyls more health damaging, indicating that brominated pesticides may deserve attention, 2) complex changes in lipid homeostasis, vitamin levels and redox status occur that need to be carefully taken into consideration, 3) cell culture experiments with prostate cells should use more than one pair of androgen sensitive/androgen insensitive cells, to avoid misleading conclusions, 4) α-tocopheryl quinone may be the most promising biomarker of oxidative stress by halogenated hydrocarbons. Overall these results provide a firm scientific basis for the work that is proposed and will follow.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
halogenated compounds, antioxidants, selenium, vitamin E	24
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Recently several factors were identified that seem to be involved in the development of prostate cancer: a high androgen level, oxidative stress, and low levels of selenium. Every compound that changes these three factors, should influence prostate cancer risk. Many scientists believe that environmental compounds may be involved. Epidemiological data identified certain occupations, farm workers, electric power workers, and Agent Orange-exposed Vietnam war veterans, at increased risk. These individuals are often exposed to high levels of halogenated hydrocarbons, like DDT, Aldrin, PCBs, and dioxins. During the last years it was shown that PCBs reduce aromatase activity. In the prostate this could result in an increase in androgen levels. Studies of PCBs liver carcinogenesis and our own work with PCBs have shown that PCBs produce oxidative stress. This may very well also happen in the prostate. Most surprisingly, however, was our finding that PCBs reduce the level of selenium and the protective enzyme Se-GSH-Px in the livers of exposed rats. If this also happens in the prostate, then men could have a hidden selenium deficiency, even though they may consume normal amounts of selenium in their diet. Thus all three risk factors for prostate cancer, androgens, oxidative stress, and selenium, may very well be influenced by PCBs, but nobody has tested this hypothesis yet. Based on these findings, our **hypothesis** is that *environmental and food contaminants, like halogenated hydrocarbons, may increase the risk of prostate cancer by regulating genes of metabolizing enzymes, resulting in increased androgens and reactive oxygen species in the prostate, and by reducing the antioxidants selenium, Se-GSH-Px, and vitamin E by yet unknown mechanisms*. To better understand the involvement of these mechanisms and to gain possible ways of protection for exposed men, we wish to (1) Measure enzyme activities and antioxidant levels in the prostate of PCB-exposed rats, (2) Identify possible co-carcinogenic factors like low selenium or high fat diets, (3) Study the mechanisms and factors involved with prostate cancer cells in culture, and (4) Develop chemopreventive strategies by feeding modified diets to PCB-exposed rats.

BODY OF THE REPORT

This is the first annual report. Because of a pending move from the University of Kentucky to the University of Iowa, experimental work was temporarily discontinued mid December 2002. During this thereby shortened time period we performed one exploratory in vivo animal experiment with three commercial mixtures of halogenated hydrocarbons, measured antioxidant levels in a second in vivo time-course experiment with the pure polychlorinated biphenyl (PCB) congener PCB77 (3,3',4,4'-tetrachlorobiphenyl), and performed cell culture experiments with prostate cell lines.

1. IN VIVO EXPERIMENT WITH 3 COMMERCIAL HALOGENATED HYDROCARBON MIXTURES

The first animal experiment was designed to establish methods, to find out whether additional parameters (for example lipid profile in serum) should be included into the studies, and to gain insight into the comparative toxicity profiles of three commercial mixtures of halogenated organic hydrocarbons. This approach was chosen, since we had proposed to analyze, among others, the effect of high fat diets. It seemed therefore important to have a better understanding of the effects of halogenated compounds on normal lipid metabolism before

performing a large (120 rats) and long term feeding study. Also, our hypothesis was that several different halogenated compounds, of which PCBs are only one member, may have negative impact on the prostate through the same mechanisms. The exponential increase in the levels of polybrominated diphenyl ethers (PBDEs) in food and humans during the recent years prompted us to include this group of halogenated compound into our exploratory studies. The very recent epidemiological finding that a certain brominated pesticide is positively correlated with prostate cancer (Dr. C. Lynch, U. of Iowa, personal communication) further justifies the inclusion of brominated compounds into our experiments. Thus the goal of this experiment was 1. to identify possibly important physiological parameters that should be tested so that we would gain a maximum of possibly important information from every future, large scale experiment, 2. to examine the differences in effect of bromination vs chlorination and biphenyl vs diphenylether structure, 3. to use minimize the number of animals needed while maximizing the chances of results. This exploratory study therefore required only 24 rats.

Study design: Male Sprague Dawley rats (5 weeks old, about 150 g, 6 animals per group) received weekly injections of equimolar amounts (0.765 mmol/kg rat/injection) of the PCB mixture Aroclor 1254, the PBB mixture Firemaster BP-6, the commercial PBDE mixture DE-71, or vehicle alone (corn oil, 1 ml/100 g rat). Animals were weighted at the beginning of the experiment and after each week. One week after the second injection animals were sacrificed and liver, thymus, spleen, kidneys, prostate, and testes removed and weighted and serum collected and analyzed. Organs were either frozen and stored at -80° C for later analysis of enzyme activities, cofactor levels, and antioxidants or/and placed in formalin for later histologic analysis. This exposure regimen was chosen in order to compare these data with those from an experiment with female rats, thus also allowing for gender comparisons, where applicable. The first statistical analysis was done by students t-test. Significant findings are indicated in Tables in **bold (p>0.05)** and **bold underlined (p>0.005)**. Results from a study with female rats (same treatment schedule) are mentioned where gender differences were observed. Individual data will be made available, if desired.

1. Body weight gain

Table 1 shows that the treatments had no strong general toxicity over the two week time course. None of the treatments resulted in a statistically significant weight loss or gain. Some reduction in weight gain was seen in PBB-treated rats in the second week, but this was below the significantly level. However, a similar reduction in weight gain was observed in female PBB-treated rats. This could indicate that Firemaster BP-6 at this concentration may cause weight gain reduction.

Table 1: Body weight gain in male SD rats

Treatment Group	beginning weight in gram ± SD	1st week average weight gain		2nd week average weight gain		total average weight gain	
		gram	*%*	gram	*%*	gram	*% ± SD*
Control	150 ± 12.44	61.3	*29*	51.8	*24*	113	*75.7 ± 6.47*
PCB	149 ± 6.96	61.7	*29*	52.3	*25*	114	*76.7 ± 7.56*
PBDE	139 ± 3.27	58.0	*29*	50.8	*26*	109	*78.0 ± 5.49*
PBB	144 ± 11.24	58.7	*29*	43.0	*21*	102	*71.0 ± 9.83*

2. Organ weights

The weight of the liver, 2 kidneys, thymus, spleen, prostate, and testes (with vas deferense and epididymis) are given in Table 2 as total weight in g or mg and as % of body weight (bw). A significant effect was seen in the liver weights, which increased in all treatment groups compared to control ($p > 0.005$). The effect was strongest with PBBs, followed by PCBs and PBDEs. PBB exposure also significantly reduced the total weight of the kidneys ($p>0.05$), but not if given as % of bw. The thymus weight was strongly reduced by PBB-treatment ($p>0.0005$), whereas the other two mixtures had no effect. The spleen was smaller in all three treatments groups compared to control, but again the effect was strongest with PBBs. Prostate and testes weights were not influenced by the compounds. Female rats had been more resistant to the treatments and had only shown a significant increase in liver weights (all mixtures) and uterus weights after PCB exposure. Interestingly the thymus weight had also been elevated in female PBB rats, although not to the level of significance.

Based non organ weight data alone the acute toxicity of the three groups of halogenated hydrocarbons towards male reproductive organs (prostate and testes) is small or not existing, even at the relatively high concentrations chosen. The immune system on the other hand, represented here in the spleen and thymus, seem to be highly susceptible to acute damage by PBBs and to a by far lesser extent by PBDEs and PCBs. The small, not significant increase in thymus weight after PBDE treatment should be further analyzed, since it was consistently observed in both genders. (If the data for thymus weight in % bw for both genders were pooled, the increase became positive for $p>0.05$).

Table 2A: Average Liver, Kidney and Thymus Weights

	Liver Weights		Kidney Weights		Thymus Weights	
	g ± SD	*% of b.w.*	g ± SD	*% of b.w.*	Mg	*% of b.w.*
Control	13.44 ± 0.60	*5.10 ± 0.20*	2.27 ± 0.26	*0.86 ± 0.1*	787 ± 70	*0.30 ± 0.035*
PCB	20.93 ± 2.49	***7.96 ± 0.98***	2.04 ± 0.24	*0.77 ± 0.07*	677 ± 111	*0.26 ± 0.038*
PBDE	15.86 ± 1.31	***6.39 ± 0.41***	2.10 ± 0.14	*0.85 ± 0.04*	837 ± 161	*0.34 ± 0.054*
PBB	21.98 ±1.70	***8.94 ± 0.64***	**1.89 ± 0.17**	*0.77 ± 0.05*	**541 ± 69**	***0.22 ± 0.023***

Table 2B: Average Spleen, Prostate, and Testes Weights

	Spleen Weights		Prostate Weights		Testes Weights	
	mg ± SD	*% of b.w.*	mg ± SD	*% of b.w.*	G	*% of b.w.*
Control	917 ± 94	*0.349 ± 0.043*	251 ± 32	*0.095* ± 0.012	3.818 ±0.319	*0.145 ±0.009*
PCB	**791 ± 80**	*0.301 ± 0.030*	216 ± 39	*0.082 ± 0.014*	3.913 ±0.328	*0.149 ± 0.015*
PBDE	**790 ± 51**	***0.318 ± 0.022***	233 ± 33	*0.095 ± 0.012*	3.733 ± 0.152	*0.151 ±0.008*
PBB	**734 ± 52**	***0.299 ± 0.025***	219 ± 49	*0.89 ± 0.019*	3.800 ± 0.193	*0.154 ±0.006*

2. Serum Parameters

Different serum parameters were analyzed to identify potential not-yet discovered targets for these compounds. Several of these parameters, for example the albumin-globulin ratio, may justify further studies, but will not be discussed in this report. Three groups of endpoints will be described in detail, since they may influence events in the prostate.

a. Iron and iron binding capacity in serum

Iron is of major importance in oxidative stress production during Fenton reactions and other redox-reactions. Elevated iron levels can cause health problems in the liver and possibly other organs. Iron deficiency on the other hand is equally unhealthy, resulting among others in anemia but also enzyme deficiencies for enzymes that require Fe. Cytochromes P450 is a major iron-dependent group of enzymes and the above described increase in liver size could stress the iron distribution in the rest of the body. We therefore measured iron levels in serum, total iron binding capacity (TIBC) and unsaturated iron binding capacity (UIBC).

No significant difference in serum iron levels could be seen among the different treatments (Table 3 below). Although the average iron level is lower in PBDE treated rats, this is mostly due to one outlier. Overall the values differed strongly among the individual animals even within the same treatment group, which becomes obvious from the very large standard deviation (SD). Despite the negative finding for iron levels, the total iron binding capacity was strongly influenced by biphenyls, both chlorinated and brominated. The same increase was seen for the unsaturated iron binding capacity, which was about doubled in PCB and PBB treated animals. This increase was in each case highly significant. PBDE treatment had no effect on TIBC or UIBC.

The major transport protein for iron in blood is transferrin. If more iron is needed for example during/after blood loss or increased cytochrome P450 (CYP) synthesis due to hepatocyte proliferation, iron is mobilized from the stores in the intestine, released into the blood, and transported by transferrin to the spleen (for hemoglobin synthesis) or liver (for CYP production). We can only speculate about the reasons for this increase in transferrin in blood. It could indicate a previous higher need for iron which resulted in increased transferrin production in the liver. Although there were some variations in spleen weight, it seems unlikely that this could have caused a decrease in hemoglobin production and a feedback signal to produce more transferrin for iron transport. However, the order of UIBC levels correlates with the order in liver weights, i.e. controls < PBDE < PCB < PBB. Although PBDEs produced a significant increase in liver weight it was much smaller then the one caused by PCBs and PBBs. This could be the reason why PBDEs did not cause a significant increase in iron binding capacity.

It is questionable whether such an increase in transferrin would have any negative health effects. It most likely is only an indicator for other health effects, like liver hypertrophy. The important result with respect to oxidative stress in other organs than the liver is that the serum iron level is obviously maintained at the normal rate. Therefore it is not expected that possible variations in iron levels, which were expected based on liver findings, will play a role in prostate toxicity by these halogenated compounds.

Table 3: Average iron level and iron binding capacity in serum (ug/dl ± SD)

	Iron	TIBC	UIBC
Control	309 ± 52.9	641 ± 152.0	333 ± 102.3
PCBs	333 ± 23.4	**992 ± 71.9**	**659 ± 71.1**
PBDEs	253 ± 69.6	611 ± 127.3	358 ± 68.0
PBBs	347 ± 70.2	**1073 ± 73.4**	**726 ± 4.4**

b. Thyroid hormone levels

Numerous publications report an influence of PCBs and, most recently, PBDEs on thyroid hormone levels, but to our knowledge no comparative in vivo study was carried out so far. In addition, thyroid hormones play an important role in development and growth. Considering that factors like "priming" during the embryonic phase and developmental roots of adult health problems are only now slowly gaining recognition as possible factors for adult diseases, we decided to add thyroid hormones to the parameters in this exploratory study which seemed the more justifiable, since no additional animals would be needed to gain this potentially valuable additional information. All together four parameters were tested, total thyroxine (TT4), free thyroxine (FT4), total triiodothyronine (TT3), and thyroid stimulating hormone (TSH). To keep costs low, human antibodies were used for the TSH determinations. The data may therefore not represent accurate total amounts, but rather relative amounts.

All treatments caused a highly significant ($p>0.005$) reduction in TT4 and FT4, with PCBs > PBBs > PBDEs (Table 4). Only PCBs also caused a highly significant reduction in TT3. A similar result had also been observed in female rats, however, with a surprising gender difference in that female rats were somewhat more sensitive to PBDEs than male rats, thus switching the order of potency to PCB > PBDE > PBB. In male rats the average hormone levels were reduced by PCBs to 18% (TT4), 27% (FT4) and 56% (TT3) of control values. Such strong reduction should trigger TSH secretion to boost thyroid hormone production. Contrary to our expectations the largest increase in TSH was seen in PBB treated rats. PCBs and PBDEs also caused an increase in TSH, but it was only significant for PCBs. In female rats the same order of TSH levels, PBB > PCB > PBDE > control was observed, but only PBB levels were significantly higher than control.

These results confirm previous reports in the literature that these mixtures influence thyroid hormone levels, but they add the information that 1. the chlorinated biphenyl mixture was more potent than the brominated biphenyl mixture at equimolar concentrations (although it should be kept in mind that the PBB mixture has a higher degree of bromination and a different congener pattern), 2. female rats are more sensitive to PBDEs than male rats, which may be important with respect to embryonic/developmental origins of adult succeptibility or development of health damage, and 3. TSH levels did not at all reflect the decrease in thyroid hormone levels, but in contrast were highest in animals that showed the lowest changes in hormone levels. TSH levels are by far more costly and difficult to determine and therefore often not analyzed. These results show that this may deprive us of important information needed to fully understand possible mechanisms involved and may result in incomplete assessment of risk. The importance of our findings for prostate cancer development remains open, but the data are now available for possible later cross-comparisons with other findings.

Table 4: Thyroid hormone and TSH levels

	TT4 (ug/dl ±SD)	FT4 (ng/dl ±SD)	TT3 (ng/ml ±SD)	TSH (u IU/ml ±SD
Control	2.84 ± 0.52	1.06 ± 0.15	0.80 ± 0.14	0.03 ± 0.02
PCBs	**0.51 ± 0.09**	**0.29 ± 0.07**	**0.45 ± 0.08**	**0.17 ± 0.10**
PBDEs	**1.30 ± 0.36**	**0.59 ± 0.06**	0.70 ± 0.08	0.15 ± 0.11
PBBs	**1.10 ± 0.14**	**0.50 ± 0.06**	0.64 ± 0.08	**0.48 ± 0.42**

c. Serum lipids

We had hypothesized that dietary fat intake may influence prostate cancer development. Indirectly this hypothesis was confirmed by the latest epidemiological study (New Engl. J. Med April 2003) that obesity and high body weight increase prostate cancer risk, assuming that a lot of the excess body weight is acquired through the over-consumption of food, especially fats. One possible mechanism for the connection between obesity and prostate cancer is that more body fat causes an increase in estrogen levels, which may act as promoter of carcinogenesis. Another factor is that fats change the uptake and retention of lipophilic compounds in the body, thus possibly resulting in a higher body burden of potentially toxic compounds. Also, changes in lipids could possibly mobilize lipophilic compounds from storage and change the distribution pattern in the body.

We analyzed the serum levels of cholesterol, HDL, VLDL, and triglycerides. Cholesterol levels were significantly ($p>0.05$) elevated in PBB treated animals (Table 5). HDL levels were strongly elevated ($p>0.005$) by all three mixtures. In both cases the order of potency was PBB > PCB > PBDE. In contrast to this, VLDL and triglyceride levels were decreased, but the effect was significant only with PBBs and PBDEs, not with PCBs. There were two gender differences that could be observed. First, female rats did not show any effects on triglyceride levels, second, female rats expressed very strong effects of all three mixtures on cholesterol levels ($p>0.005$).

The physiological basis and medical significance of these findings is not clear. At this point we can not offer any possible explanation for these changes in the serum lipid profile. Also, to our knowledge nothing is known about the binding and transport of these halogenated hydrocarbons by these lipids. Cholesterol is a building block for steroid hormones. This could be a possible connection to the prostate. Also, vitamin A and vitamin E, both considered candidates for chemoprotection, are lipid soluble and their uptake, distribution and retention possibly influenced by changes in lipid metabolism and profile.

Table 5: Serum lipids

Treatment	Cholesterol	HDL	Triglycerides	VLDL
Control	130.20 ± 20.02	98.33 ± 2.31	109.00 ± 6.68	26.50 ± 10.43
PCBs	152.67 ± 17.26	**129.83 ± 9.77**	77.83 ± 28.84	15.75 ± 5.35
PBDEs	126.67 ± 18.28	**111.00 ± 6.69**	**71.58 ± 20.65**	**14.33 ± 3.92**
PBBs	**174.60 ± 19.44**	**148.00 ± 10.15**	**61.80 ± 11.54**	**12.20 ± 1.68**

With respect to the gender differences that we mentioned, it was reported that female rats are more susceptible to liver cancer after PCB exposure than male rats whereas male rats are more susceptible to thyroid cancer than females. The different level of response to PCBs with respect to thyroid hormones (stronger in males) and cholesterol levels (stronger in females) could potentially be related to these gender differences in organ specific cancer susceptibility.

Overall we learned from this experiment so far: 1.) at equimolar levels the PBB mixture Firemaster BP-6 has the most negative effect on body and organ (liver, thymus, spleen) weights and caused the strongest changes in serum lipids and transferrin levels. The PCB mixture Aroclor 1254 was the next strongest with respect to these endpoints, but was clearly the most potent mixture with respect to thyroid hormone levels. Despite this, PBBs caused the strongest increase

in TSH. 2.) although the PBDE mixture DE-71 had the smallest effects of the three mixtures on all endpoints tested, it did significantly alter liver and spleen weight, thyroxin levels, HDL, VLDL and triglycerides. Also, the thymus may be a target of toxicity, reacting possibly with an increase, not decrease, as with the other mixtures, in organ weight. 3.) Comparison with data from a similar experiment with female rats revealed that females had a higher increase in cholesterol and HDL levels, whereas male rats had a higher TSH increase. 4.) prostate and testes weight and gross morphology were not influenced by any of the three mixtures. Further analysis of the tissues is planned for the next months.

2 *IN VIVO STUDY WITH PCB 77:* ANTIOXIDANT TIME-STUDY

Liver tissues from a previous in vivo study which had focused on liver enzymes had been preserved in a -80 freezer. We decided to use part of these tissues to gain insights into the time course of oxidative stress. In this study male and female Sprague-Dawley rats (150 -200g) had received one single injection of 300 μmol/kg PCB77. Animals had been euthanized 6 hrs, 12 hrs, 1 day, 4 days, 1 week, 2 weeks, and 3 weeks after the injection. We measured body and liver weights, glutathione, vitamin C, vitamin E and selenium levels, glutathione peroxidase, and cutochrome P450 1A1 activity. Significance was calculated by analysis of variance and Scheffe's test. Results that are significantly different from control ($p>0.05$) are indicated with an asterix (*).

Table 7 shows that the PCB77 treatment resulted in a small, but significant reduction in body weight after 3 weeks in males and after 1 and 2 weeks in females. Liver weights were increased after 4 days, but no longer statistically higher after 3 weeks in males. Females had higher liver weights already 1 day after injection of PCB77 and the livers remained enlarged throughout the experiment. The liver/body ratio was even more sensitive, indicating a treatment effect after 6 hrs and at day 1 until the end (females) or week 2 (males) of the experiment.

Table 7 Average body and liver weights at the indicated time after injection of PCB77

	Male rats			Female rats		
	Body weight (g)	Liver weight (g)	Liver/body ratio	Body weight (g)	Liver weight (g)	Liver/body Ratio
Control	313.5 ± 3.0	12.9 ± 0.5	0.041 ± 0.002	222.0 ± 1.8	8.3 ± 0.2	0.037 ± 0.001
6 hrs	307.8 ± 7.2	14.6 ± 0.1	**0.047 ± 0.001***	222.5 ± 1.5	10.1 ± 0.4	**0.045 ± 0.002***
12 hrs	297.5 ± 4.7	13.3 ± 0.4	0.045 ± 0.001	219.5 ± 0.9	9.0 ± 0.2	0.041 ± 0.001
1 day	295.5 ± 4.6	13.6 ± 0.1	**0.046 ± 0.001***	219.8 ± 5.0	**10.6 ± 0.4***	**0.048 ± 0.001***
4 days	310.5 ± 5.9	**16.4 ± 0.7***	**0.053 ± 0.002***	218.5 ± 3.3	**11.6 ± 0.5***	**0.053 ± 0.002***
1 week	311.0 ± 8.8	**17.6 ± 1.1***	**0.056 ± 0.002***	**209.5 ± 3.5***	**11.8 ± 0.9***	**0.056 ± 0.003***
2 weeks	301.0 ± 1.2	**16.6 ± 0.4***	**0.055 ± 0.001***	**204.0 ± 4.0***	**11.9 ± 0.6***	**0.058 ± 0.002***
3 weeks	**286.3 ± 4.6***	13.2 ± 0.3	0.046 ± 0.001	211.8 ± 4.0	**10.7 ± 0.4***	**0.051 ± 0.002***

Glutathione (GSH) is a major cellular antioxidant. Reduction of intracellular GSH could render the tissue extremely vulnerable to oxidative damage in the form of lipid peroxidation and

protein and DNA oxidation. An increase in the oxidized form of glutathione (GSSG) is an indicator of oxidative stress with inappropriate recycling of GSH. After PCB77 treatment, a very strong reduction of GSH in the livers of male rats could be seen as early as 12 hrs after exposure. One and two weeks after PCB exposure the GSH level was reduced to as little as 17% of normal control. No increase in oxidized glutathione (GSSG) was seen. This could indicate that GSH levels were reduced due to conjugation and addition reactions with reactive intermediates, not because of its function as cofactor for glutathione peroxidase (GPx) or glutaredoxin. In contrast to the finding in male rats, no reduction of GSH was seen in female rats.

Table 8a: Antioxidants in liver tissue of male rats after PCB77 exposure

	GSH	GSSG	Ascorbic Acid	Dehydro-Asc. Acid	α-Tocopherol	α-Tocopheryl Quinone
Control	23.6 ± 3.4	3.2 ± 0.8	6.3 ± 0.4	0.6 ± 0.1	100.0 ± 5.6	100.0 ± 36.6
6 hrs	15.2 ± 4.2	7.7 ± 2.7	5.7 ± 0.2	0.4 ± 0.2	101.8 ± 11.2	63.9 ± 21.9
12 hrs	**11.0 ± 1.7***	5.7 ± 1.8	**4.3 ± 0.6***	0.8 ± 0.4	84.3 ± 15.0	81.6 ± 29.3
1 day	**9.2 ± 4.4***	3.2 ± 1.1	5.0 ± 0.3	0.6 ± 0.1	56.3 ± 41.1	117.3 ± 51.2
4 days	**7.8 ± 2.1***	4.1 ± 1.5	**4.4 ± 0.3***	0.4 ± 0.0	78.1 ± 32.7	**713.4 ± 219.5***
1 week	**4.1 ± 1.0***	3.1 ± 2.1	**4.0 ± 0.1***	0.5 ± 0.1	110.1 ± 31.5	**987.9 ± 182.9***
2 weeks	**4.2 ± 1.4***	1.9 ± 0.9	4.7 ± 0.5	0.4 ± 0.1	96.6 ± 25.0	**1616.3 ± 556.0***
3 weeks	**10.3 ± 1.8***	5.7 ± 2.5	5.8 ± 0.7	0.5 ± 0.1	87.0 ± 18.6	**2419.1 ± 497.4***

Table 8b: Antioxidants in the livers of female rats after PCB77 exposure

	GSH	GSSG	Ascorbic Acid	Dehydro-Asc. Acid	α-Tocopherol	α-Tocopheryl Quinone
Control	13.8 ± 4.2	10.1 ± 3.1	6.8 ± 1.2	0.9 ± 0.2	100.0 ± 7.7	100.0 ± 33.7
6 hrs	13.8 ± 4.4	14.0 ± 1.0	5.9 ± 0.2	0.4 ± 0.3	106.4 ± 12.7	122.3 ± 22.5
12 hrs	15.9 ± 4.2	7.8 ± 1.4	5.4 ± 0.3	1.5 ± 1.2	73.9 ± 22.4	220.4 ± 67.5
1 day	14.1 ± 2.9	10.7 ± 1.3	**3.6 ± 0.4***	**1.8 ±1.0***	131.9 ± 16.8	84.4 ± 68.6
4 days	6.9 ± 1.2	10.3 ± 1.2	**4.6 ± 0.3***	0.5 ± 0.2	113.0 ± 19.8	118.5 ± 60.0
1 week	11.1 ± 2.7	9.0 ± 0.3	**4.5 ± 0.2***	0.5 ± 0.3	96.9 ± 29.8	265.9 ± 75.0
2 weeks	12.4 ± 6.2	8.4 ± 2.6	**4.2 ± 0.7***	0.8 ± 0.3	146.9 ± 40.8	**1006.3 ± 56.2***
3 weeks	4.1 ± 1.0	9.2 ± 0.5	4.7 ± 0.5	0.9 ± 0.1	152.9 ± 58.1	**1789.1 ± 194.9***

Other important antioxidants are vitamin E (α-tocopherol) and vitamin C (ascorbic acid). Vitamin E is the body's primary lipophilic antioxidant and its major function is to terminate the free radical induced lipid peroxidation cycle. Based on previous experiments with multiple exposures, α-tocopherol was expected to be reduced, but no significant changes were seen in male or female rats during the time of the experiment (Table 8). This could indicate that the oxidized form of vitamin E was very efficiently recycled or further metabolized. Usually α-tocopherol is oxidized by peroxyl radicals to α-tocopherol radicals, which are regenerated by vitamin C, the body's major hydrophilic antioxidant. Indeed, regeneration of oxidized vitamin E and scavenging of ROS in the aqueous phase of the cell are the major functions of vitamin C. As shown below, PCB77 treatment reduced ascorbic acid levels in the liver of both, male and female rats, a second indicator for oxidative stress in the liver. Dehydroascorbic acid, the oxidized form

of ascorbic acid, is regenerated by glutaredoxin with the oxidation of GSH to GSSG, which is regenerated by glutathione reductase, with the consumption of NADPH. Dehydroascorbic acid was significantly elevated only in female rats on day 1 after PCB treatment. Alternatively the α-tocopherol radical can react with a second peroxyl radical to form α-tocopheryl quinone. As shown in Table 8 and Fig. 1, α-tocopheryl quinone increased steadily with time in male rats after PCB77 treatment and reached a maximum of 2400% of control after 3 weeks at the end of the experiment. In female rats α-tocopheryl quinone was significantly elevated only during the last two time points, at week 2 and 3, where it reached 1800% of control values. These results confirm our previous observation that PCBs cause oxidation of Vitamin E to the corresponding quinone. This effect as well as the GSH depletion was more pronounced in males than in females.

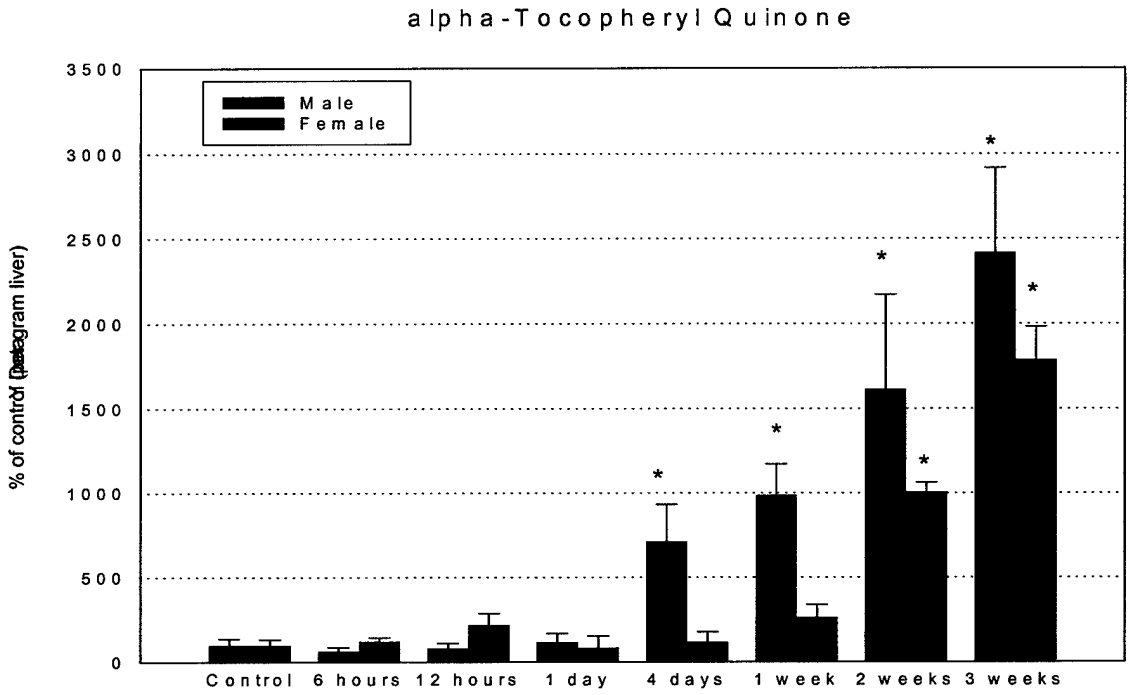

Figure 1: alpha-Tocopheryl quinone levels in the livers of rats treated with PCB77

Besides these vitamins, we also determined total hepatic selenium levels and glutathione peroxidase (GPx) activity. In contrast to our previous findings with multiple PCB exposure, total selenium was not changed in male rats (Fig. 2). Female rats had a higher baseline level than male rats and in these animals selenium was significantly reduced from day 4 on. GPx activity is generally seen as a good indicator of selenium levels. In this study it also reflected the findings with selenium in that GPx activity was much higher in females than in males and only slightly reduced in male rats from day 1 on (significant only on day 4), but significantly reduced in female rats from day 4 to the end of the experiment (Fig. 3).

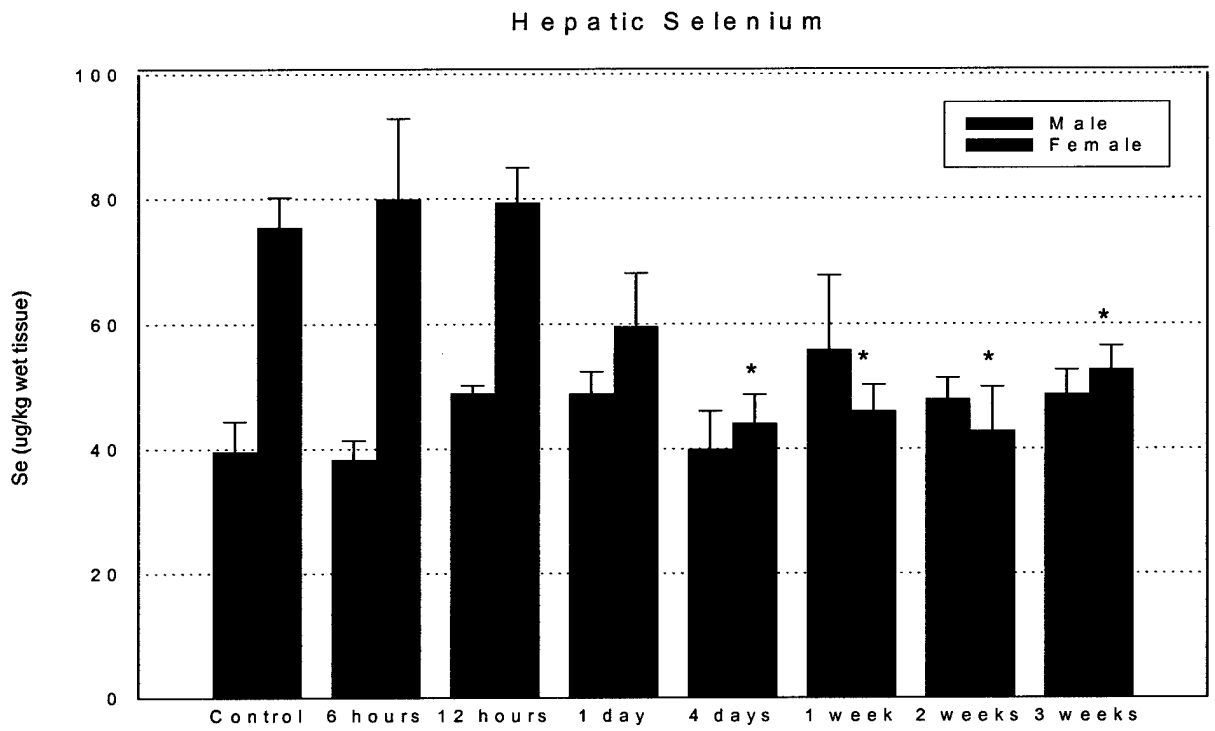

Fig. 2: Total Selenium levels in the livers of rats treated with PCB77

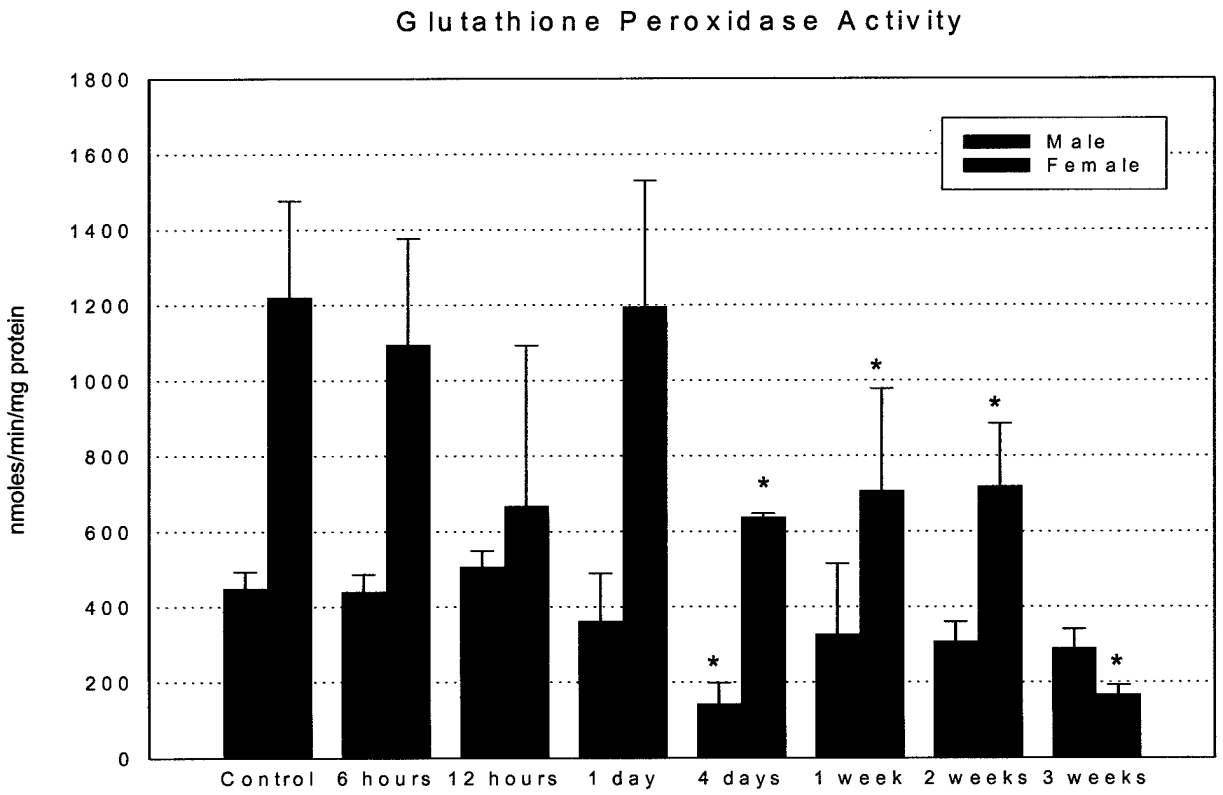

Fig. 3: Glutathione peroxidase activity in liver cytosol from rats treated with PCB77

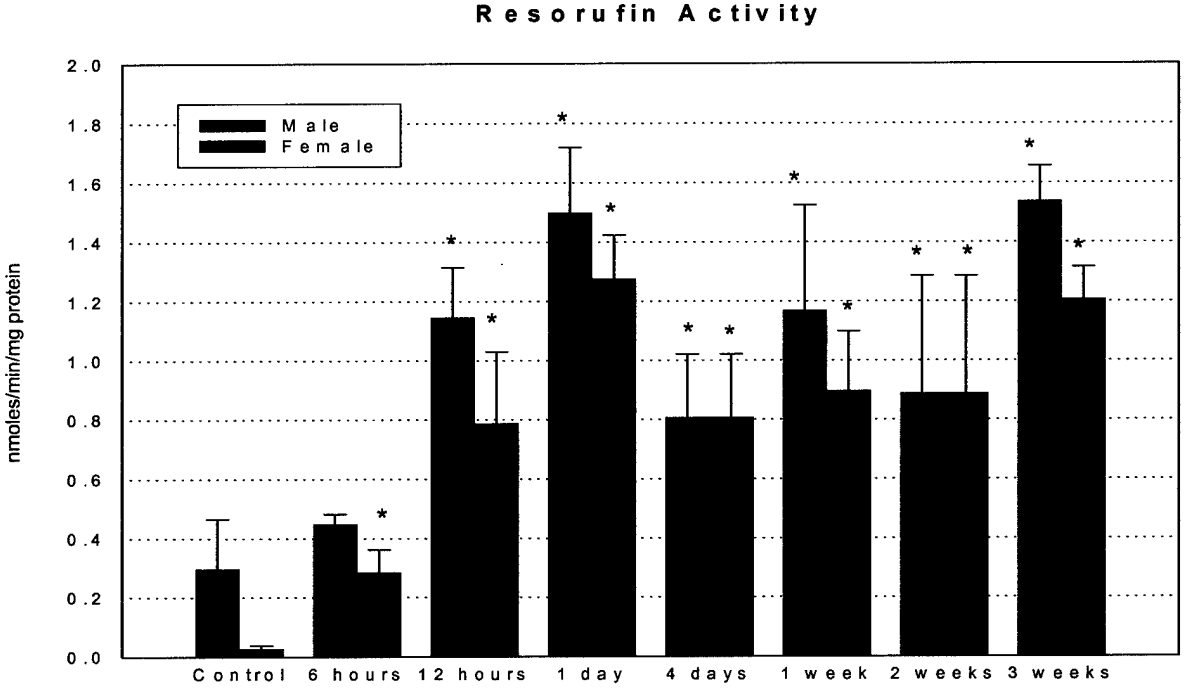

Fig. 4: Ethoxyresorufin (EROD) activity in liver microsomes from rats exposed to PCB77.

The EROD activity (Cyochrome P450 1A1) was determined as a positive control for PCB exposure and for comparison of time-lines of other endpoints with cytochrome P450 induction. As depicted in Fig. 4, resorufin activity was significantly increased in both genders as early as 6 hrs (females) after exposure to PCB77.

These results clearly show oxidative stress in the liver of PCB77 treated rats as early as 12 hrs after PCB injection in males (GSH and vitamin C reduction). Males seem to be more prone to oxidative stress than females, with the exception of selenium and GPx levels, which were more reduced in females. Most striking was the large increase in α-tocopheryl quinone. It is conceivable that vitamin E quinone may be a better indicator of PCB induced oxidative stress in tissues than other parameters. Whether this indicator of oxidative stress also indicates oxidative damage needs to be determined. Several publications (Koremura et al., 1990; Kato et al., 1989; Ohchi et al., 1987) reported an increase in total vitamin E in rats that received PCB in the diet. This increase was seen in all tissues tested, i.e. liver, kidneys, testes, brain spleen, lung, muscle, adipose tissue, and serum. The authors reported an increased uptake of vitamin E from the intestine. They also observed an increase in serum cholesterol, HDL, and VLDL and that other halogenated compounds, they tested DDT, had the same effect as PCBs on vitamin E uptake. Sato (1990) on the other hand observed a decrease in α-tocopherol in liver microsomes of PCB treated animals and a decrease in GPx activity and increase in lipid content and lipid peroxidation in liver cytosol of these animals.

Overall these data indicate that all parameters, lipids, vitamins and other antioxidants, enzyme activities and the protein and vitamin content of the diets have to be considered, if we want to fully understand the possible risks associated with the exposure to certain halogenated compounds, and to a certain extent justify our efforts described in our first, exploratory experiment.

3. CELL CULTURE EXPERIMENTS

Cell culture experiments were initiated to develop the best possible assay protocol and to obtain initial results with the test compounds and modifiers. The toxicity/cell growth assay with resazurin was chosen as a fast and sensitive method to measure the number of living cells per well in 96 well plates after exposure to test compounds under the chosen conditions and with/without media additives for different lengths of time. During these experiments we encountered unexpected problems with the assay. The problems and our efforts to overcome them as well as our results are summarized below.

As proposed, we began our experiments with two human prostate cancer cell lines, LNCap and PC-3. LNCap cells express the androgen receptor, although a mutated form that is also sensitive to estrogens, whereas PC-3 cells do not express the androgen receptor. According to this difference, LNCap is regarded as being androgen sensitive, whereas PC-3 is described as insensitive to androgens. Both cell lines were maintained in RPMI1640 medium with phenol red and 10% fetal bovine serum (FBS). This medium will be called normal medium (NM) in this report. To also measure potential growth enhancement by the compounds due to androgenic or estrogenic activity, most assays were performed in RPMI medium without phenol red and with charcoal stripped FBS, here referred to as stripped medium (SM).

Cells were seeded in 96 well plates at different concentrations and cell growth visually inspected over the following days. Based on these observations a seeding number of $5x10^3$ cells per well was chosen as the appropriate cell number, since it allows cell growth for several days without reaching confluency. In a typical experiment cells were seeded into 96 well plates. Twenty-four hours later the medium was changed for new medium with test compounds. After 4 days of exposure, medium with 10% resazurin was added to the cultures and these incubated for an additional 2-4 hours. Living cells convert the dye from its oxidized form (blue) and to a fluorescent intermediate (red), which can be measured with a plate reader spectrophotometrically or, as we did, fluorometrically at an excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Other prostate cell lines (NRP154 and NRP152)

During the work with the LNCap cells we noticed that they did not seem to respond to the androgen dehydrotestosteron (DHT) as expected. Assuming that our batch of cells had mutated, we performed the same experiments with a batch of LNCap from a neighboring laboratory, but experienced the same problem. In addition, LNCap do not adhere very strongly to the cell culture plastic, making it difficult to wash the cells or change medium during the assay without dislodging some of the cells. In the literature we located publications by Dr. David Danielpour in which he described studies with a pair of rat prostate cell lines that he had isolated and characterized. These cell lines, NRP 154 (without androgen receptor expression, equivalent to PC-3) and NRP 152 (express the androgen receptor, equivalent to LNCap) seemed promising. We contacted Dr. Danielpour and he generously agreed to provide us with his cell lines. These cells grow in DMEM-F12. The NRP152 require dexamethasone, insulin, cholera toxin, and epithelial growth factor for optimal growth. Cells were maintained in normal medium (NM) containing phenol red and normal serum. For growth experiments cells were transferred to stripped medium (SM, without phenol red and with charcoal stripped FBS) before seeding them into 96 well plates. All growth experiments were performed in SM.

Cytotoxicity and/or growth enhancement results

a. Polychlorinated biphenyls

As proposed, the PCB congeners PCB77 (3,3',4,4'-tetrachlorobiphenyl) and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) were tested in all four cell lines. PCB77 is a coplanar PCB congener and a MC-type inducer of cytochromes P450. PCB 153 is a non-coplanar congener and PB-type inducer. We also tested the commercial PCB mixture Aroclor 1254. In addition, we examined PCB118 (2,3',4,4',5-pentachlorobiphenyl) in LNCap cells. PCB118 is a mixed type inducer. Data for PCB118 are not included in this report, since it was only tested in one cell line so far. For the assays 200x concentrated stock solutions of the PCB congeners and mixture in DMSO were prepared. The final concentration of DMSO in the medium never exceeded 0.5%.

Both pure PCB congeners caused only a small reduction in cell number at the highest concentration tested (Fig. 5 & 6). Neither PCB77 nor PCB153 produced a significant increase in cell number above control in any of the cell lines tested. The mixture Aroclor 1254 was more toxic than the congeners, resulting in 100% cell death at the highest concentration, 100 μM (Fig 7). No cell growth enhancing effect was obvious at the lower concentrations.

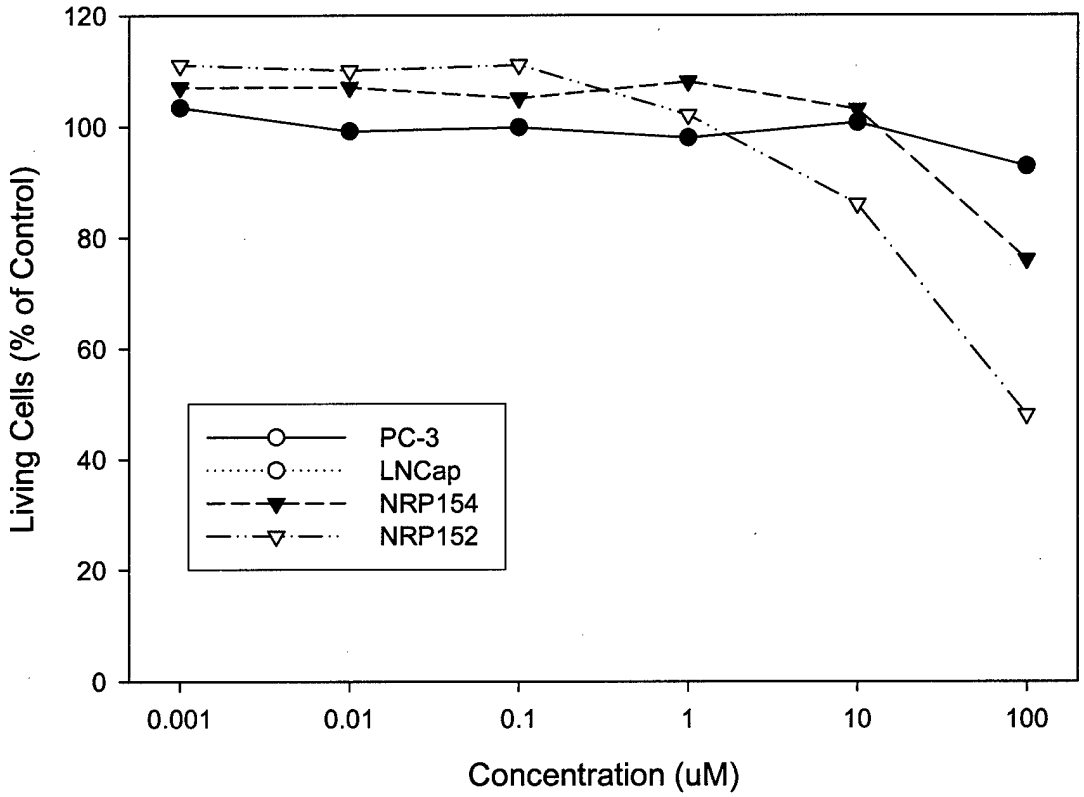

Fig. 5: PCB77 (3,3',4,4'-tetrachlorobiphenyl)

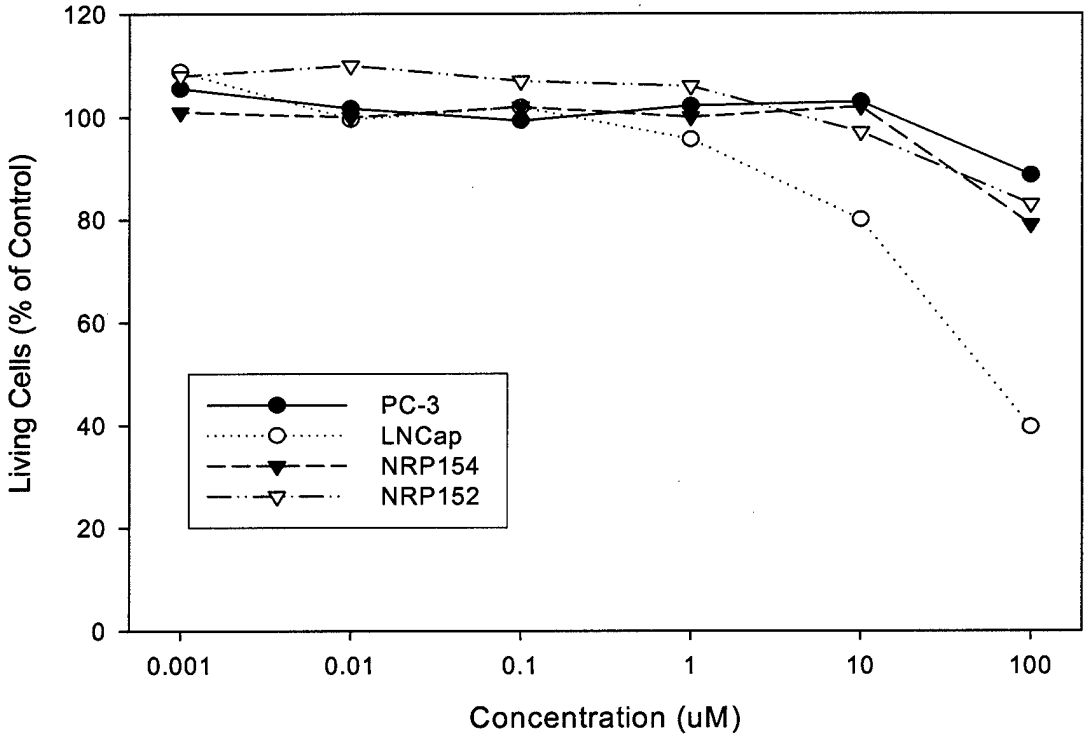

Fig. 6: PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl)

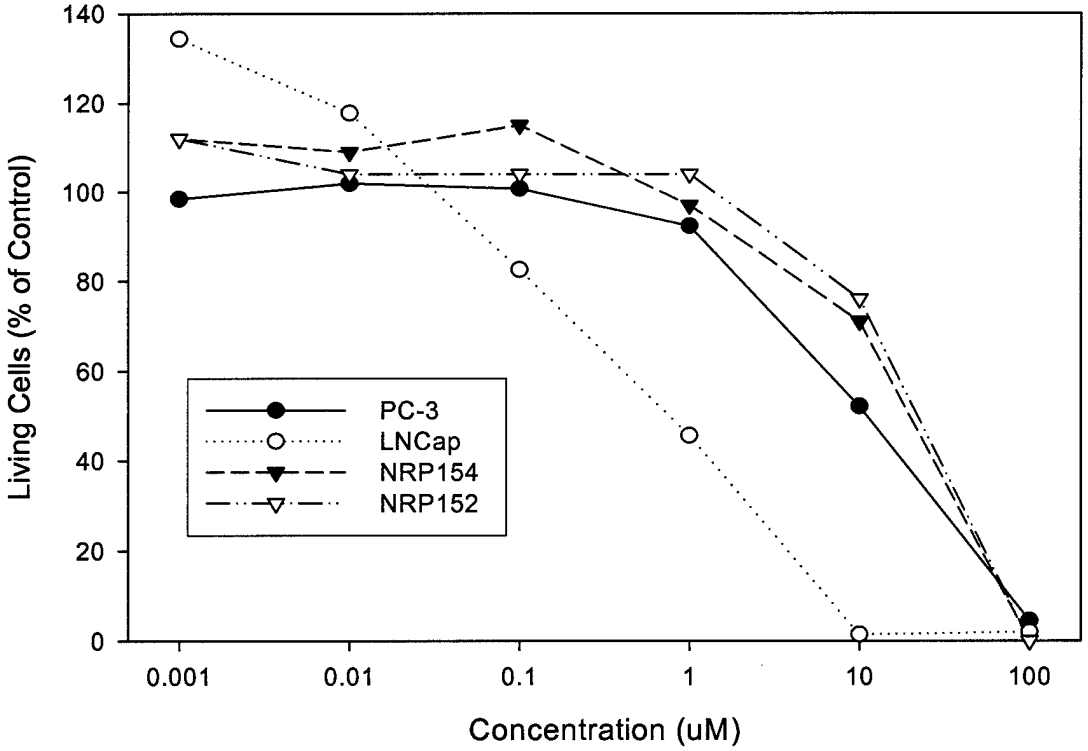

Fig. 7: Aroclor 1254

These results are somewhat surprising, since we had assumed that one of the congeners, representing the coplanar or non-coplanar type, would be more toxic. Aroclor 1254 is a mixture of many different congeners. We plan to reconstitute the mixture to a certain extent to gain further insight into this effect.

b. Antioxidants

Our hypothesis is that one mechanism of PCB toxicity is through the generation of reactive oxygen species. This hypothesis is supported by several of our in vitro, in vivo, and cell culture studies. This could mean that antioxidants should be protective against PCB toxicity. In connection with prostate cancer, the two antioxidants vitamin E and selenium are of special interest. In a first step to analyze this possibility, we tested these two antioxidants. We included vitamin C in these studies, since in vivo it recycles oxidized vitamin E and also functions as a radical scavenger in the cytosol.

Antioxidant stock solutions were prepared in water or ethanol (vitamin E) and tested in 1:10 dilution steps. Vitamin C and vitamin E did not produce any toxicity or growth enhancing effect in any of the four cell lines up to the highest concentration tested (100 μM, data not shown). The very small reduction in cell number in LNCap cells at 100 μM of vitamin E needs to be further analyzed, if such high concentrations will be used in later experiments. SeO_3Na significantly reduced the number of living cells per well at the two highest concentrations tested, 100 and 10μM (Fig. 8). All four cell lines reacted in a similar way. No growth enhancing effect was seen at lower concentrations.

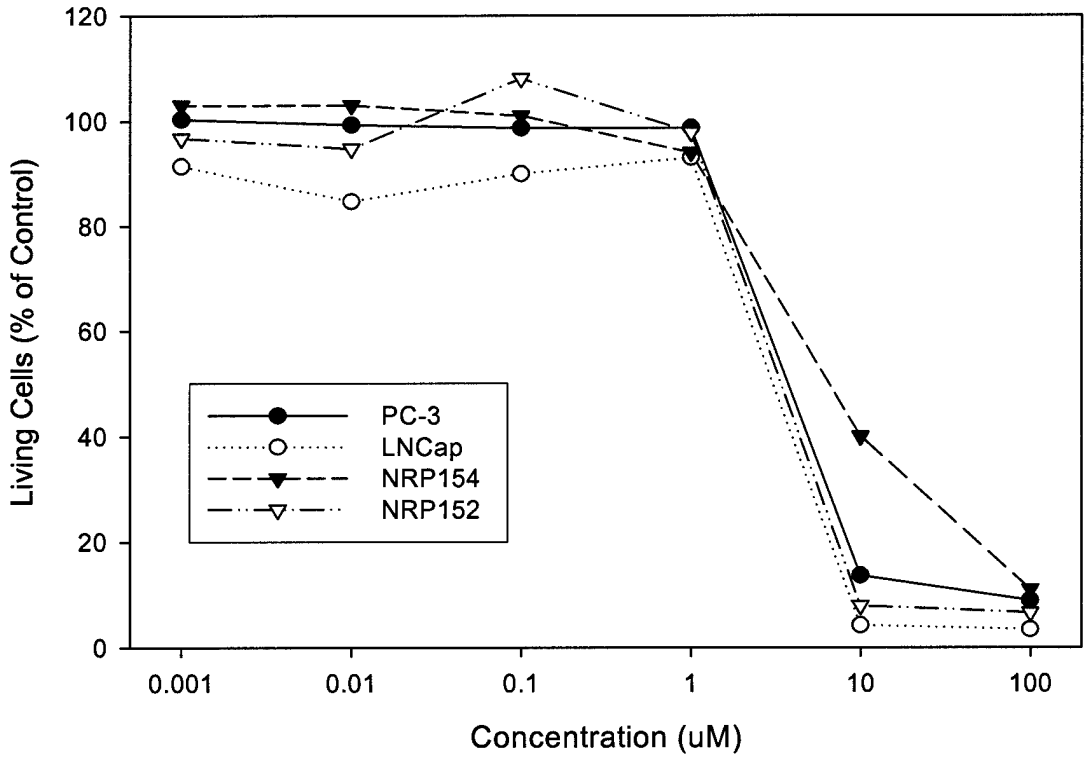

Fig. 8: Effect of SeO_3Na_2 on prostate cell lines

c. Androgens and anti-androgens

Since one of our questions is whether halogenated hydrocarbons are involved in prostate cancer development through direct or indirect activity on the androgen receptor, it was necessary to have a functioning positive and negative control in the form of an androgen and an anti-androgen for cell culture studies. Differences in response in androgen receptor negative (PC-3, NRP 154) vs androgen receptor positive cells (LNCap, NRP 152) could also be due to other differences in the cell lines besides the androgen receptor. Enhancement or blocking of an effect of test compounds by (anti)-androgens would help to exclude this possibility. We therefore tested one androgen, dehydrotestosterone (DHT), and two anti-androgens, the steroidal anti-androgen cyproterone acetate and the non-steroidal anti-androgen nilutamide for their effects on the four cell lines. Concentrations up to 1 μM (DHT) and 100 μM (both anti-androgens) were used.

DHT had no toxic effect on any of the cell lines, possibly with the exception of LNCap at the highest concentration (Fig. 9). However, DHT also did not have a growth enhancing effect in any of the cell lines in the hormone deprived SM medium. This was surprising, since it was expected that LNCap and NRP152 cells would grow better in the presence of this androgen. A possible explanation for this negative finding is that the medium/cells were not sufficiently androgen deprived. Longer exposure times in SM medium before and during the assay and lower serum concentrations are two approaches that will be used to clarify this phenomenon.

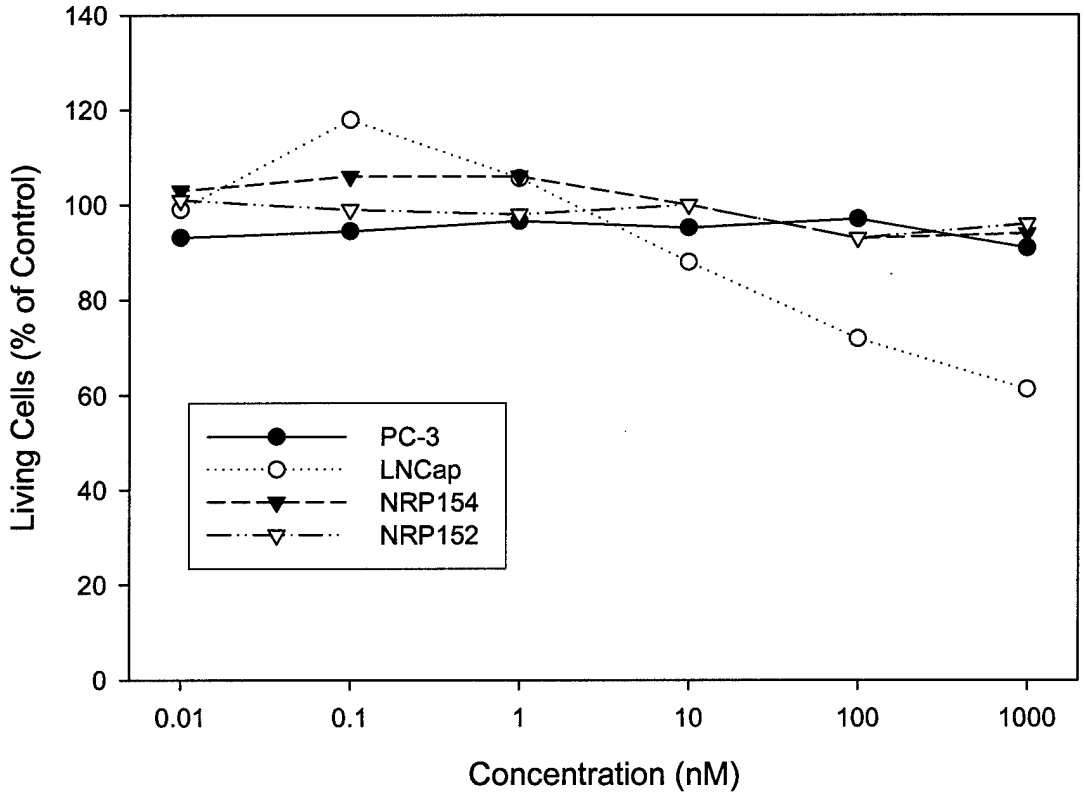

Fig. 9: Dehydrotestosterone (DHT)

The two anti-androgens gave different results in the different four cell lines. Nilutamide, the non-steroidal anti-androgen, caused a reduction of living cells per well at the highest concentration tested in two cell lines, LNCap and NRP152 (Fig. 10). These two lines are the androgen receptor positive lines. This effect may indicate that there was indeed a small remaining amount of androgens in the medium that was successfully blocked from enhancing cell growth of those cells that express the androgen receptor. The results with cyproterone acetate, the steroidal anti-androgen, were not so clear. This compound caused not only a reduction in cell number, but severe cytotoxicity in three of the four cell lines, with the exception of PC-3 (Fig. 11). Additional experiments are planned to confirm and explain this finding.

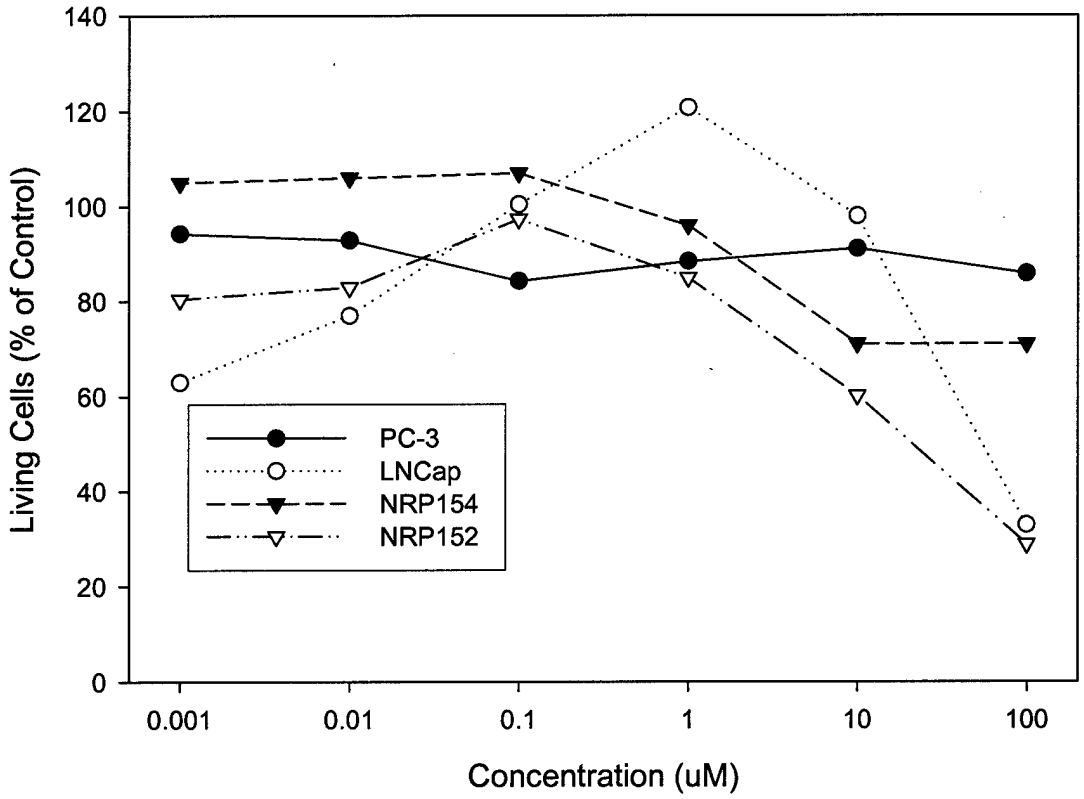

Fig. 10: Effects of Nilutamide on prostate cells

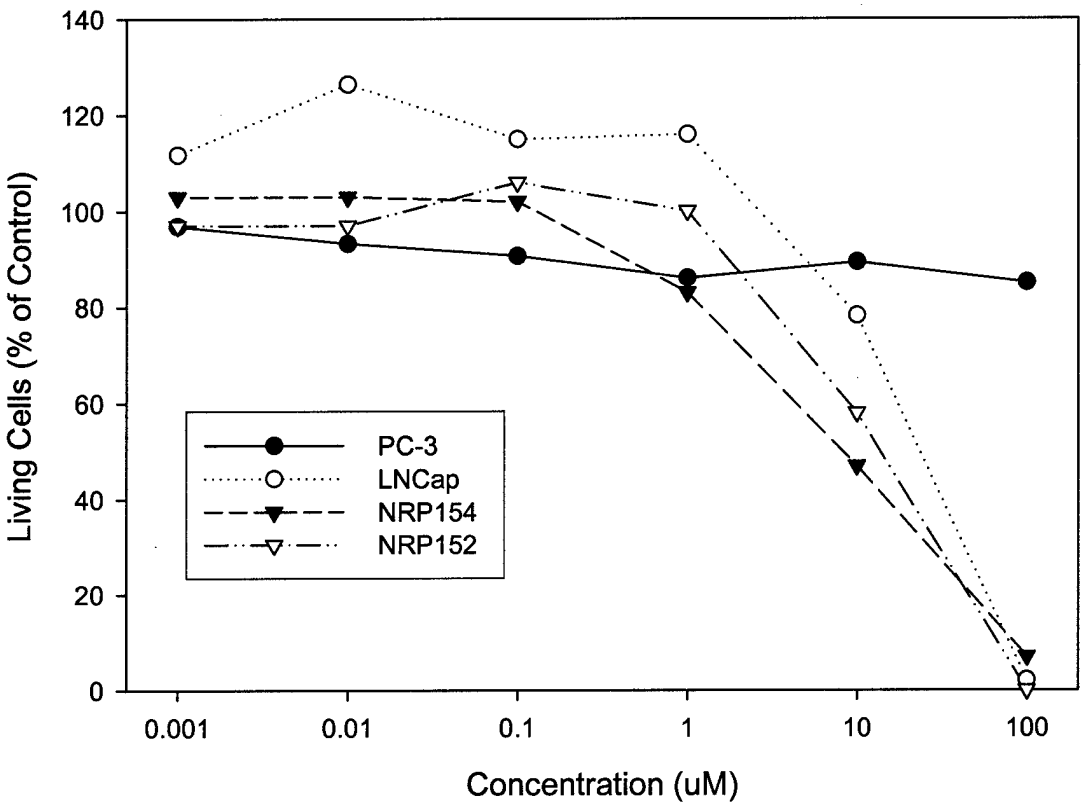

Fig. 11: Cyproterone acetate

Taken together, these first cell culture experiments show that the prostate cells are surprisingly resistant to toxic effects by PCBs in short term exposure. Also, selenium in itself is toxic at high concentrations and assay conditions have to be optimized in order to be able to see androgen/anti-androgen effects with these cells. Thus assay conditions for future experiments have to be designed carefully with respect to medium and additives, pre-exposure in specified medium, exposure duration with PCBs, and so on. It is expected, however, that the endpoint that was analyzed in these experiments, i.e. cytotoxicity, is the least sensitive, and that other endpoints (intracellular oxidative stress, enzyme activities, and so on) will cause effects at lower concentrations of our PCBs.

KEY RESEARCH ACCOMPLISHMENTS

- One in vivo exploratory experiment with three different mixtures of halogenated hydrocarbons (ongoing), one time course study with PCB77, and the initial cell culture experiments with two pairs of androgen receptor positive/negative cell lines were performed.
- At equimolar levels the PBB mixture Firemaster BP-6 has the most negative effect on body and organ (liver, thymus, spleen) weights and caused the strongest changes in serum lipids and transferrin levels compared to the PCB mixture Aroclor 1254 or the PBDE mixture DE-71.
- The PCB mixture Aroclor 1254 was the next strongest with respect to these endpoints, but was clearly the most potent mixture with respect to thyroid hormone levels. Despite this, PBBs caused the strongest increase in TSH.
- Although the PBDE mixture DE-71 had the smallest effects of the three mixtures on all endpoints tested, it did significantly alter liver and spleen weight, thyroxin levels, HDL, VLDL and triglycerides. Also, the thymus may be a target of toxicity, reacting possibly with an increase, not decrease, as with the other mixtures, in organ weight.
- Comparison with data from a similar experiment with female rats revealed that females had a higher increase in cholesterol and HDL levels, whereas male rats had a higher TSH increase.
- Prostate and testes weights and gross morphology were not influenced by any of the three mixtures.
- In a time course experiment with PCB77, oxidative stress in the liver of treated rats was observed as early as 12 hrs after PCB injection in males (GSH and vitamin C reduction).
- Males seem to be more prone to oxidative stress by PCB77 than females, with the exception of selenium and GPx levels, which were more strongly reduced in females.
- Most striking was the large increase in α-tocopheryl quinone after PCB77 injection. It is conceivable that vitamin E quinone may be a better indicator of PCB induced oxidative stress in tissues that other parameters.
- These data indicate that all parameters, lipids, vitamins and other antioxidants, enzyme activities and the protein and vitamin content of the diets have to be considered, if we want to fully understand the possible risks associated with the exposure to certain halogenated compounds.
- Cell culture experiments with two pairs of prostate cell lines, human PC-3 (androgen receptor negative) and LNCap (androgen receptor positive) and rat NRP154 (androgen receptor negative) and NRP152 (androgen receptor positive) show that these cells are surprisingly resistant to the cytotoxic effects of PCB77 and PCB153, but somewhat more sensitive to the PCB mixture Aroclor 1254 in short term exposure.
- Selenium in itself is toxic at high concentrations.
- Dehydrotestosterone did not increase cell proliferation in our cell lines under the chosen assay conditions, but the anti-androgen Nilutamine reduced cell number in both androgen receptor positive cell lines, but not in the androgen insensitive lines.
- The rat and human androgen sensitive lines LNCap and NRP152 may differ in their sensitivity to the steroidal anti-androgen cyproferone.
- Assay conditions have to be carefully optimized in order to be able to see androgen/anti-androgen effects with these cells.

REPORTABLE OUTCOMES

Degrees obtained

Shahram Shoeibi obtained a Ph.D. from the University of Tehran, Iran, with cell culture work performed during his stay in my (G.L.) laboratory during April – December 2002, which was partially funded by this grant.

Employment Opportunities received

I myself, Gabriele Ludewig, Ph.D., and the Co-I of this grant, Larry W. Robertson, Ph.D., received job offers for faculty positions at the University of Iowa's College of Public Health, Department for Occupational & Environmental Health, which we accepted. We moved during the first quarter of this year (March/April) and are now officially employed faculty members of the University of Iowa. This grant was helpful in obtaining these positions. Iowa has a relatively high rate of prostate cancer (exposure to halogenated organic pesticides?!?) and a strong research component in this area. Our new location is therefore very promising with respect to possible fruitful cooperations with our colleagues in other departments.

CONCLUSIONS

We learned from these experiments that 1.) the recently discovered environmental and human contaminant group, the polybrominated diphenyl ethers (PBDEs) seem to be less health threatening than the PCBs for the endpoints tested, but should not be overlooked, since they have health damaging effects as well, 2). Substituting bromine for chlorine makes biphenyls more health damaging, indicating that brominated pesticides may deserve attention, 3.) complex changes in lipid homeostasis, vitamin levels and redox status occur that need to be carefully taken into consideration during all future in vivo experiments, 4.) cell culture experiments with prostate cells should use more than one pair of androgen sensitive/androgen insensitive cells, if we want to avoid misleading conclusions. These initial observations regarding in vivo toxicity, prominent markers of change (i.e. α-tocopheryl quinone), and comparisons of sets of cell lines offer promising insights for future mechanistic studies and possible population investigations. Overall these results provide a firm scientific basis for the work that is proposed and represent a strong position for the research to follow.

REFERENCES

N. Kato, Y. Momota and T. Kusuhara (1989) Changes in distribution of alpha-tocopherol and cholesterol in serum lipoproteins and tissues of rats by dietary PCB and dietary level of protein. J Nutr Sci Vitaminol 35 (6), 655-60.

N. Koremura, M. Saito, T. Hasegawa and S. Innami (1990) Effect of polychlorinated biphenyls on tissue distribution and intestinal absorption of alpha-tocopherol in rats. J Nutr Sci Vitaminol 36 (5), 457-65.

H. Ohchi, T. Kusuhara, T. Katayama, K. Ohara and N. Kato (1987) Effects of dietary xenobiotics on the metabolism of copper, alpha- tocopherol and cholesterol in rats. J Nutr Sci Vitaminol 33 (4), 281-8.

M. Saito (1990) Polychlorinated biphenyls-induced lipid peroxidation as measured by thiobarbituric acid-reactive substances in liver subcellular fractions of rats. Biochim Biophys Acta 1046 (3), 301-8.