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Persistent organic pollutants have dose and CAG repeat length dependent effects on androgen receptor activity in vitro

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Abstract

Recently, the effect of exposure to persistent organic pollutants (POPs) on sperm concentration was only seen in men with a short androgen receptor (AR) gene CAG repeat. In order to investigate whether these effects could be observed also *in vitro*, we tested the impact of 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) and 1,1-*Bis*-(4-chlorophenyl)-2,2-dichloroethene (4,4'-DDE) on 5α-dihydrotestosterone activated ARs containing 16, 22 and 28 CAG repeats, respectively. Single exposure to 4,4'-DDE had the most pronounced effect on the AR activity containing 16 CAG repeats, whereas 28 CAG was the most sensitive variant when a mixture of the two compounds was added. Thus, our *in vitro* results have confirmed the *in vivo* data indicating a CAG repeat length dependent effect of endocrine disrupters on the AR activity.

Keywords: Androgen receptor activity, CAG polymorphism, Persistent organic pollutants, 4,4'-DDE, CB-153

Abbreviations and definitions

POPs: Persistent organic pollutants

AR: Androgen receptor

PCB: Polychlorinated biphenyl

4,4'-DDT: 2,2-*Bis*-(4-chlorophenyl)-1,1,1-trichloroethane

4,4'-DDE: 1,1-*Bis*-(4-chlorophenyl)-2,2-dichloroethene

CB-153: 2,2′,4,4′,5,5′-Hexachlorobiphenyl

DHT: 5α-Dihydrotestosterone

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1. Introduction

Exposure to persistent organic pollutants (POPs) has been suggested as potential cause of reproductive disturbances including reduced semen quality [1], testicular cancer [2], prolonged waiting time to pregnancy [3], imbalanced sex ratio [4-5] and altered age of sexual maturation [6]. These compounds are resistant to abiotic as well as biotic degradation and exposure therefore results in bio-accumulation. In humans POPs are stored in adipose tissue, serum and breast milk [7-8]. Following the Seveso accident in 1976, exposure of males with low concentrations of dioxin in utero and after birth through breastfeeding, resulted in a 50% reduction of sperm concentration and total sperm counts in young adulthood [9].

The most widespread POPs are polychlorinated biphenyls (PCBs) and 2,2-*bis*-(4-chlorophenyl)-1,1,1-trichloroethane (4,4'-DDT) [10]. Although the production of PCBs and 4,4'-DDTs has been banned for almost 40 years in most countries, these chemicals are still detected in animals and humans all over the world. PCBs are by-products of combustion and have also been used as electrical insulators [10] and 4,4'-DDT has been, and still is, widely used as an insecticide in the combat of malaria (http://www.atsdr.cdc.gov). 4,4'-DDT is metabolised to the persistent metabolite 4,4'-DDE (1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene), which accumulates in fatty tissue and is a marker of long-term DDT exposure. When entering the body, 4,4'-DDE inhibits androgen binding to the androgen receptor and androgen induced transcriptional activity [11]. In epidemiological studies in malaria endemic areas in Mexico and South Africa, negative effects of 4,4'-DDE on sperm motility and morphology have been reported [12-13]

With respect to PCB, similar to observations from the population exposed to DDT, PCB-contaminated rice oil in the so called Yusheng accident in 1979 in Taiwan was associated with impaired sperm quality [14]. In the Yusheng accident the exposed fathers also had a decreased male to female offspring ratio, particularly if exposed during adolescence [4].

The PCB congener, 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) is one of the most abundant congeners in biological extracts and correlates with the total lipid adjusted concentration of PCBs in plasma [10, 15]. It has in experimental studies been shown to have either no effect or androgen receptor antagonistic properties [16-17].

Recently, in an EU supported project including Greenland Inuit, Polish, Ukrainian and Swedish men (www.inuendo.dk), serum levels of 4,4'-DDE and CB-153 were measured and correlated to sperm parameters [18]. In this study, a negative correlation between CB-153 and sperm motility was observed, but, in general, no association between POP levels and sperm concentration was found. However, when also AR genotype was taken into account, high CB-153 exposure was linked to 35% reduction in sperm concentration in subjects with an androgen receptor (AR) containing a short glutamine stretch (<20 amino acids), comprising approximately 20% of the study population, [19]. The glutamine stretch is encoded by a number of CAG bases in the AR gene, commonly referred to as the CAG repeat. The CAG number varies in length between individuals from approximately 10 to 30 repeats and displays population based diversity with African Americans having on average fewer CAG repeats than Caucasians and Far-Eastern Asians [20-21]. In Caucasian populations the median CAG repeat length is 22 amino acids [22-23], which recently was demonstrated to exhibit the highest transcriptional activity as compared to CAG repeat lengths in the outer normal ranges [24] .

In order to investigate whether effects of POPs on AR activity were CAG length dependent in an in vitro setting, we tested the impact of CB-153 and 4,4'-DDE on 5α-dihydrotestosterone activated ARs containing 16, 22 and 28 CAG repeats, respectively. The effects of these two compounds were tested after single exposure and also in mixture.

2. Methods

2.1. Cell culture and transfection

African green monkey kidney cells COS-1 (ECACC, Salisbury, UK), that do not express endogenous AR, were incubated at 37°C with 5% CO_2 and grown in Dulbecco's Modified Eagle Media (DMEM) (Gibco, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (Saveen & Werner AB, Limhamn, Sweden), 2 mM L-Glutamine (PAA, Pasching, Austria) and 0.02% Gentamicin (Gibco, Invitrogen, CA, USA). Approximately 150.000 cells per well were seeded in 12-well plates and grown for 24 hours to reach 50-80% confluence.

Three different CAG stretches: 16, 22 and 28 together with 23 GGN in the pCMV4-AR plasmid had previously been constructed [24-25]. The cells were transiently co-transfected by using 2.4 µl FuGENE 6 transfection reagent (Roche diagnostics, Bromma, Sweden) in each well, with either 200 ng of the pCMV4-AR construct or with pCMV4 lacking the AR, together with 600 ng of the pGL3-basic vector containing the firefly-luciferase reporter gene driven by the human prostate specific antigen (PSA) promoter harbouring an enhancer sequence with androgen-responsive elements.

After 24 hours, the media was changed to phenol-red free DMEM, containing 10% dextran charcoal-treated stripped serum and the same supplements as mentioned above, together with 10 nM 5α-dihydrotestosterone (DHT) and one of following:

a) no POP (unexposed)
b) CB-153 in low (1 nM) or high (10 nM) concentration;
c) 4,4'-DDE, in low (1.7 nM) or high (17 nM) concentration;
d) Both CB-153 (10 nM) and 4,4'-DDE (17 nM) in high concentrations (mixture);
e) 10 x the mixture (100 nM CB-153 and 170 nM 4,4'-DDE);
f) 100 x the mixture (1 µM CB-153 and 1.7 µM 4,4'-DDE).

The hormone was dissolved in ethanol and the POPs in methanol. The final concentration of ethanol/methanol together with the POP was less than 0.4%, which did not affect the viability of the cells.

All alternatives were also tested without addition of DHT.

Low and high POP concentrations corresponded to those measured in serum lipids of the Inuendo cohorts (Table 1). The Greenland Inuit and Swedish fishermen were exposed through consumption of sea food, Ukrainians mainly through farming and Polish men represented a general European population. Higher doses of the mixture were also tested to scrutinize if the CAG length dependent effects persisted.

The samples were incubated for another 24 hours before harvest.

2.2. Reporter gene assay

The cells were washed once with Phosphate-buffered saline (Invitrogen, Stockholm, Sweden), lysed with passive lysis buffer (Promega, Stockholm, Sweden) and centrifuged for 1 minute at 12.000 g. The Luciferase activity was measured with the Dual-Luciferase reporter system (Promega, Stockholm, Sweden) in a plate luminometer according to the protocol provided by the manufacturer. The amount of luciferase from 6-8 performed experiments in duplicates was adjusted for total protein amount, determined by Quick start Bradford protein assay (Bio-Rad, Sundbyberg, Sweden).

2.4. Statistical analysis

The present study aimed to investigate whether the effect of POP exposure on the AR activity was dependent on the CAG repeat length (16, 22 and 28). Primarily, for each set of experiments with all CAG repeat variants and a certain exposure type, we evaluated whether POP exposure

induced a change in AR activity. By using Wilcoxon's test for paired data, we compared the activities with and without POPs exposure. Thereafter, for each of the three CAG lengths, ratios between AR activity with and without POP exposure were calculated and compared by use of Friedman's test. If this test showed a statistically significant difference ($p<0.05$), pair-wise comparisons of ratios were performed (16 vs. 22; 16 vs. 28 and 22 vs. 28) using Wilcoxon's test. In order to define the absolute AR activity of the three CAG repeat length variants in relation to each other, in the presence or absence of POP, the same statistical tests were used.

All analyses were carried out by SPSS software (SPSS for Windows 15.0 or 18.0; SPSS Inc., Chicago, IL).

3. Results

3.1. AR activity in response to single exposure of CB-153 or 4,4'-DDE

As compared to the other two lengths, the AR containing the 22 CAG repeat variant exhibited the highest activity, not only when stimulated with only DHT ($p<0.0001$; Fig 1), but also if CB-153 ($p\leq0.028$) or 4,4'-DDE ($p\leq0.028$) was added (Fig 4).

No CAG repeat length related relative changes in response to low concentration of CB-153 or 4,4'-DDE were found. When the activity of each CAG repeat length after exposure to high CB-153 was compared to the baseline activity, an increase was seen for 16 CAG and 22 CAG (medians: 27% and 29%, respectively), whereas a decline in receptor activity was observed for 28 CAG (median: 11%; Fig 2A). The baseline defines the line of no effect of POP exposure corresponding to the activity due to DHT only. These differences were however not statistically significant.

When the relative changes in receptor activities were compared between different CAG lengths (Friedman's test) the variants seemed to respond differently to high CB-153 ($p=0.030$; Fig 2A). Pair-wise comparisons showed that the effect of high CB-153 on the 28 CAG repeat variant differed from that on 16 CAG and 22 CAG ($p=0.028$ and $p=0.043$, respectively).

With respect to 4,4'-DDE in high concentration, 16 CAG was statistically significantly hampered with a median 23% reduction in activity compared with the baseline activity ($p=0.018$; Fig 2B), whereas no statistically significant effects were noted for 22 CAG and 28 CAG.

Comparing the three lengths as considers relative changes in activity following addition of the POP, a borderline statistically significant difference in response was found ($p=0.054$; Fig 2B). For the 16 CAG repeat variant, the relative activity after addition of 4,4'-DDE was (in median) 30% lower as compared to 22 CAG and 17% lower compared to 28 CAG ($p=0.018$ and $p=0.063$, respectively).

No POP induced AR activity was observed in the absence of DHT.

3.2. AR activity in response to mixed exposure of CB-153 and 4,4'-DDE

After exposure to CB-153 and 4,4'-DDE in a mixture of 1, 10 and 100 times the highest concentration (d, e and f; described in methods 2.1), the activity was still highest for the 22 CAG repeat variant ($p \leq 0.028$; Fig 4).

After exposure to 1 x the mixture (d), a median 24% reduction in activity of the 28 CAG variant was noted ($p=0.016$; Fig 3) compared with the baseline activity, whereas no statistically significant effect was found on 16 CAG and 22 CAG. The CAG-length dependent difference in response was also seen using Friedman's test ($p=0.018$). Pair wise comparisons revealed that the effect of the mixture was most pronounced for the 28 CAG repeat variant, which displayed (median) 13% lower relative activity as compared to 16 CAG and 32% compared to 22 CAG ($p=0.018$ and $p=0.028$, respectively).

Ten times the mixture (e) seemed to decrease the activity for all variants (medians: 16 CAG: 15%; 22 CAG: 15% and 28 CAG: 11%), but these differences were not statistically significant.

Exposure to 100 x the mixture (f) hampered the activity of all variants (medians: 16 CAG: 76%; 22 CAG: 74% and 28 CAG: 79%, $p=0.028$ for all comparisons). No statistically significant difference in effect between the lengths was noted.

4. Discussion

Our in vitro study confirmed the previous in vivo results showing a CAG length dependent effect of POPs exposure on AR activity. Short CAG repeat length and high PCB exposure has previously been associated with lower sperm concentration in an epidemiological study [19] and this finding was the starting point of current in vitro study. Our experimental data show that the AR variant containing the shortest CAG repeat indeed displayed lower relative activity than the longer variants in response to 4,4'-DDE, but in contrast to the epidemiologic result, this was in response to high 4,4'-DDE exposure and not to CB-153. An explanation for the discrepancy between the experimental and the epidemiological results could be the high correlation between CB-153 and 4,4'-DDE [26]. These congeners occur in lipid adjusted samples almost in the ratio 1:1, which could influence previous epidemiological results. Other unmeasured compounds or congeners associated with both CB-153 and 4,4'-DDE could also play a role for the epidemiological findings.

Furthermore, our in vitro results are to be considered as a "proof of concept" of the interaction between POP exposure and CAG repeat length in relation to AR activity. Unlike the in vitro situation where the activity of the AR can be directly assessed, in vivo a lot of other physiological factors as e. g. the influence of other sex steroids, hormone metabolism and the hypothalamo-pituitary-gonadal axis may have an impact on the androgenic activity. Thus, the effects observed in these two situations are not directly comparable. However, the recent finding that in utero and lactational exposure to relatively low dioxin doses can have negative effects on the reproductive system and alter the secretion of reproductive hormones, decades after exposure [9], demonstrates the importance to investigate the mechanisms underlying the impact of POPs on androgen dependent functions in males.

Since the in vivo exposure includes a combination of several compounds, we also investigated the effect of a mixture of CB-153 and 4,4'-DDE on AR activity. Interestingly, in concentrations corresponding to levels measured in serum of Greenland Inuit men, a combination of these two POPs had most pronounced effect on the activity of the AR containing a long CAG. This gene-environment interaction, as demonstrated in vitro, shows a heterogeneous picture with the variant with short CAG being mostly affected when a single compound is added and the one with long repeat following exposure to a mixture of two POPs. We also found a dose dependent negative response to the mixture. Both effects could either be due to intervention with the transcription machinery, which also has been demonstrated in previous in vitro studies with 4,4'-DDE and PCB congeners [16, 27], or a result of an inhibitory effect at the RNA-level thereby preventing protein translation. The compounds could possibly also promote degradation of the AR protein in a dose dependent fashion.

In agreement with a previous study [24] we noted that the median long CAG repeat was highest in basic activity in the presence of the natural hormone DHT, but also that it was most robust after exposure to POPs. It has been anticipated that since between 91-99% of the AR gene CAG repeat alleles in different ethnic groups are between 16-29 repeats, this could be the range that maintains maximum interaction between the transactivating domain and the hormone binding domain of the AR and has therefore been preserved during evolution as an important component of androgen induced AR signalling [28], most resistant to adverse effects of different kinds.

In conclusion, we found a CAG repeat length dependent effect of POPs exposure on the transactivating ability of the AR. The median long CAG repeat variant, also corresponding to median length among Caucasians, showed the highest activity at all types of exposure. Otherwise, the level of suppression of the AR was dependent on type of exposure and CAG number. However, the modifying effects of the repeat lengths was only seen at exposure levels

comparable to those found in exposed humans, whereas at higher concentrations, the modifying effects of the AR polymorphisms were absent.

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Table 1. POP concentrations used in the experiments, measured in serum of men in three European populations and the Greenland Inuit.

	CB-153		**4,4'-DDE**	
	High	Low	High	Low
Greenland	x		x	
Poland		x		x
Sweden	x			x
Ukraine		x	x	

CB-153; 2,2′,4,4′,5,5′-hexachlorobiphenyl. 4,4'-DDE; 1,1-*bis*-(4-chlorophenyl)-2,2-dichloroethene. High CB-153; 500 ng/g (10 nM). Low CB-153; 50 ng/g (1 nM). High 4,4'-DDE; 700 ng/g (17 nM). Low 4,4'-DDE; 70 ng/g (1.7 nM).

Legend to figures

Figure 1. The AR activity in the presence (black bars) and absence (white bars) of 10 nM DHT, where the activity of 22 CAG with DHT was set as 100%. The results are based on duplicate values from 27 experiments.

Figure 2. The relative change in AR activity in cells stimulated with DHT together with (A) CB-153 (10 nM) or (B) 4,4'-DDE (17 nM) in high concentration. Bars correspond to median values, boxes to the inter-quartile range (25th-75th percentile) and whiskers to the minimum and maximum values. Outliers are marked with ° and extreme values with *.

Figure 3. The relative change in AR activity for cells stimulated with DHT together with the mixture in 1x (10 nM CB-153 and 17 nM 4,4'-DDE), 10 x (100 nM CB-153 and 170 nM 4,4'-DDE) and 100 x (1 µM CB-153 and 1.7 µM 4,4'-DDE) the high concentration**.**

Figure 4. The DHT-induced AR activity at all POP concentrations.

Figure 1.

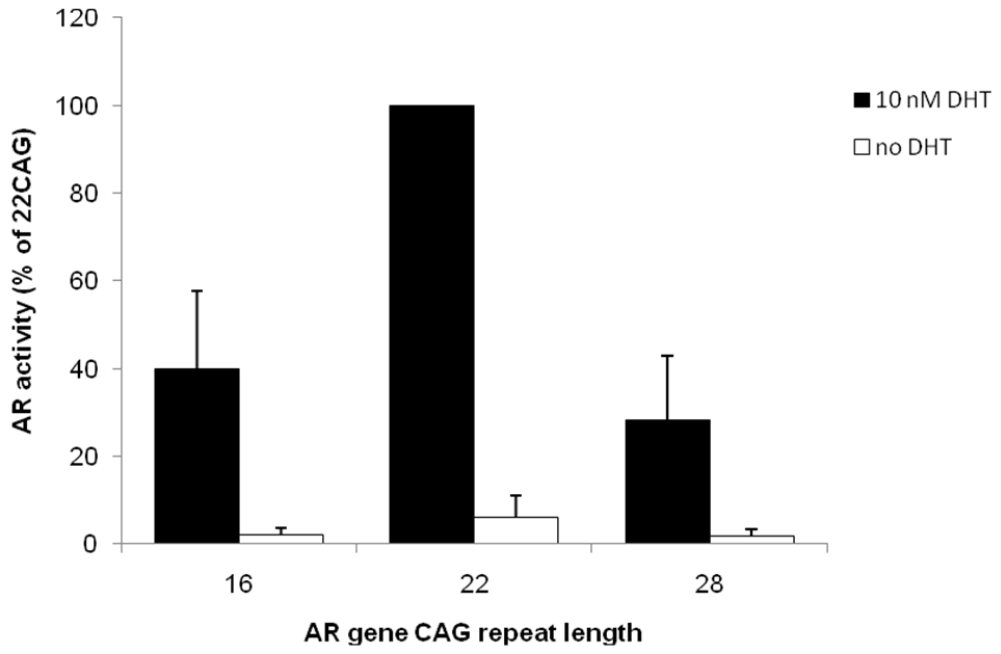

120
100
80
60
40
20
0
AR activity (% of 22CAG)
10 nM DHT
no DHT
16
22
28
AR gene CAG repeat length

Figure 2A.

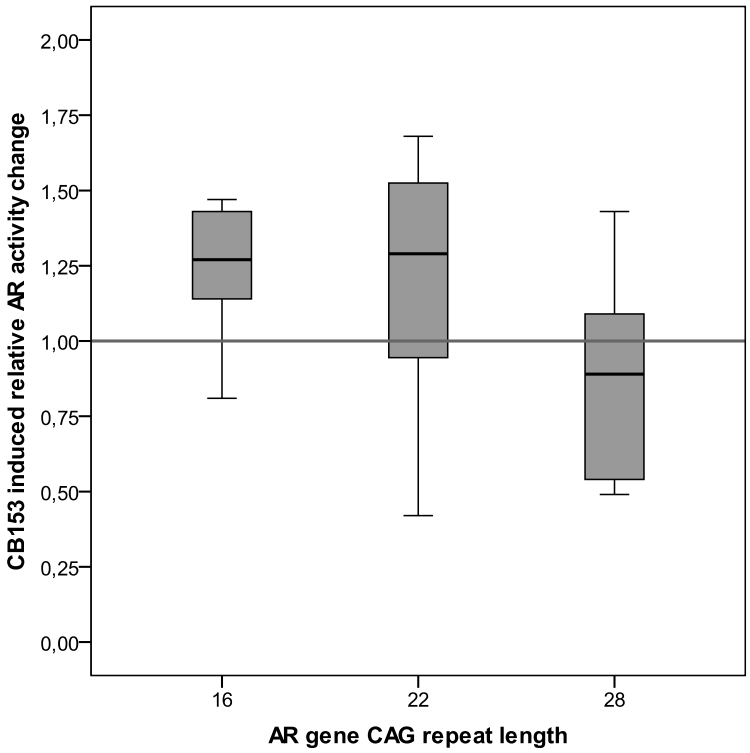

CB153 induced relative AR activity change
2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0,00
16
22
28
AR gene CAG repeat length

Figure 2B.

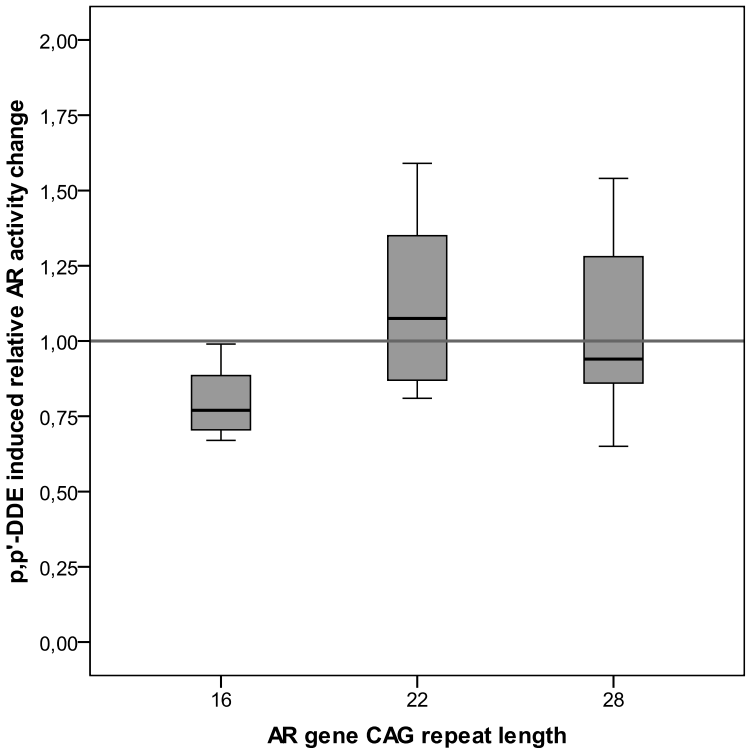

2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0,00
p,p'-DDE induced relative AR activity change
16
22
28
AR gene CAG repeat length

Figure 3.

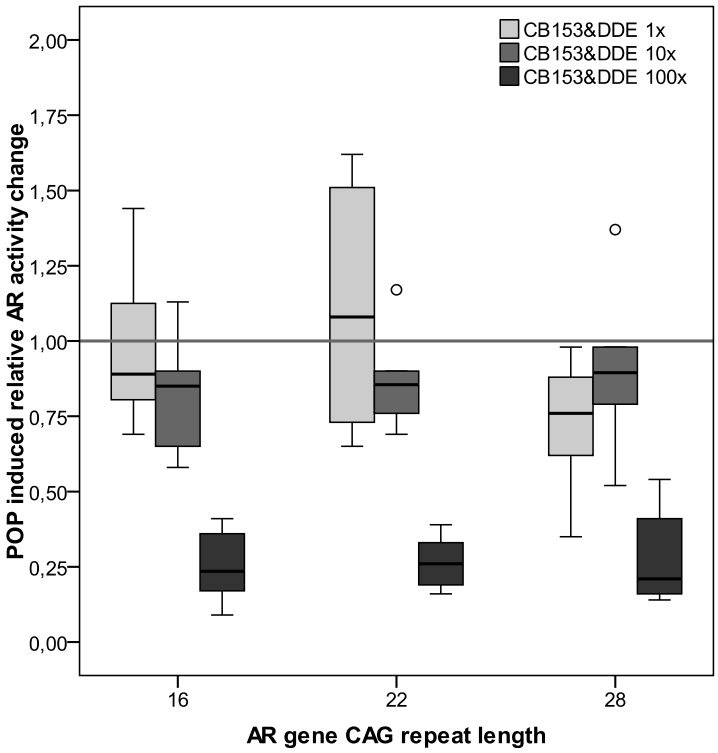
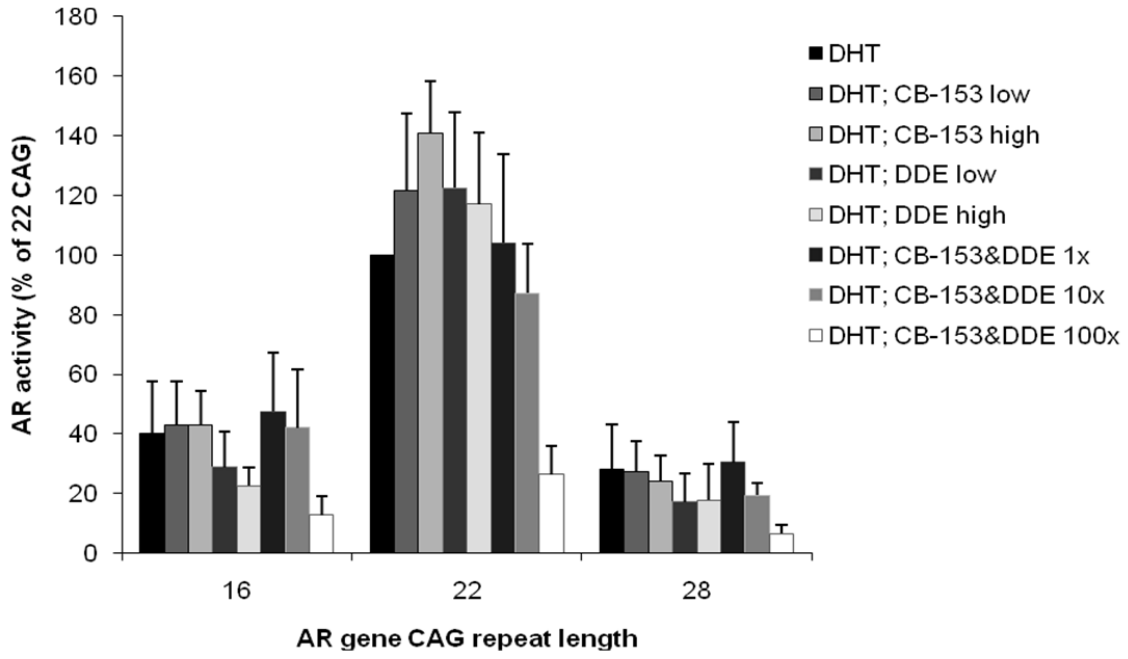

CB153&DDE 1x
CB153&DDE 10x
CB153&DDE 100x
POP induced relative AR activity change
2,00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0,00
16
22
28
AR gene CAG repeat length

Figure 4.


180
160
140
120
100
80
60
40
20
0
AR activity (% of 22 CAG)
16
22
28
AR gene CAG repeat length
DHT
DHT; CB-153 low
DHT; CB-153 high
DHT; DDE low
DHT; DDE high
DHT; CB-153&DDE 1x
DHT; CB-153&DDE 10x
DHT; CB-153&DDE 100x