UNIVERSIDADE DE LISBOA

Faculdade de Medicina de Lisboa

The Role of Sexual Steroid Hormones in Glioblastoma

Mariana Cristina Lança de Oliveira

Orientadores: Professora Doutora Cecília Santos

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Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

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Abstract

Introduction: Glioblastoma (GBM) represents about 30-40% of the Central Nervous System tumors and has 50% more incidence in men than in women. This sex biased pattern suggests that sex hormones may be of relevance for GBM's etiopathology. However, this hypothesis has not been the target of much investigation so far.

Objectives: The aim of this project was to assess the role of sex steroid hormones, estradiol (E2), progesterone (P4), dihydrotestosterone (DHT) and testosterone (T) in cell viability and apoptosis of three GBM cell lines (U-87MG, SNB19 and U-373MG),which represent different grades of GBM aggressiveness, and in a human astrocyte cell line (HASTR/ci35).

Materials and methods: The presence of estrogen, progesterone and androgen receptors (ER, PR and AR, respectively) was assessed in U-87MG, SNB19, U-373MG and in HASTR/ci35 by immunocytochemistry. Then, the effect of E2, P4, DHT and T on cell viability was assessed by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay upon incubations with physiological concentrations of hormones, during different incubation periods.

Results: We found ERα and ERβ expression in HASTR/ci35, U-87MG, SNB19, and U-373MG. ERα had a predominant cytoplasmatic distribution in all the cells lines except in U-87MG. PR had a marked expression in the U-87MG cell line but not in HASTR/ci35, SNB19 and U-373MG. AR expression was confirmed in all cell lines analysed. E2 increased the viability of HASTR/ci35 and diminished it in SNB19, pointing to a protective role in these lines. Progesterone diminished viability in U-87MG cells but had the opposite effect in the most aggressive cell lines SNB19 and U-373MG. DHT increased the viability of U-87MG, SNB19 and U-373MG. Interestingly, testosterone increased the viability of astrocytes but diminished the viability of U-87MG cells, what contradicts previous studies pointing to its effect in promoting migration, invasion and proliferation in GBM.

Conclusion: This study sets the basis for further exploring the effect of these hormones on relevant parameters of cancer such as proliferation, apoptosis, migration and invasion to further elucidate the differences observed in the effect of these hormones on cell viability, that concur with a protective effect of E2 and T, and a more controversial role of P4 in GBM cell lines. On the other hand, DHT, enhanced cell viability, what is a

preliminary indication that the higher prevalence of GBM in men might be indeed favoured by their hormonal background.

Keywords: Glioblastoma, Sex Steroid Hormones, Estradiol, Progesterone, Testosterone, Dihydrotestosterone

Resumo

Introdução: O glioblastoma (GBM) representa cerca de 30-40% dos tumores do Sistema Nervoso Central e tem 50% mais incidência em homens do que em mulheres. Esse padrão de tendência para um viés sexual sugere que as hormonas sexuais podem ser relevantes para a etiopatologia do GBM. No entanto, essa hipótese não tem sido alvo de muita investigação até ao momento.

Objetivos: O objetivo deste projeto foi avaliar o papel das hormonas esteróides sexuais, estradiol (E2), progesterona (P4), dihidrotestosterona (DHT) e testosterona (T) na viabilidade celular e apoptose de três linhas celulares de GBM (U-87MG, SNB19 e U-373MG), que representam diferentes graus de agressividade do GBM e numa linha celular de astrócitos humanos (HASTR/ci35).

Materiais e métodos: A presença de recetores de estrogénios, progesterona e androgénios (ER, PR e AR, respetivamente) foi avaliada em U-87MG, SNB19, U-373MG e em HASTR/ci35 por imunocitoquímica. De seguida, o efeito de E2, P4, DHT e T na viabilidade celular foi avaliado pelo ensaio de MTT em incubações com concentrações fisiológicas de hormonas, durante diferentes períodos de incubação.

Resultados: Verificou-se a expressão de ERα e ERβ em HASTR/ci35, U-87MG, SNB19 e U-373MG. O ERα teve uma distribuição predominantemente citoplasmática em todas as linhas celulares, exceto em U-87MG. PR teve uma expressão marcada na linha celular U-87MG, mas não em HASTR/ci35, SNB19 e U-373MG. A expressão de AR foi confirmada em todas as linhas celulares analisadas. E2 aumentou a viabilidade em HASTR/ci35 e diminuiu em SNB19, apontando para um papel protetor nessas linhas. A progesterona diminuiu a viabilidade nas células U-87MG, mas teve o efeito oposto nas linhas celulares mais agressivas SNB19 e U-373MG. A DHT aumentou a viabilidade das U-87MG, SNB19 e U-373MG. Curiosamente, a testosterona aumentou a viabilidade dos astrócitos e a diminuiu a viabilidade nas células U-87MG, o que contradiz estudos anteriores que apontam para o seu efeito na promoção da migração, invasão e proliferação em GBM.

Conclusão: Este estudo estabelece as bases para explorar ainda mais o efeito destas hormonas em parâmetros relevantes no cancro, como proliferação, apoptose, migração e invasão, para elucidar ainda melhor as diferenças observadas no efeito destas hormonas na viabilidade celular, que coincidem com um efeito protetor das hormonas E2 e T, e um

papel mais controverso de P4 em linhas celulares de GBM. Por outro lado, a DHT, aumentou a viabilidade celular, o que é uma indicação preliminar de que a maior prevalência de GBM nos homens pode ser de facto favorecida pelo seu *background* hormonal.

Palavras-Chave: Glioblastoma, Hormonas Esteroides Sexuais, Estradiol, Progesterona, Testosterona e Dihidrotestosterona

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List of Acronyms and Abbreviations

AR – Androgen Receptor

CAML – Centro Académico de Medicina de Lisboa

CNS – Central Nervous System

CP – Choroid Plexus

CSF – Cerebrospinal Fluid

DHT – Dihydrotestosterone

DMSO - Dimethyl Sulfoxide

E2 – Estradiol

ER – Estrogen Receptor

FBS - Fetal Bovine Serum

GBM – Glioblastoma

MGMT - O6-methylguanine methyltransferase

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

O6-MG - O6-methylguanine

P4 – Progesterone

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PR – Progesterone Receptor

SH – Sex Steroid Hormones

T – Testosterone

TBS – Tris-buffered Saline

TMZ – Temozolomide

1. Introduction

1.1. Glioblastoma

Glioblastoma (GBM) is a tumor that derives from glial cells, specifically from astrocytes, representing about 30-40% of the tumors of the Central Nervous System (CNS) (Bao et al., 2017; Sousa et al., 2002) and 80% of its malignant tumors (Bao et al., 2017). Despite the relatively low incidence, GBM is a pathology with a considerably low five-year survival rate (4.7%), thus having a quite reserved prognosis (Moinfar et al., 2016). Even with treatment, the overall survival is 12-18 months (Alphandéry, 2018). For that reason, GBM is the most common and the most aggressive form of astrocytoma, classified as grade IV according to the World Health Organization, characterized by an extremely high genomic instability (Nogueira et al., 2019; Louis et al., 2016; Germán-Castelán et al., 2015). About 90% of GBMs arise without any previous evidence of a precursor lesion and for that reason these are classified as primary GBMs (Ohgaki et al., 2013; Ohgaki et al., 2007). On the other hand, secondary GBMs evolve from previous lesions, usually from lower grade astrocytomas (Gessler et al., 2017). Even though both types are not distinguished histologically, they can be differentiated genetically, for example for the expression of specific mutations, such as isocitrate dehydrogenase 1 (IDH1) and p53 mutations in secondary GBMs, and hypermethylation, loss of heterozygosity on chromosome 10q, epidermal growth factor receptor amplification, p16 deletion and phosphatase and tensin homolog (PTEN) mutations in primary GBMs. Furthermore, primary GBMs are more common in older patients (mean of 62 years old) while secondary usually occur earlier in life (mean of 45 years old) and seem to present a histological pattern with less necrosis. Usually secondary GBMs develop more slowly and the mean survival rate for these is longer than for primary GBM (Gessler et al., 2017; Ohgaki et al., 2013; Ohgaki et al., 2007).

The golden standard treatment for GBM is surgical resection, radiotherapy and chemotherapy with temozolomide (TMZ) and bevacizumab for primary and recurrent cases, respectively (Mooney et al., 2019; Ozdemir-Kaynak et al., 2018). However, the treatment by itself is not sufficient and is usually accompanied by chemoresistance and neurological deterioration, and a great probability of relapse (Alphandéry, 2018; Ozdemir-Kaynak et al., 2018). TMZ crosses the blood-brain barrier due to its small size

and liposolubility and acts by adding methyl groups to specific localizations of DNA or RNA, which creates mismatched base pairs that will trigger repair mechanisms that will eventually be ineffective and lead to apoptosis. Despite this, it appears that only 20% of the concentration of TMZ in the blood is present in the brain, which can justify its low efficacy. Other than this, TMZ may also be influenced by gene expression in the tumors (Schreck et al., 2018). Studies have found this to occur because of the overexpression of O6-methylguanine methyltransferase (MGMT), which removes O6-methylguanine residues in order to repair base pair mismatches. Like mentioned before, TMZ is one of the agents that causes these mismatches and if O6-methylguanine mismatches are not repaired because of low MGMT expression, then the base pair mismatch will eventually result in failed attempts of repair ultimately resulting in cell death. However, the methylation damage caused by TMZ can be reversed by higher levels of MGMT and this is thought to be the reason for chemoresistance in some patients where elevated MGMT gene expression can confer resistance to treatment. Thus, higher levels of MGMT seem to favour tumorigenesis and lower levels may be responsible for the resistance to the most effective drug presently utilized, TMZ. Therefore, for GBM treatment, the expression of MGMT should be measured to guide a more effective treatment plan (Lee et al., 2016 & Cabrini et al., 2015).

One of the main problems with the standard treatment is the difficulty in completely resecting the entire tumor with brain surgery due to the invasive, proliferative and migratory characteristics of this type of tumor. Moreover, diagnosis does not usually occur in early stages, also enhancing this difficulty. Resistance to treatment is another major problem and it is due to the infiltrating nature, heterogeneity and tumor interactions with its environment. These factors allow the tumor to be more resistant to therapy and promote several tumor hallmarks such as angiogenesis, apoptosis, immune system escape and secretion of survival factors which ultimately also limit the efficacy of the treatment (Da Ros et al., 2018; Séhédic et al., 2015). Furthermore, these tumors are characterized by the existence of several dysregulated pathways, that cannot be blocked and/or repaired. The Cancer Genome Atlas (Chuang & Ling, 2019) identified three altered pathways in GBM: (i) RTK/Ras/ PI3K; (ii) p53; and, (iii) retinoblastoma pathways. The blood-brain barrier is also strongly linked to the drug-resistant GBM phenotype because it often prevents drugs from reaching the target (Alphandéry, 2018). Cancer cells can acquire a resistant phenotype in response to therapy, or can be intrinsically resistant (Da Ros et al., 2018). All of the three pathways lead to tumor heterogeneity, therefore a comprehensive

characterization of these tumors (Touat et al., 2017) can contribute to the development of new strategies and it becomes clear that new therapeutic targeted agents are urgently warranted (Mooney et al., 2019).

Although there are no certainties about GBM's etiopathology, it is well known that it has a larger incidence in men compared to women (Sareddy et al., 2016). Several epidemiological studies have demonstrated that primary GBM is 50% more prevalent amongst men (Yu et al., 2015; Moinfar et al., 2016; Bao et al., 2017). Other epidemiological data reported that GBM occurs in a proportion of 3:2 in men compared to women, and other revealed an incidence 1.6 times higher in men (Tamimi et al., 2017; Thakkar et al., 2014; Ostrom et al., 2013). McKinley *et. al* (2000) showed that women present a lower risk to develop GBM particularly around menopause and decreasing thereafter.

Although sex differences in GBM incidence are well-established, they are not sufficiently understood to enable sex-specific targeted treatments. However, it was found that the GBM standard treatments are also more effective in women than in men (Yang et al., 2019). Furthermore, a study developed by Kfoury *et al.* (2018) correlated sex differences in murine implanted GBM cells, revealing that only females had increased p16 and p53 activity and cell cycle arrest, while male cells would continue to proliferate and enhance tumorigenesis. This pattern suggests that hormonal and/or genetic differences could be of relevance for GBM's etiology. This is supported by the fact that several studies have demonstrated the presence of estrogen, progesterone and androgen receptors in GBM (Tavares et al., 2016; Germán-Castelán et al., 2016; Yu et al., 2015; Liu et al., 2014).

1.2. Sex Steroid Hormones

Steroids play a critical role in numerous processes. Sex steroid hormones (SHs) are lipophilic molecules, derived from a common precursor (pregnenolone), known to be implicated in varied biological functions such as development, metabolism, regulation of the hypothalamus and hypophysis, amongst many others. Since they are lipophilic, they have the capacity to move across cell membranes (Diotel et al., 2018; Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016; Regidor, 2014; Lenz et al., 2010; Bain et al., 2007; Wise et al., 2009).

Steroidogenesis (Figure 1) is a process by which cholesterol is converted to biologically active steroid hormones, the rate-limiting step in the synthesis of these

hormones (Diotel et al., 2018; Sewer et al., 2008). The first step occurs in the mitochondria, facilitated by the steroidogenic acute regulatory protein, a transporter on the outer mitochondrial membrane. After the transition to the inside of the mitochondria, the cytochrome P450 enzyme cleaves the aliphatic tail of cholesterol, originating pregnenolone, which can be converted into progesterone (P4) by 3β-HSD or into 17-hydroxypregnenolone by CYP17, which happens in the endoplasmic reticulum. This enzyme (CYP17) is able to convert progesterone into 17-hydroxyprogesterone and 3β-HSD can also convert 17-hydroxypregnenolone into 17-hydroxyprogesterone, also in the endoplasmic reticulum. Furthermore, 17-hydroxypregnenolone and 17-hydroxyprogesterone can be converted by CYP17 into dehydroepiandrosterone (DHEA) and androstenedione, respectively. 17β-HSD enzymes can catalyse the synthesis of androstenediol from DHEA and testosterone (T) from androstenedione. Aromatase (Aro) converts androstenedione and testosterone into estrone (E1) and estradiol (E2), respectively, while 5α-reductase (5α-R) converts testosterone into 5α-dihydrotestosterone (DHT). In a different step, glucocorticoids can also be formed by CYP21A2 which synthesizes 11-deoxycorticosterone and 11-deoxycortisol from progesterone and 17-hydroxyprogesterone, respectively, still in the endoplasmic reticulum. Cyp11 can synthesize corticosterone and cortisol from 11-deoxycorticosterone and 11-deoxycortisol, respectively, in the mitochondria. Finally, 11β-HSD can synthesize cortisone (Diotel et al., 2018; Sewer et al., 2008).

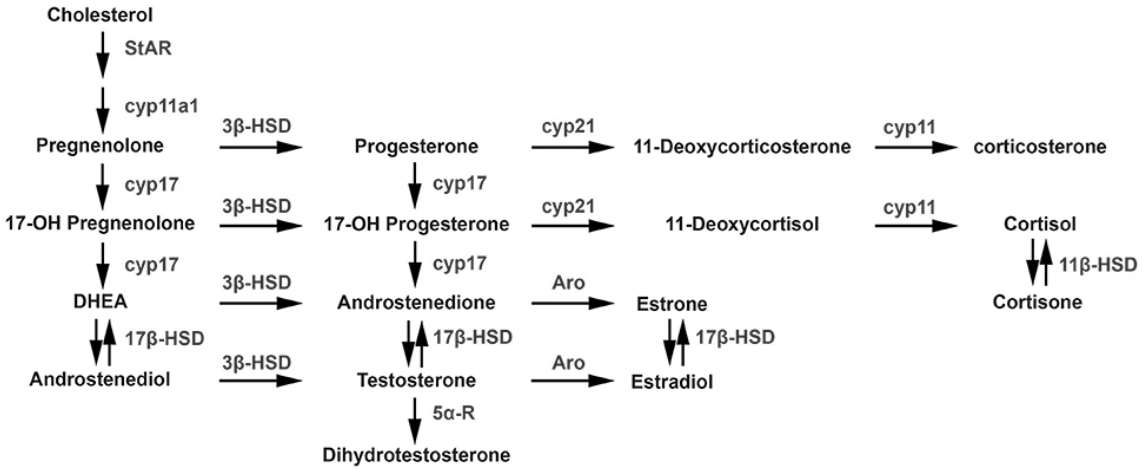


Figure 1 - Steroidogenesis pathways. Adapted from Diotel *et al*., 2018.

In the past, it was thought that steroid hormone synthesis only occurred in steroidogenic glands. However, other organs, such as the brain, adipose tissue and

intestine, can also synthetize steroid hormones. This is due to an enzyme-dependent process from steroid precursors within specific tissues (Diotel et al., 2018; Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016; Regidor, 2014; Lenz et al., 2010; Bain et al., 2007; Wise et al., 2009).

There are six families of steroid hormones: estrogens, progestins, androgens, mineralocorticoids, glucocorticoids, and vitamin D. The estrogens' family is constituted by estradiol, estrone and estriol, progesterone belongs to progestins' family, and androgens are constituted by dehydroepiandrosterone, androstenedione, testosterone (T) and 5α-dihydrotestosterone (DHT), altogether constituting the group of SHs (Acconcia et al, 2016).

Testosterone is the principal male androgen, even though DHT, a metabolite reduced by 5α-reductase from T in certain tissues, has greater affinity for the androgen receptor (AR). On the other hand, DHEA is considered a weaker androgen that can be converted to testosterone and androstenediol, or to estrogens, in other tissues. The conversion of T to estradiol, in tissues such as the bone, brain, testis or adipose tissue, occurs via P450 aromatase. It is also possible for androstenedione to be aromatized, however it is converted to estrone, which can then be metabolized to estradiol by 17β-hydroxysteroid dehydrogenase (Diotel et al., 2018; Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016; Regidor, 2014; Lenz et al., 2010; Bain et al., 2007; Wise et al., 2009).

Plasma testosterone is primarily secreted by Leydig cells around the 4^{th} and 6^{th} week of intrauterine life, which leads to sexual differentiation. During childhood, these levels remain quite low and dormant and rise to adult levels by the end of puberty, when more pronounced changes occur in the male body leading to the development of secondary sexual characteristics (Alexander, 2014). After middle age, total T levels begin to decline up to 1.6% for every year, a phase called andropause (Hiort, 2013; Stanworth et al., 2008). There are several biological actions of androgens, including development and maintenance of the male reproductive system and anabolic effects, for example on skeletal muscle and brain (Diotel et al., 2018; Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016; Regidor, 2014; Lenz et al., 2010; Bain et al., 2007; Wise et al., 2009).

In the adult female, the two most important steroid hormones are 17β-estradiol (estradiol/E2) and progesterone (P4). In addition, two metabolites of estradiol, estrone and estriol, circulate at high levels at certain phases of the menstrual cycle and during pregnancy. The biological actions of E2 can be divided into those directed towards the development and maintenance of the female reproductive system and those that have

effects on several other organs including the cardiovascular system, metabolism, and brain. Ovarian estrogen synthesis, is produced in a pulsatile manner every month, during the menstrual cycle. The peak of estrogen synthesis occurs 6 months after birth and is followed by the relative quiescence throughout childhood until the beginning of the pubertal period. Plasma estrogen pulsates at adult levels by the end of puberty and begins to decline in middle age (menopause). Circulating estrone and E2 are produced by the aromatization of androgens. Furthermore, after ovulation, the major steroid produced by the luteinized cells of the corpus luteum is P4 (Diotel et al., 2018; Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016; Regidor, 2014; Lenz et al., 2010; Bain et al., 2007; Wise et al., 2009).

Looking into SHs roles in the CNS, it is known that the activation of hormone receptors by SHs results in neuroprotective events, such as decreased reactive oxygen species, increased cell survival, reduced apoptosis, increased production of neurotrophic factors and regulation of Ca^{2+} balance (Duarte et al., 2016).

Peripheral levels of estrogens and progesterone decrease abruptly in women, whereas, in men, androgens decline is more gradual. Comparing E2 serum levels in pre- and postmenopausal women, there is a steep decrease from approximately 200–300 pmol/l to only approximately 30 pmol/l (Table 1). Similarly, P4 levels in premenopausal women are higher than in postmenopausal women who have values below 1 nmol/l (Duarte et al., 2016).

In the serum of men, T also decreases with ageing but in a more modest way than the E2 decline in women. In adult men, T is typically approximately 15 nmol/l, whereas, in elderly subjects, it is below 10 nmol/l. Serum T levels are higher in men, although in the cerebrospinal fluid (CSF), levels are similar between sexes (Duarte et al., 2016).

Moreover, E2 levels in men's CSF are higher than in serum, as well as higher than those in the women's CSF. This suggests a conversion of T to E2 by aromatase within the male brain. In addition, DHT levels in the CSF of men might also be higher than in serum, or at least very similar. Collectively, these observations reflect the occurrence of neurosteroidogenesis (Giatti et al., 2019; Duarte et al, 2016).

Table 1 – Sex steroid hormones concentrations in cerebrospinal fluid and serum. Adapted from Duarte *et al*., 2016.

	CSF			Serum		
	Men	Women		Men	Women	
		Pre-Menopausal	Post-Menopausal		Pre-Menopausal	Post-Menopausal
E2 (pmol/l)	~ 147	34.50 ± 37.50	24.20 ± 23.90	~ 46.26	~ 331.30	35 ± 38
P4 (nmol/l)	~ 0.95	~ 1.24	0.39 ± 0.24	2.51 ± 1.62	7.31 ± 1.91 Pregnant: 388 ± 25.40 Post-Partum: 50.88 ± 6.90	0.57 ± 0.35
DHT (nmol/l)	2.62 ± 0.62			1.30 ± 0.60	0.24 ± 0.10	0.14 ± 0.10
T (nmol/l)	~ 0.43	0.23 ± 0.36	~ 0.19	Adult: 10.60 ± 0.94 Elderly: 6.90 ± 2.50	~ 1.50	~ 0.71

Androgens have an important role in shaping the structure of the brain and contributing to sex differences. Estrogens are effective regulators of brain cell morphology and tissue organization through the regulation of the cytoskeleton. Brain cells' morphology is controlled by estrogens that regulate the development of neuron/neuron interconnections and dendritic spine density (Ruiz-Cortés, 2012).

The decline in SH levels along with ageing may contribute to compromised neuroprotection and set the grounds for neurodegeneration and cognitive impairments (Duarte et al., 2016). Several brain diseases, like Alzheimer and Parkinson, present sex differences regarding their prevalence and incidence, hence SHs released not only by peripheral tissues but also by neurons and glial cells may play a role in these differences that must be understood (Santos et al., 2017).

Sex steroid hormones also regulate one of the main brain barriers - the choroid plexus (CP) which is constituted by single layers of epithelial cells that also express sex hormone receptors (ER, PR and AR). Some of the functions of CP cells regulated by SH include metabolism, steroid hormone biosynthesis, circadian rhythm pathways, chemosensing and expression of neurogenic factors that may impact on neurodegenerative diseases (Santos et al., 2019; Santos et al., 2017; Quintela et al., 2013).

1.3. Sex Steroid Hormones and Cancer

Whereas SH are recognized neuroprotectants, there is an overall consensus on their carcinogenic effect in several organs and they may also have a role on the sex bias of cancer. In fact, the prevalence of cancer of various organs is higher in men than women (except for thyroid cancer). In organs like women breast and prostate, the role of sex hormones has been studied in depth as well as in non-sexual organs like stomach, esophagus or thyroid.

Higher levels of estrogens and androgens were associated with higher breast cancer risk, being more pronounced in older individuals. Estrogens are known to promote the proliferation of breast cells and to make the cells more prone to errors during replication and consequently more DNA damage, so that is one of the possible justifications for its effects. For this reason, many anti-estrogenic drugs, are sought after for breast cancer treatment. Regarding androgen levels, it was suggested that the aromatization of androgens into estrogens could explain why they also increase proliferation of breast cancer cells (Baglieto et al., 2010; Gadducci et al., 2009).

Progesterone is known to induce methylation during pregnancy, a phase when its levels are higher, which could potentially lead to silencing of genes of interest for tumorigenesis (Baglieto et al., 2010). However, its role in breast cancer still remains unclear, and some studies have found that high levels of testosterone and low levels of progesterone were associated with breast cancer development (Micheli et al., 2004).

High levels of DHT pointed to a higher risk of prostate cancer (Platz et al., 2004), while E2 seems to have a controversial role. A study indicated that in men older than 70, there was a reduced risk of developing prostate cancer due to a higher estradiol:testosterone ratio showing a protective role of E2 and the opposite for T (Black et al., 2014). However, a different study showed that estrogens can have a role in the development of prostate cancer because of ER-mediated effects in increasing toxicity and inflammation (Nelles et al., 2011). Progesterone's effect also remains controversial in prostate cancer, presenting lower values in these patients but its accumulation resulting in mutations and activation of AR (Boibessot et al., 2018).

In terms of the digestive tract, E2 was shown to diminish proliferation and migration and induce apoptosis in oesophagus and colon cancer and lowering risk while increasing apoptosis and lowering proliferation in stomach cancer. Progesterone induced a lower risk in women with colon cancer while testosterone increased overall risk in this

type of cancer. Estrogen receptor α (ERα) was abundant and ERβ was highly expressed in oesophagus and stomach cancers. Interestingly, in stomach cancer androgen receptor (AR) was highly expressed while in colon cancer it was not detected (Tang et al., 2017; Roshan et al., 2016; Wang et al., 2014; Wolmarans et al., 2014; Yi et al., 2014; Kambhampati et al., 2013; Wang et al., 2012; Kambhampati et al., 2010; Joubert et al., 2005).

Finally, in thyroid cancer women have a higher prevalence, E2 increased proliferation and ERα was highly expressed while ERβ poorly expressed (Zane et al., 2017 & Hima et al., 2016).

1.3.1. Sex Steroid Hormones in Glioblastoma

Despite the higher prevalence of GBM in men compared to women, the reasons underlying these differences still remain elusive. How SH and their receptors control the intracellular processes that favour malignant transformation and cancer progression in the CNS are still poorly studied. Moreover, the available data is often contradictory in the scarce literature that attempts to explain these differences in the incidence of the disease between men and women. The discrepancies in the results already reported could be related with the fact that previous studies have focused on the effect of only one SH, in the different models analysed, and also because of the wide range of hormone concentrations tested which was often above physiological values. Therefore, more studies in this field are essential to elucidate the SHs role in GBM. This project intents to compare the action of different SHs within the same experimental setting using physiological concentrations of SH. Besides, the use of human GBM cells instead of an animal model may be an advantage. Immortalized cell lines have several advantages such as being homogenous and genetically equal which can mean more consistent results. Other than that, they are easy and relatively fast to grow. However, they also present disadvantages because of the fact that they can divide continuously, thus having characteristics that "normal" cells do not. Moreover, their characteristics and morphology may change which is why they must be continuously monitored (Kaur et al. 2012 & Carter et al., 2010).

1.3.1.1. Estradiol

Estradiol actions are mediated by two types of receptors: estrogen receptor alpha (ERα), associated to tumor promoting effects, and estrogen receptor beta (ERβ),

considered a tumor suppressor (Sareddy et al., 2012). Low levels of both receptors were described in GBM. A study developed by González-Arenas *et al.* (2012), showed that it was possible to induce growth in astrocytoma cell lines through the ERα. A different study from Dueñas-Jiménez *et al.* (2014) explored the expression of aromatase, ERα and ERβ, and the E2 levels in astrocytomas from biopsies of human patients. The levels of E2 were increased in GBM when compared to grade II and III astrocytomas. In addition, the higher mRNA expression of aromatase in GBM was associated with a worse survival prognosis. On the other hand, lower levels of the mRNA expression of ERα in astrocytoma correlated with worse prognosis. Thus, it was possible to conclude that aromatase and ERα expression can be considered as prognostic biomarkers in patients with astrocytoma (Dueñas-Jiménez et al., 2014).

Another study realized by Manca *et al.* (2010), investigated the effectiveness of 2-methoxyestradiol (2-ME) in neuroblastoma and glioma in an animal model. The 2-ME is an anti-angiogenic, pro-apoptotic and anti-proliferative agent that derives from E2. The exposure to this compound resulted in reduction of cell number, shape modifications, retraction or absence of cytoplasmic processes, cellular growth inhibition and decreased viability. The results demonstrated that 2-ME was more effective in neural than in glial cells, and that morphological and functional differences in mitotic blockage and cellular death occurred because of changes in the microtubule system. Another study that analysed the effects of the 2-ME in GBM cells tested the concentrations of 0.2, 2 and 20 μM (all supraphysiological concentrations) in three human GBM cell lines (U-87MG, U-138MG and LN405) and in a rat glioma cell line (RG-2). The cellular viability of the cell lines was significantly reduced in more than 75% after a 6 days stimulus with 2 and 20 μM of 2-ME. Although the concentration of 0.2 μM had smaller effects (10–40% of reduction), those were significantly different in two of the cell lines tested. Apoptosis was analysed in U-87MG and RG-2 lines, and the caspase-3 activity was significantly increased with the two highest concentrations of 2-ME (Chamaon et al., 2005). In a similar study from Braeuninger *et al.* (2005), significant differences were found in GBM cells viability after 48h and 72h periods of incubation with 2 and 20 μM of 2-ME, and the effects were even more significant for the 20 μM concentration during shorter incubation periods. In this study, Hoechst 33258 staining, a fluorescent probe useful for detecting DNA, revealed a substantial number of nuclear fragmentation pointing towards late apoptosis after treatments with 20 μM of 2-ME for 24 hours or more (Braeuninger et al., 2005). In the study developed by Rivera-Delgado & Recum (2017), administration of 2-ME was able

to inhibit the migration of C166 endothelial cell line, known for being targets of anti-angiogenesis therapies.

Estradiol has also increased the survival of male, female and ovariectomized females after the implantation of GBM tumoral cells followed by the administration of E2. The effect mediated by estradiol occurred in an early stage of tumoral progression and was apparently caused by an increase in apoptosis (Barone et al., 2009). A treatment performed *in vitro* with the agonist of ERβ (LY500307), also significantly diminished GBM cells proliferation, without affecting normal astrocytes (Sareddy et al., 2016). Additionally, E2 was exogenously administered to animals with orthotopic GBM, resulting in an increase of apoptosis, cell cycle modulation and cellular response to DNA damage, as well as an improvement in animal survival. Still, it remains unclear whether the E2 effect is direct or indirect and if it is mediated by one of its receptors (Barone et al., 2009).

1.3.1.2. Progesterone

Progesterone has two isoforms of its intracellular receptor, PR-A and PR-B. A study developed by Hansberg-Pastor *et al*. (2017) pointed out that PR-B is the predominant isoform in GBM. Studies with P4 have demonstrated that it significantly diminished the viability of GBM cells. In a study from Atif *et al*. (2015), the incubation of U-87MG, U87dEGFR and U-118MG cell lines with 20, 40, and 80 μM (supraphysiological concentrations) of progesterone caused a decrease in cell viability. On the other hand, low physiological concentrations of P4 (0.1, 1, and 5 μM) induced proliferation of those GBM cell lines (Atif et al., 2015).

Studies reported that the administration of high P4 concentrations triggered more cell death than TMZ, the standard drug used for GBM treatment. Moreover, when simultaneously administered, P4 potentiated the effect of TMZ (Atif et al., 2015). These results were also corroborated in C6 glioma cells (Elmaci et al., 2019).

High-doses of medroxyprogesterone (artificial progestogen) supressed the *in vitro* growth of human GBM cells U-87MG and U-251MG and C6 glioma cells implanted in a rat model (Altinoz et al., 2018). A study using orthotopic C6 GBM cells in rats where different drugs were administered, including 10 or 20 mg/kg of P4 for 5 days, led to longer survival and better preservation of function with 10 mg/kg of P4. Also, a stimulus of 10 mg/kg better preserved the blood-brain barrier permeability, and antiangiogenic behaviour was observed for both P4 concentrations (Cheng et al., 2019). In another study

using an orthotopic GBM mouse model, high doses of P4 led to a decrease in tumor size and increased survival (Atif et al., 2019). This group performed a follow-up study with U-87MG and U-118MG cells where the same high doses of P4 inhibited modulators of glycolytic activity and induced senescence. Interestingly, low doses of P4 led to an increase in U-87MG proliferation (Atif et al., 2019).

On the other hand, the hypothesis that there is a proliferative P4 effect was corroborated in a study where U-87MG cells were implanted in the cortex of male mice, because the P4 administration (400 μg/100 g) caused an increase of tumor area and tumor infiltration in these animals (Germán-Castelán et al., 2016). Moreover, 100 nM of a membrane PR-α agonist increased cell proliferation, migration and invasion in U-87MG and U-251MG cells. This effect was abolished when the mPRα agonist was silenced (González-Orozco et al., 2018). In scratch-wound assays performed in human GBM cells D54 and U-251MG, P4 (10 nM, a physiological concentration) increased migration from 3 to 48h. Moreover, the invasion capacity was also increased at 24h (Piña-Medina et al., 2016). Progesterone might also have an important role mediated by the progesterone-induced blocking factor (PIBF). It was shown that PIBF is expressed in the cytosol of six GBM cell lines, and it is thought to supress the anti-proliferative tumoral capacity (Kyurkchiev et al., 2014). It was also demonstrated that PIBF's expression was upregulated by P4 (10 nM) in U-87MG and U-251MG human GBM cells. PIBF (100 ng/mL) increased the number of U-87MG and also increased migration and invasion of both U-87MG and U-251MG in wound-healing and transwell assays, respectively (Gutiérrez-Rodríguez et al., 2017).

1.3.1.3. Dihydrotestosterone

A significant up-regulation of the AR expression in GBM was detected in comparison to normal brain tissue in humans (Yu et al., 2015). In U-87MG cells, stimulus with DHT significantly decreased the effect of the anti-tumoral receptor TGFβ1 (transforming growth factor beta 1), whose activation significantly inhibits cellular growth and increases apoptosis, suggesting that the AR signalling pathway may counteract the effect of the TGFβ1 receptor in GBM, by promoting tumorigenesis in men through the inhibition of TGFβ1 signalling (Yu et al., 2015).

1.3.1.4. Testosterone

A study performed with the U-87MG cell line demonstrated that AR was up-regulated in glioma cell lines treated with the synthetic androgen R1881 (Bao et al., 2017). In another study performed in 2007, 10 μM (supraphysiological concentration) of testosterone significantly increased the proliferation of rat C6 glioma cells (González et al., 2007). More recently, Bao *et al.* (2017) reported that increased T levels were significantly risen in GBM patients (Bao et al., 2017).

Rodríguez-Lozano *et al*. (2019) analysed the effects of increasing T concentrations (1, 10 and 100 nM) in GBM cell lines and observed an increase in migration, invasion and proliferation of U-87MG, U251MG and D54 GBM cells, an effect that was blocked in the presence of an AR antagonist (flutamide). Another study where T98G and U138-MG GBM cells were treated with 1000 nM of testosterone, was observed an increase in proliferation (Merrit & Foran, 2007).

Table 2 – Effects of sex steroid hormones in glioblastoma.

Hormones	Effects	References
E2	↓proliferation ↑patient survival ↑ apoptosis	Sareddy *et al.* (2016); Manca *et al.* (2010); Barone *et al.* (2009); Braeuninger *et al.* (2005); Chamaon *et al.* (2005)
P4	↑survival ↑cell death ↓ viability	Atif *et al.* (2019) – concentrations used of 10, 20, 40 and 80 μM Cheng *et al.* (2019) - concentrations used of 10 or 20 mg/kg Atif *et al.* (2015) – concentrations used of 1, 5, 10, 20, 40, 80 μM
	↑ proliferation ↑ migration and invasion	Atif *et al.* (2015) & Atif *et al.* (2019) - concentrations used of 0.1, 1, and 5 μM Germán-Castelán *et al.* (2016) - concentrations used of 400 μg/100 g González-Orozco *et al.* (2018) – concentration used of 100 nM Gutiérrez-Rodríguez *et al.* (2017) - 10 nM
DHT	↓ apoptosis	Yu *et al.* (2015)
T	↑proliferation ↑migration and invasion	Rodríguez-Lozano *et al*. (2019); González *et al*. (2007); Merrit & Foran (2007)

Overall, we can infer that E2 has protective effects because it diminishes proliferation in GBM cells and increases apoptosis. There is less consensus on the role of

P4 in GBM with some studies referring opposite effects. In general, when higher and supraphysiological P4 concentrations were used, an increase in cell survival of normal cells was observed, contrarily to an increase in cell death and a decrease in viability of GBM cells. On the other hand, when lower concentrations of P4 were used, a pro-proliferative effect with an increase in migration and invasion were detected. DHT had predominantly an effect that potentiates tumorigenesis, diminishing apoptosis in GBM cells. Finally, testosterone induces proliferation as well as migration and invasion in GBM cells (Table 2).

2. Aims

The hypothesis of this project is that the sex bias seen in GBM prevalence may be due to the exposure to sexual steroid hormones that may alter cell viability of GBM cells. It is expected that the exposure to estradiol and progesterone induces a decrease in the proliferative and anti-apoptotic characteristics of the GBM cells, while the exposure to testosterone and DHT prompts the opposite effect.

Therefore, the main goal of this project was to study the role of sexual steroid hormones (estradiol, progesterone, testosterone and DHT) in three GBM cell lines (U-87MG, SNB19 and U-373MG) with different grades of aggressiveness and proliferation, in comparison with a human astrocyte cell line (HASTR/ci35), especially their effect in cell viability and apoptosis.

This study was approved by the ethics committee of Centro Académico de Medicina de Lisboa (CAML).

3. Materials and Methods

3.1. Cell Lines

Three human GBM cell lines with different grades of proliferation and aggressiveness were used in this project: U-87MG, SNB19 and U-373MG. U-87MG and U-373MG represent the least and most proliferative and aggressive forms of GBM, respectively. This classification was based on observation of the cell's pattern and rate of growth. A cell line of human astrocytes (HASTR/ci35) was also used in cell viability assays. The laboratorial experiences included immunocytochemistry, cell viability and apoptosis assays. The experiences described below were performed for each cell line, and for the different hormonal experimental conditions. Each condition was tested for different SH concentrations and incubation periods.

3.1.1. Cell Culture and Expansion

The three GBM cell lines (U-87MG, SNB19 and U-373MG) were kept in culture in a LEEC Culture Safe CO_2 Precision 190 incubator at 37°C and controlled atmosphere with 5% CO_2. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) high glucose with stable glutamine (bioWest, France) supplemented with 10% of Fetal Bovine Serum (FBS, Biochrom, Berlin) and 0.1% penicillin/streptomycin (Sigma, USA). When the confluence reached about 70- 90%, cells were trypsinized to a new flask in order to decrease cellular density and guarantee the continuous cellular expansion. This procedure consisted on removing cell culture medium, washing the cells with sterile phosphate buffered saline (PBS) 1x, and trypsinization (usually 5 minutes at 37ºC) with a trypsin/EDTA 0.25% solution added in a volume that ensured the full coverage of the cells. Once detached, equal volume of fresh cell culture medium was added into the T-flask and cells were gently resuspended with a micropipette, collected and centrifuged for 3 minutes at 1300 rpm. The supernatant was discarded and the pellet containing the cells was resuspended in 1 mL of culture medium, then transferred to a new T-flask containing 3 mL of fresh cell culture medium. The culture medium was replaced every 2-3 days.

The cell line HASTR/ci35 was kept in culture in a LEEC Culture Safe CO2 Precision 190 incubator at 33ºC in a controlled atmosphere with 5% CO_2. Cells were

grown onto collagen type I coated flasks and/or plates in DMEM with N2 supplement 1x, 10% FBS, 1% penicillin/streptomycin and 4 μg/mL blasticidin S. Overall, cell culture and expansion procedures were similar to the mentioned above, except for the centrifugation at 120 g for 3 minutes.

3.1.2. Cell Counting

Before plating for expansion or experiments and after trypsinization, cells were resuspended and 10 μL of the cellular suspension were added to 10 μL of trypan blue 0.4%, followed by homogenization. From this cell suspension, 10 μL were transferred to a Neubauer chamber (Lancing, UK), in order to proceed with the counting of viable cells (with no cells in suspension and with a stretched morphology, more expansive and rapid growth for U-373MG and more clustered and slow growth for U-87MG, while SNB19 were intermediate and, a morphology with more processes and slow growth for HASTR/ci35). The number of cells per mL was estimated as follows:

$$\frac{Cells}{mL} = \frac{Viable\ cells}{Number\ of\ quadrants} \times 2 \times 10^4 \times dilutionfactor$$

3.1.3. Cell Thawing and Freezing

Cells were thawed as quickly as possible in a water bath at 37ºC and centrifuged for 5 minutes at 1200 rpm. The supernatant was discarded, and the pellet was gently resuspended in fresh cultured medium, followed by cell culture as described in section 3.1.1.

To ensure the eternalization of the cell lines, these were frozen as follows: cells were trypsinized as described before and the pellet was resuspended in culture medium containing 40% FBS and 10% dimethyl sulfoxide (DMSO) (v/v). DMSO was used to prevent crystal water formation that can trigger cell lysis. Aliquots of approximately 0.5 – 1x10^6 cells were stored at -80ºC or liquid nitrogen.

3.1.4. Experimental setup

Before the start of all the experiments and when confluence (of 50%) was achieved, cells were serum starved in order to be synchronised to the same cell cycle

stage. This happened by substituting the cell culture medium for one without FBS. Moreover, due to the possible estrogenic activity of phenol red reported by the study from Berthois, *et al*. in 1986, the cell culture medium described in section 3.1.1 was replaced in all the experiments involving sexual steroid hormones by DMEM without phenol-red (Sigma-Aldrich, USA). This FBS-free culture medium was supplemented with 0.1% penicillin/streptomycin, 4.5 g/L glucose, 3.7 g/L $NaHCO_3$, 0.862 g/L stable glutamine, and 0.11 g/L sodium pyruvate. For the HASTR/ci35 cells, the same phenol-red-free DMEM was supplemented with 4.5 g/L glucose, 3.7 g/L $NaHCO_3$, 0.11 g/L sodium pyruvate, N2 supplement 1x, 10% FBS, 1% penicillin/streptomycin and 4 μg/mL blasticidin S.

The hormonal stimuli used for the different experiences are indicated in table 3 and are the following:

Table 3 - Concentrations of SH used in the experiments and corresponding vehicles.

	Concentration	Vehicle
E2	200 pM	EtOH* ≤ 0.00002%
	100 pM	
	10 pM	
P4	100 nM	EtOH ≤ 0.01%
	10 nM	
	1 nM	
DHT	10 nM	EtOH ≤ 0.001%
	1 nM	
	0.1 nM	
T	100 nM	EtOH ≤ 0.01%
	10 nM	
	1 nM	

*EtOH – Ethanol

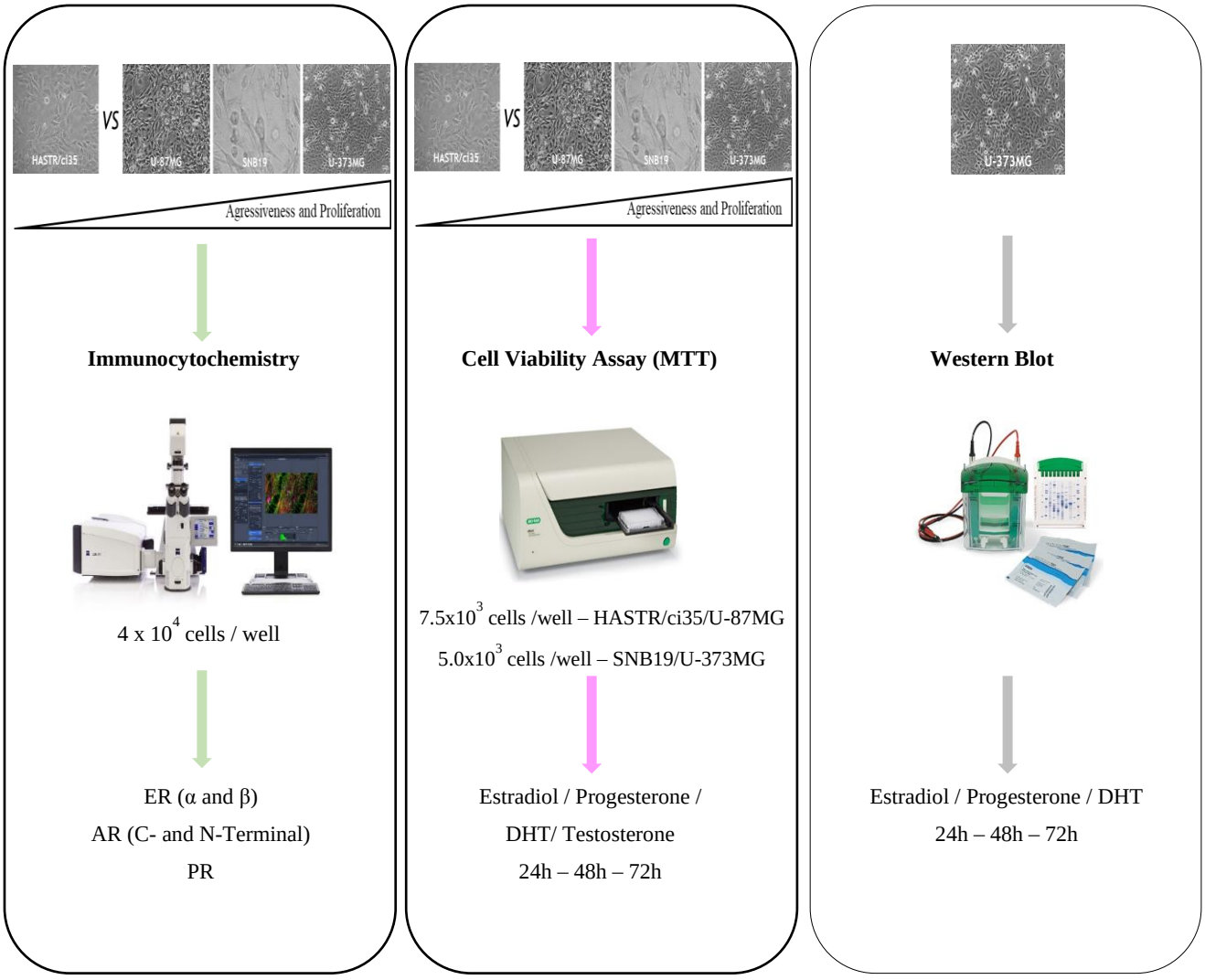


Figure 2 – Experimental setup for Immunocytochemistry, viability assay and western blot.

3.2. Immunocytochemistry and Confocal Microscopy

Immunocytochemistry was performed in order to assess the cellular expression and location of the sexual steroid hormone receptors. 4 x10^4 cells were seeded in coverslips and were grown until 60-70% confluency in 12 well-plates with duplicates for each condition. Then, coverslips were washed thrice with PBS 1x and fixed in 4% paraformaldehyde (PFA) for 10 minutes, followed by incubation for 1 hour at room temperature with a permeabilization/blocking solution containing 3% Bovine Serum Albumin and 0.2% Triton X-100 in PBS 1x. After three wash steps with PBS 1x containing 0.01% Tween-20 (PBS-T), coverslips were incubated overnight at 4ºC with the respective primary antibodies. Coverslips were washed several times with PBS-T and

incubated with secondary antibody Alexa Fluor® 488 (Invitrogen, USA) for 1 hour at room temperature. Finally, cells were washed thrice with PBS-T before nuclei staining with Hoechst 33342 (Invitrogen, USA) for 10 minutes, and mounted with Dako Mounting Medium (Dako Agilent, USA).

The coverslips were visualized under a confocal microscope LSM 710 (Zeiss, Germany), and the images were treated using the Blue Edition from ZEN 2.6 software (Zeiss, Germany).

3.3. Cell Viability Assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (GERBU, Germany). Approximately $7.5x10^3$ HASTR/ci35 and U-87MG cells and $5x10^3$ SNB19 and U-373MG cells were seeded in 96-well microplates, with triplicates for each condition, and cultured for at least 48 hours or until they reached 50% of confluence. Serum starvation and stimuli (Table 3) were performed as described in section 3.1.4, followed by incubation during 24h, 48h and 72h. After that, 110 µL of the cell culture medium were discarded, and 0.5 mg/mL of MTT solution (stock 5 mg/mL in sterile PBS 1x) were added. Relatively to the positive control (K^+, dead cells), 160 µL of culture medium were discarded, and added 50 µL of 100% ethanol (final concentration of 50%) followed by 0.5 mg/mL of MTT solution. Untreated cells were used as negative (K^-) controls, and culture medium only was used as blank. The microplates were left in the incubator until formazan crystals were formed (approximately 30 minutes for GBM cells and 120 minutes for HASTR/ci35). Then, the culture medium containing MTT solution was removed and 100 µL of DMSO were added, followed by stirring in an orbital shaker to dissolve crystals. Finally, 80 µL of each well were transferred to a new 96-well microplate and the absorbance at 570 nm was read in a microplate spectrophotometer xMark™ (Bio-Rad, USA). The results were analysed using the Prism 6 software (GraphPad, USA), using the following formula, normalized relatively to vehicle.

$$\%\, Viability = \frac{Abs_{sample} - Mean\ Abs_{blank}}{Mean\ Abs_{negative\ control} - Mean\ Abs_{blank}} \times 100$$

3.4. Western Blot

Western Blot was performed to detect the expression of Caspase-3, a protein known as an effector in apoptosis. U-373MG cells were seeded in 6-well plates until they reached more than 80% of confluence. Then, the following stimuli were added: 200 pM E2, 1 nM and 10 nM DHT, 100 nM P4, and respective vehicles. Moreover, a positive control of 1 µM staurosporine was also added. Protein extracts obtained from U-373MG cells were resuspended in ice-cold RIPA lysis buffer (NaCl 150 mM, NP-40 1%, sodium deoxycholate 0.5%, SDS 0.1%, Tris 50 mM), and kept on ice for at least 30 minutes.

Total protein containing 10% β-mercaptoethanol and loading buffer were boiled at 100ºC for 5 minutes, gently mixed and separated by SDS-PAGE using 12.5% gels and the GRS Protein Marker MultiColour (GRiSP, Portugal). Proteins were then transferred to Polyvinylidene difluoride membranes (GE Healthcare, USA) previously activated in methanol and equilibrated in water and transfer buffer, in a Trans-Blot Cell (BioRad, USA) system during 2h at 750 mA. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 hour at room temperature and washed 10 minutes with TBS containing 0.1% Tween (TBS-T). Then, the membranes were incubated overnight with primary antibody anti-caspase 3 (Santa Cruz Biotechnology, USA) diluted in TBS-T. Membranes were washed thrice for 15 minutes each in TBS-T, at room temperature, and incubated with HRP-conjugated anti-rabbit (1:20000, ThermoFisher Scientific, USA) for 1 hour at room temperature. The washing process was repeated as described above, and antibody binding was detected using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, USA). Images of blots were captured with the ChemiDoc^{MP} Imaging system (Bio-Rad, USA), and the bands densitometry was calculated using the software ImageLab™ (Bio-Rad, USA), normalized against β-Actin. For this purpose, membranes were washed with TBS-T following detection, and incubated with primary antibody mouse anti-β-Actin (1:20000, Sigma-Aldrich, USA) for 1h30 at room temperature, followed by incubation for 1h with HRP-conjugated anti-mouse (1:40000, Santa Cruz Biotechnology, USA). The washing and detection processes were performed as described above.

3.5. Statistical Analysis

Statistical analysis was carried out using Prism 6 software (GraphPad, USA). Data are presented as mean ± standard error of the mean (SEM). Comparisons between three

or more groups were assessed by one-way ANOVA. Results were considered statistically significant when p-value < 0.05.

4. Results

4.1. Immunocytochemistry

Immunocytochemistry was used to analyse the presence and cellular location of ER, AR and PR in astrocytes (HASTR/ci35) and GBM cell lines (U-87MG, SNB19 and U-373MG).

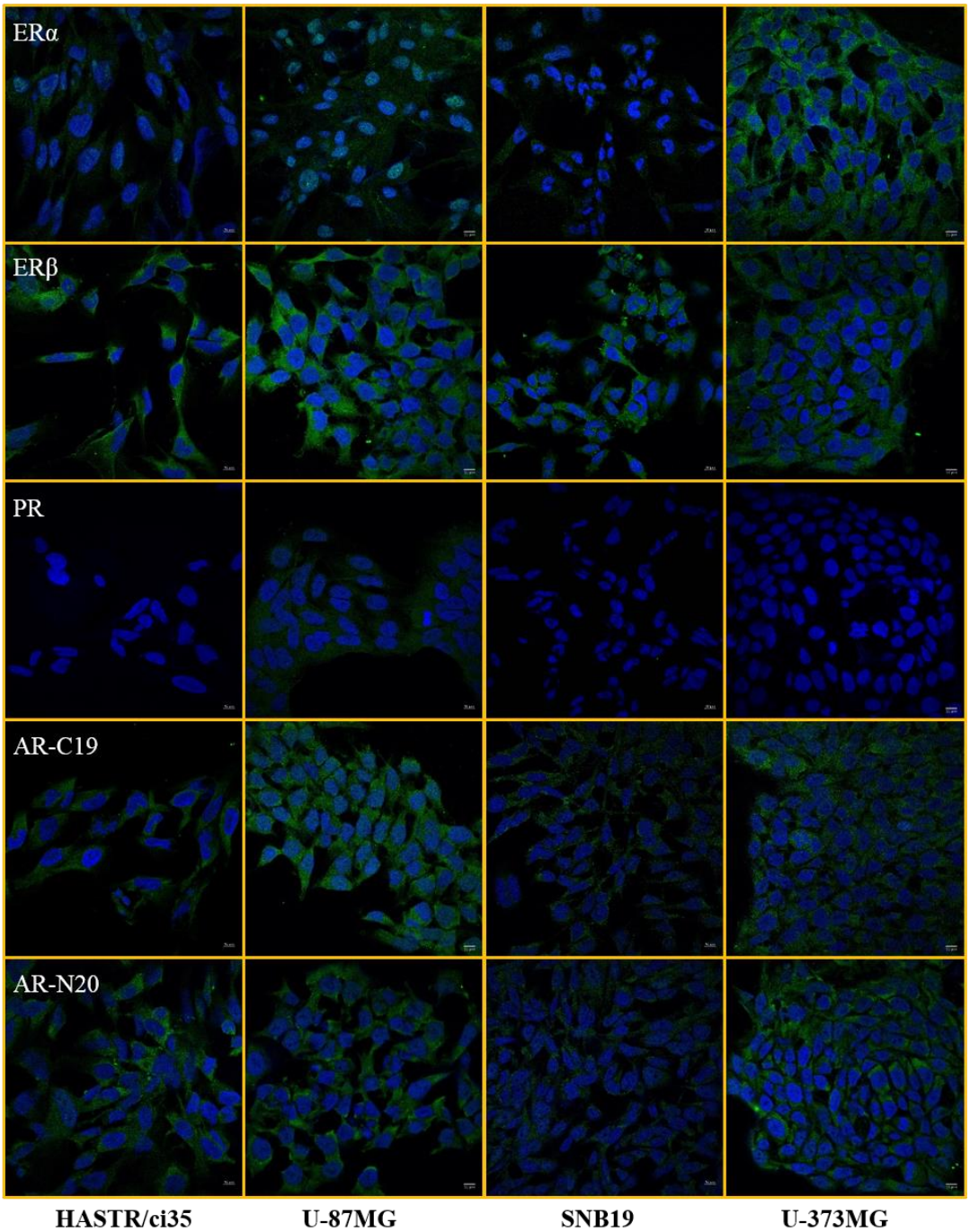


Figure 3 – Immunocytochemistry of sex hormone receptors in HASTR/ci35 and GBM cell lines. Merged images of cells incubated with primary antibodies against

receptors and nuclei were stained with Hoechst 33342 (1:1000). Primary antibodies used were: rabbit anti-ER-α (1:50), rabbit anti-ER-β (1:50), mouse anti-PR (1:50), rabbit anti-AR C-19 (1:50) and rabbit anti-AR N-20 (1:50). Secondary antibodies were: goat anti-rabbit Alexa Fluor® 488 (1:1000), goat anti-mouse Alexa Fluor® 488 (1:1000) and goat anti-rabbit Alexa Fluor® 488 (1:1000). Negative controls: absence of primary antibody (Appendixes 1-9). Scale bar: 10 μm.

The analysis of sex hormone receptors by immunocytochemistry (Figure 3) showed that ERα, ERβ, AR-C and AR-N are expressed in all the cell lines studied (HASTR/ci35, U-87MG, SNB19 and U-373MG). ERα showed a predominantly nuclear localization in U-87MG but was mostly cytoplasmatic in U-373MG. In HASTR/ci35 and SNB19 the expression of ERα was much lower than in the other cell lines. An even distribution was observed in the nucleus and cytoplasm in U-87MG and U-373MG. U-373MG was the cell line where ERα expression was more prominent. Interestingly, U-87MG was the only cell line where ERα had mainly a distinctive nuclear localization.

ERβ had a clear cytoplasmatic distribution in the four lines studied, however in HASTR/ci35, U-87MG and SNB19 labelling was stronger around the nucleus. In terms of intensity, U-373MG seems to be the line with least expression, but where ERβ was more evenly distributed in the cytoplasm.

PR expression was only observed in U-87MG line and not for HASTR/ci35, SNB19 and U-373MG. In this line, the distribution of the receptor was evenly nuclear and cytoplasmic.

Immunocytochemistry for AR was performed for the carboxi-terminal (AR-C) and for the amino-terminal (AR-N) domains. AR-C had a predominantly cytoplasmatic distribution in HASTR/ci35 while in the GBM cell lines U-87MG, SNB19 and U-373MG AR-C labelling there was some positive labelling in the nuclei as well. However, the lines where the receptor was expressed with more intensity were HASTR/ci35 and U-87MG. For AR-N, this receptor was located mainly in the cytoplasm and peri-nuclear region for HASTR/ci35 and mostly nuclear and cytoplasmatic for U-87MG, SNB19 and U-373MG. In terms of receptor intensity, a clearer and more intense expression was observed in HASTR/ci35, U-87MG and U-373MG. Thus, no major differences were noted with these 2 antibodies, as expected.

Moreover, it is important to highlight that all of the cells presented in the images taken by confocal microscopy were positive-stained with Alexa Fluor 488®, thus

indicating that 100% of the cells expressed the SHs receptors mentioned above. However, AR positive labelling in the nucleus was almost absent for AR-C in HASTR/ci35.

4.2. Effects of sex hormones on the viability of HASTR/ci35 and GBM cell lines

Once the presence of hormone receptors was established, the next step was to conduct cell viability assays to better understand the effect that different concentrations of the hormones of interest would have in the viability of normal and GBM cells, as a primary approach to understanding their effects.

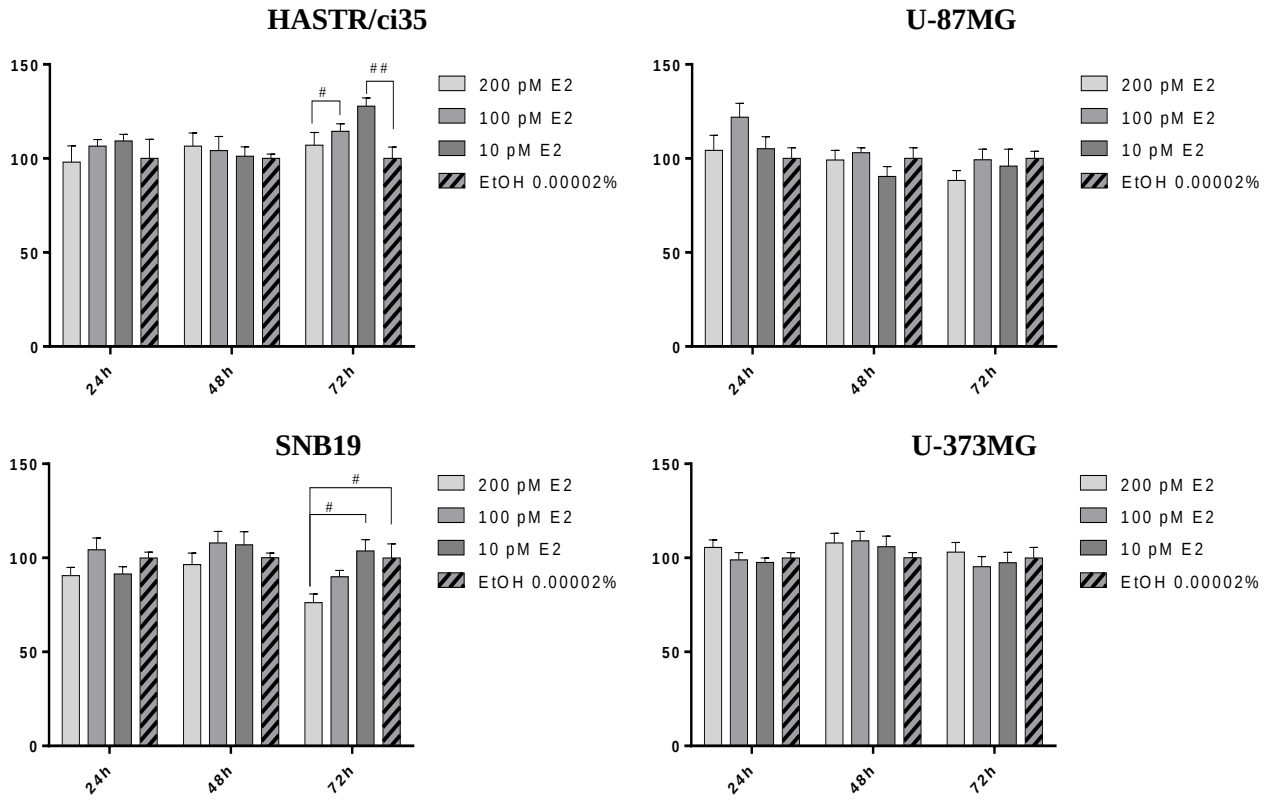


Figure 4 – Effects of of estradiol (E2) in the viability of HASTR/ci35, U-87MG, SNB19 and U-373MG cell lines determined by the MTT assay. Cells were subjected to concentrations of 200 pM, 100 pM and 10 pM and vehicle (EtOH ≤ 0.00002%) during 24h, 48h and 72h. Statistically significant differences were determined by one-way ANOVA (n=3). #p<0.05 and ##p<0.01.

The viability assay of HASTR/ci35 performed through MTT assay with E2 stimuli showed a statistically significant increase in cell viability after 72h of incubation

with 10 pM E2 compared to the vehicle ($p<0.01$). Moreover, HASTR/ci35 cell viability also significantly increased after 72h incubation with 100 pM relatively to 200 pM E2 ($p<0.05$). In the least aggressive GBM line, U-87MG, no statistically significant differences were found in this assay. Considering SNB19, it was possible to find a statistically significant decrease in cell viability with 200 pM E2 compared to vehicle and 10 pM E2 stimuli ($p<0.05$ for both), after a period of incubation of 72h. For the most aggressive cell line, U-373MG the viability assay revealed no statistically significant differences.

Analysing the results to compare normal astrocyte line HASTR/ci35 with GBM lines we see that for the incubation period of 72h, E2 concentration of 200 pM caused a decrease in SNB19 viability compared to HASTR/ci35 ($p<0.001$). For the concentration of 100 pM E2, there was also a decrease of viability of SNB19 ($p<0.01$) and U-373MG ($p<0.05$) compared to astrocytes. Lastly, for a concentration of 10 pM E2, differences were found between HASTR/ci35 and U-87MG ($p<0.001$), SNB19 ($p<0.01$) and U-373MG ($p<0.001$), decreasing viability in GBM (Appendices 10, 14 and 18).

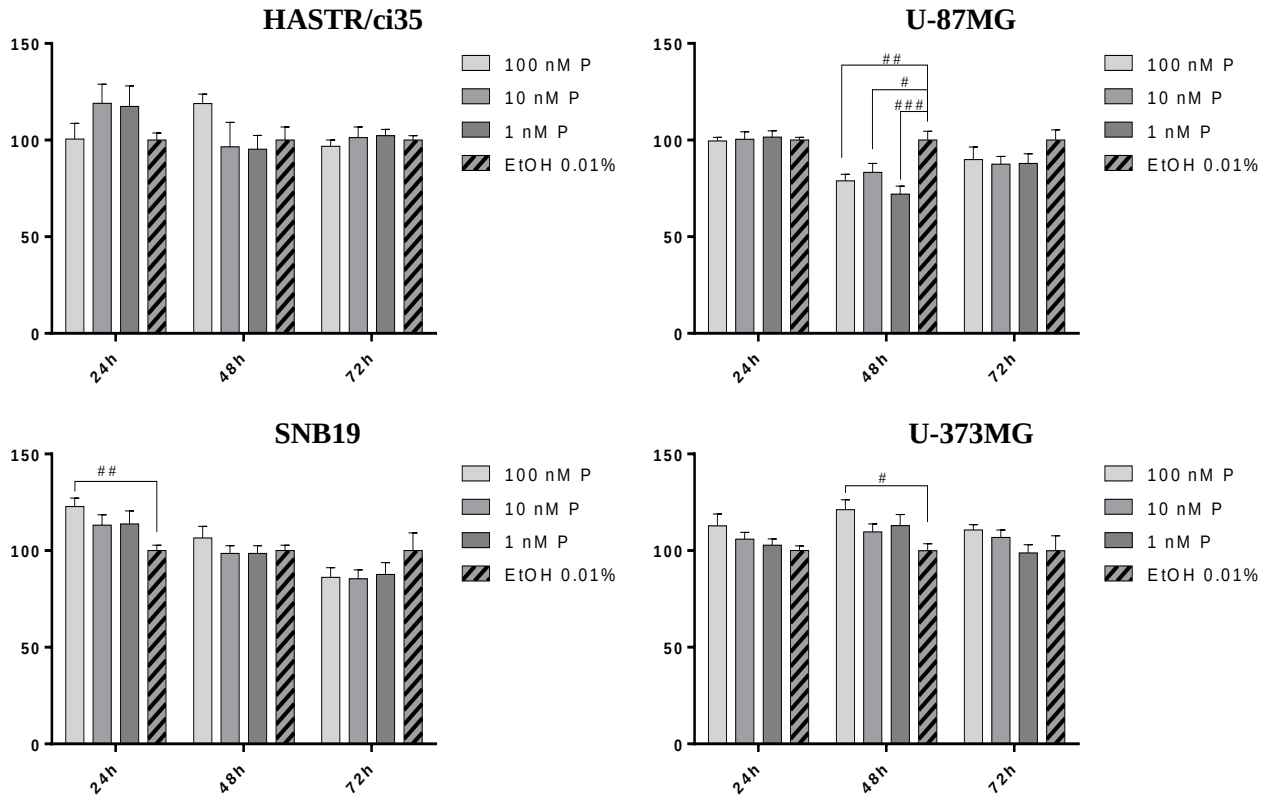


Figure 5 – Effects of progesterone (P4) in the viability of HASTR/ci35, U-87MG, SNB19 and U-373MG cell lines determined by the MTT assay. Concentrations of 100 nM, 10 nM and 1 nM and vehicle (EtOH ≤0.01%) during 24h, 48h and 72h. Positive control: 50% EtOH (not shown). Statistically significant differences were determined by one-way ANOVA (n=3). #p<0.05, ##p<0.01 and ###p<0.001

The viability assay of HASTR/ci35 performed by the MTT assay with P4 stimuli revealed no statistically significant differences between the stimuli and vehicle. As for results found for U-87MG line incubated with P4, a statistically significant decrease in cell viability was observed at 48h of incubation with concentrations of 100 nM ($p<0.01$), 10 nM ($p<0.05$) and 1 nM ($p<0.001$) relatively to vehicle. SNB19 line revealed a significant cell viability increase that occurred at 24h of incubation between 100 nM and the vehicle ($p<0.01$). Finally, in the U-373MG line, there was an increase in viability between 100 nM of P4 and the vehicle ($p<0.05$) at 48h of incubation.

When comparing the behaviour of GBM with astrocytes, for an incubation period of 24h the major differences found were for stimuli with P4 in the concentration of 100 nM between the lines HASTR/ci35 and SNB19 ($p<0.01$), where hormonal stimuli increased GBM viability while for the concentration of 10nM, there were differences between HASTR/ci35 and U-87MG ($p<0.05$) with a decrease in GBM viability. For the

incubation period of 48h, in the concentration of 100 nM ($p<0.0001$) and 1 nM ($p<0.05$) there was a decrease of viability in U-87MG (Appendices 11, 15 and 19).

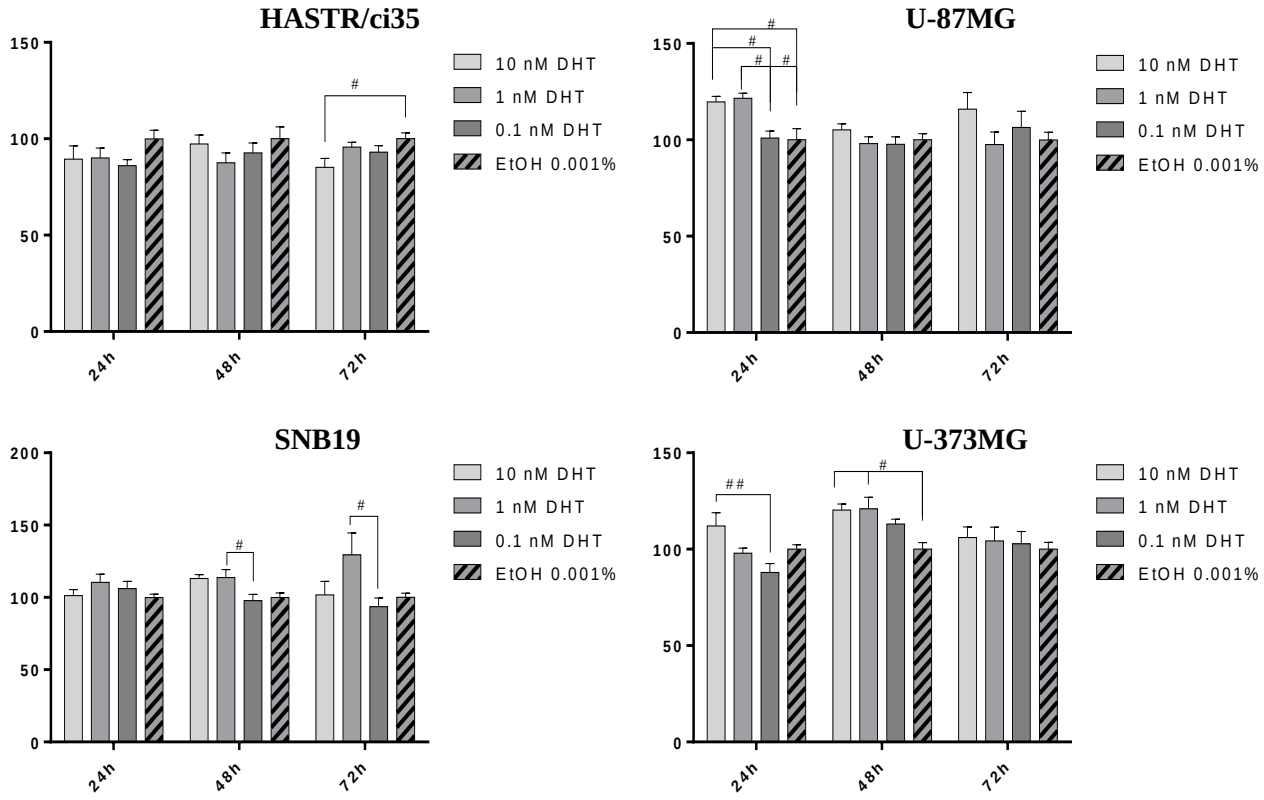


Figure 6 – Effects of dihydrotestosterone (DHT) in the viability of HASTR/ci35, U-87MG, SNB19 and U-373MG cell lines determined by the MTT assay. Concentrations of 10 nM, 1 nM and 0.1 nM and vehicle (EtOH ≤0.001%) during 24h, 48h and 72h. Positive control: 50% EtOH (not shown). Statistically significant differences were determined by one-way ANOVA (n=3). #$p<0.05$ and ##$p<0.01$.

Figure 6 shows the results of the viability assay with DHT stimuli. In the astrocyte line HASTR/ci35, a statistically significant decrease in cell viability was found at 72h of incubation between 10 nM and the vehicle ($p<0.05$). Moreover, in the U-87MG GBM cell line, there were significant differences at 24h between 10 nM and 0.1 nM ($p<0.05$), 10 nM and vehicle ($p<0.05$), 1 nM and 0.1 nM ($p<0.05$) and between 1 nM and the vehicle ($p<0.05$), all revealing an increase in cell viability towards the vehicle or the lower DHT concentrations used. The assay for SNB19 line resulted in differences at 48h and 72h between 1 nM and 0.1 nM of DHT ($p<0.05$), revealing an increase in cell viability with

the higher concentration. Finally, in the U-373MG line, the assay revealed differences after 24h of incubation between 10 nM and 0.1 nM ($p<0.01$) and, after 48h between vehicle and 10 nM ($p<0.05$) and 1 nM ($p<0.05$). All the results point to an increase in cell viability with higher DHT concentrations.

Analysing the results from GBM lines and comparing them with HASTR/ci35 we observed that for the stimuli with DHT at 24h of incubation there was an increase in the viability of U-87MG ($p<0.0001$) and U-373MG ($p<0.01$) when compared with HASTR/ci35 with a concentration of 10 nM. For the concentration of 1nM differences were found between HASTR/ci35 and U-87MG ($p<0.0001$) and SNB19 ($p<0.01$), revealing an increase in GBM viability. At 48h, 10 nM increased viability of SNB19 ($p<0.05$) and U-373MG ($p<0.001$). The same effect occurred for a concentration of 1 nM in SNB19 ($p<0.0001$) and U-373MG ($p<0.0001$). For a concentration of 0.1 nM, there was also an increase in the viability of U-373MG ($p<0.01$) compared to astrocytes. At 72h, the concentration of 10 nM lead to an increase in viability in U-87MG ($p<0.01$) and 1 nM DHT also increased viability of SNB19 ($p<0.01$) (Appendices 12, 16 and 20).

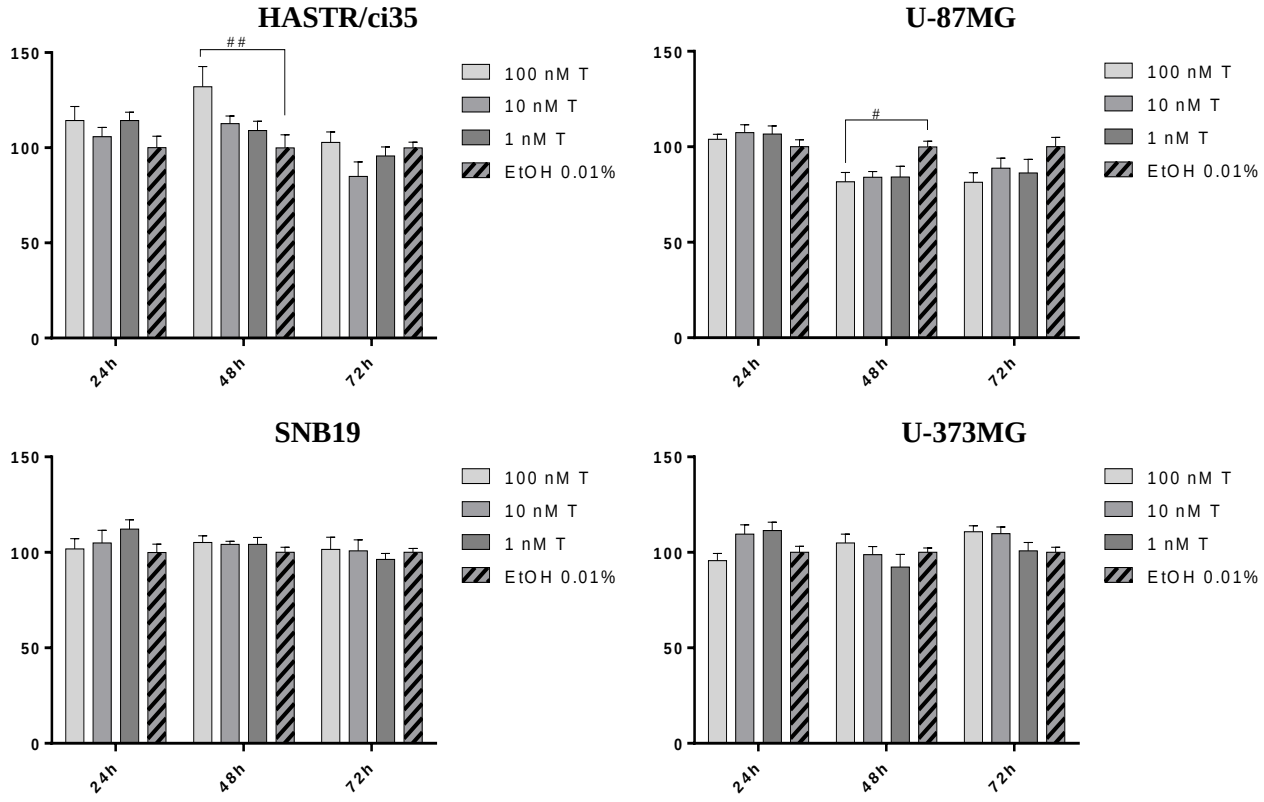


Figure 7 – Effects of testosterone (T) in the viability of HASTR/ci35, U-87MG, SNB19 and U-373MG cell lines determined by the MTT assay. Concentrations of

100 nM, 10 nM and 1 nM and vehicle (0.01%) during 24h, 48h and 72h. Positive control: 50% EtOH (not shown). Statistically significant differences were determined by one-way ANOVA (n=3). #p<0.05 and ##p<0.01.

For the effect of testosterone stimuli in cell viability, a statistically significant increase was observed in HASTR/ci35 incubated with 10 nM relatively to vehicle after 48h of incubation ($p<0.01$). For the U-87MG line, the viability assay showed there were differences at 48h between the concentration of 100nM and the vehicle 0.01% EtOH ($p<0.05$), pointing to a decrease in the viability of cells incubated with testosterone. The viability assay of GBM cell line SNB19 and U-373MG revealed no statistically significant differences between the stimuli and vehicle.

When comparing the results of viability assay of GBM cells with HASTR/ci35**,** **s**timuli of T induced a decrease of U-373MG viability ($p<0.05$) with a concentration of 100 nM. For the incubation period of 48h, 100 nM decreased the viability in U-87MG ($p<0.0001$), SNB19 ($p<0.001$) and U-373MG ($p<0.0001$). For a concentration of 10 nM ($p<0.001$) and 1 nM ($p<0.01$), the same effect occurred between HASTR/ci35 and U-87MG. Finally, at 72h of incubation, the concentration of 100 nM decreased the viability of U-87MG ($p<0.05$), while increasing the viability of U-373MG ($p<0.01$) with a concentration of 10 nM (Appendices 13, 17 and 21).

4.3. Assessment of Apoptosis in Response to Hormonal Stimuli

After the confirmation that hormonal stimuli induce significant differences in cell viability, as mentioned previously, the next step was to understand specifically if alterations in cell viability were a result of enhanced or decreased apoptosis. For that reason, we studied the expression of a specific protein, caspase-3, in order to assess if changes in apoptosis were the reason for alterations in cell viability. In order to do that, concentrations that induced significant differences in cell viability were included in this analysis. Testosterone was not included because we would not be able to guarantee that the effects observed would not be a result of the possible conversion of T to estradiol by aromatase.

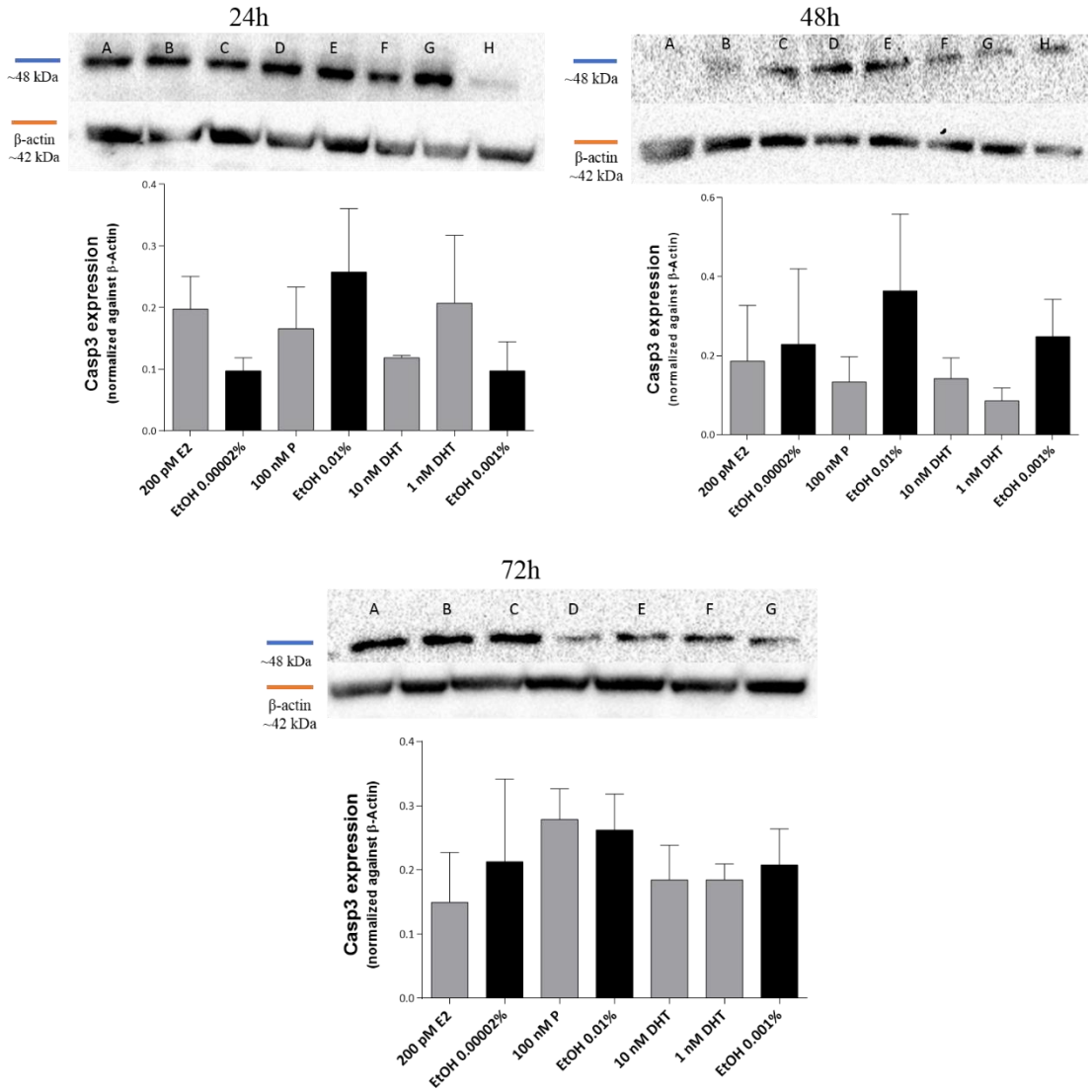


Figure 8 - Western Blot of caspase-3 in U-373MG cells stimulated for 24h, 48h and 72h with sex steroid hormones. (A) Vehicle (EtOH ≤ 0.00002%), (B) E2 200 pM, (C) Vehicle (EtOH ≤ 0.001%), (D) DHT 1 nM, (E) DHT 10 nM, (F) Vehicle (EtOH ≤ 0.01%), (G) P4 100 nM and (H) K+ (Staurosporine 1 µM). Positive control (K+). Statistically significant differences were determined by one-way ANOVA (n=3).

By using this analysis, it was not possible to observe any differences in the levels of active caspase. Analysis of Western Blots did not show statistically significant differences in U-373MG cells incubated for 24h, 48h and 72h with either 200 pM of E2, 100 nM of P, and 1nM or 10 nM of DHT and respective vehicles (Figure 8).

5. Discussion

In the past few decades, glioblastoma has become a major topic of investigation due to its devastating prognosis associated with the short survival rates for this type of cancer. This constitutes a strong driving force for more research to enhance the current knowledge about the basis of this pathology. Given the epidemiological differences in terms of incidence between men and women, studying the impact of hormones in GBM's etiology and development is certainly a relevant issue, especially because it is still a poorly understood subject. Until now, a lot of research has focused on orthotopic animal models, but most studies used supraphysiological hormone concentrations in experiments. For this reason, this study aimed to enhance the current knowledge to understand the higher incidence of GBM among men. Thus, the goal was to assess the effect of the main sex steroid hormones (estradiol, progesterone, dihydrotestosterone and testosterone) in the viability of GBM cell lines, as the levels of these hormones represent fundamental biological differences between men and women. Moreover, it was important to use physiological concentrations of these hormones in order to approach the *in vivo* environment as much as possible. We also aimed to use 3 different GBM cell lines to provide a comparison scale of cell aggressiveness, since U-87MG are considered to be the least, SNB19 the intermediate and U-373MG the most aggressive and proliferative cells. In addition, we were also able to use a human non-cancerous astrocyte cell line (HASTR/ci35) to compare to GBM cell lines conditions.

Thus, the first approach was to perform immunocytochemistry to assess if the sex steroid hormone receptors were expressed by the cell lines under study. We were able to show that both ERα and ERβ were expressed in HASTR/ci35 and in U-87MG, SNB19, and U-373MG, much like previously described in the literature (Dueñas-Jiménez et al., 2014; González-Arenas et al., 2012; Sareddy et al., 2012). Interestingly, ERα had a predominantly cytoplasmatic distribution in all the cells lines except in U-87MG, where it was mostly detected in the nucleus. According to Wan *et al*., (2018), ERα is located at the nucleus of glioma cells sampled from patients upon surgery. On the other hand, the same authors admitted that astrocytes may also express non-nuclear ERs (Wan et al., 2018). Another study revealed that in GBM, an ER-α variant (ERα36) was localized in the nucleus alone (16%), cell membrane or cytoplasm alone (8%) but the remaining (76%) was spread diffusely in the cell (Qu et al., 2019). Hence, the differences found in the ERα localization may be of interest and should be further explored, especially given

that U-87MG represents the cell line with least aggressiveness and proliferation when compared to SNB19 and U-373MG cell lines.

Many studies have dwelled in possible correlations between ER levels and malignancy stages, and Dueñas-Jiménez *et al*. (2014), found a negative correlation between the ERα expression and the level of malignancy, and otherwise a positive correlation with the survival rate of patients with gliomas. Moreover, in our analysis, U-373MG appeared to be the cell line with the most intense ERα immunoreactivity and the one with the least ERβ immunoreactivity which could be due to the fact pointed by some authors that ERα is associated to tumor promoting effects, and ERβ is considered a tumor suppressor (González-Arenas et al., 2012; Sareddy et al., 2012).

We also attempted to identify PR by immunocytochemistry, but only detected a clear expression of PR in the U-87MG cell line. This aspect may reflect a less prominent expression of these receptors within HASTR/ci35, SNB19 and U-373MG. Within the literature, some authors have identified PR in U-373MG by western blot (González-Agüero, et al., 2007). Moreover, PR was identified by mRNA and protein analysis in every case of glioblastomas analysed by González-Agüero, *et al.* (2001). In terms of PR expression in normal astrocytes, Lacroix-Fralish *et al*. (2006), observed that progesterone increased the expression of neuregulin 1 mRNA and protein, an effect that was blocked by a PR antagonist (RU-486), which also reflects the presence of PR. A possible reason for this occurrence may be due to some of the limitations associated with the use of immortalized cell lines. They are known to present as a disadvantage the fact that they often lose characteristics such as the expression of some genes (Kaur et al. 2012 & Carter et al., 2010). For this reason, this analysis should be carried out in more robust models such as in primary cells derived from tumors and in samples obtained from resected tumours. Moreover, the presence of the AR (AR-C and AR-N) was confirmed in all of the cell lines under study by immunocytochemistry. Finally, since these receptors function as transcription factors they have to translocate to the nucleus to induce gene transcription. Often, this translocation requires receptor activation upon ligand binding that can occur in the cytoplasm due to the lipophilicity of SH. For this reason, future studies should focus on immunocytochemistry assays using hormonal stimuli (Bao et al., 2017 & Sareddy et al., 2012).

Viability of GBM cells upon hormonal treatments was assessed by the MTT assay, which assesses the number of viable cells through the analysis of metabolic activity. Overall, we found that E2 increased the viability of HASTR/ci35 and diminished

it in SNB19, revealing an effect in these lines concurrent with the literature, in that E2 is recognised as a neuroprotectant for normal astrocytes, but the opposite for GBM (Rivera-Delgado at al., 2017, Sareddy et al., 2016, Dueñas-Jiménez et al., 2014, Manca et al., 2010, Barone et al., 2009). On the other hand, progesterone showed some controversial effects, much like what has been found in previous studies. It diminished the viability in U-87MG cells but had the opposite effect in the most aggressive cell lines SNB19 and U-373MG. In studies using supraphysiological P4 concentrations (20, 40, and 80 μM) there was also a decrease in U-87MG viability, but low physiological P4 concentrations (0.1, 1, and 5 μM) induced proliferation (Atif et al., 2015). Thus, we can infer that at higher concentrations, P4 may become cytotoxic to GBM cells but in lower concentrations it is potentially tumorigenic, and its effects still need to be further explored. Moreover, the unclear PR expression assessed by immunocytochemistry in U-373MG can also justify the effect P4 had in increasing U-373MG cell viability if we consider that it presents a lower PR expression in this cell line. Much like what other studies put forth, P4 seems to promote tumorigenesis by increasing SNB19 and U-373MG cells' viability, unlike in HASTR/ci35 and U-87MG.

DHT increased the viability of U-87MG, SNB19 and U-373 while diminishing it in the human astrocyte line HASTR/ci35. This points to a pro-tumorigenic role of DHT in GBM, concurrent with findings that stimulus with DHT significantly decreased the effect of the anti-tumoral receptor TGFβ1 and with data that points to an upregulation of AR in GBM cells (Yu et al., 2015).

In this study we concluded that testosterone increased the viability of astrocyte line HASTR/ci35 and diminished it in U-87MG, both with 100 nM of testosterone stimuli and after 48h of incubation. However, this would be a supraphysiological concentration and neither of the physiological stimuli used produced a significant difference. As previously stated, studies have found an AR upregulation in U-87MG (Bao et al., 2017) and revealed that 10 μM of testosterone, a supraphysiological concentration, would increase the proliferation in rat glioma cells (González et al., 2007). Using concentrations of up to 100 nM, Rodríguez-Lozano *et al*. (2019) showed an increase in migration, invasion and proliferation of U-87MG cells. In terms of proliferation, invasion and migration these data are not corroborated by our findings. However, the fact that T is converted to E2 via aromatase in several tissues, such as the brain, does not allow us to guarantee that the observed effects are indeed because of T actions and not due to E2

(Swerdloff et al., 2017; Zubeldia-Brenner et al., 2016). This was also the reason why testosterone was not included in apoptosis assays.

Analysing the results of the viability assays it was possible to compare the astrocyte line HASTR/ci35 with GBM lines and see what differences can be found (Appendices 10-21). All in all, at 24h of incubation and when compared to HASTR/ci35, P4 caused a decrease in the viability of U-87MG but an increase in SNB19. For DHT stimuli, all the GBM had an increase in viability when compared to HASTR/ci35, while T decreased the viability of U-373MG. After 48h, P4 maintained a decreasing effect on U-87MG cell viability when compared to astrocytes while DHT increased the viability of SNB19 and U-373MG and T decreased the viability of the three GBM lines. For the incubation period of 72h, E2 decreased the viability of U-87MG, SNB19 and U-373MG when compared to astrocytes while DHT increased the viability of U-87MG and SNB19. At this time period, T decreased the viability of U-87MG as it increased it in U-373MG. Interestingly, E2 only produced significant differences compared to HASTR/ci35 after 72h oh incubation as well as it was the only incubation period where T had a tumorigenic effect.

Overall, looking into the results obtained from the viability assays we can conclude that statistically significant results occurred mostly after 48h incubation followed by 24h and lastly 72h incubations. Thus, it seems that most changes induced by hormonal stimuli occur in the first 48h of incubation. Considering the different concentrations used and comparing them to controls, for E2, 200 pM and 10 pM (both within the physiologic range) were the concentrations eliciting more pronounced differences. For P4, the highest concentration of 100 nM was the one eliciting more significant results, while 10 nM and 1 nM induced less changes. All these P4 concentrations were also physiological. DHT induced most results in the two highest concentrations of 10 nM and 1 nM, both physiological as well. Finally, regarding the concentrations of T analysed, 100 nM was the only one that produced significative results. However, 100 nM T is above physiological levels and the two physiological concentrations of 10 nM and 1 nM did not cause any changes in cell viability.

The viability assay gave us important information about the influence of hormones in GBM but without revealing the mechanisms that are leading either to an increase or to a decrease in cell viability. For that reason, we considered pertinent to study apoptosis in order to understand if differences in viability were caused by an increase or decrease in apoptosis.

Apoptosis is a type of cell death where cell fragmentation and disintegration are programmed and, because of that, does not provoke inflammatory responses (Moraes et al., 2011; Walters et al. 2009). In order to study this important parameter in cancer, we aimed to detect caspase-3 through western blot assay. Caspase 3 is an effector of this mechanism and it is expressed as procaspase, with 32 kDa, that is cleaved into subunits of 17 and 12 kDa upon activation (Moraes et al., 2011; Walters et al. 2009), which correspond to the active form of this protease. The 32 kDa caspase is a zymogen and for that reason has no activity and it is constitutively expressed (Moraes et al., 2011; Walters et al. 2009). Western blot to compare protein levels requires normalization against a house keeping protein such as actin.

In the assays performed, we detected bands around 48kDa, which lead us to wonder whether the active (cleaved) subunit of caspase 3 was not present on the samples tested or whether the 48kDa subunit detected corresponded to the sum of the smaller active subunits that were not separated in the process (Moraes et al., 2011; Walters et al. 2009). Post-translational modifications have been described that could lead to the formation of heterodimers between the small units of caspase-7 (12-13 kDa) and the large unit of caspase-3 (32-34 kDa), which could help us understand why a band larger than expected was detected (UniProt, 2020; Mannick et al., 1999). As a consequence, and without obtaining statistical significance for any of the comparisons analysed, we considered these results to be inconclusive and consider the assays performed as an attempt to optimize the technique that should be further explored to assess if results produced by other authors can be seen in these cell lines (Sareddy et al., 2016; Atif et al 2015; Yu et al., 2015).

6. Conclusions and Future Perspectives

This study puts forth interesting data on the influence of sexual steroid hormones in glioblastoma cell lines. We observed differences in terms of cell viability induced by estradiol, progesterone, DHT and testosterone within the physiologic range. For that reason, an apoptosis assay was performed in order to understand if the changes in viability could be due to the induction of this mechanism of cell death, but the results obtained were inconclusive.

Overall, the results attained in this thesis set the basis for further exploring the effect of estradiol, progesterone, DHT and testosterone, as all these hormones in one or another condition elicited significant differences in cell viability. Thus, it would be of interest to analyse the effects of these SH on other relevant parameters and hallmarks of cancer in these cell lines and in cell lines derived from brain tumors obtained upon surgery that are more reliable models of the disease, using the following techniques: looking into proliferation markers such as ki67, using alternative techniques for assessing apoptosis, such as TUNEL, flow cytometry and, conducting migration assays.

Another perspective for the future is to perform immunocytochemistry assays using hormonal stimuli, to observe if there are changes in hormone receptor localization upon a hormonal stimulus, because of the different localization observed in these cell lines when compared to others studied.

Finally, a larger scale study to assess the expression of SH receptors and hormonal levels in glioma and GBM patients would have relevant information to this still neglected field of study.

7. References

- Acconcia, F. & Marino, M. (2016). Steroid Hormones: Synthesis, Secretion, and Transport. In Belfiore, A. & LeRoith, D., *Principles of Endocrinology and Hormone Action* (pp.1-31). Available in https://link.springer.com/referenceworkentry/10.1007%2F978-3-319-27318-1_4-1;
- Alexander G.M. (2014). Postnatal testosterone concentrations and male social development. *Frontiers in endocrinology*. 5, 15. https://doi.org/10.3389/fendo.2014.00015;
- Alphandéry E. (2018). Glioblastoma Treatments: An Account of Recent Industrial Developments. *Frontiers in pharmacology*, 9, 879. https://doi.org/10.3389/fphar.2018.00879;
- Altinoz, M., Nalbantoglu, J., Ozpinar, A., Emin, M., Del Maestro, R. & Elmaci, I. (2018). From epidemiology and neurodevelopment to antineoplasticity. Medroxyprogesterone reduces human glial tumor growth in vitro and C6 glioma in rat brain in vivo. *Clinical Neurology and Neurosurgery*. 173, 20-30. https://doi.org/10.1016/j.clineuro.2018.07.012;
- Alves, CH., Gonçalves, I., Socorro S., Baltazar, G., Quintela, T. & Santos, CR. (2009). Androgen receptor is expressed in murine choroid plexus and downregulated by 5alpha-dihydrotestosterone in male and female mice. *Journal of Molecular Neuroscience*. 38, 1, 41-9. https://doi.org/10.1007/s12031-008-9157-4;
- Atif, F., Patel, N., Yousuf, S. & Stein, D. (2015). The Synergistic Effect of Combination Progesterone and Temozolomide on Human Glioblastoma Cells. *PLoS One*. 10, 6. https://doi.org/10.1007/s12031-008-9157-4;
- Atif, F., Yousuf, S. & Stein D. (2015). Anti-tumor effects of progesterone in human glioblastoma multiforme: role of PI3K/Akt/mTOR signaling. *Journal of Steroid Biochemistry and Molecular Biology*. 146, 62-73. https://doi.org/10.1016/j.jsbmb.2014.04.007;
- Atif, F., Yousuf, S., Espinosa-Garcia, C., Sergeeva, E., & Stein, D. G. (2019). Progesterone Treatment Attenuates Glycolytic Metabolism and Induces Senescence in Glioblastoma. *Scientific reports*. 9, 1, 988. https://doi.org/10.1038/s41598-018-37399-5;

- Baglieto, L., Severi, G., English, G., Krishnan, K., Hopper, J., McLean, C., Morris, H., Tilley, W. & Giles, G. (2010). Circulating Steroid Hormone Levels and Risk of Breast Cancer for Postmenopausal Women. *Cancer Epidemiology, Biomarkers & Prevention*. 19, 2, 492-502. https://doi.org/10.1158/1055-9965.EPI-09-0532;
- Bain J. (2007). The many faces of testosterone. *Clinical interventions in aging*. 2, 4, 567–576. https://doi.org/10.2147/cia.s1417;
- Bao, D., Cheng, C., Lan, X., Xing, R., Chen, Z., Zhao, H., Sun, J., Wang, Y., Niu, C., Zhang, B. & Fang, S. (2017). Regulation of p53wt glioma cell proliferation by androgen receptor-mediated inhibition of small VCP/p97-interacting protein expression. *Oncotarget*. 8, 14, 23142-23154. https://doi.org/10.18632/oncotarget.15509;
- Barone, T., Gorski, J., Greenberg, S. & Plunkett, R. (2009). Estrogen increases survival in an orthotopic model of glioblastoma. *Journal of Neuro-Oncology*. 95, 1, 37-48. https://doi.org/10.1007/s11060-009-9904-6;
- Berthois, Y., Katzenellenbogen, J. A., & Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America*. 83, 8, 2496–2500. https://doi.org/10.1073/pnas.83.8.2496;
- Black, A., Pinsky, P. F., Grubb, R. L., 3rd, Falk, R. T., Hsing, A. W., Chu, L., Meyer, T., Veenstra, T. D., Xu, X., Yu, K., Ziegler, R. G., Brinton, L. A., Hoover, R. N., & Cook, M. B. (2014). Sex steroid hormone metabolism in relation to risk of aggressive prostate cancer. Cancer epidemiology, biomarkers & prevention: a publication of the *American Association for Cancer Research*, cosponsored by the American Society of Preventive Oncology. 23, 11, 2374–2382. https://doi.org/10.1158/1055-9965.EPI-14-0700;
- Boibessot, C. & Toren, P. (2018). Sex steroids in the tumor microenvironment and prostate cancer progression. *Endocrine-Related Cancer*. 25, 3, R179-R196. https://doi.org/10.1530/ERC-17-0493;
- Braeuninger, S., Chamaon, K., Kropf, S., Mawrin, C., Wiedemann, F., Hartig, R., Schoeler, S., Dietzmann, K. & Kirches, E. (2005). Short incubation with 2-methoxyestradiol kills malignant glioma cells independent of death receptor 5

upregulation. *Clinical Neuropathology*. 24, 4, 175-83. Available in: https://www.ncbi.nlm.nih.gov/pubmed/16033134;

- Cabrini, G., Fabbri, E., Lo Nigro, C., Dechecchi, M.C., & Gambari, R. (2015). Regulation of expression of O6-methylguanine-DNA methyltransferase and the treatment of glioblastoma (Review). International Journal of Oncology, 47, 417-428. https://doi.org/10.3892/ijo.2015.3026;
- Carter, M. & Shieh, J. (2010). Cell Culture Techniques. In Carter, M. & Shieh, J., *Guide to Research Techniques in Neuroscience* (pp. 281-296). Canada: Academic Press;
- Chamaon, K., Stojek, J., Kanakis, D., Braeuninger, S., Kirches, E., Krause, G., Mawrin, C. & Dietzman, K. (2005). Micromolar concentrations of 2-methoxyestradiol kill glioma cells by an apoptotic mechanism, without destroying their microtubule cytoskeleton. *Journal of Neuro-Oncology*. 72, 1, 11-6. http://doi.org/10.1007/s11060-004-2158-4;
- Cheng, Y., Yeung, W., Zhang, P., Li, N., Kiang, M. & Leung, G. (2019). Progesterone Is More Effective Than Dexamethasone in Prolonging Overall Survival and Preserving Neurologic Function in Experimental Animals with Orthotopic Glioblastoma Allografts. *World Neurosurgery*. 19, 30211-6. http://doi.org/10.1016/j.wneu.2019.01.113;
- Chuang, D. & Lin (2019). Targeted Therapies for the Treatment of Glioblastoma in Adults. *Current Oncology Reports.* 21, 7, 61. http://doi.org/10.1007/s11912-019-0807-1;
- Costa, A. R., Marcelino, H. , Gonçalves, I. , Quintela, T. , Tomás, J. , Duarte, A. C., Fonseca, A. M. and Santos, C. R. (2016), Sex Hormones Protect Against Amyloid-β Induced Oxidative Stress in the Choroid Plexus Cell Line Z310. *Journal of Neuroendocrinology*, 28, 9. https://doi.org/10.1111/jne.12404;
- Da Ros, M., De Gregorio, V., Iorio, A. L., Giunti, L., Guidi, M., de Martino, M., Genitori, L., & Sardi, I. (2018). Glioblastoma Chemoresistance: The Double Play by Microenvironment and Blood-Brain Barrier. *International journal of molecular sciences*. 19, 10, 2879. https://doi.org/10.3390/ijms19102879;
- Diotel Nicolas, Charlier Thierry D., Lefebvre d'Hellencourt Christian, Couret David, Trudeau Vance L., Nicolau Joel C., Meilhac Olivier, Kah Olivier, Pellegrini Elisabeth (2018). Steroid Transport, Local Synthesis, and Signaling

within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors. *Frontiers in Neuroscience*; 12, 84. https://doi.org/10.3389/fnins.2018.00084;

- Duarte, A.C., Hrynchak, M.V., Gonçalves, I., Quintela, T. & Santos, C.R.A. (2016). Sex Hormone Decline and Amyloid β Synthesis, Transport and Clearance in the Brain. *Journal of Neuroendocrinology*. 28, 11. https://doi.org/10.1111/jne.12432;
- Dueñas-Jiménez, J., Arellano, A., Santerre, A., Suárez, S., Sánchez, H., Romero, I., López-Elizalde, R., Venegas, M., Netel, B., Valdovinos, B. & Dueñas-Jiménez, S. (2014). Aromatase and estrogen receptor alfa mRNA expression as prognostic biomarkers in patients with astrocytomas. *Journal of Neuro-Oncology*; 119: 275-284. https://doi.org/10.1007/s11060-014-1509-z;
- Elmaci, I., Ozpinar, A., Bilir, A. & Altinoz, M. (2019). Medroxyprogesterone effects on colony growth, autophagy and mitochondria of C6 glioma cells are augmented with tibolone and temozolomide: Cell kinetic and electron microscopical studies with a broad review of the literature. *Clinical Neurology and Neurosurgery*. 177, 77-85. https://doi.org/10.1016/j.clineuro.2018.12.022;
- Farahmandlou, N., Oryan, S., Ahmadi, R., & Eidi, A. (2017). Association Of Testosterone With Colorectal Cancer (Ht29), Human Glioblastoma (A172) And Human Embryonic Kidney (Hek293) Cells Proliferation. *Acta Endocrinologica*. 13, 2, 144–149. https://doi.org/10.4183/aeb.2017.144;
- Folkerd, E. & Dowsett, M. (2013). Sex hormones and breast cancer risk and prognosis. *The Breast*. 22, 2, S38-S43. https://doi.org/10.1016/j.breast.2013.07.007;
- Gadducci, A., Biglia, N., Sismondi, P. & Genazzazi, A. (2009). Breast cancer and sex steroids: Critical review of epidemiological, experimental and clinical investigations on etiopathogenesis, chemoprevention and endocrine treatment of breast cancer. *Gynecology Endocrinology*. 20, 6, 343-360. https://doi.org/10.1080/09513590500128492;
- Germán-Castelán, L. & Camacho-Arroyo, I. (2015). Effects of progesterone on human astrocytoma progression. *Revista Mexicana de Endocrinologia Metabolismo & Nutrición*. 2, 2, 104-8. Available in: http://www.revistadeendocrinologia.com/abstract.php?id=56;
- Germán-Castelán, L., Manjarrez-Marmolejo, J., González-Arenas, A. & CamachoArroyo, I. (2016). Intracellular Progesterone Receptor Mediates the

Increase in Glioblastoma Growth Induced by Progesterone in the Rat Brain. *Achives of Medical Research*. 47, 6, 419-426. https://doi.org/10.1016/j.arcmed.2016.10.002;

- Gessler, F., Zappi, J., Konczalla, J., Bernstock, J., Forster, M., Wagner, M., Mittelbronn, M., Seifert, V. & Senft, C. (2017). Secondary Glioblastoma: Molecular and Clinical Factors That Affect Outcome After Malignant Progression of a Lower Grade Tumor. *World Neurosurgery*. 102, 49-55. https://doi.org/10.1016/j.wneu.2017.02.104;
- Giatti, S., Garcia-Segura, L.M., Barreto, G.E. & Melcangi, R.C. (2019). Neuroactive steroids, neurosteroidogenesis and sex. *Progress in neurobiology*. 176, 1-17. https://doi.org/10.1016/j.pneurobio.2018.06.007;
- González, A., Martínez-Campa, C., Mediavilla, M., Alonso-González, C., SánchezBarceló, E. & Cos, S. (2007). Inhibitory effects of pharmacological doses of melatonin on aromatase activity and expression in rat glioma cells. *British Journal of Cancer*. 97, 755-760. https://doi.org/10.1038/sj.bjc.6603935;
- González-Agüero, G., Gutiérrez, A.A., González-Espinosa, D. et al. (2007). Progesterone effects on cell growth of U373 and D54 human astrocytoma cell lines. *Endocrinology*. 32, 129–135. https://doi.org/10.1007/s12020-007-9023-0;
- González-Agüero, G., Ondarza, R., Gamboa-Domínguez. A., Cerbón, M., Camacho-Arroyo, I. (2001). Progesterone receptor isoforms expression pattern in human astrocytomas. *Brain.*56, 1, 43-48. https://doi.org/10.1016/S0361-9230(01)00590-1;
- González-Arenas, A., Hansberg-Pastor, V., Hernández-Hernández, O., GonzálezGarcía, T., Henderson-Villalpando, J., Lemus-Hernández, D., Cruz-Barrios, A., Rivas-Suárez, M. & Camacho-Arroyo, I. (2012). Estradiol increases cell growth in human astrocytoma cell lines through ERα activation and its interaction with SRC1 and SRC-3 coactivators. *Biochimica et Biophysica Acta.* 1823, 379-386. https://doi.org/10.1016/j.bbamcr.2011.11.004;
- González-Orozco, J., Hansberg-Pastor, V., Valadez-Cosmes, P., Nicolas-Ortega, W., Bestida-Beristain, Y., Fuente-Granada, M., González-Arenas A. & Camacho-Arroyo, I. (2018). Activation of membrane progesterone receptor-alpha increases proliferation, migration, and invasion of human glioblastoma cells. *Molecullar and Cellular Endocrinology*. 477: 81-89. https://doi.org/10.1016/j.mce.2018.06.004;

- Gutiérrez-Rodríguez, A., Hansberg-Pastor, V., & Camacho-Arroyo, I. (2017). Proliferative and Invasive Effects of Progesterone-Induced Blocking Factor in Human Glioblastoma Cells. *BioMed research international*. 1-10. https://doi.org/10.1155/2017/1295087;
- Hansberg-Pastor, V., González-Arenas, A. & Camacho-Arroyo-I. (2017). CCAAT/enhancer binding protein β negatively regulates progesterone receptor expression in human glioblastoma cells. *Molecular and Cellular Endocrinology.* 439, 317-327. https://doi.org/10.1016/j.mce.2016.09.018;
- Hima, S. & Sreeja, S. (2016) Modulatory role of 17β-estradiol in the tumor microenvironment of thyroid cancer. *IUBMB Life*. 68, 85–96. https://doi.org/10.1002/iub.1462;
- Hiort O. (2013). The differential role of androgens in early human sex development. *BMC Medicine*.11, 152. https://doi.org/10.1186/1741-7015-11-152;
- Joubert, A., Maritz, C. & Joubert, F. (2005). Bax/Bcl-2 expression levels of 2-methoxyestradiol-exposed esophageal cancer cells. *Biomedical. Research*. 26, 131–4. https://doi.org/10.2220/biomedres.26.131;
- Kambhampati, S., Banerjee, S., Dhar, K., Mehta, S., Haque, I., Dhar, G., Majumder, M., Ray, G., Vanveldhuizen, P. J., & Banerjee, S. K. (2010). 2-methoxyestradiol inhibits Barrett's esophageal adenocarcinoma growth and differentiation through differential regulation of the β-catenin-E-cadherin axis. *Molecular cancer therapeutics*. 9, 3, 523–534. https://doi.org/10.1158/1535-7163.MCT-09-0845;
- Kambhampati, S., Rajewski, R. A., Tanol, M., Haque, I., Das, A., Banerjee, S., Jha, S., Burns, D., Borrego-Diaz, E., Van Veldhuizen, P. J., & Banerjee, S. K. (2013). A Second-Generation 2-Methoxyestradiol Prodrug Is Effective against Barrett's Adenocarcinoma in a Mouse Xenograft Model. *Molecular cancer therapeutics*. 12, 255–263. https://doi.org/10.1158/1535-7163.MCT-12-0777;
- Kaur, G., & Dufour, J. M. (2012). Cell lines: Valuable tools or useless artifacts. *Spermatogenesis*. 2, 1, 1–5. https://doi.org/10.4161/spmg.19885;
- Kfoury, N., Sun, T., Yu, K., Rockwell, N., Tinkum, K. L., Qi, Z., … Rubin, J. B. (2018). Cooperative p16 and p21 action protects female astrocytes from transformation. *Acta neuropathologica communications*. 6, 1, 12. https://doi.org/10.1186/s40478-018-0513-5;

- Kyurkchiev, D., Naydenov, E., Tumangelova-Yuzeir, K. et al. (2014). Cells Isolated from Human Glioblastoma Multiforme Express Progesterone-Induced Blocking Factor (PIBF). *Cellular and Molecular Neurobioly*. 34, 4, 479-489. https://doi.org/10.1007/s10571-014-0031-3;
- Lacroix-Fralish ML, Tawfik VL, Nutile-McMenemy N, Harris BT, Deleo JA. (2006). Differential regulation of neuregulin 1 expression by progesterone in astrocytes and neurons. *Neuron Glia Biology*. 2, 4, 227–234. https://doi.org/10.1017/S1740925X07000385;
- Lan, Y., Zou, S., Wang, X., Lou, J., Xing, J., Yu, M. & Zhang, B. (2017). Update on the therapeutic significance of estrogen receptor beta in malignant gliomas. *Oncotarget*. 8, 46, 81686-81696. https://doi.org/10.18632/oncotarget.20970;
- Lee S. Y. (2016). Temozolomide resistance in glioblastoma multiforme. Genes & diseases. 3, 3, 198–210. https://doi.org/10.1016/j.gendis.2016.04.007;
- Lenz, K. M., & McCarthy, M. M. (2010). Organized for sex - steroid hormones and the developing hypothalamus. *The European journal of neuroscience*. 32, 12, 2096–2104. https://doi.org/10.1111/j.1460-9568.2010.07511.x;
- Liang, C., Park, A. & Guan J. (2007). *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols*. 2, 329-333. https://doi.org/10.1038/nprot.2007.30;
- Liu, C., Zhang, Y., Zhang, K., Bian, C., Zhao, Y. & Zhang, J. (2014). Expression of estrogen receptors, androgen receptor and steroid receptor coactivator-3 is negatively correlated to the differentiation of astrocytic tumors. *Cancer Epidemiology*. 38, 291-297. https://doi.org/10.1016/j.canep.2014.03.001;
- Louis, D., Perry, A., Reifenberger, G., Deimling, A., Figarella-Branger, D., Cavenee, W., Ohgaki, H., Wiestler, O., Kleihues, P. & Ellison, D. (2016). The 2016 World Health Organization Classification of Tumours of the Central Nervous System: a summary. *Acta Neuropathologica*. 131, 6, 803-820. https://doi.org/10.1007/s00401-016-1545-1;
- Manca, P. & Chisu, V. (2010). 2-methoxyestradiol induces morpho-functional changes and impairs the microtubular system in mouse neuroblastoma and rat glioma cells. *Archives Italiennes de Biologie*. 148, 1, 11-21. Available in: https://www.ncbi.nlm.nih.gov/pubmed/20426250;

- Mannick, J. B., Hausladen, A., Liu, L., Hess, D. T., Zeng, M., Miao, Q. X., ... Stamler, J. S. (1999). Fas-induced caspase denitrosylation. *Science*. 284, 5414, 651-654. https://doi.org/10.1126/science.284.5414.651;
- McKinley, B., Michalek, A., Fenstermaker, R. & Plunkett, R. (2000). The impact of age and sex on the incidence of glial tumors in New York state from 1976 to 1995. *Journal of Neurosurgery*. 93, 6, 932-9. https://doi.org/10.3171/jns.2000.93.6.0932;
- Merrit, R. & Foran, C. (2007). Influence of persistent contaminants and steroid hormones on glioblastoma cell growth. *Journal of Toxicology and Environmental Health*. 70, 1, 19-27. https://doi.org/10.1080/15287390600748807;
- Micheli, A., Muti, P., Secreto, G., Krogh, V., Meneghini, E., Venturelli, E., Sieri, S., Pala, V. & Berrino, F. (2004). Endogenous sex hormones and subsequent breast cancer in premenopausal women. *International Journal of Cancer*. 112, 2. https://doi.org/10.1002/ijc.20403;
- Moinfar, Z., Dambach, H., Schoenebeck, B., Förster, E., Prochonow, N. & Faustmann, P. (2016). Estradiol Receptors Regulate Differential Connexin 43 Expression in F98 and C6 Glioma Cell Lines. *PLOS ONE*. 11, 2. https://doi.org/10.1371/journal.pone.0150007;
- Mooney, J., Bernstock, J., Ilyas, A., Ibrahim, A., Yamashita, D., Market, J. & Nakano, I. (2019). Current approaches and challenges in the molecular therapeutic targeting of glioblastoma. *World Neurosurgery*. 129, 90-100. https://doi.org/10.1016/j.wneu.2019.05.205;
- Moraes, G., Carvalho, É., Maia, R. & Sternberg, C. (2011). Immunodetection of caspase-3 by Western blot using glutaraldehyde. *Analytical Biochemistry*. 415, 2, 203-205. https://doi.org/10.1016/j.ab.2011.04.032;
- Nelles, J. L., Hu, W. Y., & Prins, G. S. (2011). Estrogen action and prostate cancer. *Expert review of endocrinology & metabolism*. 6, 3, 437–451. https://doi.org/10.1586/eem.11.20;
- Nogueira, V., Sebastião, A., Cascalheira, J. & Valente C. (2019). Role of adenosine on the resistance to an anti-tumor agent in glioblastoma cell lines. *Frontiers*. https://doi.org/10.3389/conf.fncel.2019.01.00009;
- Ohgaki, H., & Kleihues, P. (2007). Genetic pathways to primary and secondary glioblastoma. *The American journal of pathology*. 170, 5, 1445–1453. https://doi.org/10.2353/ajpath.2007.070011;

- Ohgaki. H. & Kleihues, P. (2013). The Definition of Primary and Secondary Glioblastoma. *Clinical Cancer Research*. 19, 4, 764–772. https://doi.org/10.1158/1078-0432;
- Ostrom, Q. T., Gittleman, H., Farah, P., Ondracek, A., Chen, Y., Wolinsky, Y., Stroup, N. E., Kruchko, C., & Barnholtz-Sloan, J. S. (2013). CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2006-2010. *Neuro-oncology*. 15, 2 (Suppl 2), ii1–ii56. https://doi.org/10.1093/neuonc/not151;
- Ozdemir-Kaynak, E., Qutub, A. A., & Yesil-Celiktas, O. (2018). Advances in Glioblastoma Multiforme Treatment: New Models for Nanoparticle Therapy. *Frontiers in physiology*. 9, 170. https://doi.org/10.3389/fphys.2018.00170;
- Piña-Medina, A., Hansberg-Pastor, V., González-Arenas, A., Cerbón, M. & Camacho-Arroyo, I. (2016). Progesterone promotes cell migration, invasion and cofilin activation in human astrocytoma cells. *Steroids*. 105, 19-25. https://doi.org/10.1016/j.steroids.2015.11.008;
- Platz, E. & Giovannucci, E. (2004). The epidemiology of sex steroid hormones and their signaling and metabolic pathways in the etiology of prostate cancer. The *Journal of Steroid Biochemistry and Molecular Biology*. 92, 4, 237-53. https://doi.org/10.1016/j.jsbmb.2004.10.002;
- Qu C, Ma J, Zhang Y, et al. (2019). Estrogen receptor variant ER-α36 promotes tamoxifen agonist activity in glioblastoma cells. *Cancer Science*. 110, 1, 221–234. https://doi.org/10.1111/cas.13868;
- Quintela, T., Alves, CH., Gonçalves, I., Baltazar, G., Saraiva MJ. & Santos, CR. (2008). 5Alpha-dihydrotestosterone up-regulates transthyretin levels in mice and rat choroid plexus via an androgen receptor independent pathway. *Brain Research*. 1229, 18-26. https://doi.org/10.1016/j.brainres.2008.06.095;
- Quintela, T., Gonçalves, I., Carreto, L. C., Santos, M. A., Marcelino, H., Patriarca, F. M., & Santos, C. R. (2013). Analysis of the effects of sex hormone background on the rat choroid plexus transcriptome by cDNA microarrays. *PloS one*. 8, 4, e60199. https://doi.org/10.1371/journal.pone.0060199;
- Regidor P. A. (2014). Progesterone in Peri- and Postmenopause: A Review. *Geburtshilfe und Frauenheilkunde*. 74, 11, 995–1002. https://doi.org/10.1055/s-0034-1383297;

- Rivera-Delgado, E. & von Recum, H. (2017). Using Affinity To Provide Long-Term Delivery of Antiangiogenic Drugs in Cancer Therapy. *Molecular Pharmaceutics*. 14, 3, 899-907. https://doi.org/10.1021/acs.molpharmaceut.6b01109;
- Rodríguez-Lozano, D. C., Piña-Medina, A. G., Hansberg-Pastor, V., Bello-Alvarez, C., & Camacho-Arroyo, I. (2019). Testosterone Promotes Glioblastoma Cell Proliferation, Migration, and Invasion Through Androgen Receptor Activation. *Frontiers in endocrinology*. 10. https://doi.org/10.3389/fendo.2019.00016;
- Roshan, M. H., Tambo, A., & Pace, N. P. (2016). The role of testosterone in colorectal carcinoma: pathomechanisms and open questions. *The EPMA journal*. 7, 22. https://doi.org/10.1186/s13167-016-0071-5;
- Ruiz-Cortés, Z.T. (2012). Gonadal Sex Steroids: Production, Action and Interactions in Mammals, Steroids - From Physiology to Clinical Medicine, Sergej M. Ostojic, IntechOpen. https://doi.org/10.5772/52994. Available from: https://www.intechopen.com/books/steroids-from-physiology-to-clinical-medicine/gonadal-sex-steroids-production-action-and-interactions-in-mammals;
- Santos, C., Duarte, A. C., Costa, A., Tomás, J., Quintela, T. & Goncalves, I. (2019). The senses of the choroid plexus. *Progress in Neurobiology*. 182. https://doi.org/101680. 10.1016/j.pneurobio.2019.101680;
- Santos, CR., Duarte, AC., Quintela, T., Tomás, J., Albuquerque, T., Marques, F., Palha, JA & Gonçalves, I. (2017). The choroid plexus as a sex hormone target: Functional implications. *Frontiers in Neuroendocrinology*. 44, 103-121. https://doi.org/10.1016/j.yfrne.2016.12.002;
- Sareddy, G., Li, X., Liu, J., Viswanadhapalli, S., Garcia, L., Gruslova, A., Cavazos, D., Garcia M., Strom, A., Gustafsson, J., Tekmal, R., Brenner, A. & Vadlamudi, R. (2016). Selective Estrogen Receptor β Agonist LY500307 as a Novel Therapeutic Agent for Glioblastoma. *Scientific Reports*. 6, 24185. https://doi.org/10.1038/srep24185;
- Sareddy, G., Nair, B., Gonungunta, V., Zhang, Q., Brenner, A., Brann, D., Tekmal, R. & Vadlamudi, R. (2012). Therapeutic significance of estrogen receptor β agonists in gliomas. *Molecular Cancer Therapy*. 11, 5, 1174-1182. https://doi.org/10.1158/1535-7163.MCT-11-0960;

- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*. 9, 7, 676–682. https://doi.org/10.1038/nmeth.2019;
- Schreck, K. & Grossman, S. (2018). Role of Temozolomide in the Treatment of Cancers Involving the Central Nervous System. *Oncology Journal.* 32, 11, 555-60, 569. Available in: https://www.cancernetwork.com/role-temozolomide-cns;
- Séhédic, D., Cikankowitz, A., Hindré, F., Davodeau, F., Garcion, E. (2015). Nanomedicine to overcome radioresistance in glioblastoma stem-like cells and surviving clones. *Trends in Pharmacological Sciences.* 36, 4, 236-52. https://doi.org/10.1016/j.tips.2015.02.002;
- Sewer, M. B., & Li, D. (2008). Regulation of steroid hormone biosynthesis by the cytoskeleton. *Lipids*. 43, 12, 1109–1115. https://doi.org/10.1007/s11745-008-3221-2;
- Sousa, G., Rocha, A., Alfaiate, T., Carvalho, T., Moura, A. & Ferreira, M. (2002). Glioblastoma Multiforme… com apresentação multifocal. *Acta Médica Portuguesa*. 15, 321-324, 20;
- Stanworth RD, Jones TH. (2008). Testosterone for the aging male; current evidence and recommended practice. *Clinical Interventions in Aging*. 3, 1, 25–44. https://doi.org/10.2147/cia.s190;
- Su C, Rybalchenko N, Schreihofer DA, Singh M, Abbassi B, Cunningham RL. Cell Models for the Study of Sex Steroid Hormone Neurobiology. *Journal of Steroids and Hormonal Science*. S2, 003. https://doi.org/10.4172/2157-7536.s2-003;
- Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C., & Salameh, W. A. (2017). Dihydrotestosterone: Biochemistry, Physiology, and Clinical Implications of Elevated Blood Levels. *Endocrine reviews*. 38, 3, 220–254. https://doi.org/10.1210/er.2016-1067;
- Tamimi AF, Juweid M. (2017). Epidemiology and Outcome of Glioblastoma. In De Vleeschouwer S. *Glioblastoma*. Brisbane (AU): Codon Publications. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470003/;
- Tang, W., Liu, R., Yan, Y., Pan, X., Wang, M., Han, X., Ren, H., & Zhang, Z. (2017). Expression of estrogen receptors and androgen receptor and their clinical

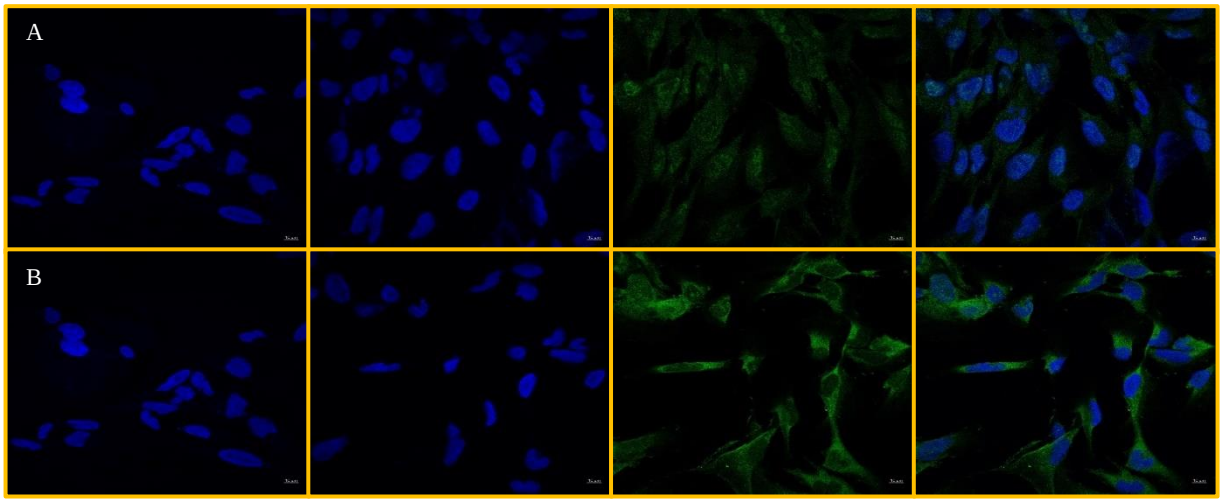
significance in gastric cancer, *Oncotarget*. 8, 40765–40777. https://doi.org/10.18632/oncotarget.16582;

- Tavares, C., Gomes-Braga, F., Costa-Silva, D., Escórcio-Dourado, C., Borges, U., Conde-Junior, A., Barros-Oliveira, M., Sousa, E., Barros, L., Martins, L., Facina, G. & Silva, B. (2016). Expression of estrogen and progesterone receptors in astrocytomas: a literature review. *Clinics (Sao Paulo, Brazil)*. 71, 8, 481-486. https://doi.org/10.6061/clinics/2016(08)12;
- Thakkar, J. P., Dolecek, T. A., Horbinski, C., Ostrom, Q. T., Lightner, D. D., Barnholtz-Sloan, J. S., & Villano, J. L. (2014). Epidemiologic and molecular prognostic review of glioblastoma. Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 23, 10, 1985–1996. https://doi.org/10.1158/1055-9965.EPI-14-0275;
- Touat, M., Idbaih, A., Sanson, M., & Ligon, K. L. (2017). Glioblastoma targeted therapy: updated approaches from recent biological insights. *Annals of oncology: official journal of the European Society for Medical Oncology*. 28, 7, 1457–1472. https://doi.org/10.1093/annonc/mdx106;
- UniProt (2020). UniProt-P4574(CASP3_HUMAN). Access in 23 of march of 2020. Available in https://www.uniprot.org/uniprot/P42574;
- Walters J, Pop C, Scott FL, et al. (2009). A constitutively active and uninhibitable caspase-3 zymogen efficiently induces apoptosis. *Biochemical Journals*. 424, 3, 335–345. https://doi.org/10.1042/BJ20090825;
- Wan, S., Jiang, J., Zheng, C. et al. (2018). Estrogen nuclear receptors affect cell migration by altering sublocalization of AQP2 in glioma cell lines. *Cell Death Discovery*. 4, 49. https://doi.org/10.1038/s41420-018-0113-y;
- Wang, J., Jiajia, L., Fang, R., Shuduo, X., Wang, L. & Xu, C. (2012) Expression of ERα36 in gastric cancer samples and their matched normal tissues. *Oncology Letters*. 3, 172–175. https://doi.org/10.3892/ol.2011.437;
- Wang, X., Huang, X., Fu, Z., Zou, F., Li, Y., Wang, Z. & Liu, L. (2014) Biphasic ER-α36-mediated estrogen signaling regulates growth of gastric cancer cells. *International Journal of Oncology*. 45, 2325–2330. https://doi.org/10.3892/ijo.2014.2660;
- Wise, P. M., Suzuki, S., & Brown, C. M. (2009). Estradiol: a hormone with diverse and contradictory neuroprotective actions. *Dialogues in clinical*

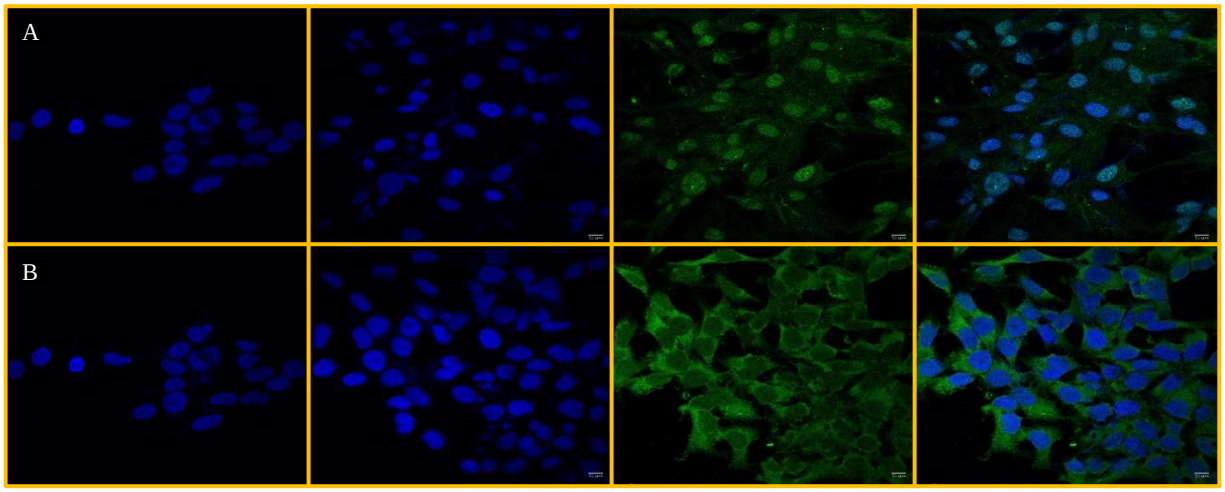
neuroscience. 11, 3, 297–303. Available in: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3181927/;

- Wolmarans, E., Mqoco, T. V., Stander, A., Nkandeu, S. D., Sippel, K., McKenna, R., & Joubert, A. (2014). Novel estradiol analogue induces apoptosis and autophagy in esophageal carcinoma cells. *Cellular & molecular biology letters*. 19, 98–115. https://doi.org/10.2478/s11658-014-0183-7;
- Yang, W., Warrington, N., Taylor, S., Whitmire, P., Carrasco, E., Singleton, K., Wu, N., Lathia, J., Barens, M., Kim, A., Barnholtz-Sloan, J., Swanson, K., Luo, J. & Rubin, J. (2019). Sex differences in GBM revealed by analysis of patient imaging, transcriptome, and survival data. *Science Translational Medicine*. 11, 473. https://doi.org/10.1126/scitranslmed.aao5253;
- Yi, J.H., Do, I., Jang, J., Kim, S.T., Kim, K., Park, S.H., Park, J.O., Park, Y.S., Lim, H.Y., Kang, W.K. & Lee, J. (2014) Anti-tumor efficacy of fulvestrant in estrogen receptor positive gastric cancer. *Scientific Reports*. 4, 1–8. https://doi.org/10.1038/srep07592;
- Yu, X., Jiang, Y., Wei, W., Cong, P., Ding, Y., Xiang, L. & Wu, K. (2015). Androgen receptor signaling regulates growth of glioblastoma multiforme in men. *Tumour Biology*. 36, 2, 967-72. https://doi.org/10.1007/s13277-014-2709-z;
- Zane, M., Parello, C., Pennelli, G., Townsend, D. M., Merigliano, S., Boscaro, M., Toniato, A., Baggio, G., Pelizzo, M. R., Rubello, D., & Boschin, I. M. (2017). Estrogen and thyroid cancer is a stem affair: A preliminary study. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 85, 399–411. https://doi.org/10.1016/j.biopha.2016.11.043;
- Zubeldia-Brenner, L., Roselli, C. E., Recabarren, S. E., Gonzalez Deniselle, M. C., & Lara, H. E. (2016). Developmental and Functional Effects of Steroid Hormones on the Neuroendocrine Axis and Spinal Cord. *Journal of neuroendocrinology*. 28, 7. https://doi.org/10.1111/jne.12401.

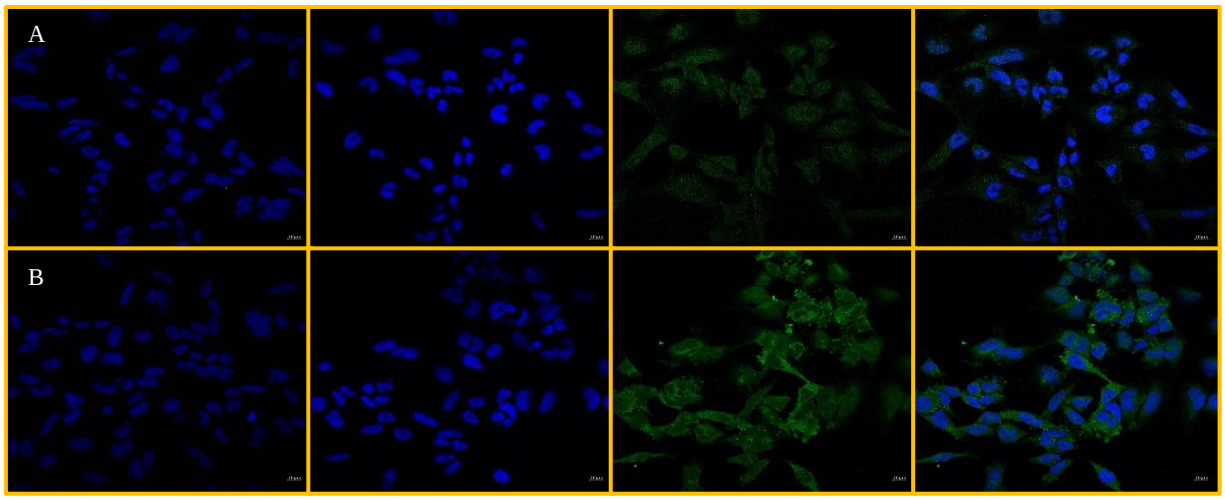
8. List of Appendices



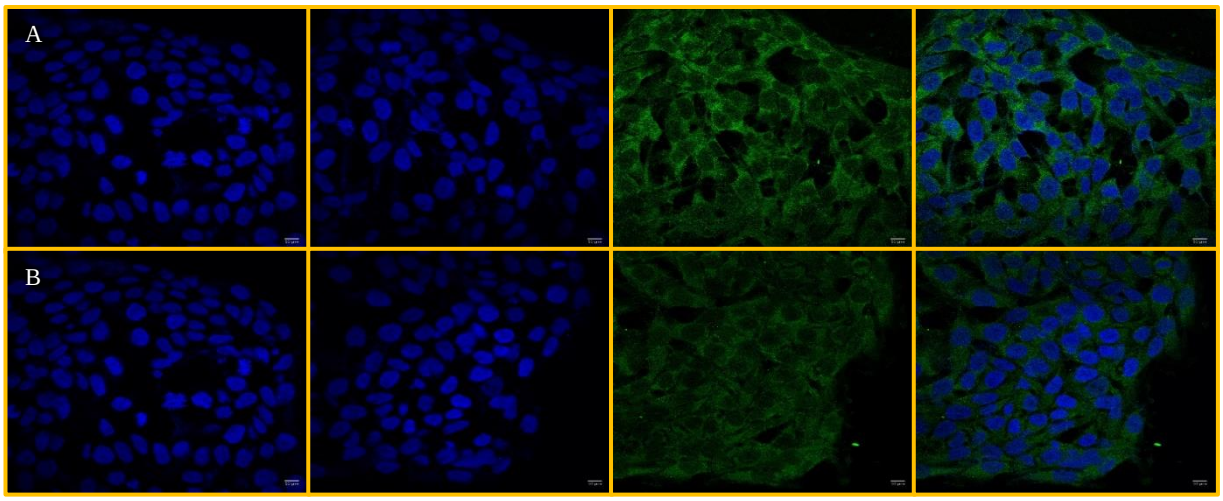
Appendix 1. Estrogen receptors expression in HASTR/ci35 cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-ER-α (1:50) and (B) rabbit anti-ER-β (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



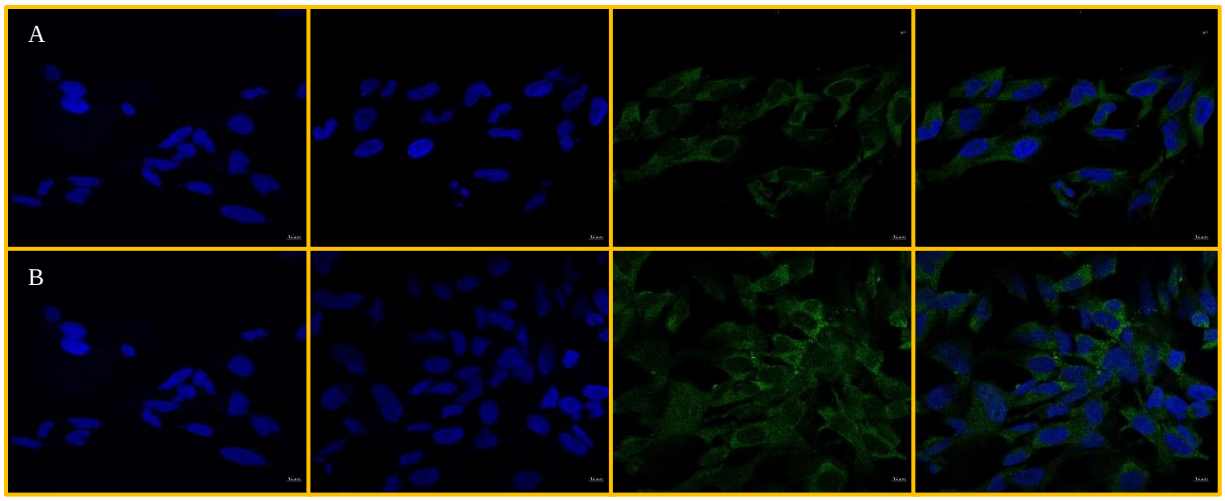
Appendix 2. Estrogen receptors expression in U-87MG cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-ER-α (1:50) and (B) rabbit anti-ER-β (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



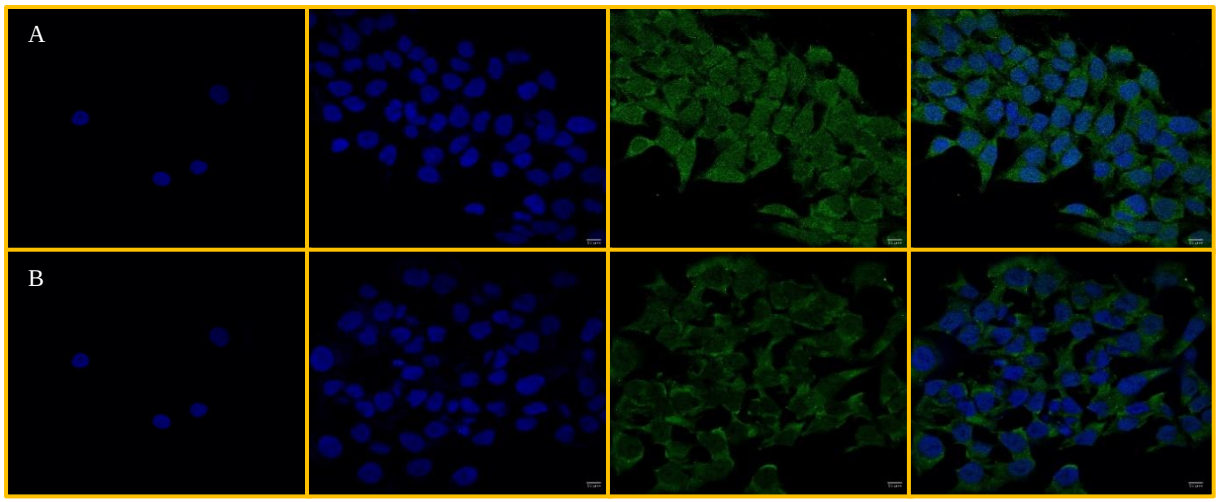
Appendix 3. Estrogen receptors expression in SNB19 cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-ER-α (1:50) and (B) rabbit anti-ER-β (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



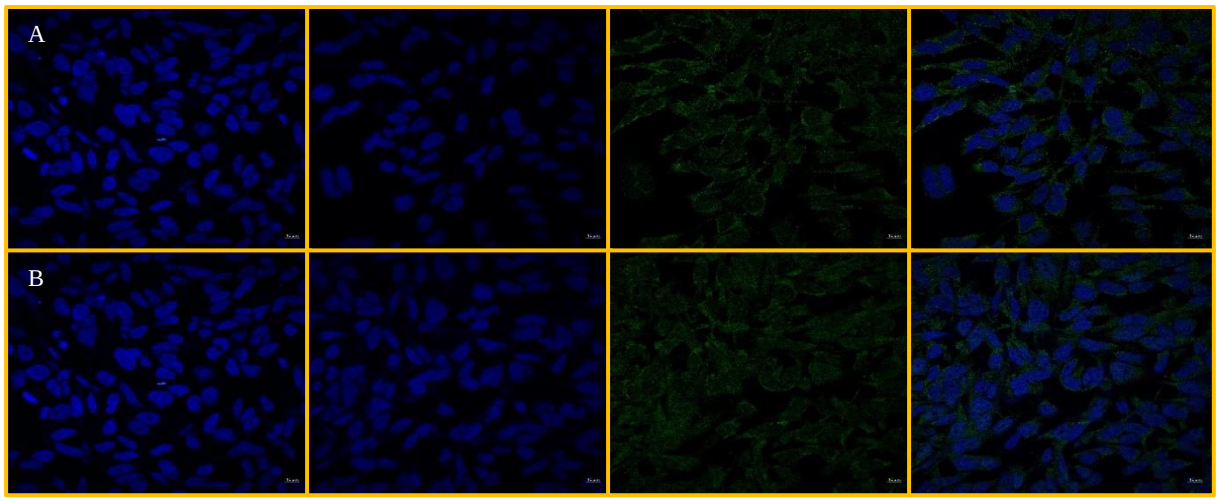
Appendix 4. Estrogen receptors expression in U-373MG cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-ER-α (1:50) and (B) rabbit anti-ER-β (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



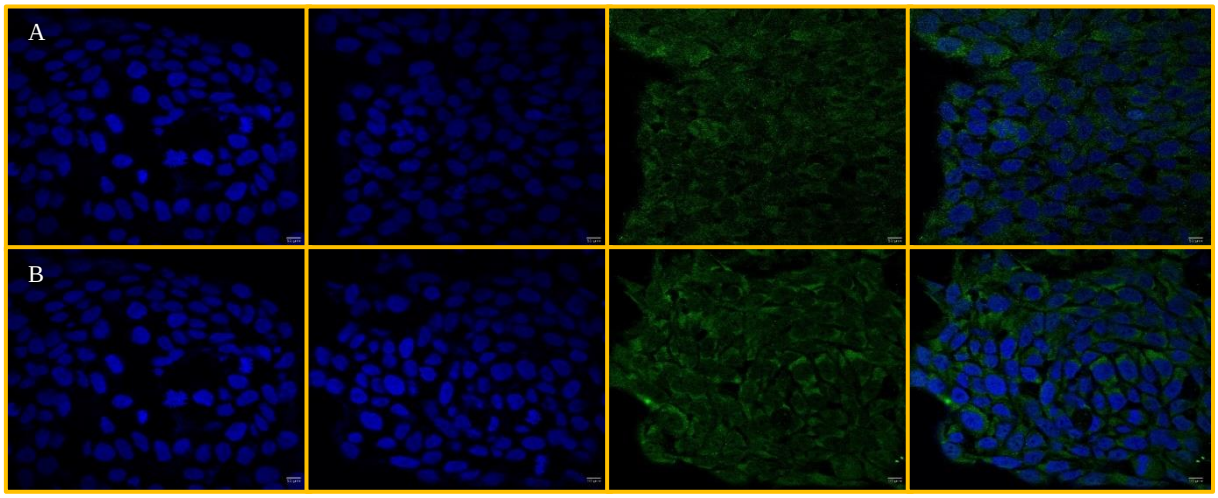
Appendix 5. Androgen receptor expression in HASTR/ci35 cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-AR C-19 (1:50) and (B) rabbit anti-AR N-20 (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



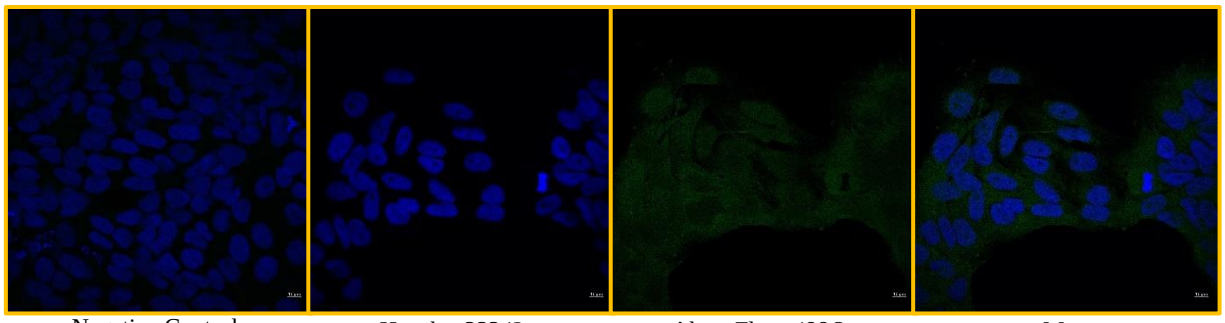
Appendix 6. Androgen receptor expression in U-87MG cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-AR C-19 (1:50) and (B) rabbit anti-AR N-20 (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



Appendix 7. Androgen receptor expression in SNB19 cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-AR C-19 (1:50) and (B) rabbit anti-AR N-20 (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.

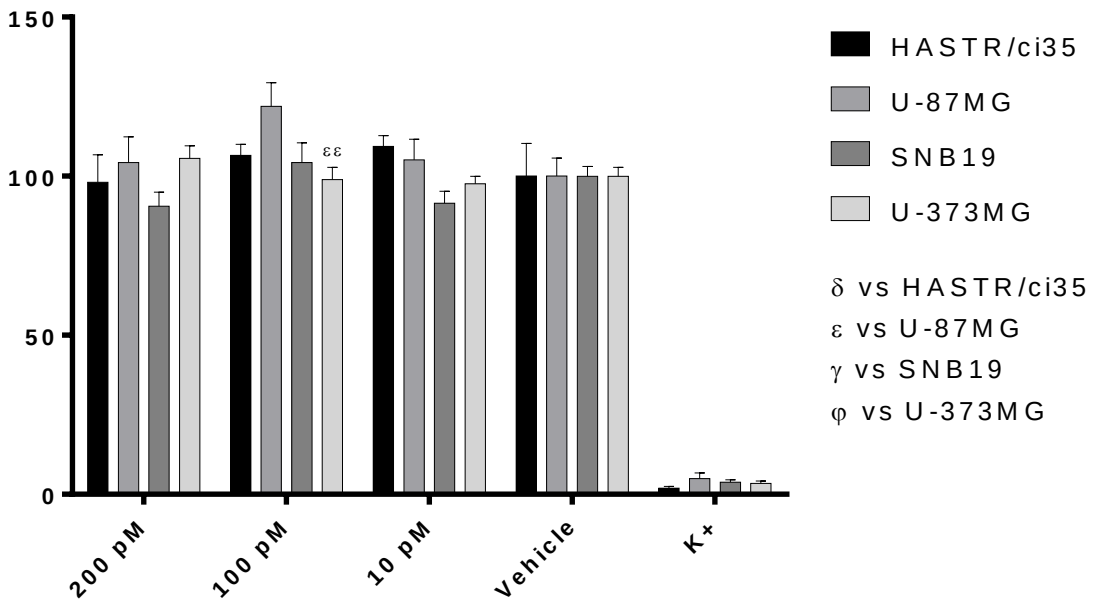


Appendix 8. Androgen receptor expression in U-373MG cell line by immunocytochemistry. Cells were stained with primary antibodies (A) rabbit anti-AR C-19 (1:50) and (B) rabbit anti-AR N-20 (1:50), and secondary antibody goat anti-rabbit Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



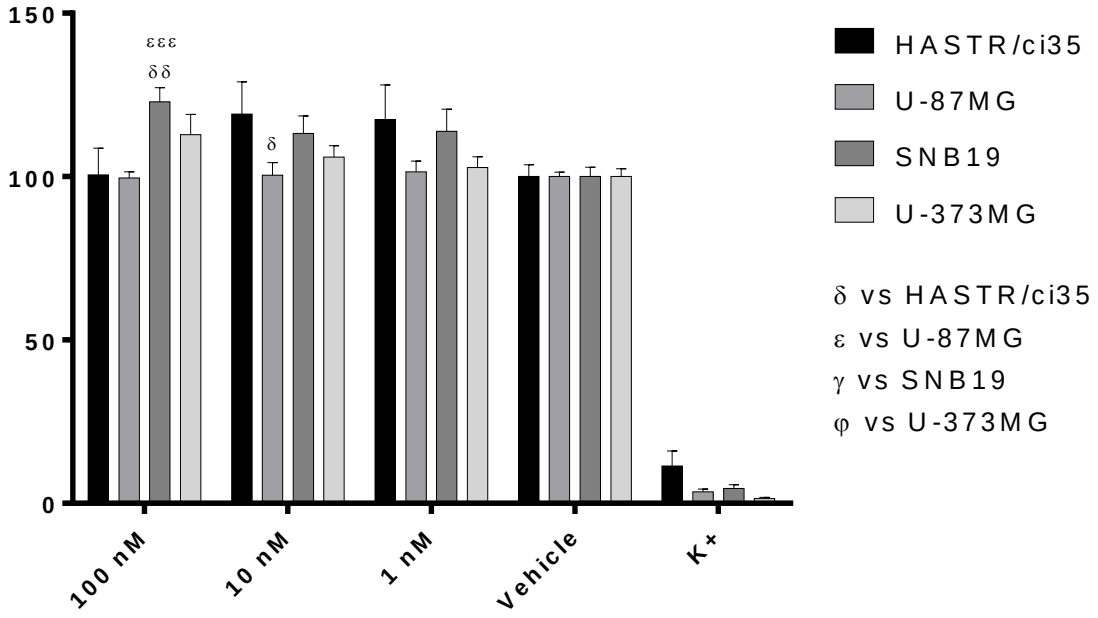
Negative Control | Hoechst 33342 | Alexa Fluor 488® | Merge

Appendix 9. Progesterone receptor expression in U-87MG cell line by immunocytochemistry. Cells were stained with primary antibody mouse anti-PR (1:50) and secondary antibody goat anti-mouse Alexa Fluor® 488 (1:1000). Nuclei were stained with Hoechst 33342 (1:1000). Negative control: absence of primary antibody. Scale bar: 10 µm.



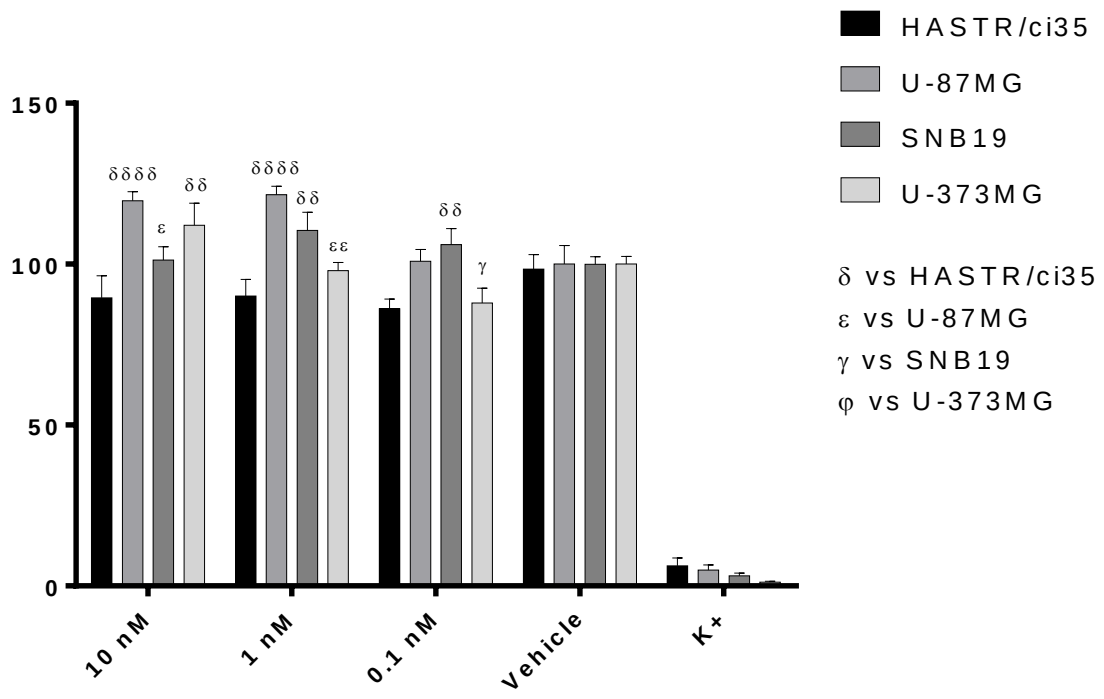
Appendix 10. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different estradiol (E2) concentrations (200 pM, 100 pM and 10 pM) and vehicle (EtOH ≤ 0.00002%) at 24h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

When comparing the stimuli for E2 with an incubation period of 24h between the 4 lines studied we found statistically significant differences within the concentration of 100 pM between the lines U-87MG and U-373MG ($p<0.01$).



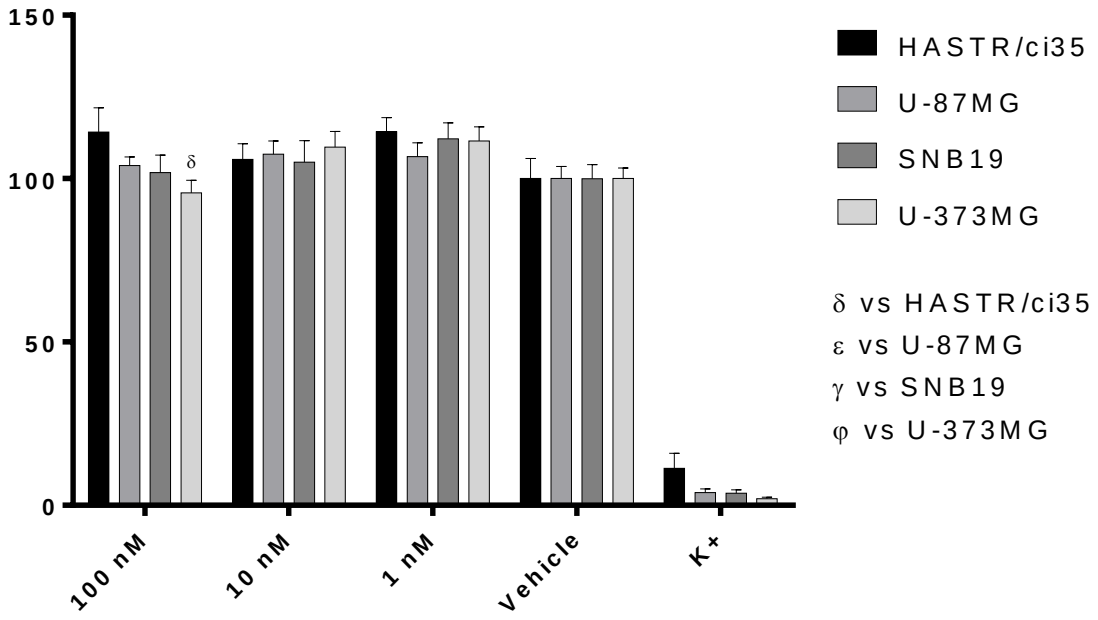
Appendix 11. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different progesterone (P4) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH $\leq$ 0.01%) at 24h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of P4 with an incubation period of 24h there were statistically significant differences in the concentration of 100 nM between the lines HASTR/ci35 and SNB19 ($p<0.01$) and between U-87MG and SNB19 ($p<0.001$). For the concentration of 10 nM, there were differences between HASTR/ci35 and U-87MG ($p<0.05$).



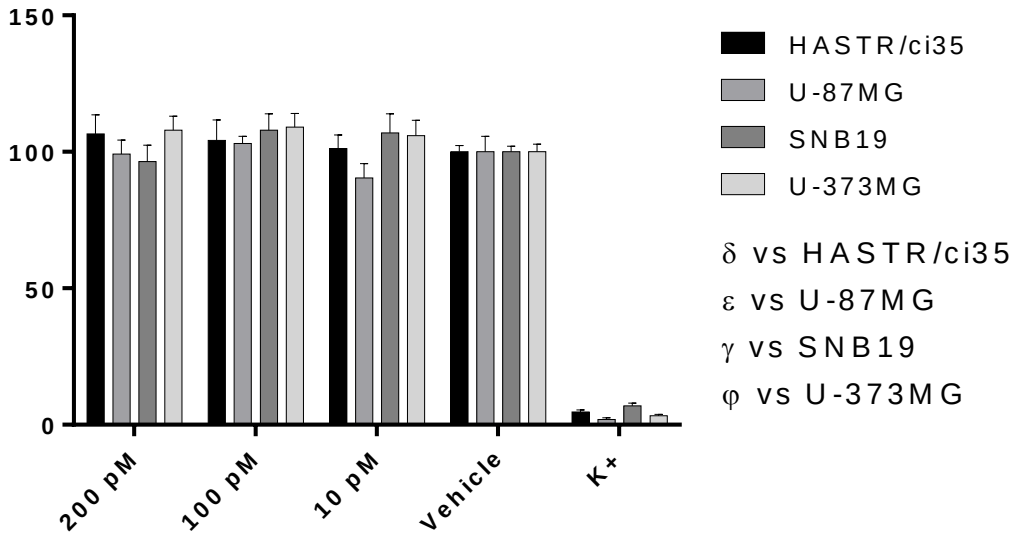
Appendix 12. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different dihydrotestosterone (DHT) concentrations (10 nM, 1 nM and 0.1 nM) and vehicle (EtOH ≤ 0.001%) at 24h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli with DHT with an incubation period of 24h there were statistically significant differences in the concentration of 10 nM between HASTR/ci35 and U-87MG ($p<0.0001$), HASTR/ci35 and U-373MG ($p<0.01$) and, U-87MG and SNB19 ($p<0.05$). For the concentration of 1 nM differences were found between HASTR/ci35 and U-87MG ($p<0.0001$), HASTR/ci35 and SNB19 ($p<0.01$) and, U-87MG and U-373MG ($p<0.01$). Finally, for the concentration of 0.1 nM, differences of found between HASTR/ci35 and SNB19 ($p<0.01$) and also between SNB19 and U-373MG ($p<0.05$).



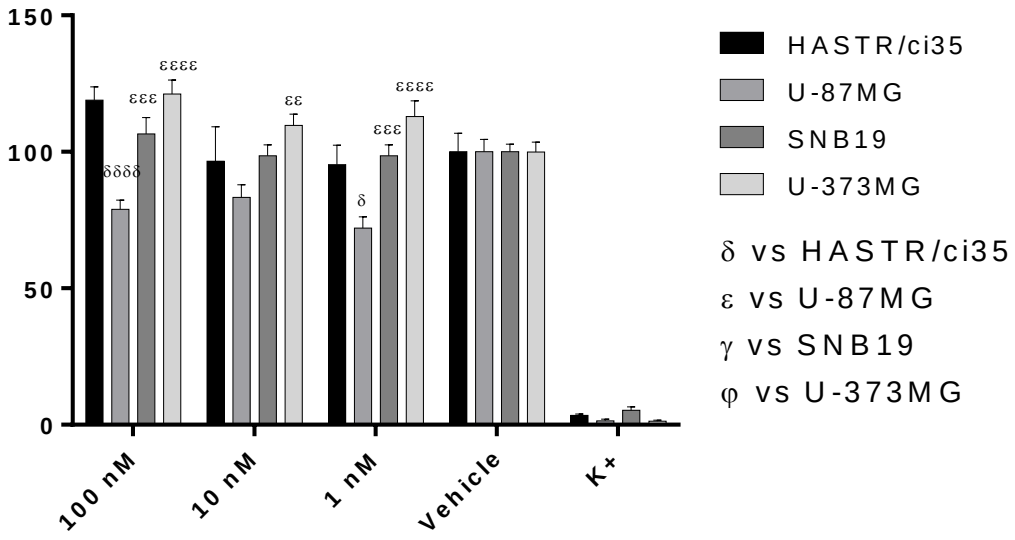
Appendix 13. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different testosterone (T) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH $\leq$ 0.01%) at 24h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

Stimuli of T were analysed within the four lines studied and after an incubation period of 24h there were statistically significant differences in the concentration of 100 nM between HASTR/ci35 and U-373MG ($p<0.05$).



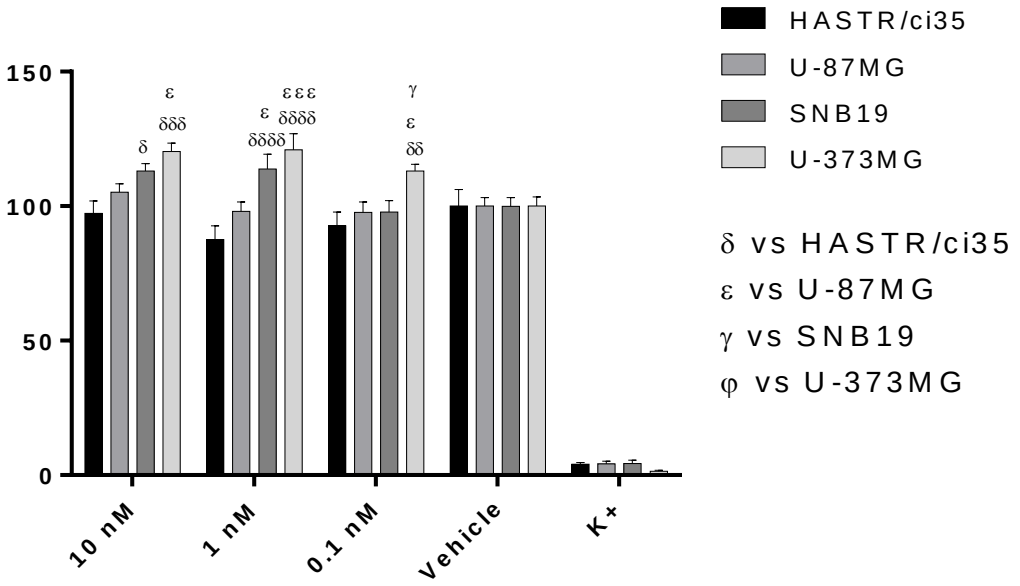
Appendix 14. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different estradiol (E2) concentrations (200 pM, 100 pM and 10 pM) and vehicle (EtOH ≤ 0.00002%) at 48h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of E2 with an incubation period of 48h there were no statistically significant differences found.



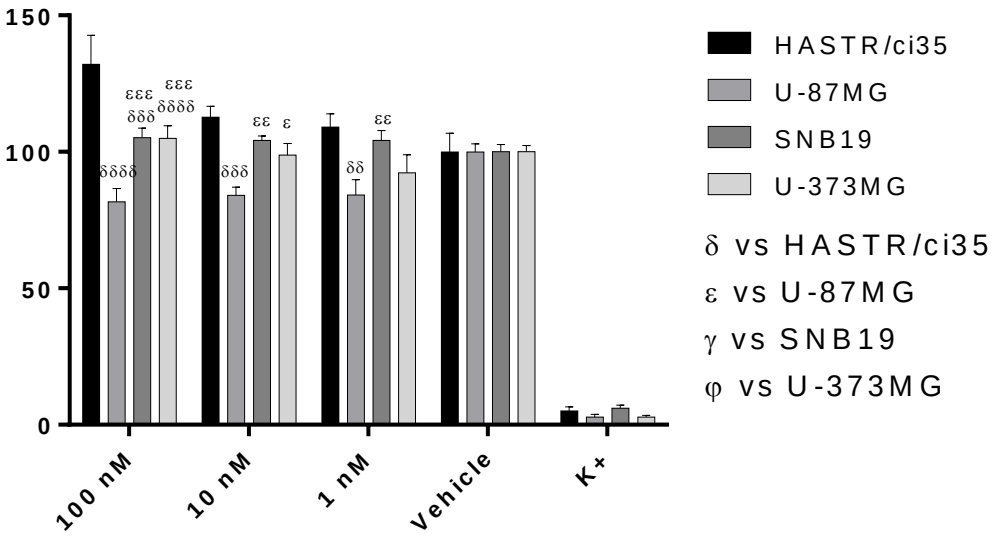
Appendix 15. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different progesterone (P4) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH $\leq$ 0.01%) at 48h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of P4 with an incubation period of 48h there were statistically significant differences in the concentration of 100 nM between the lines HASTR/ci35 and U-87MG ($p<0.0001$), U-87MG and SNB19 ($p<0.001$) and, between U-87MG and U-373MG ($p<0.0001$). For the concentration of 10 nM, there were differences between U-87MG and U-373MG ($p<0.01$). Within the stimuli with 1 nM, differences were found between HASTR/ci35 and U-87MG ($p<0.05$), U-87MG and SNB19 ($p<0.001$) and, between U-87MG and U-373MG ($p<0.0001$).



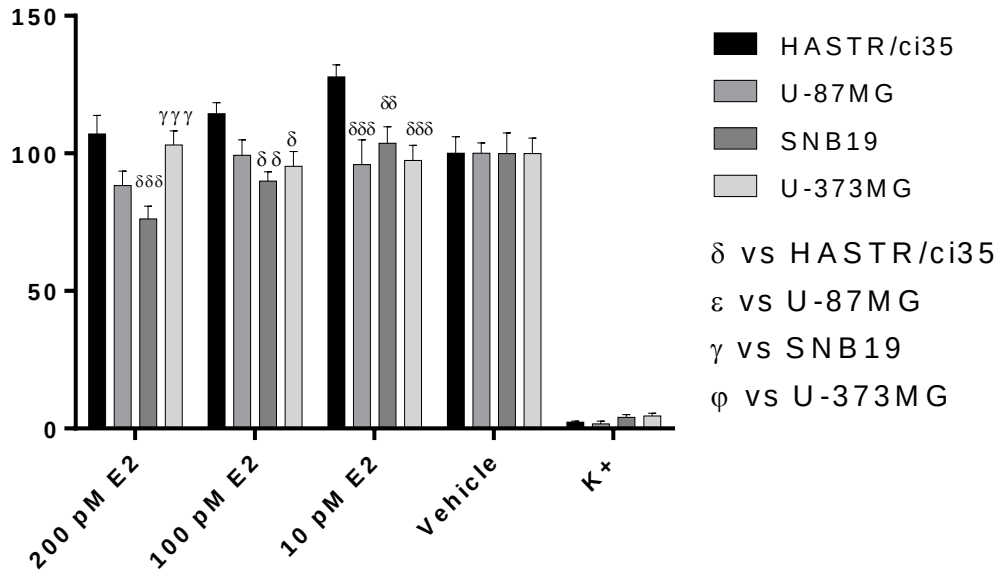
Appendix 16. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different dihydrotestosterone (DHT) concentrations (10 nM, 1 nM and 0.1 nM) and vehicle (EtOH ≤ 0.001%) at 48h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of DHT with an incubation period of 48h there were statistically significant differences in the concentration of 10 nM between the lines HASTR/ci35 and SNB19 ($p<0.05$), HASTR/ci35 and U-373MG ($p<0.001$) and, U-87MG and U-373MG ($p<0.05$). For a concentration of 1 nM, there were differences between HASTR/ci35 and SNB19 ($p<0.0001$), HASTR/ci35 and U-373MG ($p<0.0001$), U-87MG and SNB19 ($p<0.05$), and between U-87MG and U-373MG ($p<0.001$). Lastly, for a concentration of 0.1 nM, differences were found between HASTR/ci35 and U-373MG ($p<0.01$), U-87MG and U-373MG ($p<0.05$), and SNB19 and U-373MG ($p<0.05$).



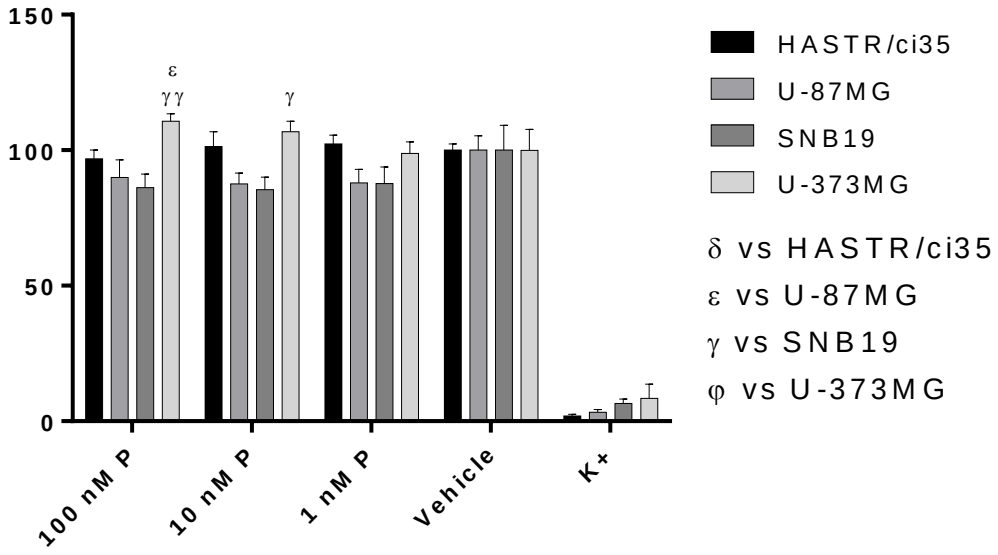
Appendix 17. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different testosterone (T) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH ≤ 0.01%) at 48h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of T with an incubation period of 48h there were statistically significant differences in the concentration of 100 nM between the lines HASTR/ci35 and U-87 ($p<0.0001$), HASTR/ci35 and SNB19 ($p<0.001$), HASTR/ci35 and U-373MG ($p<0.0001$), U-87MG and SNB19 ($p<0.001$) and, between U-87MG and U-373MG ($p<0.001$). For a concentration of 10 nM, there were differences between HASTR/ci35 and U-87MG ($p<0.001$), U-87MG and SNB19 ($p<0.01$) and, U-87MG and U-373MG ($p<0.05$). Finally, for a concentration of 1 nM, differences were found between HASTR/ci35 and U-87MG ($p<0.01$) and also between U-87MG and SNB19 ($p<0.01$).



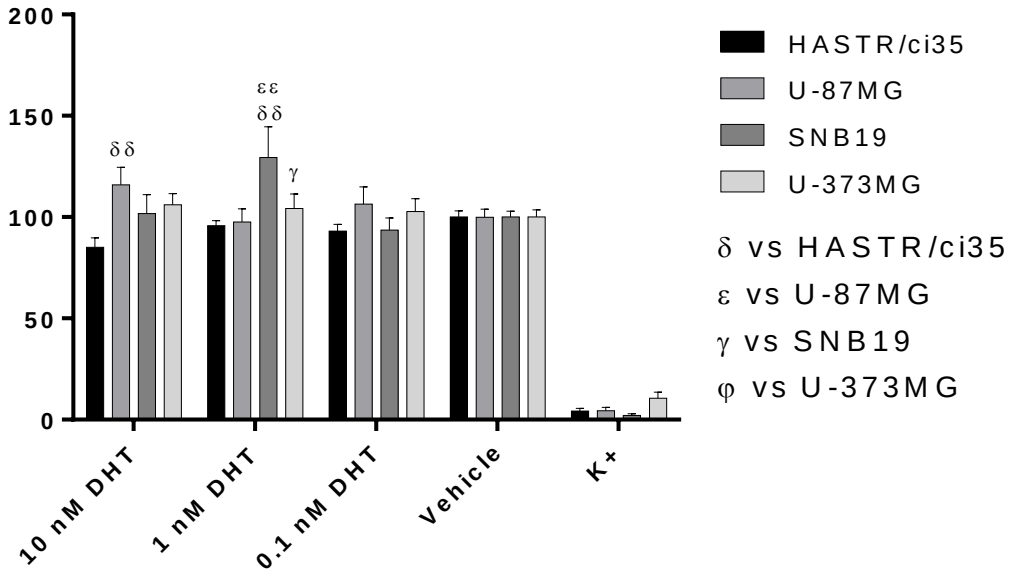
Appendix 18. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different estradiol (E2) concentrations (200 pM, 100 pM and 10 pM) and vehicle (EtOH ≤ 0.00002%) at 72h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of E2 with an incubation period of 72h there were statistically significant differences in the concentration of 200 pM between the lines HASTR/ci35 and SNB19 ($p<0.001$) and between SNB19 and U-373MG ($p<0.001$). For the concentration of 100 pM, there were differences between HASTR/ci35 and SNB19 ($p<0.01$) and between HASTR/ci35 and U-373MG ($p<0.05$). Lastly, for a concentration of 10 pM, differences were found between HASTR/ci35 and U-87MG ($p<0.001$), HASTR/ci35 and SNB19 ($p<0.01$), and HASTR/ci35 and U-373MG ($p<0.001$).



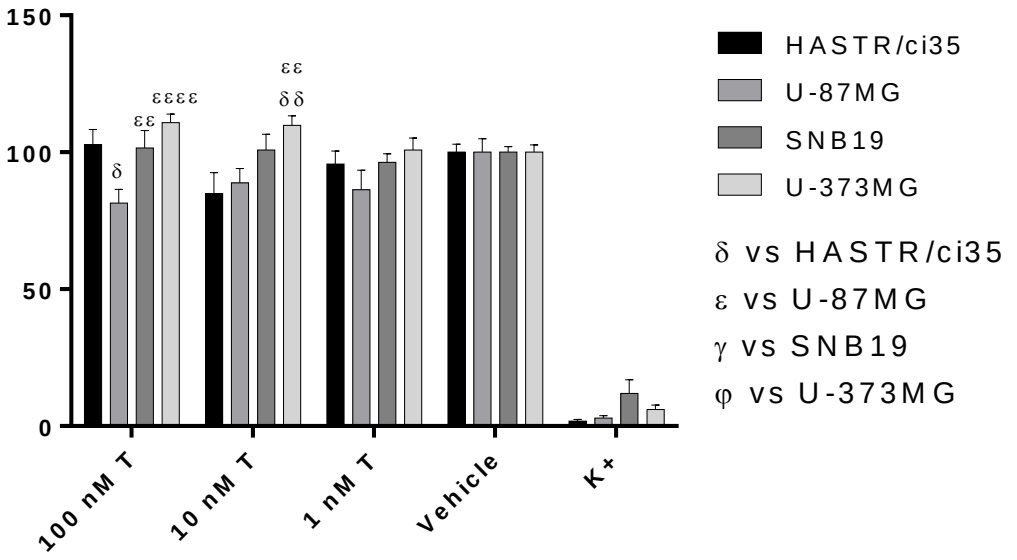
Appendix 19. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different progesterone (P4) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH $\leq$ 0.01%) at 72h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of P4 with an incubation period of 72h there were statistically significant differences in the concentration of 100 nM between the lines U-87MG and U-373MG ($p<0.05$) and, SNB19 and U-373MG ($p<0.01$). With a concentration of 10 nM, there were differences between SNB19 and U-373MG ($p<0.05$).



Appendix 20. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different dihydrotestosterone (DHT) concentrations (10 nM, 1 nM and 0.1 nM) and vehicle (EtOH ≤ 0.001%) at 72h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of DHT with an incubation period of 72h there were statistically significant differences in the concentration of 10 nM between the lines HASTR/ci35 and U-87MG ($p<0.01$). For the concentration of 1 nM, there were differences between HASTR/ci35 and SNB19 ($p<0.01$), U-87MG and SNB19 ($p<0.01$), and also between SNB19 and U-373MG ($p<0.05$).



Appendix 21. MTT assay of HASTR/CI35, U-87MG, SNB19 and U-373MG cells stimulated with different testosterone (T) concentrations (100 nM, 10 nM and 1 nM) and vehicle (EtOH ≤ 0.01%) at 72h. Control: vehicle. Positive control (K+: 50% EtOH). Statistically significant differences were determined by one-way ANOVA (n=3).

For the stimuli of T with an incubation period of 72h there were statistically significant differences in the concentration of 100 nM between the lines HASTR/ci35 and U-87MG ($p<0.05$), U-87MG and SNB19 ($p<0.01$) and between U-87MG and U-373MG ($p<0.0001$). Finally, for a concentration of 10 nM, there were differences between HASTR/ci35 and U-373MG ($p<0.01$) and between U-87MG and U-373MG ($p<0.01$).

Appendix 22. Effect of hormones in astrocyte cell line HASTR/ci35 and glioblastoma cell lines U-87MG, SNB19 and U-373MG

		HASTR/ci35			U-87MG			SNB19			U-373MG		
	Concentration	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
E2	200 pM			- (vs. 100 pM)						- - (vs. 10 pM)			
	100 pM												
	10 pM			++									
P4	100 nM					--		++				+	
	10 nM					-							
	1 nM					---							
DHT	10 nM	-			+ + (vs. 0.1 nM)						++ (vs. 0.1 nM)	+	
	1 nM				+ + (vs. 0.1 nM)				+ (vs. 0.1nM)	+ (vs. 0.1 nM)		+	
	0.1 nM												
T	100 nM		++			-							
	10 nM												
	1 nM												

Symbols: (-) decrease with statistical significance of $p<0.05$; (--) decrease with statistical significance of $p<0.01$; (---) decrease with statistical significance of $p<0.001$; (+) increase with statistical significance of $p<0.05$; (++) increase with statistical significance of $p<0.01$. Unless specified, differences are between concentration and its control (EtOH vehicle).