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Shannon, M., Wilson, J., Xie, Y., & Connolly, L. (2019). In vitro bioassay investigations of suspected obesogen monosodium glutamate at the level of nuclear receptor binding and steroidogenesis. *Toxicology Letters*, 301, 11-16. <https://doi.org/10.1016/j.toxlet.2018.10.021>

Published in:
Toxicology Letters

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
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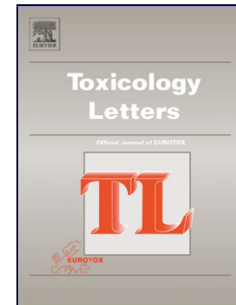
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Accepted Manuscript

Title: *In vitro* bioassay investigations of suspected obesogen Monosodium Glutamate at the level of nuclear receptor binding and steroidogenesis

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PII: S0378-4274(18)32026-5
DOI: <https://doi.org/10.1016/j.toxlet.2018.10.021>
Reference: TOXLET 10348

To appear in: *Toxicology Letters*

Received date: 24-4-2018
Revised date: 5-9-2018
Accepted date: 22-10-2018

Please cite this article as: Shannon M, Wilson J, Xie Y, Connolly L, *In vitro* bioassay investigations of suspected obesogen Monosodium Glutamate at the level of nuclear receptor binding and steroidogenesis, *Toxicology Letters* (2018), <https://doi.org/10.1016/j.toxlet.2018.10.021>

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***In vitro* bioassay investigations of suspected obesogen Monosodium
Glutamate at the level of nuclear receptor binding and steroidogenesis**

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Highlights

- Monosodium glutamate (MSG) is a suspected obesogen.
- There is a link between exposure to MSG and alterations in steroid hormone levels.
- MSG can antagonise the androgen receptor in a dose dependent manner.
- The androgen receptor plays an important role in regulating metabolic homeostasis and is an emerging target for diabetes and obesity.

Abstract

Monosodium glutamate (MSG) is a commonly used flavour enhancer in households, catering and food production. Recently it has been highlighted as a suspected dietary obesogen in epidemiological studies indicating a link between MSG consumption and weight gain. Additionally, animal studies have shown that MSG exposure has profound effects on sex steroid hormone levels and receptors; which have an important role in energy metabolism. However, the exact mechanism by which MSG exerts its effects has yet to be elucidated. Reporter gene assays (RGAs) and the H295R steroidogenesis assay have been used to investigate the endocrine disrupting potential of MSG. Receptor (ant) agonism was not observed in the MMV-Luc (oestrogen responsive) or TM-Luc (progestagen responsive) cell lines following exposure to MSG. Also, no effects on hormone production were observed. However, MSG exhibited an antagonist response in the androgen and progestagen responsive TARM-Luc cell line, with a dose dependent reduction in androgen response of 33%, 36.9% and 50.6% (in comparison to the solvent control) at 50, 250 and 500 µg/ml MSG, respectively ($P \leq 0.05$; $P \leq 0.05$; $P \leq 0.001$). No cytotoxicity or pre lethal cytotoxicity was observed at the concentrations tested. These findings demonstrate one potential pathway whereby MSG may act as a dietary obesogen.

Abbreviations: ADI; Acceptable Daily Intake, ANOVA; a one-way analysis of variance, AR; androgen receptor, dH2O; deionised water, DMSO; dimethyl sulfoxide, EAAT-1; excitatory amino acid transporter, EDCs; endocrine disrupting compounds, EFSA; European Food

Safety Authority, ELISA; enzyme-linked immunosorbent assay, HCA; high content analysis, HCS; high content screening, MSG; monosodium glutamate, MTT; thiazolyl blue tetrazolium bromide, POPs; persistent organic pollutants, RGA; reporter gene assay

Key words: Monosodium glutamate; reporter gene assay; endocrine disruptor; steroidogenesis; high content analysis; obesity; diabetes; obesogen

1. Introduction

Obesity is a global health problem, with an estimated 640 million adults in 2014 and 110 million children and adolescents in 2013 being classed as obese (Lauby-Secretan *et al.*, 2016). Although body fatness and weight gain are largely influenced by modifiable risk factors such as food consumption and exercise, further factors such as exposure to pesticides and/or persistent organic pollutants (POPs) in food or the environment may be involved (Lauby-Secretan *et al.* 2016). In particular, endocrine disrupting compounds (EDCs), which are exogenous substances or mixtures that alter the function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations (WHO/IPCS, 2002), have been linked to obesity (Legler *et al.*, 2011).

EDCs whose effects may result in metabolic disorders are termed “obesogens” or metabolic disruptors. Obesogens may act by altering the programming of adipogenesis and increasing energy storage in fat tissue, or by disrupting the neuroendocrine control of appetite and satiety (Janesick and Blumberg, 2016). Environmental exposures during early life can interfere with the epigenetic programming of gene regulation, leading to potential transgenerational effects and

influencing the risk of obesity in adulthood via adipogenesis, adiposity and body weight gain (Stel and Legler, 2015). In addition, steroid hormones and their receptors have also been linked to the homeostasis of energy metabolism (Mauvais-Jarvis, 2011).

A suspected dietary obesogen which is a widely consumed flavour enhancer is monosodium glutamate (MSG). MSG is composed of sodium mineral ion or salt, attached to glutamic acid, one of the most abundant naturally occurring non-essential amino acids naturally present in our bodies and many foods such as mushrooms, tomatoes and cheese. Once ingested, MSG is broken down into glutamate and sodium ions within the digestive tract and metabolised within the gut. The glutamate that is absorbed, is then transported in the lumen of the intestine through the EAAC-1 (the EAAT-1 or excitatory amino acid transporter) and NaDC-1 (sodium carboxylate transporter) and circulated in the bloodstream throughout the body (Burrin and Stoll, 2009; Janeczko *et al.*, 2007).

MSG is now a relevant aspect of the human diet worldwide. The UK average intake in 1991 was 580 mg/day for the general population but 2.3 g/day for excessive consumers (Rhodes *et al.*, 1991). More recent studies report an average intake of MSG of 0.4 g/day in European countries (Beyreuther *et al.*, 2007). In countries such as China, mean MSG consumption has been found to be higher, around 3.1 g/day (Shi *et al.*, 2014). In an EFSA report released in 2017, an ADI (acceptable daily intake) of 30 mg/kg bw per day, was set. Accurately monitoring levels of MSG consumption is difficult because legislation does not enforce any limit on the amount of MSG that restaurants or the food industry can add to their products. Also, food processors and manufacturers are not obligated to list the amount of MSG on their packaging (Food Standards, 2015).

The mechanism by which MSG may act as an obesogen has not yet been elucidated (He *et al.*, 2008; He *et al.*, 2011; Khalaf and Arafat, 2015; Miskowiak *et al.*, 1993). *In vivo* studies link MSG intake to disruption of steroid receptor expression and alteration of levels of hormones such as oestrogen, testosterone or progesterone (Zia *et al.*, 2014; Miskowiak *et al.*, 1993; Rodriguez-Sierra *et al.*, 1982; Nemeroff *et al.*, 1981). These studies demonstrate that MSG may disrupt steroid hormone receptors and/or steroid hormone levels.

We have previously shown that MSG decreases glucagon-like peptide 1 (GLP-1) secretion in a gut *in vitro* cellular model (Shannon *et al.*, 2017), suggesting a possible mechanism by which MSG may affect glucose regulation and satiety responses. Additionally, as discussed above, studies have demonstrated that MSG may affect sex hormone levels and receptors *in vivo*. As steroid hormones/receptors play an important role in weight homeostasis, the present study was performed to investigate the potential endocrine disrupting mechanisms of MSG at the level of nuclear receptor transcriptional activity using oestrogen, androgen, progestagen and glucocorticoid Reporter Gene Assays and on steroidogenesis using the H295R steroidogenesis model.

2. Methods

2.1 Chemicals and reagents

Cell culture reagents were supplied by Life Technologies (Paisley, UK). The standards 17 β -oestradiol, testosterone, progesterone, hydrocortisone, monosodium glutamate and forskolin were obtained from Sigma-Aldrich (Poole, Dorset, UK). Dimethyl sulfoxide (DMSO) and MTT were also supplied by Sigma-Aldrich. Lysis reagents and

luciferase assay system was purchased from Promega (Southampton, UK). Hoechst nuclear stain was provided by Thermo Scientific (UK).

2.2 Cell culture

Four reporter gene assay (RGA) cell lines, the MMV-Luc (oestrogen responsive), TARM-Luc (androgen and progestagen responsive), TGRM-Luc (glucocorticoid and progestagen responsive) and TM-Luc (progestagen responsive) were previously developed by transforming human mammary gland cell lines with the luciferase gene under the control of a steroid hormone inducible promoter (Willemsen *et al.*, 2004). The H295R human adrenocortical carcinoma cell line, used in the H295R steroidogenesis model, was obtained from the American Type Culture Collection (ATCC CRL-2128, Manassas, VA, USA).

All cell lines were routinely grown in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO₂ and 95% humidity. The Reporter Gene Assay (RGA) cell lines were cultured in cell culture medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% foetal bovine serum and 1% penicillin streptomycin. For culturing the MMV-Luc cell line, DMEM without phenol red was used due to the weak oestrogenicity of phenol red. The H295R cell line was cultured in cell culture medium containing DMEM with Ham's F-12 nutrient mixture (1:1) supplemented with 1% ITS + Premix and 2.5% NuSerum (BD Biosciences, Bedford, MA, US).

2.3 Cell viability assays

2.3.1 MTT assay

The MTT assay was performed to monitor the cytotoxic effects of test compounds in the RGA cell lines. Briefly, clear flat-bottomed 96-well plates (Nunc, Roskilde, Denmark) were seeded with 4 x 10⁵ cells/ml of the appropriate cell line. After 24 h

MSG (0.5-500 $\mu\text{g/ml}$) was added to the cells at a final dH₂O (deionised water) concentration of 0.1%. Test compounds were then incubated for 48 h. Viable cells convert the soluble yellow MTT into insoluble purple formazan by the action of mitochondrial succinate dehydrogenase. Following incubation, supernatant was discarded and 50 μl of MTT solution/well (5mg/ml stock in PBS diluted in 1:2.5 in assay media) was added and cells were incubated for a further 3 h. The supernatant was removed and 200 μl of DMSO was added to each well and incubated for 10 min with agitation at 37 °C to dissolve the formazan crystals. Optical density was measured using a Sunrise spectrophotometer at 570 nm with a reference filter at 630 nm (TECAN, Switzerland). Samples were analysed in triplicate wells and in three independent experiments. Viability was calculated as a percentage absorbance of the sample when compared with the absorbance of the solvent control (Fig.1).

2.3.2 HCA cytotoxicity

High Content Analysis was used to assess subtle pre-lethal markers of viability in the TARM-Luc cell line. Hoechst 33342 dye at a final concentration of 1.6 μM was added to each well and incubated for 10 min at room temperature and protected from light; after which cells were washed with PBS four times and evaluated on CellInsight™ NXT High Content Screening (HCS) Platform (Thermo Fisher Scientific, UK). This instrument analyses epifluorescence of individual cell events using an automated micro-plate reader analyser interfaced with a PC (Dell precision T5600 workstation). Hoechst stain was used to measure cell number and nuclear morphology including nuclear intensity and nuclear area. Data was captured for each plate at $\times 20$ objective magnification in the selected excitation and emission wavelengths of Hoechst dye (Ex/Em 350/461 nm). For each well, 25 field of view images were acquired to examine each parameter (Fig. 2 & 3).

2.4 Reporter gene assays (RGAs)

RGAs are produced by transfecting cell lines with relevant receptors and incorporating a transactivation step with a signalling protein such as luciferase. The activation of a receptor is then measured through the signalling protein, making it possible to identify both agonism and antagonism of the specific receptor. The range of RGA cell lines used in this study were developed from human mammary gland cell lines by transformation with the luciferase gene under the control of a steroid hormone inducible promoter (Willemsen et al., 2004). In the case of the TARM-Luc cell line, an additional vector was used (pSV-AR0 (coding for human AR)).

The RGA procedure has previously been described by Frizzell *et al.*, (2011). Briefly, cells were seeded at a concentration of 4×10^5 cells/ml, 100 μ l/well in white walled, clear and flat bottomed 96-well plates (Greiner Bio-One, Frickenhausen, Germany). After 24 h, MSG (0.5, 5, 50, 250, 500 μ g/ml MSG in dH₂O) and the steroid hormone standards were added to the cells at a final DMSO concentration of 0.1%. The positive controls used with their respective cell lines were: 1.36 ng/ml 17 β -oestradiol (MMV-Luc), 14.5 ng/ml testosterone (TARM-Luc), 181 ng/ml hydrocortisone (TGRM-Luc) and 157 ng/ml progesterone (TM-Luc). A solvent control (0.1%, v:v DMSO in media) was also included for each cell line. Antagonist tests were carried out by incubating the test compound with the relevant agonist hormone positive control for the cell line being tested. The cells were incubated for 48 h. The supernatant was discarded and the cells washed once with PBS prior to lysis with 30 μ l cell lysis buffer (Promega, Southampton, UK). Finally, 100 μ l luciferase substrate (Promega, Southampton, UK) was injected into each well and luciferase activity measured using a Mithras Multimode Reader (Berthold, Other, Germany). RGAs were performed in triplicate for each

experimental point and in three independent exposures. The response of the cell lines to the compound was measured and compared with the solvent and positive controls.

2.5 Steroidogenesis assay

The human adrenal carcinoma cell line H295R has all the important enzymes needed for steroidogenesis and therefore can be used to investigate effects at the level of steroid hormone production. The H295R steroidogenesis assay was performed according to previously described protocols (Gracia et al., 2007; Hecker and Giesy, 2008; Frizzell et al., 2011).

Briefly, the cells were seeded at a concentration of 3×10^5 cells/ml, 1 ml per well, in 24-well plates (BD Biosciences, Bedford, MA, US). The cells were allowed to attach for 24 h before removing the media and replacing with fresh media containing the test compounds dissolved in DMSO at a final concentration of 0.1% (v:v). Forskolin was used as a positive control at a concentration of 10 μ M. A solvent control (0.1%, v:v DMSO in media) was also included. Subsequently, the media was collected from the wells following 48 h incubation and stored at -20 °C until hormone quantification was carried out. The 48-hour incubation time allows the concentrations of these hormones to reach a plateau-phase under these conditions. The AlamarBlue® cell viability assay was carried out on the remaining cells in each well. Each experimental point was performed in triplicate with three independent exposures (Fig.1).

2.6 Hormone detection and quantification

Frozen media from the H295R steroidogenesis assay was thawed prior to hormone analysis. Oestradiol, testosterone and progesterone levels in the media were quantified by enzyme-linked immunosorbent assays (ELISAs) (Immunodiagnosics, Marburg, Germany). These highly specific kits are based on the principle of

competitive binding and are intended for the quantitative *in vitro* diagnostic measurement of oestradiol (0–2000 pg/ml), testosterone (0–16 ng/ml) and progesterone (0–40 ng/ml) in serum and plasma, with sensitivities of 9.714 pg/ml, 0.083 ng/ml and 0.045 ng/ml respectively. ELISA kits were carried out according to manufacturer's instructions with the exception of the standard curves which were prepared in the same culture medium used for the H295R assay. The intra-assay coefficient of variation was less than 10%. Standard curves were included on each ELISA plate. The mean absorbance obtained from each standard was plotted against its concentration using dose–response curves generated with GraphPad PRISM 5 software.

2.7 Statistical Analysis

All values shown are expressed as mean \pm standard deviation (SD) of three independent exposures for the compound tested. Data from the cell viability, reporter gene and steroidogenesis assays were analysed using Microsoft Excel and GraphPad PRISM 5 software (San Diego, CA). A one-way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test was used to determine significant differences between treatments and the corresponding controls. The mean concentrations were tested for significant difference at the 95% confidence level, a *P*-value of ≤ 0.05 was considered as significant ($P \leq 0.05$ *, $P \leq 0.01$ ** and $P \leq 0.001$ ***).

3. Results

3.1 Cell viability and cytotoxicity

MSG was assessed for cytotoxicity by the MTT assay in the MMV-Luc (oestrogen responsive), TM-Luc (progestagen responsive), TGRM-Luc (glucocorticoid and progestagen responsive) and TARM-Luc (androgen and progestagen responsive) cell

lines. At the various concentrations of MSG tested (0.5-500 µg/ml), no cytotoxicity was observed (*Fig. 1*).

Viability of the H295R cells following 48 h exposure to MSG (0.5-500 µg/ml) was investigated using the AlamarBlue® assay. Cytotoxicity was not observed at any of the concentrations tested (*Fig. 1*). The viability of the cells at the sample concentrations did not differ significantly from the solvent control ($P > 0.05$).

In addition to the MTT assay, subtle pre-lethal markers of viability of the TARM-Luc cell line was investigated due to MSG causing a significant decrease in transcriptional activity in this cell line following exposure to 50-500 µg/ml MSG. However, no significant change in cell number, nuclear area or nuclear intensity was observed by HCA ($P > 0.05$) (*Fig. 2 & 3*).

3.2 Reporter gene assay

No agonist response was observed for MSG at any of the test concentrations (0.5-500 µg/ml) in the MMV-Luc, TM-Luc, TGRM-Luc or TARM-Luc. Additionally, no antagonist response was seen for MSG at any of the test concentrations in the MMV-Luc, TM-Luc or TGRM-Luc cell lines. However, MSG appeared to exhibit an antagonist response in the androgen and progestagen responsive TARM-Luc cell line, with a dose dependent reduction in androgen response of 33%, 36.9% and 50.6% (in comparison to the solvent control) at 50, 250 and 500 µg/ml MSG, respectively ($P \leq 0.05$) (*Fig. 4*). At these concentrations of MSG no reduction in cell viability was observed in the MTT assay (*Fig. 1*). Additionally, as determined by HCA, the cell health markers of cell number, nuclear area and/or nuclear intensity did not significantly differ from the solvent control. Therefore these results appear to be true antagonism of the androgen receptor.

3.3 Steroidogenesis

MSG did not induce any significant changes in the production of oestradiol, testosterone or progesterone as determined by the H295R steroidogenesis assay. The mean concentration of hormones in the treated media did not differ significantly from the solvent control ($P > 0.05$) (Fig.5).

4. Discussion

The current cellular *in vitro* bioassay study on MSG exposure at the level of steroid nuclear receptor signalling and steroidogenesis reports no observed effects on steroid hormone production and no (ant)agonism of the oestrogen, progestogen or glucocorticoid receptors. However, while MSG did not exhibit an agonist response in the androgen receptor, it did induce an antagonist dose dependent response in the androgen receptor. Analysis of all MSG test concentrations confirmed no assay cytotoxicity.

The androgen receptor plays an important role in regulating metabolic homeostasis and is an emerging target for diabetes and obesity. For example, it has been established that testosterone, an androgen receptor agonist, presents anti-obesity actions mediated via the androgen receptor (Mauvais-Jarvis, 2011). Clinical and epidemiological evidence also strongly indicates a major role for sex steroid hormones in the determination of anatomical specificities of fat distribution i.e. the specific region in the body where the fat accumulates, in humans (Dieudonne *et al.*, 1998). Adipocytes (fat cells) express the androgen receptor, suggesting that androgens may contribute to the control of adipose tissue development. Androgen receptor mRNA has been

demonstrated in human preadipocytes and adipocytes (Dieudonne *et al.*, 1998), with its expression increasing during adipogenesis.

Androgen receptor antagonism has also been linked to a number of detrimental health effects including influencing normal male sexual differentiation and/or fertility (Wong *et al.*, 1995). Men presenting genetic androgen resistance linked to CAG repeats in the androgen receptor gene, which leads to a decrease in androgen receptor-mediated gene transcription, have elevated visceral fat; indicating that a reduction in androgen receptor transcriptional activity, may encourage weight gain in the stomach area (Zitzmann *et al.*, 2003). Men undergoing androgen suppression treatment for prostate cancer present increased body fat mass and serum insulin (Ramasamy *et al.*, 2012). Additionally, clinical trials have shown reduced body fat mass during testosterone replacement therapy (Stanworth and Jones, 2010). Navarro *et al.*, (2016) also highlight that the androgen receptor plays a role in stimulating the incretin effect of the gut hormone GLP-1 and demonstrate that androgen receptor antagonists can decrease glucose-stimulated insulin secretion. Winborn *et al.*, (1987) showed that androgen receptors are present in the stomach and gastrointestinal tract of baboons. Therefore, antagonism of the androgen receptor by MSG may lead to obesogenic effects through disruption of metabolic homeostasis, anti-obesity protection and GLP-1 gut hormone signalling.

Steroidogenesis is a complex process regulated by numerous enzymes and genes which can be disrupted at any step (Hilscherova *et al.*, 2004). Each steroid hormone plays an important role in the maintenance of weight and glucose homeostasis, potentially contributing to the pathogenesis of diabetes and obesity. For example, oestradiol plays a role in regulating energy metabolism and in particular the two oestrogen receptors, ER α and ER β (Mauvais-Jarvis, 2011). Modulation of these

receptor genes within insulin-sensitive tissues shows that oestradiol participates in glucose homeostasis (Mauvais-Jarvis, 2011). Therefore, disruption of oestradiol levels or oestrogen receptors could potentially impact upon glucose homeostasis, resulting in hyper/hypoglycaemia. Low levels of testosterone are an independent risk factor for obesity and epidemiological/clinical studies support the notion that testosterone deficiency in men leads to the development of metabolic syndrome (Stanworth and Jones, 2009). However, the underlying mechanism by which androgens regulate homeostasis is very complex and further exploration is required (Stanworth and Jones, 2009). Progesterone plays a role in both weight and glucose homeostasis with increased levels of progesterone being linked to weight gain (Galletti and Klopfer, 1964; Lof et al., 2009). Progesterone also plays a role in insulin secretion, having the ability to inhibit glucose-stimulated insulin secretion from isolated rat islets in a dose-dependent manner (Straub *et al.*, 2002). Disruption of progesterone could potentially lead to altered insulin levels, adversely affecting blood glucose regulation increasing diabetes risk (Diabetes UK, 2015).

In the current *in vitro* bioassay study, no significant difference in oestradiol, testosterone or progesterone hormone production levels were observed when compared to the controls. Similarly, an animal study whereby Wistar rats fed with MSG, also showed no significant alterations in blood testosterone and oestradiol concentrations ($P > 0.05$) (Ibegbulem *et al.*, 2016). However, other animal studies such as Nemeroff *et al.*, (1981) and Zia *et al.*, (2014) report alterations in oestradiol, progesterone and testosterone levels. Miskowiak *et al.*, (1993) reported that subcutaneous perinatal administration of MSG to the rat resulted in growth inhibition, obesity, weight decrease in pituitary glands and testes plus lowered testosterone levels. Further studies in rats suggest that MSG may present effects through disruption

of steroid receptor expression and altering hormones levels such as testosterone, or progesterone. For example, neonatal rats treated orally with MSG presented decreased hypothalamic oestrogen receptor expression (Rodriguez-Sierra *et al.*, 1982). Upon maturation of the treated neonates, the adult rat presented altered serum levels of oestradiol and testosterone. These differing results may be due to differences between *in vitro* and *in vivo* studies, neonate or adult models, sex differences, exposure doses and duration. It is also important to note that animal studies employing subcutaneous injection of MSG are rarely applicable in human patho-toxicological investigations (Husarova and Ostatnikova, 2013). Consequently, epidemiological studies and *in vitro* investigations may provide more relevant and useful findings.

5. Conclusion

This *in vitro* bioassay study shows that MSG can antagonise the androgen receptor in a dose dependent manner and highlights one possible mechanism through which MSG may exert its impact on obesity risk. Additional concerns of these findings include the potential disruption of normal male sexual differentiation and/or fertility; elevated visceral fat; dysregulation of adipose tissue development and the disturbance of anti-obesity protection mediated by testosterone via the androgen receptor.

6. Conflict of Interest

There is no conflict of interest.

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Figures

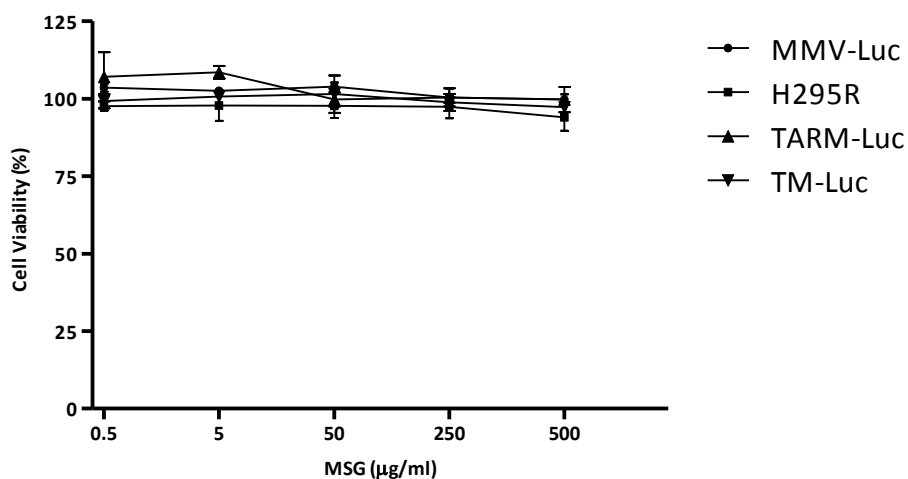


Fig. 1: Viability of the RGA cell lines following exposure to MSG (0.5-500 µg/ml) for 48 h compared to the solvent control, as determined by the MTT assay (for the RGA cell lines) and Alamar Blue assay (for the H295R cell line). The TARM-Luc cell line is specific for the detection of androgens and progestagens, MMV-Luc for oestrogens, TM-Luc for progestagens and H295R cells for oestradiol, testosterone and progesterone. Values are means \pm SEM for three independent exposures in triplicate ($n = 3$). $P \leq 0.05$ (*) and $P \leq 0.001$ (***) represent significance.

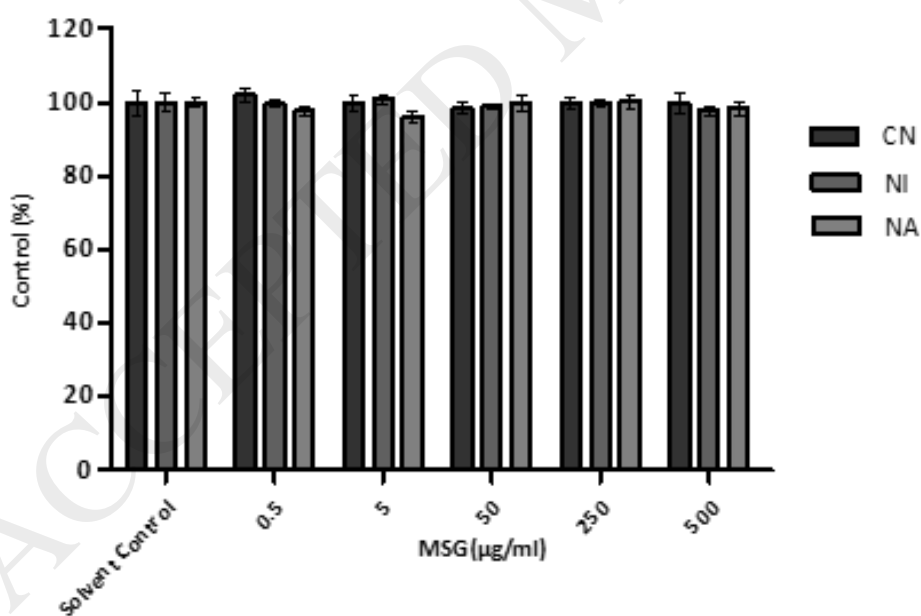


Fig. 2: Cytotoxic effects of MSG at a concentration range of 0.5- 500 µg/ml following 48 h incubation in the TARM-Luc cell line (specific for the detection of androgens and progestagens). A number of endpoints were measured including cell number, nuclear

area and nuclear intensity. Data is expressed as a percentage of solvent control (dH₂O) for each parameter. Values are means \pm SEM for the three independent exposures ($n = 3$).

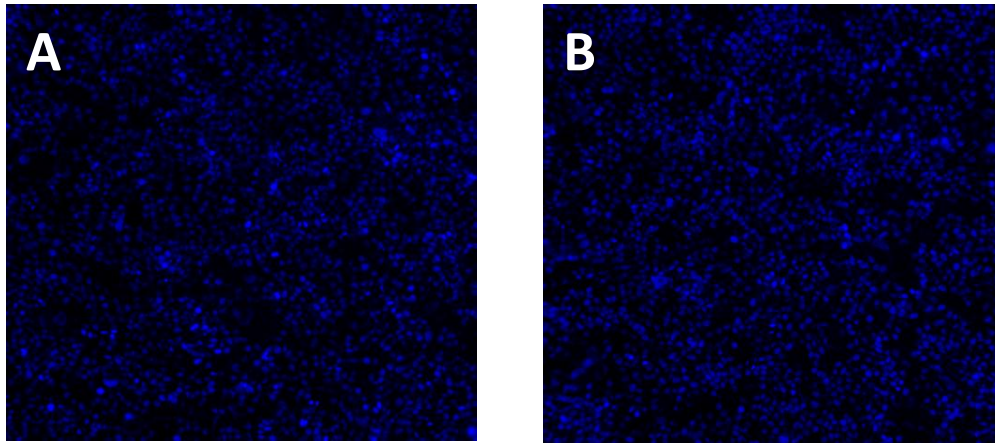
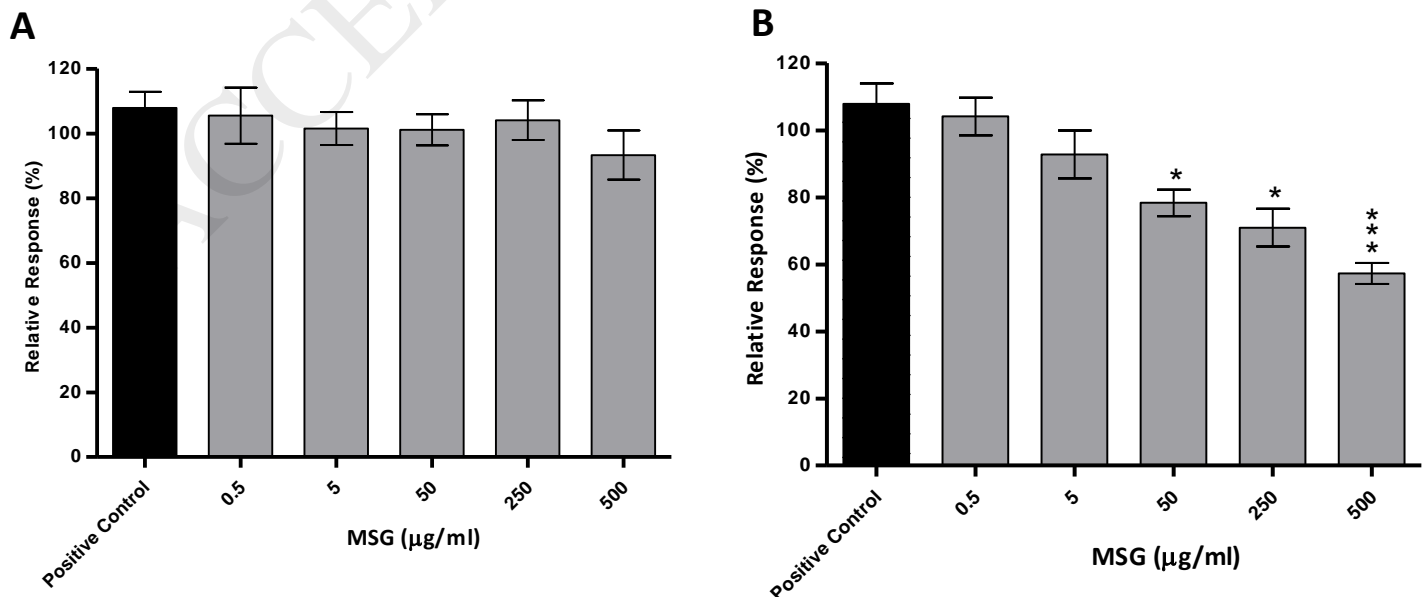


Fig. 3: HCA images for a) solvent control and b) MSG-treated (500 $\mu\text{g/ml}$) following 48 h exposure to the TARM-Luc cell line (specific for the detection of androgens and progestagens). Each image was acquired at X 20 objective magnification using Hoechst dye (blue; nuclear staining).



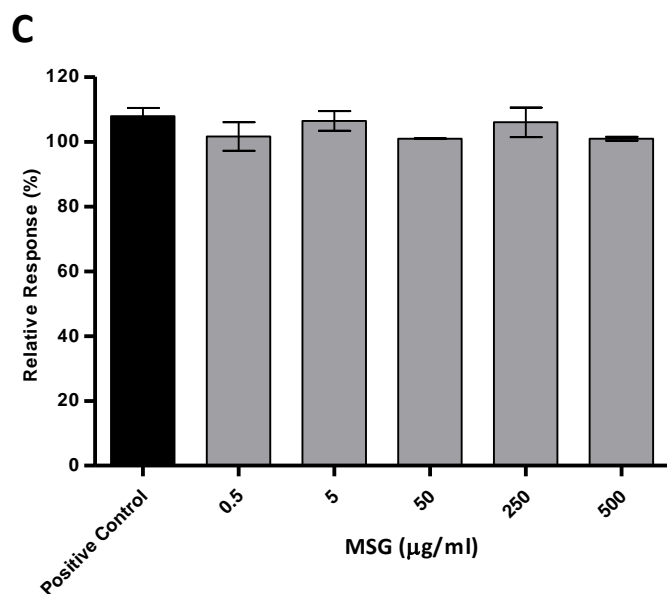


Fig. 5: Antagonist effects in the a) MMV-Luc, b) TARM-Luc cell line, and c) TM-Luc cell line cell lines, following exposure to 0.5- 500 $\mu\text{g/ml}$ MSG. Antagonism is measured in the presence of the agonist hormone (relative positive control) (1.36 ng/ml 17 β -estradiol, 14.5 ng/ml testosterone and 157 ng/ml progesterone). Response is expressed as the percentage response \pm SEM for three independent exposures in triplicate ($n = 3$). $P \leq 0.05$ (*) and $P \leq 0.001$ (***) represent significance.

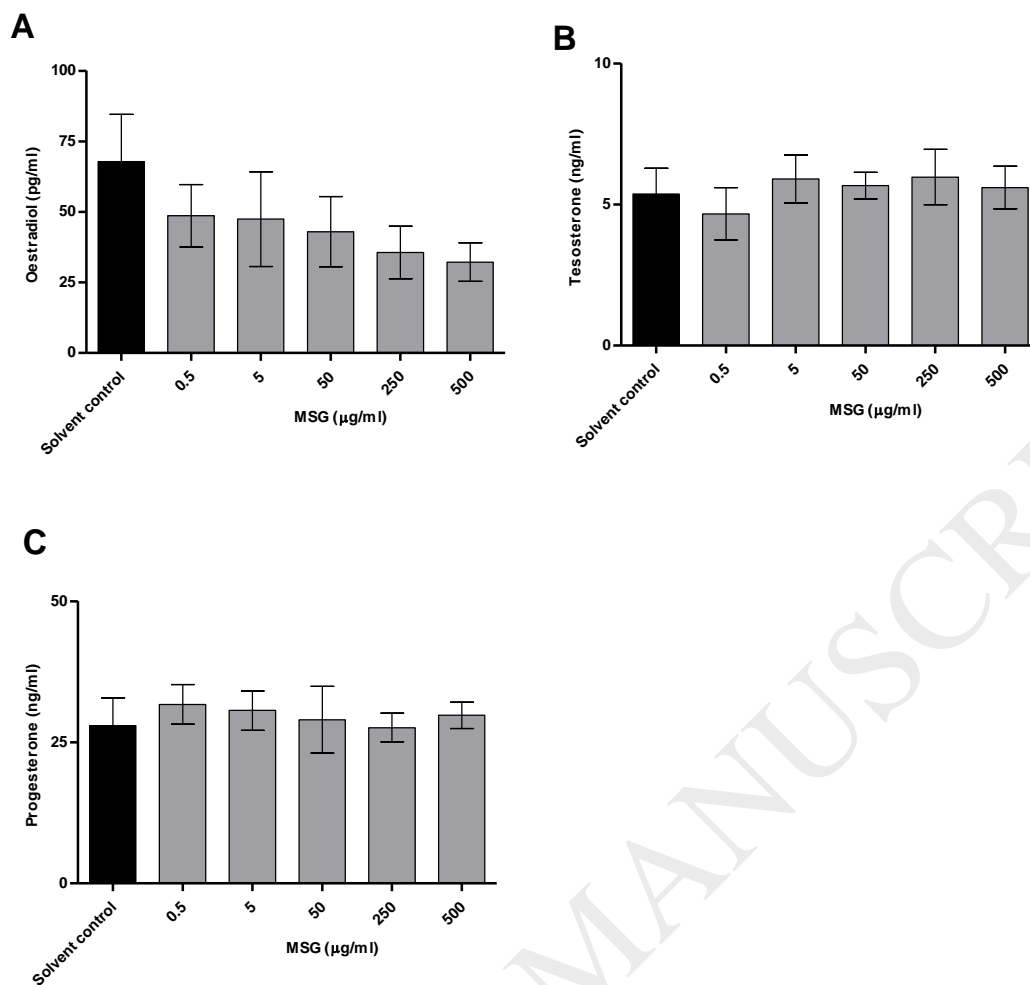


Fig. 5: Production of A) oestradiol, B) testosterone and C) progesterone, by H295R cells following exposure to 0.5-500 µg/ml MSG for 48 h. Values are means \pm SEM for three independent exposures in triplicate ($n = 3$).