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Toxicological effects of food additives – Azo dyes

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Toxicological effects of food additives – Azo dyes

Toxikologiska effekter av livsmedelstillsatser – azofärger

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ABSTRACT

Background: Azo dyes are widely used in foods, pharmaceuticals and cosmetics. They have been tested in several screenings regarding their effects on human health, but very little data is available on endocrine disrupting effects. The aim of this study has been to screen the effects of four azo dyes in different toxicological assays in the human adrenocortical H295R cell line. These results could be of importance for the evaluation of health effects of azo dyes.

Methods: Using different *in vitro* models, we have examined the effects of azo dyes on oxidative stress, sex hormone production and gene expression of transport protein. The oxidative stress response was studied using luciferase reporter assay, the sex hormone production was studied with ELISA and gene expression was studied using real-time PCR. Further, we have used MTS assay to investigate the general cytotoxicity induced by azo dyes.

Results: This study show that Brilliant Black down regulate the gene expression of the transport protein BCRP in H295R cells and that sunset yellow induces oxidative stress in H295R cells.

Conclusion: This project presents a toxicological screening of azo dyes using multiple toxicological endpoints. We conclude that azo dyes can induce oxidative stress response and alter gene expression of transport proteins, in H295R cells. However, further investigation is needed to clarify the toxicity of azo dyes and the mechanisms for these effects.

Keywords: Endocrine disruptor, Human adrenocortical (H295R) cell line, azo dyes, MTT assay, BG1luc4ER cell line, BCRP (Breast Cancer Resistance Protein)

TABLE OF CONTENTS

ABBREVIATIONS

AR	Allura Red AD
BCRP	Breast Cancer Resistance Protein
BB	Brilliant Black BN
DMEM	Dulbecco's Modified Eagle Medium
E2	Estradiol
RPMI	Phenol Red Medium Invitrogen
SY	Sunset Yellow FCF
TA	Tartrazine
T	Testosterone

1. INTRODUCTION

Food additives are natural or synthetic substances added to foodstuffs to improve properties such as antioxidants (to prevent deterioration caused by oxidation), preservatives or sweeteners.

In the European Union (EU) all food additives are given labelling codes commonly referred to as “E-number”. Each food colour authorised for use in EU is subject to a rigorous scientific safety assessment.

Within food additives, 43 are food colours approved by EU which 9 of them are azo dyes.
In this project the human adenocarcinoma cell line H295R, was used to screen toxicological effects of four azo dyes in human adrenal gland. BG1luc4ER ovarian carcinoma cell line was supposed to be used to screen agonistic and antagonistic effects of the substances on oestrogen receptor.

1.1 Azo dyes

Food colours are food additives that are added to food stuff mainly to make up for colours losses during food processing, to enhance natural colours or to add colour to food that would be colourless or coloured differently (EFSA topic, food colours).

1.1.1 *Properties*

Increasingly, natural food colours are being used in foods. However azo dyes are widely used, not just in foodstuff, but also used in pharmaceutical products or cosmetics to be more stable than natural colours. Azo dyes are stable in the whole pH range of food, heat stable, they do not fade exposed to light or oxygen and they are water-soluble. However, azo dyes are not soluble in oil or fat.

As azo dyes are highly water soluble, they do not accumulate in the body, and are metabolised mainly in the liver (by azo reductases) and excreted in the urine. As azo dyes are very strong colour, foods normally are coloured with dyes in levels of mg dye/kg food. The European Food Safety Authority (EFSA), The Panel on Food Additives and Nutrient Sources added to Food (ANS) at the has specified an

Acceptable Daily Intake (ADI) for each azo colorant, which is the amount of a specific colour that may be consumed safely, every day, throughout a lifetime.

1.1.2 *Chemical structure*

Azo dyes are organic compounds which may be used to impart colour to a substance. Dyes are classified according to colour, origin, chemical structure, and kind of material to which they are applied. The most precise and scientific classification of dyes is based on their chemical structure. Azo dyes all contain an azo group, -N=N-, but some contain two (diazo), three (triazo) or more.
Aromatic azo compounds (R = R'= aromatic) are usually stable and have vivid colours such as red, orange, and yellow, that fact can be explained by side groups around the azo bond help to stabilise the N=N group by making it part of an delocalised system often absorb visible frequencies of light.

In this project we have used sulphonated azo dyes, widely used as colouring agents in food stuff, paper, textiles etcetera.

1.1.3 *Safety*

Some azo dyes have been banned for food use due to toxic side effects. These are not due to the dye itself, but to degradation products of the dyes.

Azo linkage may be reduced; this reaction is carried out by an enzyme named azo-reductase. It is a non-specific enzyme, found in various micro-organisms (like intestinal bacteria) and present in various organs like liver, kidney, lung, and etcetera (Brown and DeVito, 1993). A small number of aromatic amines coming from degradation products (such as aniline, toluene, benzidine, naphthalene) have been found to be mutagenic or carcinogenic and subsequently, some dyes were no longer permitted as food dyes. Sulphonated dyes, mainly mono-, di- and trisulphonated compounds are world-wide permitted for use in foods, cosmetics and as drugs for oral application (Danish EPA-Environmental Protection Agency).

European Food Safety Authority (EFSA) has recently performed a series of re-evaluations on the safety of food additives authorized in EU. As part of its systematic re-evaluation, EFSA has carried out new risk assessments of all food colours, especially azo dyes, since Allura red AC has been suspected to produce significant increase of DNA migration in different tissues (Tsuda *et al.*, 2001), though ANS Panel concluded these results were not expected to mean carcinogenicity, as in vivo carcinogenicity studies were negative in mice and rats. Despite this fact, another

recently study from the same group has found positive findings on comet assay in mice but not in rats (Shimada *et al.*, 2010) and it has been suggested there is a pattern of effect related among sulphonated azo dyes structurally related that would require further investigation concerning effects due to metabolites, degradation products or dye itself on genotoxicity and carcinogenicity.

In this project we have used sulphonated mono azo dyes, namely Allura Red AC (E129), Tartrazine (E102), Sunset Yellow FCF (E110), and Brilliant black BN (E151). They are currently approved in the European Union.

Food additives have been related to adverse reactions, especially in asthma (Dipalma, 1990; Lockey, 1977) , and have been subject of debate and concern among population. Concerning about tartrazine effects in asthma patients, it was shown in a bibliographical review of hazard assessment of Tartrazine (M.Ould Elhkim *et al.*, 2007) many clinical trials have been carried out to assess effects and reporting adverse reactions following tartrazine ingestion. However, the exact mechanism why tartrazine increases allergic reactions or asthma is still not fully understood (Randhawa *et al*, 2009). But overall, there is no clear evidence that tartrazine aggravates asthma or avoiding tartrazine makes it better (Ardern, 2012).

Also it has been suggested exposure to azo dyes are associated with increased risk for hyperactivity effects on child behaviour, or increase ADHD (Attention Deficit Hyperactivity Disorder) (Schab *et al.*, 2004). The Southampton study (McCann *et al.*, 2007), commissioned by Britain Food Standards Agency (FSA), tested the effects of azo dyes mixture with sodium benzoate (E211), a common preservative, in 3-year-old and 8/9-year-old children.

The Southampton study report that a mix of additives commonly found in children's food, increases the mean level of hyperactive in children aged. However, "*this study does not prove that colours used actually cause increased hyperactivity in children, it provides supporting evidence for a link"* said Professor Leuan Huges, chair of the COT (Committee on Toxicology). Moreover, the ANS Panel (Food Additives and Nutrient Souces added to Food) concluded that the scientific evidence that is currently available did not substantiate a causal link between these individual colours and possible behavioural effects.

After Southampton study, the European Parliament and the Council of the European Union have made a political decision that food stuff containing 6 azo dyes must be labled with the text "May have an adverse effect on activity and attention in children" (Regulation (EC) No 1333/2008).

Foods containing one or more of the following food colours	information
Sunset yellow (E110)	*"May have an adverse effect on activity and attention in children"*
Quinoline yellow (E104)	
Carmoisine (E122)	
Allura red (E129)	
Tartrazine (E102)	
Ponceau 4R	

Table1: These additives were those included in the two mixtures given to the children of Southampton study, and sodium benzoate (E211). Food or drinks containing any of six artificial colourings may be linked to hyperactive behaviour in children have to carry warnings. (Parliament, Council, The, & Union, 2008)

1.1.4 *Azo dyes studied*

In the United States, food colours additives are named by FD&C (abbreviation from Federal Food, Drug, and Cosmetic Act) accompanied by the colour itself and a number, approved for FDA (U.S Food and Drug Administration). Food colours can also carry other names like Colour Index International (C.I.) where colorants are listed according to Colour Index Generic Names and Colour Index Constitution Numbers and list the manufacturer, physical form and uses.

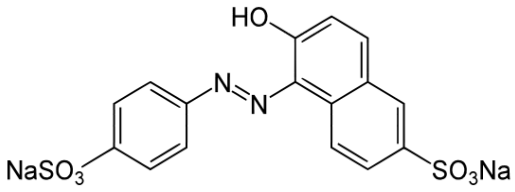
Describing the following substances used in this project, it is important to mention a couple of toxicological terms.

ADI as abbreviation from Acceptable Daily Intake is the amount of a substance used on foodstuff that can be ingested daily over lifetime without health risk. ADIs are expressed usually in mg (of the substance)/kg body weight per day (WHO food safety glossary).This value has been assessed from NOAEL results (No Observable Adverse Effect Level) extrapolated from experimental animals to man. NOAEL could be defined as the highest tested dose or concentration without adverse effect.

1.1.4.1 Tartrazine (E102):

- ✓ FDA: FD&C Yellow Nº5

- ✓ Other names: C.I. 19140, Acid Yellow 23, Food Yellow 4
- ✓ ADI: 7,5mg/kg bw/day
- ✓ IUPAC name: Trisodium(4E)-5-oxo-1-(4-sulfonatophenyl)-4-[(4-

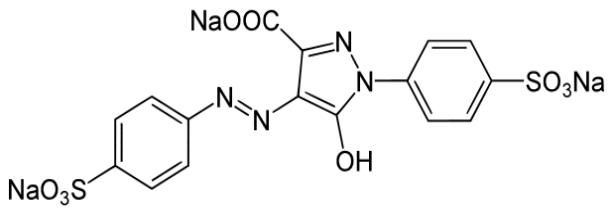


sulfonatophenyl)hydrazono]-3-pyrazolecarboxylate

- ✓ Chemical structure:

1.1.4.2 Sunset Yellow FCF (E110)

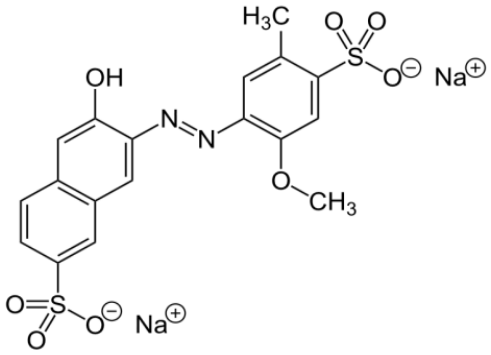
- ✓ FDA: FD&C Yellow Nº6
- ✓ Other names: C.I. 15985, Orange Yellow S
- ✓ ADI: 1 mg/kg bw/day
- ✓ IUPAC name: Disodium 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonate
- ✓ Chemical structure:



1.1.4.3 Allura red AC (E129)

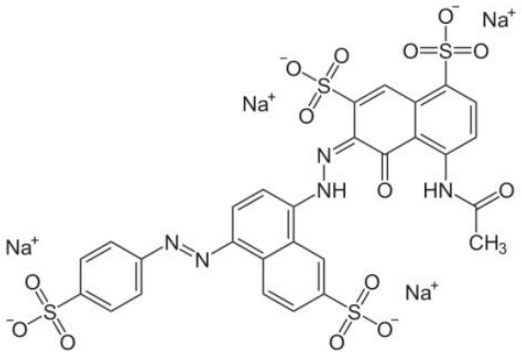
- ✓ FDA: FD&C Red Nº40

- ✓ Other names: C.I. 16035, Food Red 17
- ✓ ADI: 7 mg/kg bw/day
- ✓ IUPAC name: Disodium 6-hydroxy-5-((2-methoxy-5methyl-4-sulfophenyl)azo)-2-naphtalenesulfonate
- ✓ Chemical structure:



1.1.4.4 Brilliant Black BN (E151)

- ✓ FDA: not approved by FDA
- ✓ Other names: C.I. 28440, Brilliant Black PN, Brilliant Black A, Black PN, Food Black 1, Naphthol Black, C.I. Food Brown 1
- ✓ ADI: 5 mg/kg bw/day
- ✓ IUPAC name: Tetrasodium (6*Z*)-4-acetamido-5-oxo-6-[[7-sulfonato-4-(4-sulfonatophenyl)azo-1-naphthyl]hydrazono]naphthalene-1,7-disulfonate
- ✓ Chemical structure:



1.2 The H295R cell line

The cell line named H295R comes from a 48-year old black woman adrenocortical carcinoma (Gazdar *et al.*, 1990). These cells maintain the capacity to synthesize most of the steroid hormones characteristic of three phenotypically distinct zones of the adult adrenal cortex:

- ✓ Zone glomerulosa
- ✓ Zone fasciculate
- ✓ Zone reticularis

In the adult adrenal cortex, a battery of oxidative and other enzymes located in both the mitochondria and endoplasmatic reticulum of the three phenotypically distinct zones are involved in the biosynthesis of steroid hormones (Gazdar *et al.*, 1990). This biosynthesis cortex involves the coordinated transcription of the numerous genes encoding steroidogenic enzymes. Chemical agents that alter expression of these steroidogenic enzymes have the potential to alter hormone biosynthesis. Because of its unique steroidogenic capability the adrenal cortex has been suggested to be the most common and perhaps the most susceptible endocrine target organ for Endocrine-Disrupting Chemical (EDC) (Sanderson, 2006).

The endocrine system regulates vital functions such as metabolism, tissue functions growth and development, carrying out all these function by regulation the secretion of almost all hormones.
Nowadays we are surrounded close to 800 chemicals capable to interfere in hormonal system, such as interacting with receptors, biosynthesis pathway, and etcetera. And the knowledge gathered so far it has been shown these substance then can alter endocrine system interfering on organ development or tissue function and make humane more susceptible to endocrine disease.

In the last years number of endocrine disease not explained by genetic factors have been increased and related to exposure to EDC. Right now just few numbers of chemicals are defined as EDC, but nevertheless there is still a gap of knowledge in front of new increasing EDC. We can find this EDC in food, wildlife, on environment, where we are exposed every day to unknown mixtures and uncountable EDC.

In front of this global concern about increasing incidence of endocrine related disease, better and further information of EDC would help to prevent these disorders. Certainly more research is required, we need to know where the exposures are coming from (Bergman *et al.*, 2012).

According to the World Health Organization (WHO), an endocrine disruptor is defined as “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations”.

1.3 Aim of project

Overall the aim of this study was to investigate the toxicological effects of azo dyes using multiple *in vitro* assays for different toxicity end points.

The human adrenocortical cell line H295R is used in a wide range of biomedical research, including studies of endocrine disruptors. The cell line is also used to perform mechanistical studies of endocrine disruptors (studies on enzyme activity and expression of key genes in the steroidogenic pathway). One of the strengths with the H295R cell line is that is expresses all steroidogenic enzymes, thus making it possible to study alterations in the production of both oestrogens and androgens.

Azo dyes are present in many consumer products and there is a knowledge gap regarding the full toxicity profile of these substances. The aim of his work has been to screen the toxicological effects of four azo dyes in multiple *in vitro* assays to provide data on the potential toxicity of azo dyes.

2. METHODS AND MATERIAL

2.1 Test chemicals

Four azo dyes were used in this study: Tartrazine, Allura Red AC, Brilliant Black BN and Sunset Yellow FCF, which were purchased from Sigma-Aldrich (St. Louis, MI, USA).

Stock solutions of 20mM (dissolved in water) were used for further dilution to experimental concentration that ranged from 20mM to 20µM. This concentration range was initially selected based on toxicity data from previous studies.

2.2 Cell culture and treatment - experimental design

The NCI-H295R cell line (ATCC, Manassas, VA, USA) is derived from a human female adrenocortical carcinoma. Is a useful tool since it expresses most of the important steroidogenic enzymes, such as CYP11A, CYP11B, CYP 17, CYP 19, CYP 21 and produces many steroid hormones (androgens, oestrogens, glucocorticoids, mineralocorticoids).

Briefly, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mixture F-12 Ham (Sigma) supplemented with 1% ITS Plus Premix (BD Biosciences), 2.5% Nu-Serum, 1% L-glutamine (Gibco) and 1% antibiotic (Gibco, Invitrogen, Carlsbad, CA,USA). Culture medium was changed two-three times a week and the cells were subcultured once a week.

The human ovarian adenocarcinoma cell line BG1luc4E2 transfected with an ER responsive luciferase reporter gene, was cultured in RPMI medium and 220µl Gentamycin (50mg/ml). The cells were transferred into 150cm^2 flasks containing estrogen free DMEM media with 150µl Gentamycin (50mg/ml) to each one 24 hours before to plate them on a 96 well plate. Medium was changed 24hours later after addition of gentamycin to remove dead cells and were subcultured twice a week.

All cells were cultured as monolayers in a humid environment at 37ºC with 5% CO_2 and were detached from flask for subculturing using Gibco Trypsin-EDTA (1x) (Invitrogen, Carlsbad, CA, USA), when the cells reached suitable confluence.

Cells were cultured and exposed to azo dyes in 96, 24, 6 well plates, in a range of concentration from 1 µM up to 1mM using serial dilution from stock solution (20mM) of each dye depending on the assay. Cells treated with the same volume of water were used as negative control.

Cell density was determined using a haemocytometer.
When H295Rcells were seeded, depending on plates used, 1.7×10^4, 2.2×10^4 and 3×10^4 cells were seeded in each well in 96 well plates, 5×10^4 cells per well in 24 well plate, and 6 well plate (cell suspension was 1×10^6 cells/ml). In case of BG1luc4E2 cells were seeded in 96 well plate 4×10^4 cells seeded in each well.

2.3 Cytotoxicity. MTS test

To assess the general cytotoxicity, H295R cells were seeded in 96-well plates with 3×10^4 or $2{,}2 \times 10^4$ cells in each well and 100µl of medium per well. After 24 hours grown

in 37ºC and 5% CO_2, cells were exposed to 0, 1µM, 10 µM, 100 µM, 500 µM, 1000µM and 6.67 mM from each dye for 24 hours. The positive control was 10% DMSO, known to induce cytotoxicity in this cell line.

Cell viability was examined by CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega Corporation, Madison, WI) after 24h of cell treatment by measuring the capacity of the cells to reduce a tetrazolium compound(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT) to formazan. The absorbance signal generated is directly proportional to the number of living cells in the culture.
The cells were washed 2 to 3 times, to ensure that excessive dye did not interfered with the assay, and filled with 100 µl PBS. 20µl of CellTiter 96® AQueous One Solution Reagent was added to each well of cells. The absorbance was recorded after one hour incubation at 490 nm utilizing Wallac Victor[2].

2.4 RNA isolation

H295R cells were grown in 6-well plates at 37ºC and 5% CO_2 until ≈80% confluence. The cells were treated with 1mM Brilliant Black BN for 24h. At the end of chemical exposure, the cells were harvested and the RNA was extracted using RNeasy Mini Kit (Qiagen).

For nucleic acid extraction, after removal of the medium, cells were washed in PBS, in order to no interfere on absorbance values, then were lysed in culture plate with 600µl of Buffer RLT and RNA was isolated as described in RNeasy Mini kit protocol (Qiagen). Briefly, lysed cells were diluted with 70% ethanol. The mixture was transferred to an RNA spin cup and centrifuged for 15 seconds. The filtrate was discarded and the spin cup was washed with 700µl Buffer RW1, washed again with 500µl Buffer RPE. After each wash cycle, the samples were centrifuged and the filtrate was discarded. After the final wash, 40µl RNase-free water was added directly to the fiber matrix inside the spin cup, and centrifuged at full speed for 1 minute. The purified RNA was stored at -20ºC. An appropriate dilution of RNA sample (1:100) was prepared for RNA quantification. The absorbance of RNA was measured at 260 nm. To determine the concentration of the isolated RNA samples was used RiboGreen® RNA-Specific Quantitation Kit with DNase I (Invitrogen, Carlsbad, CA, USA).

The concentration of total RNA was estimated using A260 value and standard curve, was equivalent to 22 µg/mL for control's sample and 18.9 µg/ml for Brilliant Black's sample.

2.5 cDNA preparation

RNA was used to prepare cDNA, using Verso^{TM} cDNA Syntesis kit (ThermoScientific). Total RNA (25ng) was combined with 4µl 5x cDNA synthesis buffer, 500µM dNTP mix, Random hexamers as RNA primers, 1µl RT Enhancer, 1µl Verso Enzyme Mix, and diethylpyrocarbamate (DEPC)-treated water to a final volume of 20µl for reverse transcription. The reaction mixture was incubated at 42ºC for 30 min. and was terminated by incubation at 95ºC for 2 min. Samples were either used directly for PCR or were stored at -20ºC until PCR.

2.6 Real-time PCR

Real-time PCR (quantitative PCR) was performed using DyNAmo SYBR Green qPCR kit (Thermo Scientific), containing 2x Master mix (contains modified *Tbr* DNA polymerase, SYBR Green I fluorescent dye, optimized PCR buffer, 5mM $MgCl_2$, dNTP mix including dUTP), primer mix solution 1:10, nuclease-free water (Invitrogen) and cDNA template put together to a final volume of 20µl. DNA template did not exceed 10ng/µl in the final reaction.

The thermal cycling program, carried out by Rotor-GeneTM 3000, included an initial denaturation step at 95ºC for 10 min, followed by 55 cycles of denaturation (95ºC for 10s), primer annealing (at 55ºC for 15s), and cDNA extension (72ºC for 20s) For quantification of PCR results Ct (the cycle at which the fluorescence signal is first significantly different from background) was determined for each reaction. Ct values for each gene of interest were normalized to the endogenous control gene, TATA-binding protein (TBP). Normalized values were used to calculate the degree of gene expression as a “fold change” compared to normalized control values. Gene expression was measured in triplicate for each control and exposed cell culture samples.

2.7 Determination of hormone levels in cell culture medium

Enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and colour change to detect the presence of a substance. This assay is useful for determining serum antibody concentration, but it can also be used in toxicology as a drug screening.

Herein, competitive ELISA was performed to measure hormone production through concentration of hormones in cell culture medium after the treatment by using DEMEDITEC Diagnostics GmbH for Estradiol and Testosterone kits.

H295R cells were seeded in 24 well plates with 5×10^4 cells in each well. After 72 hours incubating, cells were exposed to 1 mM and 6.67 mM concentration of Allura Red AC, Brilliant Black BN, Tartrazine, and Sunset Yellow for 24 hours (37ºC, 5%CO_2). All experiments were performed in triplicate. Untreated cells were used as negative control. ELISA test was performed twice for 1mM and 6.67mM concentration and once for 1mM concentration.

The working ranges of these assays for the standard curve of steroid hormones in H295R medium were: E2: 0;3;10;50;200 pg/mL and T: 0;0,2;0,5;1;2;6;26 pg/ml. Media extracts were diluted 1:10 for E2 while for T samples did not require dilutions.

2.8 Oxidative Stress Response. Dual-Luciferase® Reporter Assay.

Nrf2 plays an important role in the transcriptional regulation of a set of genes induced by oxidative stress.

H295R cells seeded on 96 well plates, 1.7×10^4 cells in each well, were transfected with a Nrf2 responsive luciferase plasmid using Lipofectamine (Invitrogen) in accordance with the protocol provided by the manufacturer. Cells were co-transfected with a *Renilla* vector to standardize for transfection efficiency. Following transfection, cells were cultured for 48 hours and then treated with azo dyes (1 mM) for 24 h. Following treatment, cells were washed with PBS and afterwards lysed with Passive Lysis Buffer. Firefly and *Renilla* luciferase activities were analysed using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized to the respective *Renilla* luciferase activity.

2.9 CellTiter-Glo® luminescent cell viability assay

This assay was performed to asses general cytotoxicity levels in a new cell line in order to set up an E2-agonist test. It determines the number of viable cells measuring ATP levels detected by luminescence. The amount of ATP is directly proportional to number of cells present in culture.

BG1luc4ER cell line was seeded on 96-well plate, 4×10^4 cells in each well (2×10^5cells/ml) and treated with serial dilutions 1:10 from 1M stock solution. Brilliant Black BN stock solution was reduced to half due to solubility problem at 1M concentration. Cells were incubated for 18-24h (37ºC and 5%CO_2). Following the protocol, CellTiter-Glo® Reagent (Promega) equal to the volume of cell culture medium was added in each well and the luminescence was measured using Wallac Victor^2 as plate reader.

2.10 E2-Agonist test. Lumi-cell® ER assay

This test is used for screening of agonistic and antagonistic effects of substances on the estrogen receptor. BG1luc4ER cell line has been stably transfected with a luciferase reporter gene for activation of the estrogen receptor (ER).

The aim of this test was to identify if the chemicals used could induce or inhibit the estrogen receptor, and thereby act as endocrine disruptors.

Finally it could not perform it owing to the cells lost their response under estradiol treatment (used for standard curve). Due to technical problems, this assay could not be completed for the azo dyes.

2.11 Statistical analysis

Statistical analysis was performed by Student´s t-test in EXCEL. Statistically significant differences (SSD) from control groups were evaluated by a two-tailed T-test analysis, where $P<0.05$ were considered significant.

All experiments were done twice, and within an individual experiment each concentration was tested in triplicate.

All results are presented as means with their standard deviations (SD).

3. RESULTS

3.1 Proliferation/toxicity test

3.1.1 *H295R cell viability*

The result of the MTS cytotoxicity assay after azo dyes exposure is shown in *fig 1.* 10%DMSO was used as a positive control, since H295R cells are highly sensitive at this compound. The negative control in this case was vehicle treated cells. All substances were shown to have a cytotoxic effect on cells at highest concentration used, 6.67mM.

The assay was performed twice, and in both assays Tartrazine was shown to induce cytotoxicity at 1mM concentration. Although this figure shows statistically significant differences on 1mM, Brilliant Black BN was not considered cytotoxic in this concentration, since the cell viability was >85% as compared to the vehicle treated

control. In the following assays, each dye was used in the highest concentration that caused a cell viability decrease of maximum 15%.

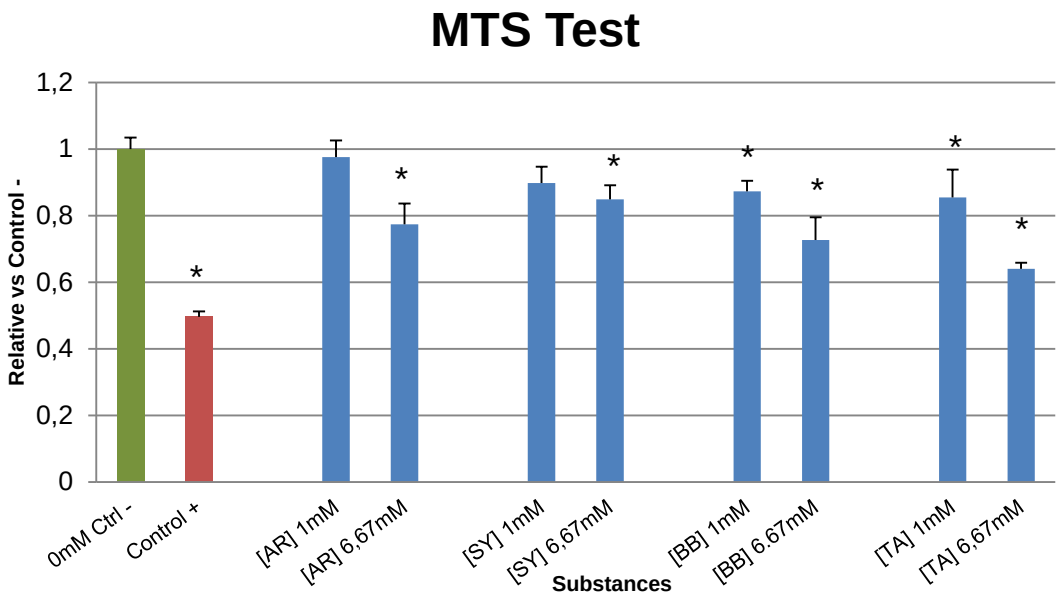


Fig 1. The result of toxicity assay after azo dyes exposure. Results are expressed as relative versus control negative. *Statistical significance compared to untreated control (P-value<0.05) Toxic levels were established under 85%cell viability respect negative control. [X]: concentration used from each dye.

3.1.2 *BG1luc4ER cell viability*

This test was performed to determine cytotoxicity of azo dyes in BG1luc4ER cells. The results are presented in *fig 2* where the data shows statistically significant differences at 1mM Sunset Yellow with 80% cell viability. Nevertheless is important to notice Sunset Yellow FCF treated cells looked stressed under microscope.

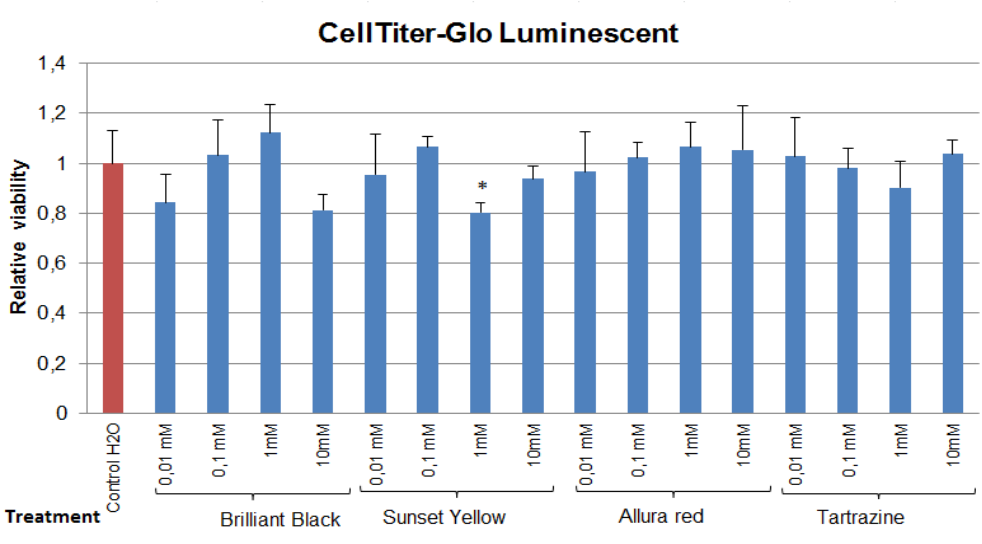


Fig 2. Cell viability assay with BG1luc4ER cell line. Negative control used was water. Results expressed as relative compared to the control.

3.2 Hormone production

3.2.1 *Estradiol*

H295R cells were seeded in 24 well plates, with 5×10^4 cells in each well and exposed with azo dyes in the concentrations 1 mM and 6.67 mM. Following 24 hours treatment, the cell culture medium was collected and the estradiol concentration was measured using ELISA. The results are presented in figure 3. Cells treated with 6.67 mM showed a significant decrease of estradiol as compared to the vehicle treated control. This could be due to cytotoxic effects of the azo dyes in this high concentration.

Brilliant Black in the concentration 1 mM decreases the estradiol level in medium compared to vehicle treated control. No statically significant changes were observed for Sunset Yellow FCF, Allura Red AC or Tartrazine at 1mM.

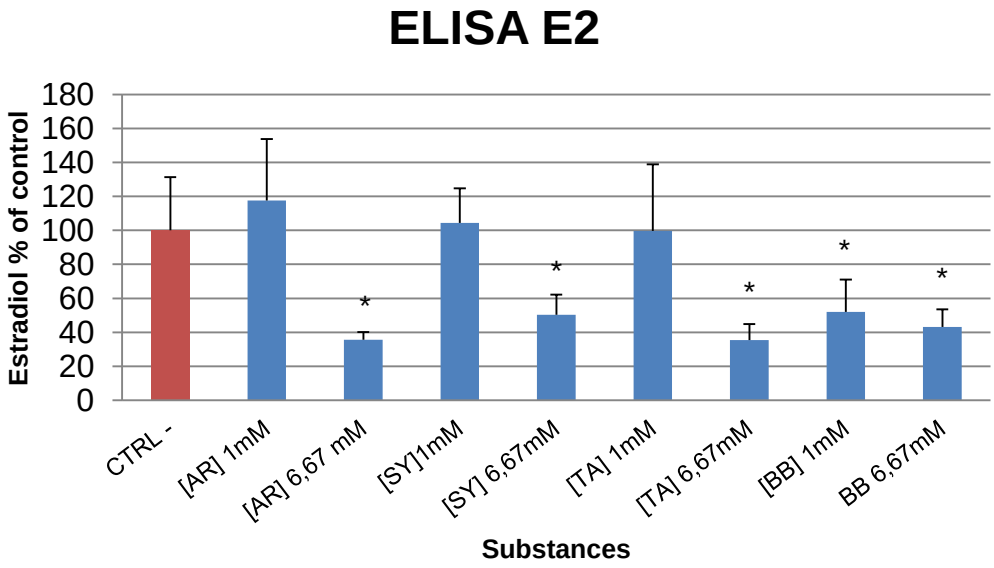


Fig 3. Elisa E2 hormone levels in culture medium were expressed as percentage (%) respect control levels. * Indicates a Statistically significant difference ($P<0.05$; in a two tailed test).

3.2.2 *Testosterone*

The same procedure as ELISA E2 was performance in this ELISA. H295R cells were cultured in 12 well plates (5×10^4 cells/well) and treated with azo dyes in the concentration of 1 mM for 24 hours. Following the treatment, cell culture medium was collected and the testosterone level was measured using ELISA.

The testosterone level was significantly decreased following treatment with Brilliant Black, while no statistically significant changes were observed for the other dyes (figure 4).

The production of E2 and T was reduced approximately 70% following treatment with 1 mM Brilliant Black (*Fig 3, fig 4*).

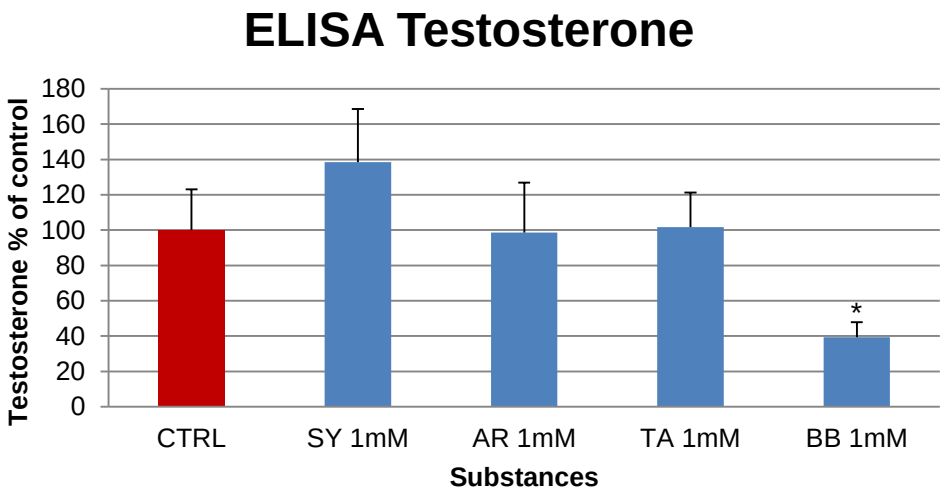


Fig 4. Results from ELISA testosterone hormone production in medium. Control was untreated cells. *Indicates a Statistically significant difference (P<0.05; in a two tailed test).

3.3 qRT-PCR: BCRP gene expression

BCRP (Breast Cancer Resistant Protein) is a xenobiotic transporter playing a role in protecting the organism from potentially harmful xenobiotics, preventing cytotoxic agents from reaching lethal levels within cells (Doyle *et al.*, 2003). Similar to P-gp, BCRP is also highly expressed in organs important for the absorption and distribution of drugs and xenobiotics (Ni Z *et al.,* 2010).

Although to prevent lethal levels within cells from cytotoxic agents, can also efflux cancer drugs by pumping them out of cell involved to give a resistance to chemotherapeutic agents (Qian *et al.,* 2013, Dankers *et al.*, 2012, Imai, *et al.*, 2005).

To study the effects of Brilliant Black on BCRP gene expression in the human adrenocortical carcinoma cell line H295R, we analysed the gene expression using quantitative real time PCR.

Cells were cultured in 6 well plates, and were exposed to Brilliant Black BN in the concentration of 1mM for 24h.The other dyes were left out after results of ELISA, where just Brilliant Black BN presented effects on hormone levels in medium. That fact indicated a possible effect on this drug transporter, either blocking the pump or inhibiting hormone production within cells.

Following treatment, cells were harvested and RNA was extracted. The RNA was reversely transcribed to cDNA and used for real time PCR. Real time PCR was performed with primers specific for BCPR. The gene expression was measured as fold change compared to the vehicle treated control. BCRP gene expression was significantly decreased following treatment with 1 mM Brilliant Black (*figure 5*).

The results presented in *Figure 5* was obtained from one run, due to the other assays performed were failed because of contamination on genetic material, such as DNA and primers.

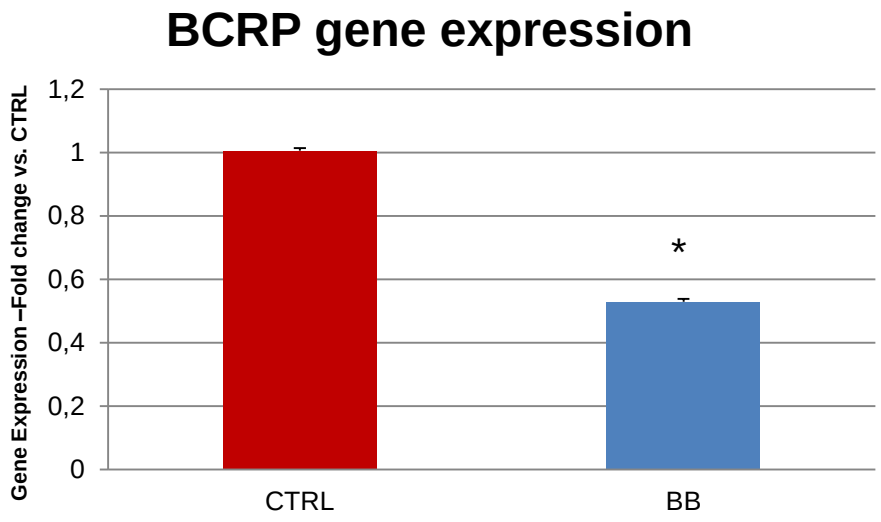


Fig 5. Effects of Brilliant Black BN (BB) on BCRP gene expression. Results are expressed mean ±SD. Error bars represent the standard deviation. *Statistical significance compared to control ($p<0.05$). TBP (TATA-box binding protein) was used as gene control.

3.4 Oxidative Stress Response

Human adrenocortical H295R cells were transfected with a Nrf2 responsive luciferase plasmid. Under oxidative stress Nrf2 signalling pathway is activated to enhance the expression of antioxidant enzymes (Numazawa *et al.*, 2003). Following transfection, the cells were cultured for 48 hours prior to treatment with azo dyes in the concentration of 1 mM. After the treatment, the luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega). The luciferase reporter activity was standardized for transfection efficiency using a renilla luciferase plasmid. The results obtained are shown in *Figure 6*.

A statistically significant effect was shown for Sunset Yellow FCF comparing with 5% water control (vehicle treated cells). We conclude that Sunset Yellow induces oxidative stress response in the H295R cell line.

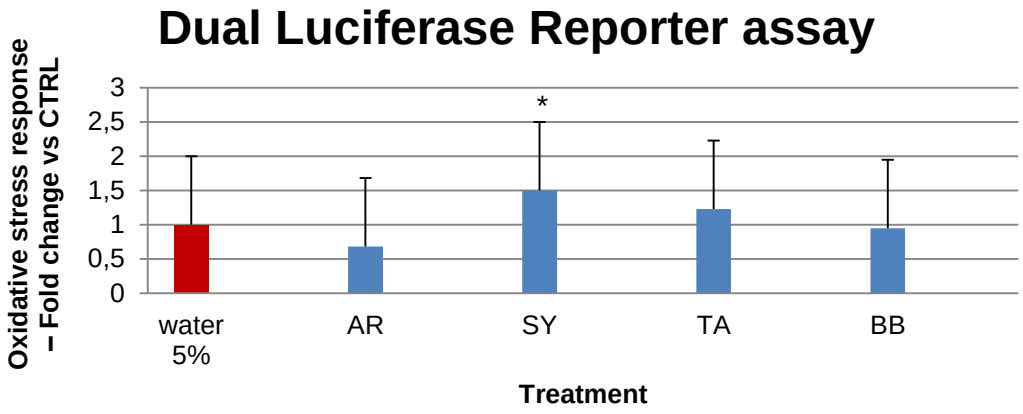


Fig.6 Luciferase emits light during conversion of substrate to metabolites, whose gene expression is regulated by Nrf 2 activity, that increase its defences system under oxidative stress.
Dual Luciferase reporter assay results shown in fold change ± SD compared to the H_2O control. The results are based on triplicates and the asterisk indicate statistical significance ($p<0.05$) in comparison with the control.

4. DISCUSSION

The aim of this project was to screen toxicological effects of four azo dyes in different human cell lines derived from adrenal cortex and ovaries.

The two cell lines can be used to investigate different aspects of endocrine disruption (altered hormone production, substances acting as xenoestrogens, alterations in gene expression etcetera).

It is important to note that concentrations used in this study are relatively high as compared to the concentrations in normal consumption of these food colours.

The observed decrease of E2 and T in cell culture medium after treated with Brilliant Black BN could be explained by two different mechanisms, either Brilliant Black decrease the production of E2 and T or the Brilliant Black treatment decreases the efflux of E2 and T, e.g. via altered BCRP activity.

BCRP functions as an efflux pump, and a decrease of the BCRP gene expression could explain why we observed decreased levels on E2 and T after Brilliant Black treatment. E2 and T can be effluxed by BCRP (Dankers *et al.*, 2012) and Brilliant Black BN is decreasing the gene expression of BCRP and consequently the level of E2 and T in cell culture medium.

Localization of Bcrp in endocrine organs together with the efficient allosteric inhibition of the efflux pump by steroid hormones are suggestive for a role for Bcrp in hormone regulation (Dankers *et al.*, 2012).

In previous studies (Imai *et al.*, 2003, 2005) it has been suggested that estrogen down-regulates BCRP expression. Estrogen-mediated regulation of Bcrp might therefore be responsible for the accumulation of estrogen within cells.

Axon *et al.*, 2012 carried out an study where Tartrazine and Sunset Yellow were identified to be activators of human ER in MCF-7 cells transfected with $(ERE)_3$-pGL3 in a range of compounds with capacity to modulate human ER transcriptional activity, nevertheless these food colours have not been reported to be xenoestrogens. Furthermore, expression of drug transporter could be transcriptionally repressed by ERα activation, where Imai *et al.*, 2005 demonstrated that expression of ERα are important for BCRP down-regulation mediated by estrogen.

A possible mechanism for the oxidative stress induced by Sunset Yellow FCF could be related by the increase in testosterone level. In the ELISA assay, treatment with Sunset Yellow resulted in a slight (however not statistically significant in our assay) increase in

the testosterone level. Previous studies have shown a relationship (Alonso-Alvarez *et al.*, 2007) where testosterone might play a role on resistance effect to free radicals explained by different mechanism. Hence, it is suggested there is a link between testosterone levels and oxidative stress response.

Overall, the results in this study indicate that Brilliant Black might have an effect on the steroidogenic pathway in H295R cells and that the effect might be mediated via a decreased gene expression of BCRP.

In the light of all data considered, further research is needed to gain more knowledge on the toxicity of azo dyes.

5. CONCLUSIONS

In this project the human H295R cell line was used as a system for detection of effects on gene expression, hormone production and oxidative stress in the adrenal cortex when exposed to high concentrations of azo dyes.

The different results obtained from these substances would need more testing, in order to prove and demonstrate possible long term effects as well as to verify the outcomes shown.

Some assays, such as qRT-PCR should be repeated due to results presented were from one run, even ELISA test gave different results in the case of tartrazine.

These substances would also need to be tested in E2-agonist test to prove any estrogenic activity since tartrazine and sunset yellow were identified as activators of human ER in a screening assay (Axon *et al.*, 2012).

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