Newly Arising Endocrine Disrupters: UV Filters in Cosmetics

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I wish to thank the Ministry of Environment of Japan and the organizers to invite me to this wonderful country. We all know that the PCBs are going down, but we still have a lot of chemicals around. Chemicals have become part of our culture. We use them, we eat them, we have them in the environment. But do we know where they really are, do we know where they are present, do we know whether they are dangerous or not? We do not have much information on that.

Maybe the story on the endocrine disrupting UV filters will help us a little bit to unravel this. I will give you some context on these UV filters. Then, I go over to the identification *in vitro* and *in vivo*, and then I will talk about the risk assessment.

Why did we come up with UV filters? You all know that we have a thinning out of the ozone layer. What do we do? We just increase sun protection factors that will help.

There is also increased use of UV filters in cosmetics, in day and night creams, even in lotions, hair sprays, hair gels, shampoos, bubble baths, lipsticks and so on, but they are often not declared. Then they are used as cosmetic product protection and not declared.

There is another big amount of chemicals used for production of technical products. We do have UV filters in plastics, in paints, fibers, carpets, curtains, everywhere, and we do not have the information what they are and how much there is in there.

Just to give you an example, this nice lady looks so nice because she is using this day cream and it says there is UV filter in there, and the marketing says that this is rejuvenating youth. She is also using the hairspray that has a UV hair protectant, and her children walk around with T-shirts that have UV protection in them.

Lately, when I went to one of the largest department and food chain stores in Switzerland I detected the tissue softener Exelia Suncare. What does it mean? I turn around the bottle, it says if you wash your clothes one time you get the sun protecting factor eight, five times gives you 20, and 10 times gives you the protecting factor to your clothes not to yourself, of 30, but by that time the bottle is empty and it is highly recommended to continue using it because your clothes might otherwise loose sun protection.

What about sun protection factor in UV screens? They have increased from the 1950s up to today from 1-2 to 30 or 60+ that we use today. Of course, the amount of chemicals added to these products has also increased. But there is no indication how much is in the cream, because it is dependent on the vehicle.

The UV filters have become in the meantime ubiquitous pollutants. They are present in human milk, in lakes and fish, and they are also present in organic solvents. Even in organic solvents of high purity used in massspectrometry, we do find peaks of 4-methylbenzylidene camphor. We also find them in lakes like in Lake Zurich and in Lake Hutten, depending on the season.

This is the GCMS analysis of a sample of human milk. A colleague who just had a baby brought some human milk and here are the identification peaks of the UV filter OMC (Octylmethoxy cinnamate) – standard and the peaks in the human milk.

I show you here the first 6 filters we had been testing. The red-ones are estrogenic, the green ones anti-androgenic. Just a single one out of these 6 was not active. You also see the very different chemical groups that show endocrine activity.

Now to the screening tests. First you do an ***in vitro*** screening. To test estrogenicity we used the MCF-7 breast cancer cells we received from Ana Soto (Tufts University, Boston, US). The androgenic/antiandrogenic activity we measured with MDA-KB2 cells from Earl Gray at U.S.

Upon estrogens or substances that act like estrogens MCF-7 cells proliferate. The MDA – kb2 cells that have a luciferase reporter gene produce luminescence upon androgens.

You see here the peak growth of the cells following addition of 17 β Estradiol and here following the UV filter 4-methylbenzyliden camphor. Of course the concentrations used for the two are very different. When you in addition to the estrogenic substances add an antiestrogen in nM concentration cell growth is no longer observed. The same is true for the negative control, that is medumi without substances.

If you look at the EC50, the concentrations where you have half maximal growth; then all of the UV filters we have been looked at so far, are in the μ M (micromolar) range, while 17 β Estradiol is in the pM (picomolar) range. There is a one million times difference between the activity of Estradiol and the estrogenic chemicals.

Measuring anti-androgenicity, the MDA-kb2 cells expressing the human androgen receptor – on this figure we have here the relative intensity of luminescence (y – axis) and here the concentration of our substance (x – axis). You first add 0.5 or 0.1 nM of dihydrotestosterone to the wells of the plate and thereafter your substance. With the antiandrogen flutamide in increasing amounts you see a fall off of the activity here at higher concentrations. Such a fall off is also seen with the 2 UV filters benzephenone-3 and homosalate. These 2 substances are therefore considered to be antiandrogenic. Taken together we can say that the *in vitro* estrogenic and antiandrogenic effects we see with UV filters (expressed as EC50`s) are well in the range of other substances in the environment.

Then, this past summer we detected another UV filter, 3-benzylidene camphor, which is about 5 times stronger active than 4-methylbenzylidene camphor on the MCF-7 cells.

Let us turn now to the ***in vivo*** **assays.** So far we have the uterotrophic assay working, and I will show you some data on that. These are our Long Evans rats, a very happy group of animals. On this slide you see the uterine weight development in our Long Evans rats and in our hairless rats that I shall present to you later.

From day 20-26 there is no increase in uterine weight in both species and then suddenly the uterine weight is increasing upon hormonal influence. If you treat the animal during the period from postnatal day 21-24 or 20-23, with estrogens or xenoestrogens, uterine weight will increase prematurely.

Our results with the first UV filters. Here you see the growth curve of the immature uterus following ethinyl estradiol . The UV screens act at much higher concentrations with an increase in weight. Also, the concentrations you need in this assay and the EC50s reach much higher values than you obtain in cell cultures.

You should not take these EC50s and calculate chronic exposure risk assessment as has been done by the European Commission for Cosmetics and Nonfood Products. This test is an identification test and by no means a test to calculate risk assessment.

This is 3 benzylidene camphor and here you see the EC50 of this substance is about 7 times lower than the one for 4-methylbenzylidene camphor.

If you want to see how this test looks like, you see here to the left a control uterus and the ovaries. The weight is 22mg.On the right, you see an uterus that has been exposed 3 times to 37 mg/kg 3-benzylidene camphor. The treatment doubled the weight that is now 45mg and we can see all these many ingrowing blood vessels. 3-benzylidene camphor shows significant effects on uterine weight down to the level of 4 mg/kg. This is, as far as I know the lowest amount needed for an acute effect by a substance in the environment. You can buy this by the kilo, and you can also throw it down the drain and nobody would care.

I will just go briefly over our results on effects via transdermal route. These are our hairless rats, and

this female likes it to be in a bath that is slightly warm. We add the substances to the olive oil at concentrations that are admitted by the law. The rat is wearing a collar so she cannot scratch her face and lick her paws that would allow an oral exposure. After the bath we let the animals dry, then we clean them and put them back in the cage.

Here you see the results of one of the substances: 4-methylbenzylidene camphor. The uterine weight increases according to the concentration in the medium. It increases up to 5%. 4% is allowed in sunscreens. If the concentration is higher than 5% the animal is losing weight. At 10%, it is not surviving. We do not understand this event as yet. In these uteri we also measure the mitosis index using bromodeoxyuridine uptake.

Following the positive identification tests we now switch over to **risk assessment.** We do RA with an extended one-generation test. We did this test first with 4-methylbenzylidene camphor that was the most active UV filter tested at the time.

4-methylbenzylidene camphor was given to the animals in feed for 10 weeks. Then we mate them and we look at the offspring at postnatal day 1, postnatal day 14, around puberty, when they are young adults, and also we let some of them grow older.

What did we see? First we looked at the survival rates at postnatal day 2 and 14 and we see a very strong reduction at the higher doses and even at intermediate doses we have a reduction in survival. We crossed the highest dose of 70mg/kg (100mg/kg in feed) because of high perinatal toxicity.

What is interesting is that we do have a greater effect in the females. The females apparently are suffering more, and we now closely observe the litters with regard to sex ratio at birth.

First we wanted to know whether this is a pre- or a postnatal event. We can already exclude a possible high postnatal impact. We do a crossfostering experiment i.e. we give control pups (no prenatal exposure) to lactating mothers exposed to 47mg/kg. You see here the control males and females and these were the exposed males and females. In this experiment the pups with exclusively postnatal exposure perfectly survived suffering only from a small reduction in weight.

This tells us that the prenatal effect maybe toxicologically more decisive. We also see a relative weight decrease in both male and female thymus at birth. As yet we do not understand this perinatal toxicity of 4-MBC. Let us move on to later life toxicity that appears to be more connected to the endocrine disrupting activity.

We looked at developmental toxicity, puberty and reproductive organ development. What we see in puberty is shown on this slide. 2 big series of treated animals were done in summer 2001 and in spring 2002. The results from these series indicate a significant shift in puberty towards later development. Results are very close and significant also for the 7mg/kg (low dose). They are not season-dependent. We also do see differences in testis weight early, at postnatal day 14, and in young adult animals. Note that testis decrease at postnatal day 14, relative and absolute weight, and in the young adults we did see a weight increase.

Male body weight remains unchanged. There is one treatment group that is slightly different. Seminal vesicles are increased and ventral prostate shows decreased weight.

Now let us switch to gene expression. We looked at estrogen dependent gene expression in the steady state animals. There are some changes at distinct dose levels in uterus and prostate in gene expression. We use real time PCR and cyclophilin as a reference gene.

We are looking at progesterone receptor, IGF-1 receptor, ER alpha and beta and AR receptor mRNAs. Some effects are seen in the uterus at different doses for IGF-1 and progesterone receptor. And in ventral prostate we also see effects for IGF - 1 and AR.

Though we also have looked in the brain, I like to remind you on presented data yesterday evening, that the fetal testis is producing in early life testosterone. The hormone is entering neurons in the brain

where it is aromatised to estradiol, that is binding to the estrogen receptor in cns neurons. Resulting proteins or mRNAs will masculinize the brain.

We dissected brain tissue of the **ventromedial hypothalamic nucleus**, which is a region that is very closely connected to female sex behavior and also part of the medial preoptic area which is also a sexually dimorphic region in the brain. The estrogen receptor alpha mRNA was greatly different between offspring of control and exposed mothers. There is also a significant difference in female and male controls, the males having a much lower level of progesterone and estradiol alpha receptor mRNA expression as compared to females. There are in addition significant changes in ER alpha and progesterone receptor expression in animals that had been brought up on different 4-MBC containing feed. Interesting enough, levels of estradiol –and progesterone receptor mRNA expression in exposed females approach levels seen in control males.

In the **medial preoptic area,** we see a different pattern of mRNA expression. There is no significant difference between male and female patterns and also less differences between control and exposed animals. We do not see much in the pre-proenkephalin but we do see differences in the progesterone- and estrogen alpha – receptor mRNAs in the preoptic area. That means each individual brain region at each treatment might differ in estrogen induced mRNA expression patterns.

We did not see extensive changes in the patterns of estrogen induced mRNA in peripheral organs of steady state adult animals. We therefore wanted to see how the system would be reacting to the hormone when we deprive it from sex hormones by gonadectomy two weeks before such a hormonal challenge is done.

You see the results here. This is the uterine Progesterone and IGF –1 receptor mRNA expression two weeks after gonadectomy followed by an injection of 10 or 50 microgramms of 17 β estradiol and lasting for 6 hours. While in the control animal the increase in PR and IGF-1 receptor mRNA is high and dose dependent, we see a significantly lower and dose dependent increase in the 4-MBC exposed animals with less response in the animals exposed to the higher dosage of 4–MBC.

The last slide here is IGF receptor mRNA expression in ventral prostate. The picture is different insofar as upon challenge with 17 β estradiol. We see here a repression of IGF –1 receptor mRNA expression in controls and a dose dependent smaller repression with higher dosages of 4-MBC. The sensitivity to 17 β estradiol of the system appears to lessen in UV filter exposed animals, also at low dose exposure.

These are about all of our data, but I would not like to stop until I show you some picture of effects of the 3BC in fish. We see the first publication of Henrik Holbech et al. from Denmark showing that the 3-benzylidene camphor injection in male trout induces dose dependently vitellogenin in male trout.

You see here the increase in vitellogenin following 17 β estradiol and following different doses of 3-benzylidene camphor. This substance when gaining access to the environment might, or I am almost sure it will have some effects on these animals. Let us come to the end. What did we really do?

We identified endocrine activity in UV filters, but I have to say again that so far we have only looked at UV filters in cosmetics. This might just be the tip of the iceberg. We might have a lot more UV filters in technical products as I told you, and as adjuvants in other preparations that we do not even know.

From the 30 UV filters admitted for use in sunscreens, 6 out of 8 were tested positive *in vitro*, and 4 out of 8 filters were tested positive *in vivo*.

In risk assessment, we realized that 4-MBC is greatly toxic in the perinatal period. So far, we have identified a LOAEL (lowest observed adverse effect level) of 7 mg/kg of body weight. The steady state gene expression levels in periphery and brain show also that there are effects at this level and there is definitely a change in sensitivity to the hormone when these animals are developmentally exposed to these substances.

The outlook: we shall continue our identification process *in vitro* and *in vivo* and we would like to push the risk assessment to obtain a NOAEL for these substances as so far we only have a LOAEL of 7mg/kg body weight. We shall continue with low dose experiments and also our animals are already feeding on 3-benzylidene camphor in a new developmental toxicity study. Thank you for your attention.